Polarized Raman Spectroscopy: Application to Bone Biomechanics

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ABSTRACT

Raman spectroscopic studies have shown that the properties of the organic matrix and the orientation of the mineral and matrix components of bone have a large influence on its properties. We employ polarized Raman microspectroscopy to monitor the changes in the orientation of mineral crystallites during tensile loading of bovine femora in the elastic regime. We load tissue in a custom-built dynamic mechanical tester that fits on the stage of a Raman microprobe and can accept hydrated tissue specimens. Parallel and perpendicular polarization components of the Raman spectra along the long axis of the diaphysis are obtained. We propose that the orientation and structure of mineral crystallites change on deformation of bone tissue by tensile loading.

Keywords: Raman, bone, biomechanics, polarization

1. INTRODUCTION

Raman microspectroscopy has been established as a powerful tool for monitoring molecular deformation in a range of natural and synthetic crystalline materials and polymers.[1] Polarized Raman spectroscopy has been an often used tool to study orientation in crystalline solids[2] and in both natural and synthetic fibers at the ultrastructural level.[3] In crystals, when molecular orientation is known relative to the polarization of the laser beam, the depolarization ratio is strongly influenced by molecular alignment; therefore, it can provide additional structural information. In the mineralized tissue literature, the first use of polarized Raman spectroscopy was to study dental enamel crystallite orientation.[4] It has also been employed to examine healthy[5] and carious[6] enamel.

The Raman spectrum is sensitive to changes in mineral parameters such as crystal structure, crystallite size and deviations from stoichiometry[7, 8] and to changes in protein conformation.[9] Because these ultrastructural parameters change with age and mechanical loading, Raman spectroscopy is a powerful tool for assessing the effects of these parameters on the bone tissue. Biomechanical studies on the bone have focused on relating the biology and the mechanics of the tissue. Because of the hierarchical nature of bone, the biomechanical testing of bone at different levels of scale (1µm, 1mm, 1cm, etc.) can be used to isolate changes at each level which affect the bone quality. It is well-known that the organization of the bone ultrastructure i.e., the collagen structure and cross-linking, the mineral type and crystal alignment and collagen-mineral interfaces, determine the mechanical behavior of bone.[10] However, the correlation between the chemical structure and the mechanical properties of bone has been more difficult to evaluate.

We have previously shown spectroscopic changes during compressive and tensile loading of bone. These studies demonstrated that mechanical damage caused spectral shifts and that different changes in the organic and inorganic region occur in response to mechanical loading and deformation.[11-13] Changes in the mineral component of the bone in the elastic regime are attributed to changes in mineral anion-cation species on loading.

Recently, polarized Raman spectroscopic imaging has been used by Kazanci et al. to study the orientation and composition of cortical bone tissue.[14, 15] The study demonstrated that the orientation of both mineral and collagen can be elucidated by examining the polarization components of phosphate v_1 (P-O symmetric stretch) and amide I (carbonyl stretch). These workers pointed out that because the crystallites are oriented with their c-axes along the length of collagen fibrils phosphate v_1 should be strongly polarized along this axis. Similarly, because the carbonyl groups are oriented perpendicular to the collagen chain, amide I is strongly polarized in the direction perpendicular to the collage fibril orientation. In the present study, we use polarized Raman microspectroscopy to evaluate changes in the orientation of mineral crystallites in bone tissue under tensile loading in the elastic regime.

2. METHODOLOGY

2.1 Specimen Preparation

Three bovine bone specimens were milled from a bovine femur obtained from a local abattoir and stored at -80˚C until use. Each specimen was machined to about 3 cm in length and 2mm x 2mm in cross sectional area, while being irrigated with Calcium-buffered saline. To prepare the specimens for mechanical testing, the ends were embedded in a cold-setting acrylic and the exposed section was kept moist with gauze soaked in phosphate-buffered saline. A custom-made fixture was used to align the specimen. The thawed specimens were loaded onto a custom designed mechanical testing apparatus.[13] Under computer control, a DC motor was used to extend the specimen, while a load cell measured the applied load. The specimen was photo-bleached for 15 minutes at 200 mW prior to the experiments. The specimen was kept moist throughout the experiment by dripping phosphate buffered saline onto it.

2.2 Polarized Raman Spectroscopy

The Raman microspectroscopy system employed in the present study consisted of a research grade microscope (E600, Nikon USA), a 2 Watt 532 nm laser (Millennia II, Spectra Physics, Mountain View, CA), and an f/1.8 axial transmissive spectrograph (HoloSpec, Kaiser Optical Systems, Inc., Ann Arbor, MI).[13] The spectrograph was fitted with a 512 x 512 pixel back-illuminated EMCCD (iXon, Andor Technology, Belfast, Northern Ireland). The mechanical tester containing the specimen was placed on the micrometer stage with the laser line focused parallel to the long axis of the diaphysis.[13]

All Raman spectra were acquired through a 100X, 0.90 NA plan apochromatic objective (Nikon USA) at an exposure time of 30s. The polarization direction of the beam was selected using a half-wave plate. The collected Raman scatter was passed through an analyzing polarizer and directed onto the slit of the spectrograph. A wedge depolarizer after the analyzer eliminated intensity artifacts caused by the polarization dependence of the grating transmission efficiency.

In all experiments the polarization of the incident laser beam was maintained parallel to the long axis of the diaphysis, i.e. along the proximal-distal (pd) direction. The analyzer was adjusted to pass either the proximal-distal (pd), i.e. parallel, or the medial-lateral (ml), i.e. perpendicular component of the Raman scatter. The intensities (I) of the two possible polarization components of the Raman scatter are described by their excitation and detection polarizations along the conventional anatomical directions: $I_{pd,pd}$ and $I_{pd,ml}$ respectively. The polarization directions are shown in figure 1.
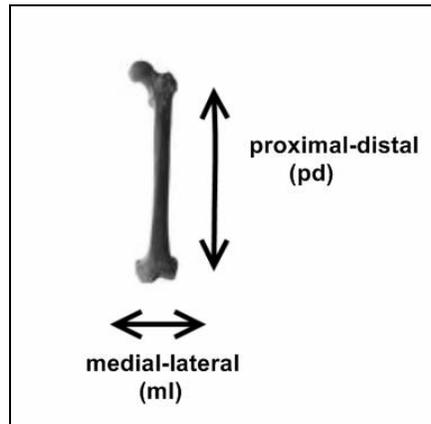

Fig 1. Polarization directions with respect to the long axis of the diaphysis of the bone

2.3 Mechanical Testing

The three bovine bone samples were loaded in tension in a series of discrete steps. The specimens were first loaded to 12.5 MPa, unloaded back to 0 MPa, then loaded to 19.75 MPa and unloaded to 0 MPa. Polarized Raman spectra, as

explained earlier, were taken from three different locations on the bone specimen at each loading point. One of the specimens broke during the second load cycle.

2.4 Data Analysis

The wavenumber scale of the spectrograph was calibrated against the emission lines of an argon lamp discharge. Intensities were corrected for polarization dependence of the optics by calibration against cyclohexane.[16] The spectra were corrected for spectrograph image curvature as previously described.[17] Dark current subtraction and white light correction (flat-fielding) were performed using locally written scripts on MATLAB (Math Works, Inc.). Spectra were analyzed using GRAMS/AI 7.01 (Thermo Galactic, Waltham, MA). Peak fitting was performed using GRAMS/AI 7.01 (ThermoGalactic, Madison, WI) and the intensities of the characteristic mineral band, phosphate ν_1 (959 cm^{-1}), were measured. Depolarization ratios, i.e., the ratio of the intensity of the perpendicularly polarized component to that of the parallel polarized component, were calculated. Two-factor Anova without replication was used to calculate significance in the shift in peak position and width on tensile loading. Paired t-test was performed on the depolarization ratio data from the first loading cycle.
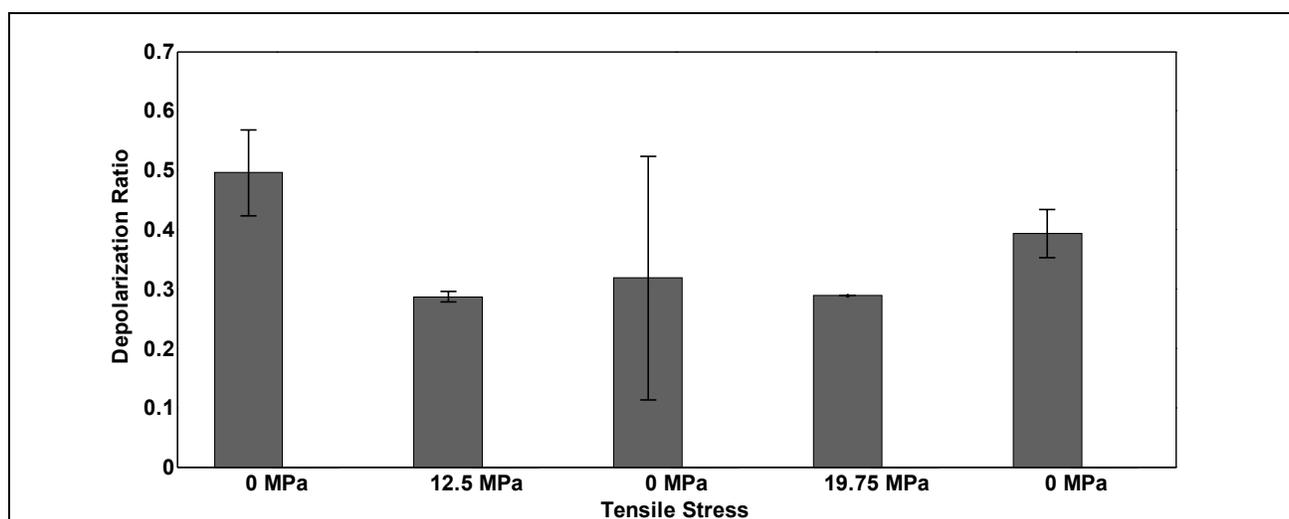
3. RESULTS

Fig. 2. Depolarization ratio of the phosphate ν_1 band along the proximal-distal direction as a function of the applied tensile stress (N=3 at first three loads, N=1 at 19.75 MPa and N=2 at final load)

Figure 2 compares the depolarization ratios calculated for the phosphate ν_1 band (958 cm^{-1}) along the proximal-distal direction at the various tensile load points. Our preliminary data indicates that as the bone specimen is loaded in tension, the depolarization ratio ($I_{pd,ml}/I_{pd,pd}$) decreases to about 0.6 times the original ratio in the first loading case (p=0.02). This significant decrease in depolarization ratio suggests that the mineral crystals have become more well-ordered when loaded in tension. As the bone specimen is unloaded, the increase in depolarization ratio is insignificant (p=0.4), indicating that the mineral crystals do not retain their original orientations.

Figure 3 compares the phosphate band peak positions of the polarized Raman scatter at the different load points. Changes in peak position are observed on tensile loading of the bone tissue (p=0.12). The small sample size and low signal-to-noise ratio make it difficult to ascertain if the bone mineral crystallites undergo a very small change in response to stress. The phosphate peak positions for the two polarization components of the Raman scatter are not significantly different (p=0.8).
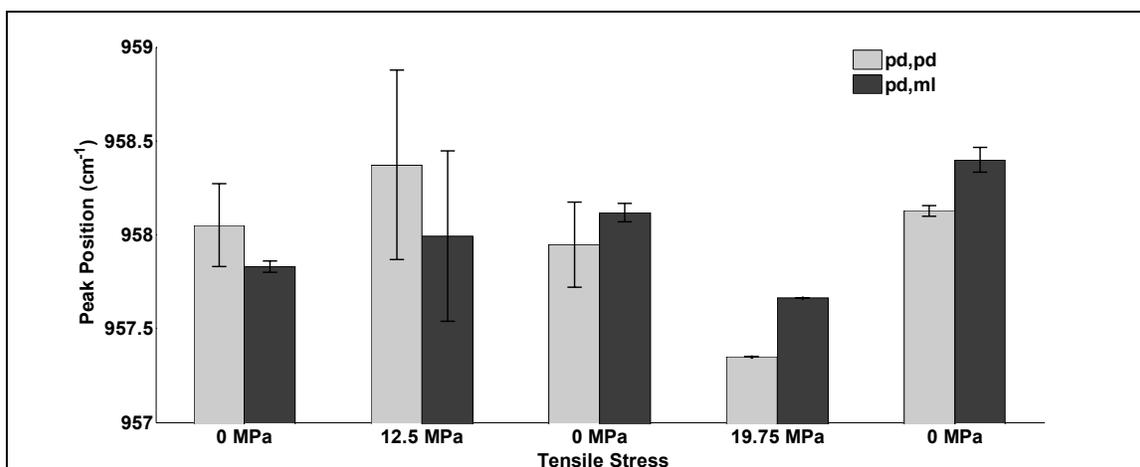
Fig. 3. Peak position of the phosphate ν_1 band as a function of applied stress. The measurements from the parallel (pd,pd) and perpendicular (pd,ml) components of the Raman scatter are reported. (N=3 at first three loads, N=1 at 19.75 MPa and N=2 at final load)

In the first loading cycle, the peak position of the pd,pd component increases by 0.3 cm^{-1} and that for the pd,ml component increases by about 0.16 cm^{-1} as the stress is increased from 0 MPa to 12.5 MPa. On unloading, the peak position for the pd,pd component decreases by 0.4 cm^{-1} and that for the pd,ml component increases by 0.12 cm^{-1}. In the second loading cycle, the peak position reduces by 0.6 cm^{-1} for the pd,pd component and by about 0.4 cm^{-1} for the pd,ml component when the bone tissue is loaded to 19.75 MPa. During the final unloading, the peak positions for both the pd,pd and pd,ml components increase by about 0.7 cm^{-1}.
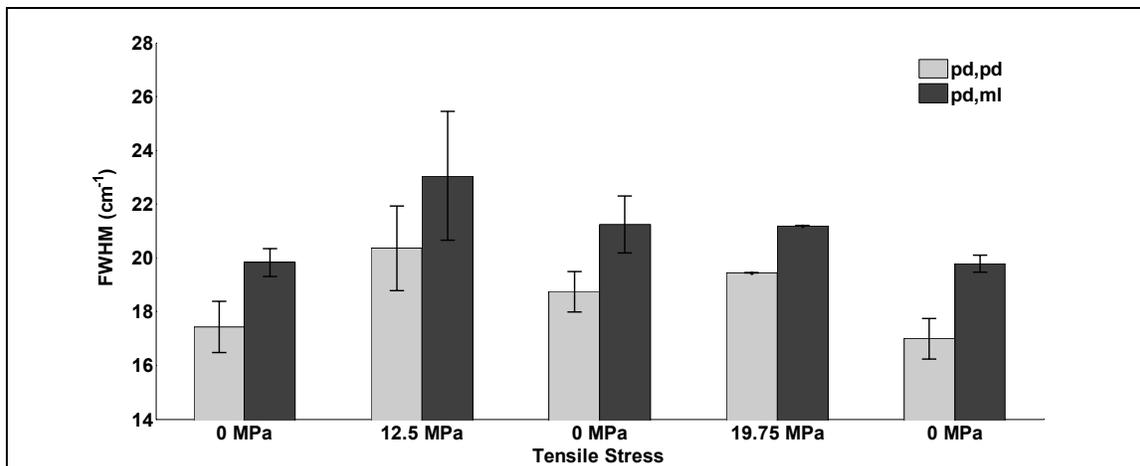

Fig. 4. Full width at half maximum (FWHM) of the phosphate ν_1 band as a function of applied stress. The measurements from the parallel (pd,pd) and perpendicular (pd,ml) components of the Raman scatter along the proximal-distal direction are reported. (N=3 at first three loads, N=1 at 19.75 MPa and N=2 at final load)

Figure 4 compares the full width at half maximum (FWHM) for the phosphate band at different tensile stresses. The two polarization components show significantly different FWHM's ($p<0.01$) and undergo significantly different shifts in width at different applied loads ($p<0.01$). The width of the phosphate band increases by about 2.9 cm^{-1} and 3.2 cm^{-1} for the pd,pd and pd,ml components of the phosphate band as the bone is loaded from 0MPa to 12.5 MPa. On unloading to 0 MPa, the width decreases by 1.6 cm^{-1} and 1.8 cm^{-1} for pd,pd and pd,ml components respectively. When loaded up to 19.75 MPa, the width increases by 0.7 cm^{-1} for the pd,pd component and decreases by 0.05 cm^{-1} for the pd,ml

component. Unloading to 0 MPa decreases the width by 0.24 cm^{-1} and 1.4 cm^{-1} for the pd,pd and pd,ml components respectively.

4. DISCUSSION

Kazanci et al.[14] showed that the phosphate bands are strongly polarized parallel to the direction of the long axis of normal human cortical bone tissue. For murine cortical femora, the depolarization ratio of the phosphate ν_1 band at zero load is $\simeq 0.1$, indicating highly oriented mineral crystallites [data not shown]. In this study, a depolarization ratio of about 0.5 is measured for bovine bone tissue at the starting zero load point. This indicates that the mineral crystallites in bovine bone are less well-ordered than the crystallites in murine cortical bone. This suggests that we were probing plexiform tissue.

Tensile loading of the bovine bone samples causes the phosphate bands to be more strongly polarized, which is reflected in a decrease in the depolarization ratio in the first loading step. This suggests that the mineral crystallites are becoming more ordered on extension of the bone tissue. It is well-known that the bone collagen axis is preferentially aligned parallel to the diaphysis of a long bone. Mineral crystallites in bone tissue are mainly in the gap regions within the collagen fibril and also decorate the outer surface. The crystallographic c-axes of the crystals align along the long axis of the fibrils.[18, 19] Other studies have reported that the mineral particle orientation and the degree of alignment trace the orientation of the mineralized collagen fibrils within the bone material.[20-22] We suggest that the increase in orientation of the mineral crystallites at higher loads is due to collagen chains becoming extended, more aligned and tighter on application of tensile loads. The mineral crystallite orientation is increased because crystallites remain bonded to a matrix that is more-ordered with tensile loading. Comparison of the depolarization ratio of the phosphate band before and after loading suggests that the mineral crystallite ordering is not completely reversible in the elastic loading regime.

That the mechanical properties of bone tissue are dependent on the properties of the mineral crystallites and the matrix proteins as well as the interactions between them is well-known.[23] Our present data confirms that bone mineral crystallites undergo change in response to stress. The broadening of the phosphate band at higher stresses is similar to that seen at increasing pressure.[24] The change in width can be attributed to both the strain on the crystallite and the change in its ordering on tensile loading. It is highly unlikely that these shifts in width reflect a change in the mineral crystallinity because we see that these changes are partly reversible on unloading. It should be noted that the bone mineral crystallites are highly substituted, small and therefore, may not behave in a manner similar to a perfect, infinite crystal lattice.

Different widths of the phosphate band are observed in the proximal-distal and medial-lateral directions of the Raman scatter. These different Raman shifts in the parallel and perpendicular components are a consequence of the generation of biaxial strains in the specimen on uniaxial loading. There is greater load-induced broadening perpendicular to the collagen axis. It is known that increased pressure decreases anion-cation spacing in the a,b crystallographic plane in flourapatite.[7,8] We propose that the same behavior occurs in bone mineral, resulting in band width dispersion in the polarization direction perpendicular to the collagen axis. We see very small changes in peak position of up to 0.8 cm^{-1} on tensile loading and unloading of the bovine bone tissue. Further experiments on a larger number of samples are necessary to comment on the effect of mechanical loading on the phosphate peak position.

Peak width appears to be a more sensitive metric for the change in mineral crystallites on tensile loading of bone tissue. The observed changes in the phosphate band width should be a consequence of a combination of the change in mineral crystallite structure and the change in their ordering due to the applied stress.

5. CONCLUSIONS

Milled bovine bone samples were elastically loaded in tension and the spectral changes in the phosphate band component were followed using polarized Raman spectroscopy. Significant changes in broadening of the phosphate bands were observed at increased tensile loads and these were partially reversible on unloading of the bone tissue. Further, a decrease in the depolarization ratio of the phosphate band was observed on tensile loading, indicating that the mineral crystallites were becoming more ordered.

Thus, polarized Raman spectroscopy has been shown to be a powerful technique to study deformation of structure and orientation simultaneously in bone tissue. Our results suggest that mechanical loading of bone not only distorts the

mineral crystallite structure but also its orientation. These changes are only partially reversible in the elastic loading regime.

6. ACKNOWLEDGEMENT

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