Published in final edited form as:

Calcif Tissue Int. 2011 August ; 89(2): 111–122. doi:10.1007/s00223-011-9497-x.

Measuring Differences in Compositional Properties of Bone Tissue by Confocal Raman Spectroscopy

Jeffry S. Nyman^{1,2,3,4}, Alexander J. Makowski^{1,4}, Chetan A. Patil⁴, T. Philip Masui⁵, Elizabeth C. O'Quinn², Xiaohong Bi⁴, Scott A. Guelcher⁵, Daniel P. Nicollela⁶, and Anita Mahadevan-Jansen⁴

Jeffry S. Nyman: jeffry.s.nyman@vanderbilt.edu

¹Department of Veterans Affairs Tennessee Valley Healthcare System Nashville, TN

²Department of Orthopaedics and Rehabilitation Vanderbilt University Medical Center Nashville, TN

³Center for Bone Biology Vanderbilt University Medical Center Nashville, TN

⁴Department of Biomedical Engineering Vanderbilt University Nashville, TN

⁵Department of Chemical and Biomolecular Engineering Vanderbilt University Nashville, TN

⁶Materials Engineering Department Southwest Research Institute San Antonio, TX

Abstract

The full range of fracture risk determinants arise from each hierarchical level comprising the organization of bone. Raman spectroscopy is one tool capable of characterizing the collagen and mineral phases at a near sub-micron length scale, but the ability of Raman spectra to distinguish compositional differences of bone is not well defined. Therefore, we analyzed multiple Raman peak intensities and peak ratios to characterize their ability to distinguish between the typically less mineralized osteonal tissue and the more mineralized interstitial tissue in intra-cortical human bone. To further assess origins of variance, we collected Raman spectra from embedded specimens and for 2 orientations of cut. Per specimen, Raman peak intensities or ratios were averaged among multiple sites within 5 osteons and 5 neighboring interstitial tissue. The peak ratios of ν_1 phosphate (PO_4) to Proline or Amide III detected the highest increases of 15.4% or 12.5%, respectively, in composition from osteonal to interstitial tissue. The coefficient of variance (COV) was less than 5% for each as opposed to a COV of $\sim 8\%$ for the traditional $\nu_1\text{PO}_4/\text{Amide I}$, a peak ratio that varied the most between transverse and longitudinal cuts for each tissue type. Although embedding affected Raman peaks, it did not obscure differences in most peak ratios related to mineralization between the 2 tissue types. In studies with limited sample size but sufficient number of Raman spectra per specimen for spatial averaging, $\nu_1\text{PO}_4/\text{Amide III}$ or $\nu_1\text{PO}_4/\text{Proline}$ is the Raman property that is most likely to detect a compositional difference between experimental groups.

Correspondence: Jeffry S. Nyman Vanderbilt Orthopaedic Institute Medical Center East, South Tower, Suite 4200 Nashville, TN 37232.

Disclosures: None

Keywords

Raman; Embedding; Cortical Bone; Collagen; Mineral

Introduction

Since there is a disproportionate increase in fracture risk with respect to the age-related decrease in bone mass [1], bone fragility or osteoporosis is viewed as a problem of inferior bone quality in addition to a problem of low bone mass. In other words, there are other determinants of fracture resistance, besides bone mineral density [2, 3]. These determinants can exist at each level within the bone hierarchy [4]. As an example at the level of the collagen fibril, the strength and toughness of bone decreases when copper deficiency [5, 6] or an inhibitor of lysyl oxidase [7] disrupts enzymatic crosslinking. The complex organization of bone necessitates the use of different characterization tools to span multiple length scales (ultra, nano, micro, meso, and macro) in order to understand the causes of bone fragility.

As an optical technique, Raman spectroscopy generates spectra in which the position of the peaks reflects the change in energy of the scattered laser light as it interacts with specific molecular sub-units within the sample, and the scattering intensity of the peaks is proportional to the prevalence of the sub-unit. Thus, the measured spectra provide a molecularly specific biochemical fingerprint of the material that can be analyzed to provide quantitative information of the sample's composition. Confocal Raman spectroscopy systems are capable of probing the physiochemical properties of bone tissue at a spatial resolution of <5 microns, thereby providing compositional characterization of microscopic features such as primary or secondary lamellae [8] and tissue immediately surrounding the lacuna [9]. In bone tissue, chemical groups within the mineral and collagen phases generate distinct Raman peaks (Figure 1), which can be analyzed to quantify compositional differences. Subsequently, ratios of relative peak intensity can then be calculated to quantify higher order characteristics such as mineralization [10].

The use of Raman spectroscopy in studies of bone quality has several advantages including 1) spectral acquisition in a reflective mode allowing for non-destructive processing [8, 11], 2) sensitivity to damage accumulation [12], 3) microscopic resolution ‘mapping’ of compositional properties via imaging systems [10], and 4) the potential for *in vivo* assessment of tissue characteristics [13]. Not surprisingly then, there are now numerous studies applying Raman to investigate bone tissue with the majority using the ratio of the ν_1 phosphate peak ($\nu_1\text{PO}_4$) to the Amide I peak to identify differences in mineralization (Table 1). However, certain processing and embedding methods have been found to affect ratios of Raman peaks [14], while other studies have suggested that the intensity of the Amide I band is affected by the orientation of the collagen fibrils [15, 16]. Since collagen orientation is heterogeneous in bone tissue [17], there is potential for large variance in the peak ratio $\nu_1\text{PO}_4/\text{Amide I}$ when using standard Raman systems without depolarizers, and thus other peak ratios may be more suitable for detecting differences in tissue mineralization, especially since sample size is often limited. For example, the use of proline/hydroxyproline

bands have been offered as viable indicators of collagen content in bone [18]. To the best of our knowledge, there has not been a systematic study to identify which of Raman-derived peak ratios detect the greatest compositional difference in bone tissue with the least variance irrespective of sample processing, embedding, and orientation of cut.

Therefore, we analyzed the ability of multiple peak intensities and multiple peak ratios to differentiate between interstitial tissue and secondary osteonal tissue, based on the fact that interstitial tissue is known to be, on average, more mineralized [19, 20] with higher modulus than osteonal tissue [21]. To assess origins of variance in the Raman features related to mineralization, we examined the effect of embedding and fixation on Raman spectroscopy's ability to differentiate tissue types. Data was collected from non-embedded, non-fixed samples, samples immersed in ethanol for 72 hours, and samples immersed in ethanol and embedded in an acrylic polymer. Then in a secondary experiment, we investigated the effect of specimen orientation on the peak intensities of interest by collecting spectra from multiple osteonal and interstitial sites for both a transverse and longitudinal cut of bone (i.e., orthogonal planes). We hypothesized that collagen peaks least affected by the orientation of bone tissue demonstrate statistically significant differences in bone composition (namely, mineral-to-collagen ratio) between osteonal and interstitial tissue with a relatively small sample size of 7 and sufficiently sampling of sites within each specimen.

Material and Methods

Specimen Preparation

Using a surgical osteotomy saw, a cross-section, ~80 mm in axial length, was cut from the diaphysis of 7 cadaveric femurs. As reported by the Vanderbilt Anatomical Donation Program, the four Caucasian, female donors were either 95, 86 (two), or 82 years of age, and the three Caucasian, male donors were either 94, 80, or 48 years of age. A low speed, diamond embedded, circular saw (660, South Bay Technology, Inc., Torrance, CA) was used first to cut each diaphysis into two cross-sections, and then to extract a longitudinal strip from the medial quadrant (nominally 6 mm × 6 mm × 40 mm) of the proximal half. Next, the saw was used to transversely cut the medial strips of bone into 3 cross-sections of approximately equal length. All precision cuts were made under irrigation, and bone specimens were stored at -20 °C until further processing.

Two of the 3 sections per donor were immersed in 70% Ethanol (EtOH) for 24 hours and then further dehydrated in 100% EtOH for 48 hours. Note that these specimens were not stored in alcohol after dehydration nor were spectra collected from the bone while immersed in alcohol. One of the dehydrated sections per donor was then embedded in polymethyl/ butyl methacrylate (PMMA). In doing so, the bones were immersed in methyl methacrylate (Fisher Scientific), N-butyl methacrylate (Sigma), methyl benzoate (Fluka), and polyethylene glycol 400 (Fluka) for 2 days. The bones were placed in a new mixture of the polymer solution plus the addition of a catalyst, 1g of wet benzoyl peroxide (Fisher), for 2 days. After removing water by passing a new mixture of the polymer with the catalyst through calcium chloride, N, N-dimethyl-p-toluidine was added to initiate polymerization of the PMMA surrounding the bone specimen at the bottom of a scintillation vial, which was then capped and stored at -20 °C within 4-8 minutes. After 48 hours, the PMMA block was

retrieved, and the low speed saw cut excess material such that the surface of bone was nearly parallel when the PMMA block was glued (cyno-acrylate) to a plastic slide (41500, Exact Technologies, Inc., Oklahoma City).

We ground the transverse side of each section on wet silicon carbide papers of successive grits (800, 1200, and 4000). For one donor, the longitudinal side of an extra section was ground and polished. The grinding involved attaching the bone by suction to the oscillating head of the Exakt grinding wheel (Apparatebeuh GmbH & Co., Germany). Thus, we produced specimens with parallel surfaces of transverse cuts of bone for each donor and each processing technique (non-processed, alcohol, or alcohol & embedded) plus 1 extra smooth surface in the longitudinal orientation (non-processed). Lastly, all ground surfaces were polished by hand on synthetic cloth (South Bay Technology, Inc., Torrance, CA) with 5 and 0.05 micron alumina slurry and then vacuum sealed until Raman analysis.

Confocal Raman Spectroscopy

Raman spectra were acquired from the polished surface of the bone tissue in air using a standard confocal Raman microscope (Ramanscope Mark III, Renishaw, Hoffman Estates, IL) with a 785 nm laser diode source (Innovative Photonic Solutions, Monmouth Junction, NJ). A 50× objective (numerical aperture of 0.75) focused the light to a 2-5 μm spot just below the surface of the bone tissue. The Raman scattered light was then passed through a 35 μm slit and collected by a spectrograph having a spectral resolution of 1 cm^{-1} . Additional optics were not added to alter the inherent polarization of the system; tissue site selection and measurement protocol were designed to reduce effects of polarization bias on the collected data. The wavenumber axis of the spectrograph was regularly calibrated with a silicon reference (i.e., peak at 520 cm^{-1}) such that the intensities were averaged over 5 pixels per wavenumber on the CCD of the spectrograph. Each acquired spectra consisted of three accumulations each with an integration time of 10 s and a binning of 3 wavenumbers. All medial specimens were positioned with periosteal side to the left and the endosteal side to the right under the objective, and the laser orientation did not vary throughout the course of spectral acquisitions.

Calculating Raman Properties

Background fluorescence in the spectra was subtracted by a modified polynomial fitting algorithm [22], and the raw peak intensities were calculated for ν_1 Phosphate at 960-962 cm^{-1} ($\nu_1\text{PO}_4$) [10, 23], ν_2 Phosphate at 431-433 cm^{-1} ($\nu_2\text{PO}_4$) [10], ν_4 Phosphate at 584-589 cm^{-1} ($\nu_4\text{PO}_4$) [10, 23], Type-B carbonate at 1071-1072 cm^{-1} (Carb) [23], Proline at 855-857 cm^{-1} [23, 24], Amide III at 1247-1248 cm^{-1} [10, 23], CH_2 -wag at 1451-1452 cm^{-1} [10, 23], and Amide I at 1666-1667 cm^{-1} [10, 23] (Figure 1) using custom developed software (Matlab, Mathworks, Inc., Natick, MA). In addition, we calculated 15 peak ratios (e.g., $\nu_1\text{PO}_4/\text{AmideI}$, $\nu_2\text{PO}_4/\text{AmideIII}$, $\nu_4\text{PO}_4/\text{Proline}$, $\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$, and $\text{Carb}/\nu_4\text{PO}_4$) as well as the inverse of the full width of the $\nu_1\text{PO}_4$ peak at half-max ($\nu_1\text{FWHM}^{-1}$) in which the maximum intensity was normalized.

Site Selection within Tissue

We selected osteons for Raman analysis within the intra-cortical region. Since the diameter of the cement line and the diameter of the Haversian canal (pore) of secondary osteons vary within this region, we systematically chose 1 large osteon with an average sized pore, 1 large osteon with a large pore, 2 average sized osteons with an average sized pore, and 1 average sized osteon with a small pore. Two Raman spectra were collected and averaged per osteon, and the locations were approximately equidistance from cement line and Haversian canal and away from lacunae (Figure 2). In the rare case that spectra exhibited strong PMMA peaks (Fig. 1) in the embedded samples, spectra were re-acquired at another site. Next, for each osteon, 2 Raman spectra were collected and then averaged from outside intact cement lines and within the neighboring interstitial tissue, which were likely remnants of secondary osteons given the age of the donors. Thus, each donor gave representative Raman measurements for 2 different tissue types, osteonal and interstitial (Figure 2), within each transverse cut or the one longitudinal cut because the signal intensity of each peak and the peak ratios were averaged among the 5 osteons as well as among the 5 interstitial patches per specimen.

Statistical Analysis

A two-way analysis of variance (ANOVA) tested for the effect of tissue type and processing method on the peak intensities or peak ratios of interest. Since in the post hoc analysis (Holm-Sidak method for pair-wise comparisons) there were no differences in the peak intensities, irrespective of tissue type, between bone specimens immersed in alcohol for 72 hours and non-processed bone specimens, these groups were combined bringing the sample average per donor closer to the population average of the property. Note that there is an inherent variance (heterogeneity) in the physiochemical properties of bone tissue at the micron length scale. Moreover, this averaging did not change the group size, which remained equal to the number of donors ($N=7$). Thus, we tested for differences in peak intensities and peak ratios between osteonal and interstitial tissue using a one-way ANOVA (SigmaStat v3.5, Systat Software Inc., San Jose, CA) for non-embedded and embedded specimens, separately. For the one donor, statistically significant differences between transverse and longitudinal cuts within each tissue type (n equals 5 distinct sites per type) were determined using a two-way ANOVA. Statistically significant differences were considered at $p<0.05$.

Results

Effects of Tissue Type and Embedding on Peak Intensities

There were differences in peak intensities between the type of tissue (osteonal or interstitial) for only Proline and $\nu_1\text{PO}_4$ bands (Table 2). The method of processing the bone specimens affected the intensity of all the Raman peaks of interest, and differences between the type of tissue did not depend on processing method (i.e., interaction was not significant as shown in Table 2). The post hoc analysis revealed that embedding in PMMA, not immersion in alcohol alone, caused these effects on Raman peak intensities.

In the analysis of the combined non-processed and ethanol-soaked bones (herein referred to as non-embedded), the Proline signal intensity was greater in the osteonal tissue than in interstitial tissue (Figure 3). There were no differences in the other collagen-associated Raman peaks between the tissue types (Figure 3). With respect to the mineral phase, only $\nu_1\text{PO}_4$ intensity was greater in the interstitial tissue than in the osteonal tissue (Figure 4). Similar trends existed for $\nu_4\text{PO}_4$ and $\nu_2\text{PO}_4$, but differences between the tissue types were not statistically significant at the given sample size of 7 (Figure 4). The inverse of the full width of the $\nu_1\text{PO}_4$ peak at half-max ($\nu_1\text{FWHM}^{-1}$) (crystallinity) was greater in the interstitial tissue than in the osteonal tissue (Figure 4). The Type-B carbonate peak intensity did not vary between tissue types (p -value = 0.533).

Differences in Raman Properties (Peak Ratios) between Tissue Types

Since peak intensities depend on laser power and the optical components in the Raman system (i.e., light throughput), not just tissue composition, Raman peak ratios rather than peak intensities are customarily used to detect differences in the composition of bone tissue (Table 1). Except for $\nu_4\text{PO}_4$ /Amide I and $\nu_4\text{PO}_4$ /Amide III, embedding had no effect on the Raman properties related to the mineral-to-collagen ratio ($p > 0.1$ for embedded vs. non-embedded as determined by two-way ANOVA). Moreover, this lack of an effect did not depend on tissue type ($p > 0.5$ for the interaction in the ANOVA). Dividing the mineral peak – ν_1 vibration mode – by the Proline peak or the Amide III peak detected a 15.4% or 12.5% increase, respectively, in mineralization going from osteonal to interstitial tissue (non-embedded in Table 3). Of the peak ratios, $\nu_1\text{PO}_4$ /Proline provided the lowest coefficient of variance (COV) for both tissue types. In contrast, the traditional $\nu_1\text{PO}_4$ /Amide I measured a 9.7% increase in mineralization (Table 3), and this peak ratio had the highest COV such that the difference in this Raman property between the types of tissue was not statistically significant at $n=7$ (Table 3). Embedding affected all the Raman properties related to Type B carbonate substitution ($p < 0.05$ for embedded vs. non-embedded as determined by two-way ANOVA). Unexpectedly, Carb/ $\nu_1\text{PO}_4$ was 6.4% lower in interstitial than in osteonal tissue for the non-embedded bone (Tables 3). $\nu_1\text{FWHM}^{-1}$, on the other hand, was greater in interstitial than osteonal tissue, regardless of whether the bone was embedded or non-embedded. In fact, for most peak ratios, embedding did not obscure differences in peak ratios between osteonal and interstitial tissues (Supplemental Table 1).

Effect of Orientation on Peak Ratios

Besides embedding material, another potential source of variance in Raman properties is the orientation of the bone. Raman analysis of 1 donor (multiple osteonal and interstitial sites) revealed that the greatest difference in peak ratio between a transverse cut and longitudinal cut of bone was $\nu_1\text{PO}_4$ /Amide I, irrespective of tissue type (Table 4). Moreover, there were differences in most peak ratios between the 2 orientations of the bone, except for $\nu_1\text{PO}_4$ /Proline, $\nu_4\text{PO}_4$ /Proline, and $\nu_1\text{FWHM}^{-1}$. The higher peak ratios in the longitudinal cut than in the transverse cut did not depend on whether the tissue was osteonal or interstitial ($p > 0.05$ for the interaction term in the two-way ANOVA). Exceptions included Carb/ $\nu_1\text{PO}_4$ in which differences between orientations of cut were only observed for osteonal tissue and $\nu_1\text{FWHM}^{-1}$ in which differences between tissue types were only observed in the transverse cut (Table 4).

Discussion

Coupling Raman-derived properties of bone with biomechanical properties can potentially explain the difference between a weak or brittle bone and a strong or ductile bone (i.e., provide structure-function relationships). Despite the growing application of Raman microspectroscopy to bone tissue (Table 1), there has not been an extensive analysis of the ability of relevant peaks to quantify known compositional differences in bone tissue. In this study, various Raman peak intensities were used to quantify the known difference in mineralization between osteonal and interstitial bone from 7 donors. In addition, sources of variance related to processing were considered. Normalizing the ν_1 phosphate or the ν_2 phosphate peak by the Proline peak, not the traditional Amide I peak, provided the most striking difference (Tables 3) in bone composition between the tissue types. Moreover, this trend was consistent for 2 orthogonal surfaces of the bone (Table 4) and had the lowest variance of all the Raman properties (Table 3). Normalization by Amide III also detected differences in composition between the 2 tissue types with COV range of 3-4%. Embedding bone typically increases variance of most Raman peak ratios, but differences in the compositional properties were still detectable in samples embedded in PMMA (Supplemental Table 1).

The $\nu_1\text{PO}_4$ /Proline and $\nu_1\text{PO}_4$ /Amide III ratios essentially characterize the mineral-to-matrix ratio of bone tissue, but given that mineral accumulates in the matrix over time, they are related to the degree of mineralization. The gold standard for measuring the degree of mineralized bone (DMB-AI) is contact micro-radiography of thin bone sections in which gray level values are calibrated to mineralization using an aluminum (Al) step-wedge. Recently, Bergot et al. [20] acquired microradiographs of transverse cortical bone sections from 99 female and 94 male donors (N=193) ranging from 20 years to 90 years of age; and using image processing software, they were able to report the DMB-AI for interstitial and osteonal tissue, separately. Among all donors, osteonal was 88.79 ± 7.07 DMB-AI and interstitial was 90.56 ± 7.14 DMB-AI. Thus, the variability, or COV, was 7.88% and 7.96%, respectively, and the percent difference between osteonal and interstitial ranged from 2% to 3.5% ($p < 0.0001$) [20]. For all the presently reported Raman peak ratios, the variance was less than and the percent difference greater than that indicated by micro-radiography (Table 3). The expectation is that all the Raman-derived measurements of the mineral-to-matrix ratio can detect a difference in mineralization between osteonal and interstitial tissue; but with 7 donors, $\nu_1\text{PO}_4$ /Amide I failed to do so (albeit $p = 0.065$ as shown in Table 3).

One possible explanation for the higher variance in $\nu_1\text{PO}_4$ /Amide I is the strong dependence of Amide I intensity on the orientation of the collagen fibrils relative to the preferential orientation of the incident laser light polarization. This phenomenon is most evident in polarized Raman spectroscopy, where filters are used to explicitly control the polarization of the incident laser light and the collected backscatter. In a polarized Raman analysis of embedded cortical bone from 1 donor, Kazanci et al. [15] observed that $\nu_1\text{PO}_4$ /Amide I varied between transverse and longitudinal surfaces of bone when the lamellar structure was parallel to the polarizing direction. In the present study, we utilized a standard Raman spectrometer without added polarization-sensitive optics, but the instrument retains a small degree of sensitivity to polarization effects. Therefore, not surprisingly, a similar, more

mutated, increase in $\nu_1\text{PO}_4/\text{Amide I}$ was observed from longitudinal to transverse cuts. The origin of this phenomenon is likely due to distinct differences in the orientation of collagen fibrils between the 2 orthogonal cuts of bone. Thus, when collagen fibril orientation shifts among lamellae [25], the orientations of the Amide I chemical motif as well as the c-axis of the crystal also change, thereby differentially affecting $\nu_1\text{PO}_4$ and Amide I peak intensities and adding variance that is independent of composition. Indeed, when Hofmann et al. [23] examined multiple orientations of osteons (cut 0° through 90° in increments of 15°) by standard Raman microspectroscopy, they observed a banding pattern of Amide I (normalized to background PMMA) that matched the lamellar structure of bone and the intensity changed with orientation of cut. In their study, the ν_1 phosphate intensity had a more homogeneous distribution relative to Amide I. We observed that the other peak ratios had less apparent dependence on orientation of the bone than $\nu_1\text{PO}_4/\text{Amide I}$, though statistically significant differences existed (Table 4). Ideally, all bone sections in any study should have the same orientation of cut to minimize this undue source of variance.

Care should also be given to orientating each bone section in a consistent fashion (e.g., medial pointed left) under the objective when the Raman system has some sensitivity to polarization effects. As a demonstration, we collected spectra at the same site before and after a 90° rotation for 1 transverse section of human cortical bone. There were 5 unique osteonal sites and 5 unique interstitial sites for a total of 10 spectra. The most striking change in the spectra upon rotation was a decrease in the Amide I intensity, especially in the interstitial tissue. Confirming what was observed comparing peak ratio measurements from transverse cuts to those from longitudinal cuts of bone (Table 4), rotation of the bone specimen by 90° affected the $\nu_1\text{PO}_4/\text{Amide I}$ the most and $\nu_1\text{PO}_4/\text{Proline}$ the least. Additional collection of Raman spectra from osteonal and interstitial sites of bone from multiple donors is necessary though to make statistical inferences on the extent to which specimen rotation (i.e., change in collagen fibril orientation) affects each Raman property.

There is a caveat to the use of alternative collagen peaks to normalize the mineral peaks. The peak intensity of Proline and Amide III within transverse cuts was 6.7% and 4.0% less, respectively, in the interstitial than in the osteonal tissue (Figure 3). If this decrease is strictly due to collagen content, then we could hypothesize the amount of collagen secreted by osteoblasts changed over time. To best of our knowledge, such a phenomenon has not been reported in the literature. In the case of Proline, hydroxylation of the amino acid over time could also explain the difference in the Proline intensity between tissue types, but an increase in hydroxyproline is not known to occur as the tissue ages or matures. Like Amide I, the Amide III can be sensitive to protein confirmation given the role of amide moieties in crosslinking [26]. Possibly, the accumulation of mineral within the collagen matrix during secondary mineralization could be affecting the conformation of the collagen molecule and thus changing the intensity of the collagen peaks (Figure 3).

The higher $\nu_1\text{FWHM}^{-1}$ observed in the interstitial site than in the osteonal site of transverse cuts suggests that mineral becomes more crystalline over time. Raman-derived crystallinity has been found to correlate with Fourier Transform Infrared (FTIR)-derived crystallinity with crystallinity being higher in sites near the cement line (older tissue) than in sites near the Haversian canal (younger tissue) [27]. The lower Carb/ $\nu_1\text{PO}_4$ in the interstitial site than

in the osteonal site was an unexpected finding in that this ratio was observed to increase in rat bone with aging [28] as well as with an increase in the tissue age (identified by flourochrome labeling of mineralizing fronts at multiple time points) [29]. The discrepancy between rat and human tissue may be due to differences in the period of secondary mineralization between species. A long period of time can elapse (on the order of decades, not months) following the secretion of osteoid in human bone relative to rodent bone. Therefore, over an extended period of time, the accumulation of mineral in the organic matrix at interstitial sites, which increases the intensity of ν_1 phosphate, may exceed the number of Type B substitutions of carbonate for phosphate that can occur within the mineral lattice at this site. Interestingly, Yerramshetty et al. observed that Carb/ $\nu_1\text{PO}_4$ in human cadaveric bone – with no distinction between the tissue types – increased with the age of the donor, and it was inversely proportional to $\nu_1\text{FWHM}^{-1}$ [30]. Certain bones are embedded prior to analysis by Raman microspectroscopy so that they can be polished in order to identify features (e.g., lamellae of a mouse femur). The embedding media infiltrates the microscopic pores, and potentially alters the Raman spectrum. Thus, there is a concern that the process of embedding the bone may obscure real differences in Raman properties. Previous work by Yeni et al. [14] investigated the effects of alcohol and several embedding media on Raman peak ratios as measured from the cortex of mouse humeri and found that 70% EtOH increased $\nu_1\text{PO}_4/\text{Amide I}$ and that methyl methacrylate decreased $\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$ and increased the FWHM of $\nu_1\text{PO}_4$ with no effect on Carb/ $\nu_1\text{PO}_4$. In the present work, immersion in alcohol did not affect any of the Raman properties of human tissue, nor did it affect peak intensities, but there were differences in the dehydration steps and storage conditions between the studies. Also unlike the previous study, PMMA increased the mineral-to-matrix ratio when phosphate (ν_1 , ν_2 , or ν_4) was normalized by Amide I, but this can be due to differences in the formulation of the polymer. Most importantly, statistically significant differences in composition between osteonal and interstitial tissue were not obscured by the embedding process (Supplemental Table 1).

Presently, we found that the traditional Raman measurement of $\nu_1\text{PO}_4/\text{Amide I}$ did not detect differences in composition between osteonal and interstitial tissue for a sample size of 7, even though its variability is similar to what has been reported when mineralization is quantified by micro-radiography for a large sample size. Therefore, normalization by Amide I may not always be suitable in quantifying compositional differences in bone, especially when using typical Raman systems that do not specifically include optics such as depolarizers to minimize polarization bias. However, use of such optics comes at the expense of light throughput. Therefore, measurement protocol and tissue site selection can be designed to average out polarization bias, as described in this study. Nonetheless, the Amide I band has potential in explaining anisotropic differences in mechanical properties due to its potential sensitivity to collagen fibril orientation. There are other collagen peaks that can be used in place of Amide I in order to quantify the mineral-to-collagen ratio. Proline was the least affected by orientation of bone specimen; and when used to normalize the ν_1 or ν_2 vibration mode of the phosphate group, it detected the increases in composition between osteonal and interstitial tissue with a variance that was less than would be expected from DBM measurements by micro-radiography (Tables 3 and 4). Dividing $\nu_1\text{PO}_4$ by Amide III and $\text{CH}_2\text{-wag}$ also detected less mineralization in the osteonal tissue relative to

the interstitial tissue, irrespective of embedding in PMMA. To minimize variance in the Raman-derived peak ratios when using standard Raman microscopes, specimens should be oriented in the same manner under the objective of the microscope; and moreover, to account for inherent heterogeneity in both composition and structure, spectra should be collected from a sufficient number of spatially distinct sites within a given specimen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Study was supported in part by the following NIH grants NCI U54 CA-126505 and NIAMS R21 AG-029413.

References

1. Kanis JA, Johnell O, Oden A, Dawson A, De Laet C, Jonsson B. Ten year probabilities of osteoporotic fractures according to BMD and diagnostic thresholds. *Osteoporos Int.* 2001; 12:989–995. [PubMed: 11846333]
2. Schnitzler CM. Bone quality: a determinant for certain risk factors for bone fragility. *Calcif Tissue Int.* 1993; 53(Suppl 1):S27–31. [PubMed: 8275376]
3. Turner CH. Biomechanics of bone: determinants of skeletal fragility and bone quality. *Osteoporos Int.* 2002; 13:97–104. [PubMed: 11905527]
4. Rho JY, Kuhn-Spearing L, Zioupos P. Mechanical properties and the hierarchical structure of bone. *Med Eng Phys.* 1998; 20:92–102. [PubMed: 9679227]
5. Opsahl W, Zeronian H, Ellison M, Lewis D, Rucker RB, Riggins RS. Role of copper in collagen cross-linking and its influence on selected mechanical properties of chick bone and tendon. *J Nutr.* 1982; 112:708–716. [PubMed: 6121843]
6. Jonas J, Burns J, Abel EW, Cresswell MJ, Strain JJ, Paterson CR. Impaired mechanical strength of bone in experimental copper deficiency. *Ann Nutr Metab.* 1993; 37:245–252. [PubMed: 8311418]
7. Oxlund H, Barckman M, Ortoft G, Andreassen TT. Reduced concentrations of collagen cross-links are associated with reduced strength of bone. *Bone.* 1995; 17:365S–371S. [PubMed: 8579939]
8. Akkus O, Polyakova-Akkus A, Adar F, Schaffler MB. Aging of microstructural compartments in human compact bone. *J Bone Miner Res.* 2003; 18:1012–1019. [PubMed: 12817753]
9. Lane NE, Yao W, Balooch M, Nalla RK, Balooch G, Habelitz S, Kinney JH, Bonewald LF. Glucocorticoid-treated mice have localized changes in trabecular bone material properties and osteocyte lacunar size that are not observed in placebo-treated or estrogen-deficient mice. *J Bone Miner Res.* 2006; 21:466–476. [PubMed: 16491295]
10. Carden A, Morris MD. Application of vibrational spectroscopy to the study of mineralized tissues (review). *J Biomed Opt.* 2000; 5:259–268. [PubMed: 10958610]
11. Bi X, Patil CA, Lynch CC, Pharr GM, Mahadevan-Jansen A, Nyman JS. Raman and mechanical properties correlate at whole bone- and tissue-levels in a genetic mouse model. *J Biomech.* 2011; 44:297–303. [PubMed: 21035119]
12. Timlin JA, Carden A, Morris MD, Rajachar RM, Kohn DH. Raman spectroscopic imaging markers for fatigue-related microdamage in bovine bone. *Anal Chem.* 2000; 72:2229–2236. [PubMed: 10845368]
13. Schulmerich MV, Dooley KA, Morris MD, Vanasse TM, Goldstein SA. Transcutaneous fiber optic Raman spectroscopy of bone using annular illumination and a circular array of collection fibers. *J Biomed Opt.* 2006; 11:060502. [PubMed: 17212521]
14. Yeni YN, Yerramshetty J, Akkus O, Pechey C, Les CM. Effect of fixation and embedding on Raman spectroscopic analysis of bone tissue. *Calcif Tissue Int.* 2006; 78:363–371. [PubMed: 16830201]

15. Kazanci M, Wagner HD, Manjubala NI, Gupta HS, Paschalis E, Roschger P, Fratzl P. Raman imaging of two orthogonal planes within cortical bone. *Bone*. 2007; 41:456–461. [PubMed: 17602910]
16. Kazanci M, Roschger P, Paschalis EP, Klaushofer K, Fratzl P. Bone osteonal tissues by Raman spectral mapping: orientation-composition. *J Struct Biol*. 2006; 156:489–496. [PubMed: 16931054]
17. Bromage TG, Goldman HM, McFarlin SC, Warshaw J, Boyde A, Riggs CM. Circularly polarized light standards for investigations of collagen fiber orientation in bone. *Anat Rec B New Anat*. 2003; 274:157–168. [PubMed: 12964206]
18. Morris, MD. Emerging Raman applications and techniques in biomedical and pharmaceutical fields. Springer; New York: 2010. *Raman Spectroscopy of Bone and Cartilage*; p. 347-364.
19. Boivin G, Bala Y, Doublier A, Farlay D, Ste-Marie LG, Meunier PJ, Delmas PD. The role of mineralization and organic matrix in the microhardness of bone tissue from controls and osteoporotic patients. *Bone*. 2008; 43:532–538. [PubMed: 18599391]
20. Bergot C, Wu Y, Jolivet E, Zhou LQ, Laredo JD, Bousson V. The degree and distribution of cortical bone mineralization in the human femoral shaft change with age and sex in a microradiographic study. *Bone*. 2009; 45:435–442. [PubMed: 19501681]
21. Rho JY, Roy ME 2nd, Tsui TY, Pharr GM. Elastic properties of microstructural components of human bone tissue as measured by nanoindentation. *J Biomed Mater Res*. 1999; 45:48–54. [PubMed: 10397957]
22. Lieber CA, Mahadevan-Jansen A. Automated method for subtraction of fluorescence from biological Raman spectra. *Appl Spectrosc*. 2003; 57:1363–1367. [PubMed: 14658149]
23. Hofmann T, Heyroth F, Meinhard H, Franzel W, Raum K. Assessment of composition and anisotropic elastic properties of secondary osteon lamellae. *J Biomech*. 2006; 39:2282–2294. [PubMed: 16144702]
24. Nie S, Bergbauer KL, Ho JT, Kuck JF Jr, Yu NT. Applications of near-infrared fourier transform raman spectroscopy in biology and medicine. *Spectroscopy*. 1990; 5:24–32.
25. Giraud-Guille MM. Twisted plywood architecture of collagen fibrils in human compact bone osteons. *Calcif Tissue Int*. 1988; 42:167–180. [PubMed: 3130165]
26. Bandekar J. Amide modes and protein conformation. *Biochim Biophys Acta*. 1992; 1120:123–143. [PubMed: 1373323]
27. Gourion-Arsiquaud S, Burket J, Havill L, Dicarlo E, Doty S, Mendelsohn R, van der Meulen M, Boskey A. Spatial Variation in Osteonal Bone Properties Relative to Tissue and Animal Age. *J Bone Miner Res*. 2009
28. Akkus O, Adar F, Schaffler MB. Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone. *Bone*. 2004; 34:443–453. [PubMed: 15003792]
29. Donnelly E, Boskey AL, Baker SP, van der Meulen MC. Effects of tissue age on bone tissue material composition and nanomechanical properties in the rat cortex. *J Biomed Mater Res A*. 2010; 92:1048–1056. [PubMed: 19301272]
30. Yerramshetty JS, Lind C, Akkus O. The compositional and physicochemical homogeneity of male femoral cortex increases after the sixth decade. *Bone*. 2006; 39:1236–1243. [PubMed: 16860007]
31. Yerramshetty JS, Akkus O. The associations between mineral crystallinity and the mechanical properties of human cortical bone. *Bone*. 2008; 42:476–482. [PubMed: 18187375]
32. de Carmejane O, Morris MD, Davis MK, Stixrude L, Tecklenburg M, Rajachar RM, Kohan DH. Bone chemical structure response to mechanical stress studied by high pressure Raman spectroscopy. *Calcif Tissue Int*. 2005; 76:207–213. [PubMed: 15742234]
33. Ramasamy JG, Akkus O. Local variations in the micromechanical properties of mouse femur: the involvement of collagen fiber orientation and mineralization. *J Biomech*. 2007; 40:910–918. [PubMed: 16678186]
34. Silva MJ, Brodt MD, Wopenka B, Thomopoulos S, Williams D, Wassen MH, Ko M, Kusano N, Bank RA. Decreased Collagen Organization and Content Are Associated With Reduced Strength of Demineralized and Intact Bone in the SAMP6 Mouse. *J Bone Miner Res*. 2006; 21:78–88. [PubMed: 16355276]

35. Freeman JJ, Wopenka B, Silva MJ, Pasteris JD. Raman spectroscopic detection of changes in bioapatite in mouse femora as a function of age and in vitro fluoride treatment. *Calcif Tissue Int.* 2001; 68:156–162. [PubMed: 11351499]
36. Kavukcuoglu NB, Arteaga-Solis E, Lee-Arteaga S, Ramirez F, Mann AB. Nanomechanics and Raman spectroscopy of fibrillin 2 knock-out mouse bones. *J Mater Sci.* 2007; 42:8788–8794.
37. Kavukcuoglu NB, Denhardt DT, Guzelsu N, Mann AB. Osteopontin deficiency and aging on nanomechanics of mouse bone. *J Biomed Mater Res A.* 2007; 83:136–144. [PubMed: 17390367]
38. Kavukcuoglu NB, Patterson-Buckendahl P, Mann AB. Effect of osteocalcin deficiency on the nanomechanics and chemistry of mouse bones. *J Mech Behav Biomed.* 2009; 2:348–254.
39. Wallace JM, Golcuk K, Morris MD, Kohn DH. Inbred strain-specific response to biglycan deficiency in the cortical bone of C57BL/6J129 and C3H/He mice. *J Bone Miner Res.* 2009; 24:1002–1012. [PubMed: 19113913]
40. Kohn DH, Sahar ND, Wallace JM, Golcuk K, Morris MD. Exercise alters mineral and matrix composition in the absence of adding new bone. *Cells Tissues Organs.* 2009; 189:33–37. [PubMed: 18703871]
41. Balooch G, Balooch M, Nalla RK, Schilling S, Filvaroff EH, Marshall GW, Marshall SJ, Ritchie RO, Derynck R, Alliston T. TGF-beta regulates the mechanical properties and composition of bone matrix. *Proc Natl Acad Sci U S A.* 2005; 102:18813–18818. [PubMed: 16354837]
42. Ager JW, Nalla RK, Breeden KL, Ritchie RO. Deep-ultraviolet Raman spectroscopy study of the effect of aging on human cortical bone. *J Biomed Opt.* 2005; 10:034012. [PubMed: 16229656]
43. Shen J, Fan L, Yang J, Shen AG, Hu JM. A longitudinal Raman microspectroscopic study of osteoporosis induced by spinal cord injury. *Osteoporos Int.* 2010; 21:81–87. [PubMed: 19436936]
44. McCreddie BR, Morris MD, Chen TC, Sudhaker Rao D, Finney WF, Widjaja E, Goldstein SA. Bone tissue compositional differences in women with and without osteoporotic fracture. *Bone.* 2006; 39:1190–1195. [PubMed: 16901772]
45. Tarnowski CP, Ignelzi MA Jr, Morris MD. Mineralization of developing mouse calvaria as revealed by Raman microspectroscopy. *J Bone Miner Res.* 2002; 17:1118–1126. [PubMed: 12054168]

Figure 1.

Typical Raman spectra are shown for human intra-cortical bone tissue (non-processed, immersed in alcohol, or immersed and then embedded). The embedding media (PMMA) has overlapping peaks with bone tissue, but the peaks are labeled for only the bone spectra. Conventionally, mineral-to-collagen ratio is calculated as ν_1 Phosphate peak per Amide I peak, but presently we investigated the ability of other peak ratios to distinguish between osteonal and interstitial tissue.

Figure 2. Sites of osteonal and interstitial tissues are indicated for transverse and longitudinal cuts of bone.

Figure 3.

There were not many differences in the signal intensities (mean $\pm$ standard deviation) of the Raman peak associated collagen between osteonal and interstitial tissue.

Figure 4.

Of the Raman peaks (mean $\pm$ standard deviation) associated with mineral, the intensity of v_1 phosphate was greater in interstitial than in osteonal tissue for transverse cuts of bone. There was also a difference in crystallinity between the tissue types.

Table 1

The method of identifying compositional differences by confocal Raman spectroscopy varies among studies of bone tissue.

Bone	Species	Conditions	Laser (nm)	Factor(s)	Raman Property	Reported trends	Ref.
Femur	Human	Wet	632.8	Anatomical location and age	$\nu_1\text{PO}_4/\text{CH}_2$, Carb/ $\nu_1\text{PO}_4$, $\nu_1\text{FWHM}$	There was a loss in compositional heterogeneity in bone tissue after the 6 th decade.	[30]
Femur	Human	Wet	N.A.	Mineral phase	$[\nu_1\text{FWHM}]^{-1}$	Strength and modulus of bone tissue directly correlated with crystallinity, whereas ductility had an inverse relationship.	[31]
Femur	Human	Wet	785	Tissue type	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$, $\nu_1\text{FWHM}$	Mineral maturation persisted for 2 decades unless remodeled.	[8]
Femur	Mouse/Rat	Powder	514.5	Pressure	Raman shift of $\nu_1\text{PO}_4$ Amide I , Carb, CH_2 stretch, and CH_2 -wag	Vibration frequency of mineral and, collagen species shifted to a higher wavenumber when high pressure was applied to tissue.	[32]
Femur	Mouse	Wet	633	Micromechanical Properties and Collagen orientation	$\nu_1\text{PO}_4/\text{CH}_2$ -wag Carb/ $\nu_1\text{PO}_4$ $[\nu_1\text{FWHM}]^{-1}$	Raman properties listed did not explain micromechanical property differences due to observed differences in collagen orientation.	[33]
Femur	Mouse	Dry	532	Skeletal fragility	CH_2 -wag/ $\nu_1\text{PO}_4$ Amide I / $\nu_1\text{PO}_4$, Carb/ $\nu_1\text{PO}_4$, $\nu_1\text{FWHM}$, CarbFWHM	SAMP6 mice, which have brittle bones compared to SAMR1 controls, had reduced collagen-to-mineral ratio	[34]
Femur	Mouse	Dry	514	Aging and <i>in vitro</i> fluoridation	$\nu_1\text{FWHM}$	$\nu_1\text{FWHM}$ decreased with aging and Fluoride treatment, this apparent increase in crystallinity was associated with a decrease in rotation to failure.	[35]
Femur	Mouse	Dry	785	Fibrillin 2	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$, $[\nu_1\text{FWHM}]^{-1}$	Mice deficient in fibrillin 2 had lower tissue-level modulus and hardness in mid-cortical region and lower carbonate substitution in the endosteal region than wild-type mice.	[36]
Femur	Mouse	Dry	785	Osteopontin and age	$\nu_1\text{PO}_4/\text{Amide I}$	Osteopontin-deficient mice had less mineral content in young bone (< 12 weeks) than wild-type, but no difference in older group.	[37]
Femur	Mouse	Dry	785	Osteocalcin	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$, $[\nu_1\text{FWHM}]^{-1}$	Mice deficient in osteocalcin had less Type-B carbonate substitution and greater hardness than wild-type mice in a location-dependent manner.	[38]

Bone	Species	Conditions	Laser (nm)	Factor(s)	Raman Property	Reported trends	Ref.
Femur	Rat	Wet	532	Aging	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$, [$\nu_1\text{FWHM}$] ⁻¹	Mineralization, crystallinity, and Type-B carbonate substitution increased with age whereas elastic work decreased.	[28]
Femur	Rat	Dry	785	Labeled tissue age and mineralization	$\nu_1\text{PO}_4/\text{Amide I}$ Carb/ $\nu_1\text{PO}_4$	Periosteal rat bone shows tissue age spatial gradient of lower mineral to collagen ratio and carbonate substitution that corresponds to mechanical properties.	[29]
Tibia	Mouse	Dry	785	Biglycan deficiency	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$, [$\nu_1\text{FWHM}$] ⁻¹ , Peak area ratio 1660/1690	Mice deficient in biglycan had greater mineral-to-matrix ratio and less mature-to-immature crosslink ratio than wild-type controls. Differences in crystallinity and carbonated substitution depended on mouse strain and age.	[39]
Tibia	Mouse	Dry	785	Exercise and biglycan deficiency	$\nu_1\text{PO}_4/(\text{Proline}+\text{Hydroxyproline})$ Peak area ratio 1660/1690, Carb/ $\nu_1\text{PO}_4$	Collagen crosslinking explained fatigue changes in exercised wild-type mice. Exercised Biglycan deficient mice recovered mechanical properties, type B carbonate and collagen crosslinking.	[40]
Tibia	Mouse	Dry	632.8	TGF β signaling	Maps of $\nu_1\text{PO}_4$ and C-H stretch intensity	Inverse relationship existed between TGF β signaling and mineralization.	[41]
Tibia/Femur	Mouse	Wet/Dry	785	MMP2 knockout	$\nu_1\text{PO}_4/\text{Amide I}$ $\nu_1\text{PO}_4/\text{Amide III}$ Carb/ $\nu_1\text{PO}_4$ [$\nu_1\text{FWHM}$] ⁻¹ $\nu_1\text{PO}_4/\text{Proline}$	Raman properties significantly explained whole bone and tissue level biomechanical properties independently of μCT quantities.	[11]
Humerus	Human	Wet	244	Aging	Peak area at 1610 and at 1655, Peak area ratio 1610/1655	Changes in collagen conformation correlated with age and fracture toughness.	[42]
Humerus/Femur	Rat	Wet	632.8	Spinal Cord Injury induced Osteoporosis	$\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$ Carb/ $\nu_1\text{PO}_4$	Rats recovering from spinal cord injury had greater mineral-to-matrix ratio that increased with time (1-5 weeks), as well as increased carbonate substitution in the femur at 5 weeks.	[43]
Femoral head; Iliac crest	Human	Dry	785	Fracture risk	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$, Carb/Amide I	Bone from patients sustaining a hip fracture had higher carbonate/amide I area ratio than bone from non-fracture controls.	[44]
Calvaria	Mouse	Dry	785	Developmental time course	$\nu_1\text{PO}_4/\text{Amide I}$, Carb/ $\nu_1\text{PO}_4$	Mineral composition in mouse calvaria varied during growth from embryonic stage to 6 months of age.	[45]

Table 2

P-values are given for the effect of two factors on Raman peak intensities as determined by a two-way ANOVA.

	Tissue type ^a	Process ^b	Tissue type × Process ^c
$\nu_1\text{PO}_4$	0.012	<0.001	0.602
$\nu_2\text{PO}_4$	0.161	<0.001	0.772
$\nu_4\text{PO}_4$	0.153	<0.001	0.804
Carb	0.456	<0.001	0.711
Amide I	0.668	<0.001	0.953
Proline	0.028	<0.001	0.690
Amide III	0.191	<0.001	0.752
$\text{CH}_2\text{-wag}$	0.204	<0.001	0.902

^a osteonal or interstitial

^b no processing, dehydrated in ethanol, or dehydrated in ethanol and embedded

^c interaction in which difference between osteonal and interstitial depends on processing method

Table 3

Differences in each Raman property (mean $\pm$ standard deviation) between osteonal and interstitial tissue are given for the transverse cuts along with coefficient of variance (COV) as a percentage. Specimens were a combination of non-processed and ethanol-soaked bones.

Peak ratios	Osteonal	%COV	Interstitial	%COV	p-value
ν_1 PO ₄ /Amide I	11.79 $\pm$ 0.89	7.55	12.94 $\pm$ 0.90	6.93	0.085
ν_1 PO ₄ /Proline	13.38 $\pm$ 0.47	3.52	15.45 $\pm$ 0.43	2.81	<0.0001
ν_1 PO ₄ /Amide III	8.99 $\pm$ 0.33	3.62	10.11 $\pm$ 0.47	4.61	<0.0001
ν_1 PO ₄ /CH ₂ -wag	6.91 $\pm$ 0.34	4.90	7.68 $\pm$ 0.44	5.72	0.012
ν_2 PO ₄ /Amide I	2.80 $\pm$ 0.24	8.62	2.98 $\pm$ 0.26	8.57	0.351
ν_2 PO ₄ /Proline	3.18 $\pm$ 0.13	4.04	3.55 $\pm$ 0.14	3.87	0.003
ν_2 PO ₄ /Amide III	2.14 $\pm$ 0.09	4.42	2.32 $\pm$ 0.14	6.12	0.035
ν_2 PO ₄ /CH ₂ -wag	1.64 $\pm$ 0.09	5.74	1.77 $\pm$ 0.13	7.13	0.153
ν_4 PO ₄ /Amide I	2.27 $\pm$ 0.18	7.78	2.42 $\pm$ 0.18	7.32	0.248
ν_4 PO ₄ /Proline	2.58 $\pm$ 0.09	3.64	2.90 $\pm$ 0.08	2.83	<0.0001
ν_4 PO ₄ /Amide III	1.73 $\pm$ 0.07	3.84	1.90 $\pm$ 0.09	4.87	0.009
ν_4 PO ₄ /CH ₂ -wag	1.33 $\pm$ 0.07	5.07	1.44 $\pm$ 0.09	5.96	0.064
Carb/ ν_1 PO ₄	0.217 $\pm$ 0.008	3.65	0.204 $\pm$ 0.008	4.14	0.005
Carb/ ν_2 PO ₄	0.915 $\pm$ 0.038	4.18	0.889 $\pm$ 0.050	5.59	0.339
Carb/ ν_4 PO ₄	1.126 $\pm$ 0.043	3.83	1.087 $\pm$ 0.046	4.22	0.200
ν_1 FWHM ⁻¹	0.0501 $\pm$ 0.0004	0.75	0.0509 $\pm$ 0.0003	0.62	0.003

Table 4

Differences in each Raman property (mean $\pm$ standard deviation) between transverse and longitudinal cuts of bone are given for osteonal and interstitial type of tissue.

Peak Ratio	Osteonal			Interstitial			ANOVA p-value	
	Transverse	Longitudinal	%Diff ^a	Transverse	Longitudinal	%Diff	Orientation	Type
$\nu_1\text{PO}_4/\text{Amide I}$	11.0 $\pm$ 1.5	17.9 $\pm$ 1.4	12.0	12.8 $\pm$ 2.2	19.4 $\pm$ 2.4	10.3	<0.001	0.067
$\nu_1\text{PO}_4/\text{Proline}$	12.8 $\pm$ 1.2	14.2 $\pm$ 1.0	2.6	15.8 $\pm$ 1.5	15.0 $\pm$ 0.8	-1.2	0.531	0.002
$\nu_1\text{PO}_4/\text{Amide III}$	8.46 $\pm$ 0.59	10.44 $\pm$ 0.66	5.2	10.34 $\pm$ 0.94	11.32 $\pm$ 0.58	2.3	<0.001	<0.001
$\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$	6.50 $\pm$ 0.59	8.16 $\pm$ 0.54	5.7	7.83 $\pm$ 0.78	8.56 $\pm$ 0.51	2.2	<0.001	0.006
$\nu_2\text{PO}_4/\text{Amide I}$	2.66 $\pm$ 0.35	4.42 $\pm$ 0.40	12.4	2.88 $\pm$ 0.59	4.84 $\pm$ 0.57	12.7	<0.001	0.163
$\nu_2\text{PO}_4/\text{Proline}$	3.10 $\pm$ 0.28	3.50 $\pm$ 0.23	3.0	3.54 $\pm$ 0.44	3.74 $\pm$ 0.19	1.4	0.041	0.022
$\nu_2\text{PO}_4/\text{Amide III}$	2.05 $\pm$ 0.14	2.58 $\pm$ 0.16	5.7	2.32 $\pm$ 0.28	2.82 $\pm$ 0.14	4.8	<0.001	0.008
$\nu_2\text{PO}_4/\text{CH}_2\text{-wag}$	1.58 $\pm$ 0.14	2.02 $\pm$ 0.14	6.1	1.76 $\pm$ 0.23	2.13 $\pm$ 0.12	4.8	<0.001	0.056
$\nu_4\text{PO}_4/\text{Amide I}$	2.16 $\pm$ 0.26	3.40 $\pm$ 0.30	11.1	2.38 $\pm$ 0.34	3.76 $\pm$ 0.51	11.3	<0.001	0.096
$\nu_4\text{PO}_4/\text{Proline}$	2.52 $\pm$ 0.22	2.69 $\pm$ 0.18	1.6	2.93 $\pm$ 0.20	2.90 $\pm$ 0.10	-0.3	0.405	0.001
$\nu_4\text{PO}_4/\text{Amide III}$	1.67 $\pm$ 0.10	1.98 $\pm$ 0.13	4.3	1.92 $\pm$ 0.12	2.19 $\pm$ 0.09	3.2	<0.001	<0.001
$\nu_4\text{PO}_4/\text{CH}_2\text{-wag}$	1.28 $\pm$ 0.11	1.55 $\pm$ 0.11	4.8	1.46 $\pm$ 0.10	1.65 $\pm$ 0.08	3.1	<0.001	0.007
Carb/ $\nu_1\text{PO}_4$	0.221 $\pm$ 0.006	0.204 $\pm$ 0.008	-2.0	0.200 $\pm$ 0.008	0.200 $\pm$ 0.002	0.03	0.009	<0.001 ^b
Carb/ $\nu_2\text{PO}_4$	0.910 $\pm$ 0.022	0.825 $\pm$ 0.030	-2.4	0.893 $\pm$ 0.057	0.804 $\pm$ 0.010	-2.6	<0.001	0.233
Carb/ $\nu_4\text{PO}_4$	1.12 $\pm$ 0.02	1.07 $\pm$ 0.03	-1.1	1.07 $\pm$ 0.02	1.04 $\pm$ 0.02	-0.8	0.002	0.002
$\nu_1\text{FWHM}^{-1}$	0.0495 $\pm$ 0.0009	0.0503 $\pm$ 0.0003	0.4	0.0511 $\pm$ 0.0003	0.0506 $\pm$ 0.0005	-0.3	0.553	0.001 ^b

^aPercent difference for each property was calculated as follows: 100*(Longitudinal – Transverse)/(Longitudinal + Transverse)/2

^bThe interaction between orientation and type was statistically significant.