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POST-YIELD NANOMECHANICS OF HUMAN CORTICAL BONE IN COMPRESSION USING SYNCHROTRON X-RAY SCATTERING TECHNIQUES

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Abstract

The ultrastructural response to applied loads governs the post-yield deformation and failure behavior of bone, and is correlated with bone fragility fractures. Combining a novel progressive loading protocol and synchrotron X-ray scattering techniques, this study investigated the correlation of the local deformation (*i.e.*, internal strains of the mineral and collagen phases) with the bulk mechanical behavior of bone. The results indicated that the internal strains of the longitudinally oriented collagen fibrils and mineral crystals increased almost linearly with respect to the macroscopic strain prior to yielding, but markedly decreased first and then gradually leveled off after yielding. Similar changes were also observed in the applied stress before and after yielding of bone. However, the collagen to mineral strain ratio remained nearly constant throughout the loading process. In addition, the internal strains of longitudinal mineral and collagen phases did not exhibit a linear relationship with either the modulus loss or the plastic deformation of bulk bone tissue. Finally, the time-dependent response of local deformation in the mineral phase was observed after yielding. Based on the results, we speculate that the mineral crystals and collagen fibrils aligned with the loading axis only partially explain the post-yield deformation suggesting that shear deformation involving obliquely oriented crystals and fibrils (off axis) is dominant mechanism of yielding for human cortical bone in compression.

Keywords

Bone; synchrotron; internal strain; macro strain; post-yield

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INTRODUCTION

Osteoporotic and age-related bone fractures due to bone mass loss and deteriorated tissue properties have been a major concern in the health care of postmenopausal women and elderly populations (Melton, et al., 2005). It is commonly accepted that bone fragility could be estimated using its toughness (a measure of resistance to fracture), which is mainly determined by the post-yield properties of the tissue (McCalden, et al., 1993, Wang, et al., 2002).

The post-yield behavior of bone mainly involves three major mechanisms: microdamage accumulation, plastic strain accretion, and increased viscous response (Leng, et al., 2009, Nyman, et al., 2009a, Nyman, et al., 2009b). Nonetheless, the mechanism of the post-yield behavior of bone at ultrastructural levels is still unclear. Lack of such knowledge has significantly hindered mechanistic understanding of bone fragility fractures.

Recent developments in synchrotron X-ray scattering techniques have allowed researchers to measure the internal strain of mineral and collagen phases in bone under load (Almer & Stock, 2005, 2007, Gupta, et al., 2006, Gupta, et al., 2005). The results of tensile tests of bovine cortical bone indicated that the internal strain of both mineral and collagen phases increased linearly with the applied strain. However, the magnitude of the internal strain was significantly smaller than that of the macroscopic strain and was considerably different between the mineral and collagen phases (Gupta, et al., 2006, Gupta, et al., 2005). Similarly, compressive tests of canine fibula under dry condition also demonstrated a significant difference in the internal strain between the mineral and collagen phases (Almer & Stock, 2005, 2007). Since these studies were mainly focused on the *in situ* behavior of the mineral and collagen phases, how such a behavior is related to the bulk behavior of bone still remains unclear.

In this study, a novel progressive loading protocol and synchrotron X-ray scattering techniques were combined to investigate the relationship of the internal strain of the mineral and collagen phases with the bulk post-yield deformation of bone. One advantage of this methodology is that the evolution of macroscopic properties with increasing deformation of bone could be determined simultaneously with the *in situ* behavior of the mineral and collagen phases.

MATERIALS & METHODS

Specimen preparation

Five human cadaveric femurs of middle-aged males (51±2 years old) were collected from the National Disease Research Initiatives (NDRI, Philadelphia, PA) and screened for diseases and drug treatments that affect the tissue (*e.g.*, bisphosphonates, etc.). Since only male and middle-aged donors were included in this study, the confounding effects of gender and aging could be ignored. A cylindrical compressive specimen (3.0mm in diameter and 5.0mm long) was prepared from the anterior quadrant of mid-diaphysis of each femur. The loading axis to the specimens was parallel to the long axis of the femurs.

Synchrotron X-ray scattering

The diffraction of X-ray from ordered layers of atoms/molecules having a characteristic (lattice) spacing of d can be described using the modified Bragg's equation:

$$E_b = \frac{k}{2d \sin(2\theta/2)} \quad (1)$$

where E_B is the X-ray energy in keV, 2θ is the angle between the incident and diffracted beams and k is a constant ($=12.39$). For bone, the average band spacing of collagen fibrils ($d \sim 67\text{nm}$) can be measured using the small-angle X-ray scattering regime (SAXS), whereas the interplanar spacing of mineral crystallites ($d \sim 0.1\text{--}0.3\text{nm}$) can be measured by the wide-angle X-ray scattering regime (WAXS). The diffracted X-ray beam forms a so-called Debye cone projecting circular rings on an area detector. The corresponding radius (r) of the rings is related to the characteristic spacing by the Bragg's law. The cone angle (denoted as the azimuthal angle, η) defines the crystal orientation with respect to the loading direction (Figure 1).

WAXS pattern of bone is a full spectrum of reflection rings in all orientations (Figure 2A) and can be transformed to a 2-D distribution in terms of η and r (Figure 2B). When load is applied to bone, the lattice of mineral crystalline unit cells contracts along the loading direction and expands in the transverse direction due to Poisson effects. This causes a corresponding distortion of the circular (unstrained) Debye ring into an elliptical shape. By measuring the radius (r_η) vs. azimuth (η) profiles at different applied stress levels, one can determine the 'invariant' orientation value η^* and corresponding radius r^* , at which all profiles converge (Figure 2C). The internal strain (ϵ_η) of the mineral crystals is measured from the difference between the radii (r_η) and r^* , whose details are described elsewhere in the literature (Almer & Stock, 2005):

$$\epsilon_\eta = \frac{r_\eta - r^*}{r^*} \quad (2)$$

SAXS measurements of the collagen strain were implemented following the similar principles described above. The characteristic spacing of collagen fibrils is $d \sim 67\text{nm}$, which leads to periodical diffraction peaks along the fibril axis with spacing $d_n = d/n$, where n is the order of the spacing (Figure 2D). The collagen strain at the macroscopic strain (ϵ_i) is calculated using the formula derived elsewhere (Almer & Stock, 2005, Noyan & Cohen, 1987).

$$\epsilon_n(\epsilon_i) = \frac{d_n(\epsilon_i) - d_n(0)}{d_n(0)} \quad (3)$$

Mechanical loading protocol

A progressive loading protocol (Nyman, et al., 2007, Wang & Nyman, 2007) was used to determine the evolution of bulk bone properties simultaneously with *in situ* strain measurements using synchrotron X-ray scattering techniques. This loading scheme subjects bone specimens to a series of load-dwell-unload-dwell cycles with a progressive increment of strain until failure (Figure 3). In each loading cycle, the specimen was first loaded in displacement control at a constant loading rate (0.6mm/min) to a prescribed displacement level (from **a** to **b**), held at the displacement level for 150 seconds (from **b** to **c**: stress relaxation), unloaded in load control at constant rate to zero force (from **c** to **d**), held for 150 seconds at zero force (from **d** to **e**: viscoelastic creep), and then reloaded in displacement control at the same rate to the next designated strain level (from **e** to **f**). The synchrotron X-ray scattering measurements were conducted between **b** and **c** such that the *in situ* behavior of the mineral and collagen phases could be determined at the strain level.

The following properties were quantified at each loading cycle (Figure 3): 1) The applied strain at each cycle (ϵ_i) was determined as the strain measured at the beginning of the stress relaxation dwelling of the cycle (*i.e.*, point **b**); 2) the instantaneous modulus (E_i) was

estimated as the slope between the following two points; one at the end of stress relaxation dwelling (*i.e.*, point *c*) and the other at the end of anelastic deformation dwelling (*i.e.*, point *e*); 3) the applied stress (σ_i) was measured at the corresponding applied strain (ϵ_i); 4) the permanent strain (ϵ_{ip}) as the residual strain value at the end of anelastic creep dwelling (*i.e.*, point *e*); and finally 5) the viscoelastic time constant (τ_i) was estimated by fitting the stress-time curve during the relaxation dwelling with an exponential equation ($\sigma_{i0}e^{-t/\tau_i}$) as described elsewhere (Leng, et al., 2009).

Experimental procedure

A servo-hydraulic materials testing system (MTS Model 858) was aligned with the 1-ID beam line of the Advanced Photon Source, Argonne National Laboratory (Figure 1). During loading, the change (δ) in the gage length ($L = 5.0\text{mm}$) was recorded using an extensometer attached to the loading platens to measure the macroscopic strain of the specimens (*i.e.*, $\epsilon = \delta/L$). Hydration was maintained throughout the test by dripping normal saline to the specimens. A monochromatic high-energy ($E \sim 80\text{keV}$) X-ray beam with a size of approximately $0.1\text{mm} \times 0.1\text{mm}$ was incident on to the center of specimen perpendicular to the loading axis. A large area detector (GE rad detector) was placed at a sample-detector distance of $z_{s-d} \sim 1.0\text{m}$ to record the WAXS pattern, while a smaller area detector (Bruker 6500 CCD) was placed further downstream ($z_{s-d} \sim 5\text{m}$) to record the SAXS pattern. WAXS and SAXS data (Figure 3A and 3D) were recorded sequentially at the beginning of the stress relaxation dwelling period of each cycle. The total recording time was about one second for WAXS and ~ 10 seconds for SAXS measurements. Due to the time constraint to switching between SAXS and WAXS sensors, only WAXS data were recorded at the end of the dwelling period to estimate the *in situ* viscoelastic response of bone. For simplicity, in this study we only measured the internal strains in the loading direction, which was deemed crucial for understanding the local deformation in bone.

Statistical analysis

Student *t*-test (unpaired) was performed to detect the difference in the internal strain between the collagen and/or mineral phases at different microscopic strains. Significant differences were considered only when $p \leq 0.05$.

RESULTS

The internal strains of the longitudinal oriented mineral and collagen phases increased almost linearly with the macroscopic strain (Figure 4A), peaked at $\sim 0.7\%$ strain for collagen and ~ 0.33 for mineral before yielding ($\sim 1.2\%$ microscopic strain). However, when the internal strains were normalized with the macroscopic strain (Figure 4B), a toe region (disproportional change) was observed at the initial load before the proportional changes were reached. In fact, the toe region was most likely an artifact due to the initial engagement between the loading platens and the specimen. After yielding, the internal strains sharply decreased initially and then saturated gradually to $\sim 0.3\%$ for the collagen and $\sim 0.15\%$ for the mineral phase (Figure 4A). It was also observed that both internal strains were less than the macroscopic strain, suggesting that the *in situ* deformation of longitudinal mineral crystals and collagen fibrils only partially accounted for the macroscopic deformation of bone.

Similarly, the applied stress also increased linearly with the macroscopic strain prior to yielding, decreased after yielding and then gradually saturated afterward (Figure 5A). The plot of the internal strains vs. the applied stress showed that the internal strains of both longitudinal mineral and collagen phases were positively related to the applied stress during both pre and post-yield deformation of bone (Figure 5B). However, the internal strains were

markedly reduced after yielding even though bone was loaded at the same stress levels, suggesting that the longitudinally oriented mineral and collagen phases were partially disengaged in load bearing after yielding.

The absolute difference between the longitudinal collagen strain (ϵ_c) and the mineral strain (ϵ_m) changed with the macroscopic strain before and after yielding (Figure 6A). However, such changes disappeared when the collagen to mineral strain ratio (ϵ_c/ϵ_m) was calculated, suggesting that the relative deformation between the two phases was independent of the varying bulk behavior of bone (Figure 6B).

Moreover, the internal strains of the longitudinal oriented mineral and collagen phases exhibited a bimodal change with the bulk elastic modulus of bone (Figure 7). Prior to yielding a negative relationship was observed, suggesting that the internal strains increased with the modulus loss. After yielding, however, a positive relationship was discerned, showing that the internal strains decreased with the modulus loss.

After yielding, the internal strains decreased acutely first and then gradually to a relatively consistent value (Figure 8), suggesting that the longitudinal mineral and collagen strains did not change linearly with the plastic deformation of bone.

Finally, stress-relaxation induced reductions in the longitudinal mineral strain were observed at and after yielding of bone (Figures 9A). This change coincided with the viscous response of bulk bone specimens, showing that the relaxation time-constant (or viscosity of bone) reached to saturation after yielding (Figures 9B).

DISCUSSION

Several important observations were obtained regarding the relationship between the bulk bone behavior and the accompanied local deformation of the mineral crystals and collagen fibrils aligned in the loading direction under compression.

First, the local, internal strains of the longitudinally oriented mineral crystals and collagen fibrils after yielding explain little of the overall macroscopic post-yield deformation suggesting that local strain of off-axis oriented crystals and fibrils dictate failure behavior of bone. The internal strains of the longitudinally oriented mineral crystals and collagen fibrils vary considerably prior and after yielding of bone under compression (Figure 4), but the relative deformation between the two phases exhibit little correlation with the bulk behavior of bone (Figure 5). After yielding, the internal strains decrease almost linearly with decreasing stress and increasing modulus loss (Figures 5 and 7), but did not change to the same extent as the change in plastic and viscous deformations (Figures 8 and 9). Considering the observation that the collagen to mineral strain ratio is relatively consistent prior and after yielding of bone (Figure 6B), it is presumable that there exists no relative deformation between the longitudinally oriented mineral and collagen phases throughout the bulk deformation of bone. Since such relative deformations would be changed due to microdamage formation and/or permanent deformation, the above results actually suggest that the deformation of the mineral and collagen phases oriented in the loading direction under compression may not dictate the post-yield behavior of bone.

In fact, previous studies have shown that shear deformation (cross hatched microcracks) is the major mechanism of the post-yield deformation of human cortical bone (Ebacher, et al., 2007, Leng, et al., 2009). Moreover, it has been reported that large portions of mineral crystals and collagen fibrils are also oriented in different directions other than the longitudinal axis of bone (Ascenzi, et al., 2003, Ascenzi & Lomovtsev, 2006, Fratzl, et al., 1992, Green, et al., 1987, Hasegawa, et al., 1994, Hofmann, et al., 2006, Martin & Ishida,

1989, Riggs, et al., 1993). For instance, it has been found that even in individual lamellae the mineral and collagen phases are grouped in small patches that are randomly oriented in all directions (Ascenzi and Lomovtsev, 2006). In fact, the local shear deformation in oblique orientations may actually dominate the microdamage accumulation and post-yield behavior of bone in compression (Ebacher, et al., 2007). Thus, it is presumable that the local bone tissue consisting of the longitudinally oriented mineral and collagen phases are unlikely damaged under compression, but deform elastically in conformation with the deformation of surrounding tissues even after yielding of bone.

Next, the strain ratio between the macroscopic strain and the internal strains of both mineral and collagen phases obtained from this study (*i.e.*, 12:7:3.3) are a little different compared with that (12:5:2) reported by a previous study (Gupta, et al., 2006). Such differences are most likely due to the distinct experimental conditions. For instance, bovine bone was tested in tension in Gupta's study, whereas this study tested human bone in compression.

The large disparity between the internal and macroscopic strains is most likely due to the limited deformation of the longitudinally oriented mineral and collagen phases compared with those oriented in oblique directions to the loading axis, which actually dominate the bulk deformation of bone.

The mismatch between the mineral and collagen strains verifies that bone is a composite material, consisting of hard mineral crystals and more compliant collagen fibrils. In fact, several composite models have been proposed to study the contribution of bone constituents to the bulk mechanical behavior (Ascenzi, et al., 2003, Fritsch & Hellmich, 2007, Gupta, et al., 2006, Raspanti, et al., 1996, Weiner, et al., 1999). The collagen/mineral strain ratio actually provides important reference data for establishing a legitimate representation of bone ultrastructure.

Finally, it is intriguing to note that the time-dependent changes in the internal strain of longitudinal mineral crystals occur only at and after yielding of bone (Figure 9A). In fact, this behavior coincides with the bulk viscous behavior of bone, in which the viscous response (*i.e.*, time constant) of bone reaches a full manifestation after a transition period prior to yielding (Figure 9B). The results imply two possibilities: either the time-dependent changes are merely a reflection of the viscous deformation of surrounding tissues that are damaged, or the tissue containing the longitudinal mineral crystals itself behaves viscoelastically. Based on the earlier discussion, we believe that the former scenario is more likely because the tissue with the longitudinally oriented mineral and collagen phases remains intact, thus unlikely exhibiting large viscous response itself.

This study has several limitations. First, all tests were on human cortical bone in compression. Thus, the results may not be representative of other species and loading modes. Next, in this study we only discussed the internal strains of the mineral crystals and collagen fibrils that are oriented in the loading (longitudinal) direction. Since a large portion of mineral and collagen phases in bone are oriented in the other directions, this information may not fully reflect the local deformation of bone. To estimate the internal strains of mineral and collagen phases as the function of orientation, methodologies need to be developed for a full spectrum of analyses. This important issue will be addressed in our future study. Next, the WAXS and SAXS measurements were implemented sequentially during the loading process, respectively. Thus, the internal strain measurements of mineral and collagen phases were not synchronized but with a time delay for switching the sensors. Nonetheless, the possible error could be ignored for relative comparisons of experimental data since the measurements were conducted in the exact sequence for all specimens during stress relaxation dwells. Finally, due to the dwelling time constraint, only the mineral strain

was measured at both beginning and end of each stress relaxation dwell to estimate the time-dependent change of local tissue deformation in this study.

CONCLUSION

The results of this study indicate that the longitudinally oriented collagen fibrils and mineral crystals may not exhibit apparent damage which explains the overall post-yield permanent deformation. Since bone is a composite with the mineral and collagen fibrils being oriented in all directions, the microdamage accumulation and post-yield deformation of bone in compression is most likely realized in the region where the mineral and collagen phases are oriented oblique to the loading axis.

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Figure 1. MTS 858 mechanical testing system used for in-situ X-ray measurement of internal strains in bone under load and the schematic representation of Bragg's law of X-ray diffraction in transmission X-ray diffraction geometry.

Figure 2.

Example of WAXS/SAXS data and analysis: (a) Typical WAXS pattern, with definitions of radius r and azimuthal orientation η given, where $\eta = 90^\circ/270^\circ$ is the applied loading direction. (b) Pattern from (a) transformed to Cartesian $r - \eta$ coordinates, with selected hydroxyapatite reflections shown. (c) Radial peak position for HA (002) reflection as a function of η for different applied compressive loads, increasing in magnitude monotonically from A–E, with definitions of η^* , r^* given. (d) Typical SAXS pattern, with 1st and 3rd order collagen peaks indicated with arrows, along with characteristic elliptical halo E .

Figure 3.

Typical stress-strain curve of the progressive loading scheme: For the i th diagnostic cycle, (a–b) loading to a prescribed strain level; (b–c) stress relaxation dwell (150s); (c–d) unloading to zero force; (d–e) strain relaxation dwell (150s); and (e–f) reloading for the next diagnostic cycle.

Figure 4.

(A) Average internal strains of mineral and collagen phases measured at each loading cycle as the function of the applied macroscopic strain; (B) Ratio of the internal strains (both mineral and collagen phases) with respect to the macroscopic strain in the pre-yield deformation of bone, showing that a toe region existed at small deformation before reaching a linear relationship (a constant ratio) between the internal and macroscopic strains. (* denotes the statistically significant difference between the mineral and collagen strains; N=5)

Figure 5.

(A) Bulk stress measured at each loading cycle as the function of the applied macroscopic strain. (B) Bulk stress vs. the internal strains of the mineral and collagen phases in bone.

Figure 6.

Absolute difference (A) and ratio (B) between the mineral and collagen strains, showing that the relative difference (strain ratio) between two phases is relatively consistent although their absolute difference varies with the macroscopic strain, thus implying that the bone tissue comprising the mineral crystals and collagen fibrils oriented in the loading direction may still be intact in both pre and post-yielding deformations.

Figure 7.

Correlation of the elastic modulus loss with the internal strains: (A) collagen fibrils and (B) mineral crystals, showing the distinct trends prior and after yielding of the tissue. It is noted that the modulus values at small strain levels are not available due to the difficulty in acquiring the data.

Figure 8.

Correlation of the plastic strain of bulk tissue with the internal strains: (A) collagen fibrils and (B) mineral crystals, showing an exponential decay of internal strains with respect to the plastic strain sustained by the tissue.

Figure 9.

Time-dependent changes of the internal strain of mineral crystals at the beginning and end of the stress relaxation dwelling period in each diagnostic cycle (A) after yielding, which coincided with the bulk viscous response (time constant) of bone. (* denotes the statistically significant difference in the mineral strains between the early and late relaxation dwelling period; N=5)