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Applying Full Spectrum Analysis in the Raman Spectroscopic Assessment of Fracture Toughness of Human Cortical Bone

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Abstract

A decline in the inherent quality of bone tissue is a contributor to the age-related increase in fracture risk. Although this is well known, the important biochemical factors of bone quality have yet to be identified using Raman spectroscopy (RS), a non-destructive, inelastic light scattering technique. To identify potential RS predictors of fracture risk, we applied principal component analysis (PCA) to 558 Raman spectra ($370\text{ cm}^{-1} - 1720\text{ cm}^{-1}$) of human cortical bone acquired from 62 female and male donors (9 spectra each) spanning adulthood (21 – 101 yo). Spectra were analyzed prior to R-curve, non-linear fracture mechanics that delineates crack initiation (K_{init}) from crack growth toughness (K_{grow}). The traditional ν_1 Phosphate peak per Amide I peak (mineral-to-matrix ratio) weakly correlated with K_{init} ($r = 0.341$, $p = 0.0067$) and overall crack growth toughness ($J-int$: $r = 0.331$, $p = 0.0086$). Sub-peak ratios of the Amide I band that are related to the secondary structure of type I collagen did not correlate with the fracture toughness properties. In the full spectrum analysis, one principal component (PC5) correlated with all of the mechanical properties (K_{init} : $r = -0.467$, K_{grow} : $r = -0.375$, and $J-int$: $r = -0.428$; $p < 0.0067$). More importantly, when known predictors of fracture toughness, namely age and/or volumetric bone mineral density (vBMD), were included in general linear models as covariates, several principal components helped explain 45.0% (PC5) to 48.5% (PC7), 31.4% (PC6), and 25.8% (PC7) of the variance in K_{init} , K_{grow} , and $J-int$, respectively. Deriving spectral features from full spectrum analysis may improve the ability of RS, a clinically viable technology, to assess fracture risk.

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Conflict of Interest

The authors have no conflict of interest to report.

Keywords

Bone Quality; Raman Spectroscopy; Toughness; Multivariate; Bone mineral density; Fracture risk

1. Introduction

As a natural material, healthy bone tissue has exceptional mechanical properties, especially given the combination of relatively low density, high strength, and significant post-yield toughness. Since the overall fracture resistance of bone arises from each level of its organizational hierarchy^{1,2}, numerous changes at multiple length-scales can contribute to an age- or disease-related increase in fracture risk. The relative contribution of changes in composition and organization of the matrix to this increase is difficult to assess, but in general, the bone matrix is an important contributor to fracture resistance as suggested by mouse models in which the deletion of matrix genes cause low bone toughness^{3–5} or by genetic mutations affecting collagen I structure such as osteogenesis imperfecta, a brittle bone disease^{6,7}. Presently, *in vivo* assessment of sub-microstructural features of bone composition and matrix organization as indicators of bone fragility is still in the developmental stage.

Of the spectroscopic techniques applied *ex vivo* to study disease and drug effects on bone quality⁸, Raman spectroscopy (RS) has clinical feasibility in the near term (infrared spectroscopy is susceptible to diffuse reflectance artifacts⁹). As applied to bone, RS quantifies such characteristics as the mineral-to-matrix ratio (phosphate-to-proline or other organic peak), carbonate substitutions (carbonate-to-phosphate), crystallinity (line-width of the phosphate band), and collagen secondary structure (sub-peak ratios derived from Amide I band), all of which are uniquely different than X-ray-derived parameters that are only dependent on the presence of mineral. RS does not require special preparation of the bone and is non-destructive. In fact, several groups have acquired Raman spectra from bone through skin *in vivo*^{10–12}. While previous studies demonstrate the feasibility of applying RS to human bone *in vivo*, a critical barrier to clinical utility is a meaningful analysis of the acquired spectra that differentiates strong, tough bone from fragile, brittle bone.

As described in a recent review by Mandair and Morris¹³, a number of studies have found significant relationships between RS properties and the fracture resistance of bone (primarily from rodents). However, aside from correlations between RS and nanoindentation measurements from primate bone tissue^{14,15}, there is surprisingly little evidence that Raman analysis helps explain mechanical properties of human bone at the apparent-level (i.e., fracture resistance independent of bone size). The first study comparing Raman to mechanical properties found only significant correlations between RS-derived crystallinity and certain mechanical properties of cadaveric bone tested in tension (negative for failure strain but positive for failure stress)¹⁶. A more recent paper reported that the ratio of a shoulder peak at $\sim 1640\text{ cm}^{-1}$ and the Amide I peak at $\sim 1670\text{ cm}^{-1}$ was sensitive to collagen damage and negatively correlated with post-yield toughness of bovine cortical bone (three-point bending)¹⁷. Of note, this is not the typical sub-band ratio used to determine the so-called collagen crosslink (maturity) ratio from the Amide I band¹³. Lastly, in a recently

published pilot study involving trabecular bone (2 female donors), a significant inverse relationship was found between ultimate strain and carbonate substitution for tensile and bending tests of individual trabeculae¹⁸. Thus, there is some evidence that RS properties are related to mechanical properties of bone, but there is no consensus on which properties are most important for assessing the fracture resistance of human bone.

In case-control studies to identify significant associations between tissue composition by spectroscopy and fracture risk, a clear understanding of how spectroscopic measurements relate to fracture resistance has also not yet emerged^{19,20}. As an example, one study reported higher carbonate-to-phosphate ratio, as determined by RS, in the cortex of iliac crest biopsies from fracture cases compared to non-fracture controls (age-matched women)²¹, while in another iliac biopsy study matching both age and areal bone mineral density between the cases, the carbonate-to-phosphate ratio of the cortex, as determined by Fourier transform infrared (FTIR) imaging, was significantly lower for women with fractures than for women without fractures²². While a number of differences between the studies may explain this discrepancy (e.g., time between fracture and biopsy collection), spectroscopy biomarkers of bone fragility are not readily identifiable partly because of other important contributors to fracture resistance (e.g., trabecular bone volume fraction and volumetric bone mineral density).

Since the application of multivariate statistics to Raman spectra of bone has been useful in previous studies^{23–27}, we hypothesized that full spectrum analysis could potentially be more accurate than traditional peak ratios, including sub-band ratios, in predicting the fracture toughness of human cortical bone. As a first step to integrating the full spectrum into fracture prediction, we applied principal component analysis (PCA), an unsupervised statistical analysis approach, to Raman spectra of bone. In comparison to traditional RS properties, the principal component or spectral traits were then tested for their ability to explain the variance in fracture toughness properties while accounting for known determinants.

2. Materials and Methods

2.1 Study Design

Fresh frozen human cadaveric femurs from 62 donors (30 male donors, aged 21 – 98 years old, mean $\pm$ standard deviation: 63.5 ± 23.7 years; and 32 female donors, aged 23 – 101 years old, 64.4 ± 21.3 years) were obtained from the Musculoskeletal Transplant Foundation (Edison, NJ), the Vanderbilt Donor Program (Nashville, TN), and the National Disease Research Interchange (Philadelphia, PA). There was no explicit exclusion of donors with osteoporosis, diabetes, and/or history of bone modifying treatments, and common causes of death included cardiac arrest, anoxia (respiratory failure), pneumonia, and trauma. For five donors (< 67 yo), the cause of death was listed as lung cancer (2 donors), colon cancer, pancreatic cancer, and breast cancer. The cortical bone samples were extracted from the lateral quadrant of the distal mid-shaft using a low-speed diamond-embedded saw (South Bay Technologies, San Clemente, CA) and machined into a single-edge notched beam (SENB) sample with an endocortical notch, sharpened by a razor blade and oriented in the radial direction, such that crack propagation would occur normal to osteon direction (Fig.

1A). SENBs samples were then scanned with micro-computed tomography (μ CT50; Scanco Medical AG, Brüttisellen, Switzerland) at a 5 μ m voxel size using the same settings (tube voltage: 90 kVp; beam current: 200 μ A; 1000 projections per 360° rotation; integration time: 400 ms). After post-processing the μ CT images with a Gaussian filter to suppress image noise ($\sigma = 1.8$ and support = 3), apparent volumetric bone mineral density (vBMD) was determined in a volume of interest selected ahead of the notch tip (Fig. 1B). By performing multiple extraction steps sequentially as well as conducting μ CT soon after Raman analysis without re-freezing the sample, we minimized the number of freeze-thaw cycles to 3.

2.2 Raman Spectroscopy: Data Acquisition

All Raman spectra were acquired using a confocal Raman microscope (Renishaw InVia Raman Microscope, Renishaw, Hoffman Estates, IL) with a 785 nm laser diode (Innovative Photonic Solutions, Monmouth Junction, NJ). A 50X objective (N Plan, NA=0.75, Leica, Wetzlar, Germany) was used to focus the light into a ~ 1 μ m spot at 35 mW on the sample. Raman scattered light was collected through the same objective with a 180-degree collection geometry, then passed through a 35 μ m slit and dispersed by a holographic grating (1800 lines/mm) onto a thermoelectrically cooled (-70 °C) deep-depleted, couple-charged diode (CCD) that provided 1 cm^{-1} spectral resolution. The spatial (lateral) resolution of the confocal Raman microscope is ~ 1 μ m. The inherent laser polarization (extinction ratio of 1:200) was aligned upright according to Renishaw specifications and confirmed with known polarizers and silicon standard intensity at 520 cm^{-1} . Daily silicon measurements and laser power at the sample before and after data collection ensured wavenumber calibration and light throughput.

The long axis of the SENB was aligned parallel to laser polarization. To minimize the time required for collection of Raman spectra using extended scans, which take >10 times longer than static scans, only nine spectra were acquired per sample. An extended scan uses a single moving grating to continuously collect Raman data without compromising spectral resolution, and for the InVia system, is necessary to acquire a full spectrum (range of 1500 cm^{-1}). The locations were a 3 $\times$ 3 grid with a ~ 250 μ m linear spacing centered within the intended crack propagation region (Fig. 1B). Spectral acquisitions for each of the 9 sites included a 10 second exposure with 7 accumulations.

2.3 Raman Spectroscopy: Property Calculations

The presence of any cosmic rays was removed using a custom MATLAB script (R2016a, Mathworks, Natick, MA). Raman spectra were also processed to remove background fluorescence via a least-squares, modified polynomial fitting algorithm²⁸ and to minimize the influence of system noise (unrelated to bone) with a fourth-order Savitzky-Golay filter²⁹. Next, spectral intensity values between 370 cm^{-1} and 1720 cm^{-1} (Fig. 1B) were interpolated (i.e., piecewise cubic interpolation) to align the wavenumber axis across all spectra while maintaining a spectral resolution of 1 cm^{-1} . The edges of the peaks were identified by derivative analysis; and then, a linear baseline subtraction³⁰ was conducted to ensure no residual fluorescence contributed to peak height, namely for proline (at 856–858 cm^{-1}), phosphate mineral ($\nu_1\text{PO}_4$ at 961–962 cm^{-1} and $\nu_2\text{PO}_4$ at 431–433 cm^{-1}), and carbonate (CO_3 at 1072–1073 cm^{-1}) peaks (Fig. 1B). All these selected intensities were then

used to generate peak ratios of bone composition: mineral-to-matrix ratio ($\nu_1\text{PO}_4/\text{Amide I}$, $\nu_1\text{PO}_4/\text{Proline}$ and $\nu_2\text{PO}_4/\text{Amide III}$), Type-B carbonate substitution ($\text{CO}_3/\nu_1\text{PO}_4$), and crystallinity (the inverse of the line-width of the peak normalized $\nu_1\text{PO}_4$ at half the height from baseline or half-maximum; FWHM^{-1})³¹.

Further analysis of the Amide I band involved determining sub-band peak ratios that are sensitive to the secondary structure of collagen I. First, the Amide I band was again subjected to a fourth-order Savitzky-Golay filter to remove residual noise (grey line in Fig. 2), and then band shoulders were determined at the minima of the second derivative of the spectrum (namely at ~ 1606 , ~ 1636 , and $\sim 1688\text{ cm}^{-1}$), and the two peak ratios of interest were extracted ($1668'/1636'$ and $1668'/1688'$) (red X's in Fig. 2). Second, four sub-bands were fitted to the spectrum between $1580\text{--}1720\text{ cm}^{-1}$ using Gaussian-Lorentzian mixed functions. To start, each peak was centered on the 4 points that were identified by the second derivative analysis, and the Gauss/Lorentzian mixture was set to 50/50. Then, a non-linear constrained optimization algorithm in MATLAB determined the final position of each peak within a $\pm 5\text{ cm}^{-1}$ window as well as the final mixture for each peak that minimized the weighted root mean square error between the experimental data and the sum of the 4 sub-bands (grey line in Fig. 2). Over the 558 spectra that were collected, the average positions of the sub-bands (with percentage of Gaussian) were (mean $\pm$ SD cm^{-1}): 1607.8 ± 0.5 (27 ± 35), 1637.9 ± 0.3 (61 ± 14), 1667.8 ± 0.2 (78 ± 4), 1689.4 ± 0.4 (99.7 ± 1.6). These RS sub-band locations were similar to the ones reported by Buckley et al. for antler and sheep cortical bone samples and who attributed differences in sub-band ratios as being reflective of differences in the secondary structure of type 1 collagen³². The sub-band ratios of interest were calculated as the ratio of the peak height (blue X's in Fig. 2) of the 3rd over the 2nd sub-band ($1668/1638$) and the 3rd over the 4th sub-band ($1668/1689$).

Finally, principal component analysis (PCA) was performed on the standardized variables or correlation matrix of the 558 spectra (i.e., intensities converted to z-scores) between 370 and 1720 cm^{-1} . After this standardization of the Raman spectra, the mean intensity among the 558 spectra for a given wavenumber was zero with unit variance.

2.4 Fracture Toughness Testing

The dimensions of the SENB specimens, the description of the R-curve tests, and the calculations of the fracture toughness properties are provided in our companion paper³³. Briefly, cracks were propagated using a three-point bending configuration (Fig. 1B) and a progressive loading scheme (Fig. 1C). Crack extension (a) was quantified from each successive unloading slope as described in ASTM E1820³⁴. Determination of crack initiation toughness (K_{init}) encompassed the elastic-plastic mechanical behavior in the form of the J-integral (J-int)³⁵. Crack growth toughness (K_{grow}) was determined from slope of the linear regression between $K_J (= (J \times E)^{0.5}$ where E is elastic modulus of bone) vs. the square root of a . Certain tests could not be used to calculate K_{grow} due to brittle instability (i.e., little-to-no post-yield displacement). This was the case for 11 of the 62 total samples. There were no obvious characteristics in the donor information or in the SENB dimensions that might explain the instable crack growth of these 11 samples with both female donors (23,

37, 53, 54, 80, 85, and 87 years old) and male donors (57, 61, 86, 88 years old) being represented.

2.5 Statistical Analysis

Correlations between the mean (9 per donor) of the Raman properties or PCs and fracture toughness properties or age were tested for significance using Pearson's linear correlation coefficient (r) at a significance level of 0.05 in which the data were bootstrapped because the normality assumption in parametric testing did not hold for most properties. Age, vBMD, and Raman outcomes were considered as independent predictors in backward, stepwise, linear regressions with the fracture toughness parameters as dependent variables to determine which combination of parameters best explain the variance in fracture toughness properties (i.e., highest adjusted R^2 ; Table 2). As discussed in our previous fracture toughness studies involving the same cohort^{33,36}, sex either did not explain (K_{grow} and J_{int}) or weakly explained (K_{init}) fracture toughness with respect to age, a known predictor of mechanical properties of human bone. Therefore, as before, sex was not included as a covariate in the multiple linear regressions. The goal was to have at least 15 unique donors per explanatory variable (e.g., age, vBMD, and Raman property) or interaction term (age $\times$ Raman property) in the general linear models³⁷.

To verify that the PC loadings did not necessarily depend on the data used for the model, we performed a 10-fold cross-validation. Precisely, the data set ($n=62$ donors) was partitioned into 10 groups (8 groups of 6 donors with 54 spectra and 2 groups of 7 donors with 63 spectra) so that there were no differences in K_{init} between the groups. Of the 10 groups, one group was retained as the validation set for testing the model, and PCA was performed on the remaining 9 groups as the training set. The loadings of this model were used to compute the scores of the spectra in the validation set. The cross-validation process was then repeated 10 times, with each of the 10 groups used exactly once as the validation set.

3. Results

3.1 Correlations with Fracture Toughness

Examining the mean among the 9 sites distributed in the region of the intended crack propagation (Fig. 1b), Type-B carbonate substitution ($\text{CO}_3/\nu_1\text{PO}_4$) was the only selected Raman property that correlated with age (Table 1). In addition, $\text{CO}_3/\nu_1\text{PO}_4$ only correlated with crack growth toughness (K_{grow}) and did so quite weakly (Table 1), even though each fracture toughness property decreased with age (Table 1). Furthermore, the selected mineral-to-matrix ratios did not explain more than 12% of the variance in any of the fracture toughness outcomes reaching statistical significance only for $\nu_1\text{PO}_4/\text{Amide I}$ and $\nu_2\text{PO}_4/\text{Amide III}$ (Table 1). None of the peak ratios within the Amide I band, whether calculated by band fitting (1668/1638 or 1668/1689) or directly as an intensity ratio (1668'/1636' or 1668'/1688'), correlated with age or the fracture toughness properties.

The plot of the cumulative sum of variance (Supplemental Fig. 1) indicated that 7 PCs accounted for ~95% of the total variance in the Raman intensities between 370 cm^{-1} and 1720 cm^{-1} (i.e., 1351 intensities per spectrum could be reduced to 7 uncorrelated spectral

traits). Thus, correlations with mechanical properties were tested for principal component 1 (PC1) through PC7. From this data reduction scheme, only PC5 significantly correlated with all 3 fracture toughness properties (Table 1). Moreover, the correlation coefficient between PC5 and each mechanical property was higher than those between traditional RS properties and the corresponding mechanical property as well as between volumetric bone mineral density (vBMD) and most mechanical properties (with K_{init} being the one exception). PC6 was the only other spectral trait to correlate with a fracture property (Table 1), while PC7 was the only other one to correlate with age.

PC5 derived from 10-fold cross-validation significantly correlated K_{init} , K_{grow} , and J_{int} , (Fig. 3) indicating that the PC correlations (Table 1) were not the result of over-fitting. The loadings of PC1 through PC7 were fairly consistent across the 10 different PCAs (Supplemental Fig. 2).

3.2 Multivariate Explanation of Fracture Toughness

After including age as a covariate, changes in the Amide I band ($1668'/1688'$) became a significant *negative* predictor of crack initiation toughness (Table 2). However, this sub-peak ratio along with age only explained 26.1% of the variance in K_{init} whereas, vBMD and age explained 41.1% of the K_{init} variance. $\nu_1\text{PO}_4/\text{Amide I}$ (Supplemental Table 1), PC5 and PC7 (Table 2) each remained significant predictors of K_{init} when age was included as a covariate, but again the strength of their association with K_{init} was not as high as the association between vBMD and K_{init} (as indicated by the standardized β coefficients across models in Table 2). As with vBMD, $\nu_1\text{PO}_4/\text{Amide I}$ had a positive relationship with K_{init} (Supplemental Table 1). Of the Raman-derived parameters, only $1668'/1688'$ (Supplemental Table 1), PC5 (Table 2), and PC7 (Table 2) significantly explained K_{init} variance when included along with age and vBMD together as covariates.

vBMD did not significantly predict K_{grow} when age was included as covariate. In fact, only PC6 helped age explain the variance of this fracture toughness property. The interaction between PC6 and age was significant indicating that the contribution of PC6 to crack growth toughness changed with age. Related to the energy dissipated during fracture of the notched bone, J_{int} was best explained by age, vBMD, and PC7 again with vBMD providing a positive contribution. Without vBMD included as a covariate, other Raman-derived properties helped age explain the variance in this fracture toughness property, but the composite spectral trait PC7 had the strongest association (Table 2). The sub-peak $1668'/1688'$ also had some predictive ability for J_{int} , but this depended on the age of the donor (Table 2).

4. Discussion

Deriving biochemical signatures from multivariate analysis of the full Raman spectrum may be an effective strategy for the development of RS as a diagnostic tool of fracture risk. Applying PCA to a set of spectra acquired from 62 cadaveric cortical samples yielded several spectral signatures that correlated with the fracture toughness properties of bone. In particular, explaining only 6% of the total variance in the overall Raman spectra, PC5 significantly correlated with crack initiation toughness, crack growth toughness, and overall

energy dissipated during cracking; whereas, standard peak ratios correlated with 1 or 2 fracture toughness properties (Table 1). Moreover, in linear regression models that included known determinants of fracture toughness (age and vBMD), the PCs were significant covariates meaning they added value (Table 2). This is not to say that mineral-to-matrix ratios, carbonate-to-phosphate ratios, or sub-peak ratios of the Amide I are not useful and have no potential as predictors of fracture risk. Rather, incorporating the wealth of information hidden in Raman spectra of bone tissue in prediction algorithms is potentially a valuable strategy for *in vivo* fracture risk assessment.

Unlike other multivariate approaches (e.g., support vector machines, canonical correlation analysis, partial least squares regression), the PC or spectral trait is not trained to correlate with a mechanical property. Thus, over-fitting is less of an issue, but a PCA model needs to be robust (i.e., informed by numerous bone spectra from numerous donors) for PCs to be effective predictors. Increasing the number of donors and the number of spectra acquired would undoubtedly improve the generality of the model. Nonetheless, PC5 significantly correlated with each fracture toughness property in a 10-fold cross-validation (Fig. 3).

The present study is of course not the first to apply PCA to Raman spectra of bone. In the earliest applications of multivariate analysis in RS of bone, Morris and colleagues found that factor analysis of PCs could reveal subtle differences in mineralization within osteonal tissue (human)³⁸ and among fetal and post-natal calvaria (mouse) obtained at different ages³⁹. Applying PCA without ‘z-scoring’ (instead excluded wavenumbers with intensities of less than 100 counts), Goodyear et al.⁴⁰ reported differences in several PCs between cortical and trabecular bone for the femur and tibia of 5-mo. old C57BL6 mice (also reported differences in peak ratios). PCA (‘z-scoring’ not specified) also found differences in bone tissue (mandibular cortex) between sham-operated and ovariectomized rats 8 months after surgery (also reported significant differences in peak ratios)⁴¹. PCA on “mean centered” spectra was applied to two sets of Raman spectra that were acquired from discarded proximal femurs (hip replacement surgery) using different acquisition protocols (point-by-point vs. “macro laser spot” generated by scanning mirrors), and spectral features related to the first principal component (explained 92.5% of total variance in Raman intensities) were similar between the 2 acquisitions⁴².

Recently, when applied to select regions of decomposed Raman spectra that were acquired with *in vivo* SORS (phalangeal bones), PCA revealed a trend of the first component being different between osteoporotic patients on bisphosphonate therapy and controls¹². The application of PCA to Raman spectra from nail fragments of 213 women found no correlation between PCs and areal BMD of the hip and spine⁴³. Without z-scoring though, the loadings of PC1 are similar to the mean Raman spectrum of bone^{40,42} or fingernail⁴³ as the prominent peak intensities dominate the Raman variance even if mean normalized. As shown in the present study, applying z-scoring allows all intensities across the spectrum to influence the PC loadings, and notably the fifth component, not the first, correlated with fracture toughness.

The collection of 9 spectra at a 1 μm spot size is one important limitation of the present study that likely affected our ability to find multiple correlations between Raman peak ratios

(as well as sub-peak ratios) and mechanical properties of bone. Bone composition is heterogeneous due to osteonal remodeling, and the average of 9 spectra may not fully capture the overall bulk composition for each human sample. Moreover, polarization bias (i.e., sensitivity to collagen fibril orientation) is more prominent with higher numerical aperture of the objective^{44,45}. Thus, both the use of a 20× objective and the collection of spectra from a greater number of sites (>9) may have yielded significant correlations between sub-peak ratios of Amide I and the mechanical properties of cortical bone as was done in a previous Raman study involving mechanical testing of cortical bone¹⁷. Also, other methods of locating sub-peak ratios (local maxima instead of minima in the second derivative spectra) may prove useful. Nonetheless, such data acquisition would also be expected to improve the ability of full spectrum analysis to predict mechanical properties.

There are also inherent limitations in cadaver studies, namely the lack of information on lifestyle and medical history of the donors. Thus, we do not know the extent to which alcoholism, cardiovascular disease, osteoporosis, bone altering drugs, diabetes, etc. influenced the observed correlations. Nonetheless, the causes of death for the present cohort were common to the general population suggesting the variance in mechanical properties was influenced by common age-related health issues. To the best of our knowledge, there are no reports of sex-related differences in fracture toughness of cortical bone partly because the analysis of machined samples removes sex-related differences in the thinning of the cortex with aging. As we previously reported in our companion paper involving other analyses of the SENB specimens³⁶, intra-cortical porosity was not dependent on the sex of the donor. Still, moving toward clinical RS assessment of bone, medical history and gender should be considered as underlying factors affecting the ability of multivariate-derived spectral traits to predict fracture resistance.

PC5 loadings exhibit distinct peaks (Fig. 4) in the Amide I band, in the shoulder after the carbonate peak, and at the proline peak; whereas, PC1 loadings were distributed across the range of wavenumbers (Fig. 4). Such characteristics cannot be directly attributed to being determinants of fracture toughness, based on PCA. Nonetheless, previous studies indicate that the collagen network and collagen crosslinking are important to fracture toughness of bone (human, bovine, and murine)^{46–48}. Possibly then, a small change in the Amide I band did not greatly contribute to the overall variance in the Raman spectral intensities, but still helped explain fracture toughness properties (Table 2).

Overall, finding strong correlations between fracture toughness and bone characteristics is rather difficult because there are multiple factors across the length scales of bone's hierarchical organization contributing to fracture toughness properties, especially those derived from a non-linear elastic, R-curve approach. In our companion studies on the fracture toughness of bone, age combined with bound water fraction and pore water fraction (surrogate of porosity) explained 47.0% of the variance in K_{init} ³³ while age combined with local heterogeneity in mineralization (derived from quantitative back-scatter electron images) explained 42.5% of the variance in the same property³⁶. Given that all fractures are the result of cracks coalescing and propagating to failure, a multifactorial diagnostic approach is likely necessary to ultimately predict an individual's true risk of fracture. For example, a disease-related deterioration in trabecular architecture may be increasing the risk

of a vertebral fracture in which case an imaging technique would be useful. Raman spectroscopy may contribute to this comprehensive prediction of fracture risk as it probes bone tissue at the nano-structural level of organization.

5. Conclusions

Being sensitive to collagen orientation, collagen crosslinking, collagen denaturation, mineral relative to the organic matrix, carbonate substitutions, and crystallinity, a multivariate approach to full spectrum analysis has the potential to combine this information into a meaningful signature of bone quality. In the present study, traditional Raman properties either did not or weakly correlated with fracture toughness properties of human cortical bone. Instead, PCA provided spectral traits that helped explained the variance in crack initiation toughness, crack growth toughness, and overall energy dissipated when including age and volumetric bone mineral density as significant covariates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The overall study design. Each bone sample extracted from the lateral quadrant of the femoral mid-shaft was machined into a single-edge notched-beam specimen for fracture toughness testing (**A**). μ CT imaging was used to measure the apparent volumetric bone mineral density ahead of the notch (white box) (**B**). Nine Raman spectra were acquired from the surface in the same region (**B**). R-curve testing was then used to evaluate the fracture resistance in terms of the J-integral as a function of crack extension. Representative curves depict the behavior of high (filled circles) and low (open circles) fracture toughness samples (circles) (**C**).

Figure 2.

Sub-band analysis of Amide I. Identified from the second derivative of the spectrum between 1580 cm^{-1} and 1720 cm^{-1} , the shoulder peaks and inflection points (red X) served as the starting position for 4 sub-bands that were fit to the Amide I band (blue X). The sub-bands could move within a 10 cm^{-1} window ($\pm 5\text{ cm}^{-1}$).

Figure 3.

10-fold cross-validation to validate that PC5 as a potential predictor of fracture toughness. In the linear regression, the PC5 score for each donor was determined from a PCA that excluded the donor plus 5 or 6 other donors depending on the test set.

Figure 4.

Principal component loadings overlaying a Raman spectrum of bone (black). In contrast to the broad loadings of PC1 (blue), the loadings of PC5 (red) exhibited several sharp peaks (denoted by a star symbol) at the Proline peak, a shoulder of the Carbonate band, and a shoulder of the Amide I band.

Table 1

Significant Pearson's correlation coefficients in **bold** and corresponding p-values below (in *italics*) as calculated from bootstrapped data.

Characteristic	Property	Age	K _{init}	K _{grow}	J-int
Age		1	-0.476 (<i>0.0001</i>)	-0.413 (<i>0.003</i>)	-0.357 (<i>0.004</i>)
Bone mineral density	vBMD	- (<i>0.913</i>)	0.460 (<i><0.001</i>)	- (<i>0.245</i>)	0.206 (<i>0.043</i>)
Mineral-to-matrix	ν ₁ PO ₄ /AmideI	- (<i>0.102</i>)	0.341 (<i>0.007</i>)	- (<i>0.215</i>)	0.331 (<i>0.009</i>)
	ν ₁ PO ₄ /Proline	- (<i>0.502</i>)	- (<i>0.308</i>)	- (<i>0.200</i>)	- (<i>0.498</i>)
	ν ₂ PO ₄ /AmideIII	- (<i>0.358</i>)	- (<i>0.185</i>)	- (<i>0.982</i>)	0.290 (<i>0.022</i>)
Carbonate substitution	CO ₃ /ν ₁ PO ₄	0.547 (<i><0.001</i>)	- (<i>0.144</i>)	-0.278 (<i>0.048</i>)	- (<i>0.274</i>)
Crystallinity	1/FWHM(ν ₁ PO ₄)	- (<i>0.341</i>)	- (<i>0.753</i>)	- (<i>0.134</i>)	- (<i>0.611</i>)
Amide I sub-peak ratios ^a	1668/1638	- (<i>0.417</i>)	- (<i>0.743</i>)	- (<i>0.386</i>)	- (<i>0.318</i>)
	1668/1689	- (<i>0.542</i>)	- (<i>0.492</i>)	- (<i>0.731</i>)	- (<i>0.529</i>)
	1668'/1636'	- (<i>0.243</i>)	- (<i>0.870</i>)	- (<i>0.299</i>)	- (<i>0.323</i>)
	1668'/1688'	- (<i>0.061</i>)	- (<i>0.352</i>)	- (<i>0.463</i>)	- (<i>0.284</i>)
Selected spectral traits ^b	PC5	0.447 (<i><0.001</i>)	-0.467 (<i><0.001</i>)	-0.375 (<i>0.007</i>)	-0.428 (<i><0.001</i>)
	PC6	- (<i>0.332</i>)	- (<i>0.501</i>)	-0.348 (<i>0.012</i>)	- (<i>0.561</i>)
	PC7	-0.494 (<i><0.001</i>)	- (<i>0.920</i>)	- (<i>0.491</i>)	- (<i>0.295</i>)

^aFigure 2 provides the locations of these sub-peaks. The ratios with the prime (') were derived from sub-band fitting.

^bPC1 through PC4 did not correlate with age or mechanical properties (p>0.176)

Table 2

Best-fit general linear models showing combinations of parameters (age, volumetric bone mineral density, RS properties, and PCs) that significantly explain the variance in the fracture toughness properties of human cortical bone. All interactions are first entered in the model and successively eliminated when not significant ($p < 0.05$) until only significant covariates remain.

Fracture property	Explanatory variables	Linear models ^a	Adj-R ² (%)
K_{init}	age + vBMD	age ($\beta = -0.47, p < 0.001$)	41.1
	age + RS	age ($\beta = -0.33, p = 0.008$)	28.4
		age ($\beta = -0.64, p < 0.001$)	28.3
	age + vBMD + RS	age ($\beta = -0.35, p = 0.001$)	45.0
		age ($\beta = -0.63, p < 0.001$)	48.5
K_{grow}	age + vBMD	age ($\beta = -0.41, p = 0.003$)	17.1
	age + RS	age ($\beta = -0.23, p = 0.025$)	31.4
	age + vBMD + RS	age*PC6 ($\beta = 0.36, p = 0.008$)	31.4
		Same as above	
J	age + vBMD	age ($\beta = -0.35, p = 0.001$)	13.9
	age + RS	age ($\beta = -0.48, p = 0.034$)	20.2
		age ($\beta = -0.56, p < 0.001$)	22.9
	age + vBMD + RS	age ($\beta = -0.56, p < 0.001$)	25.8
		age*1668'/1688' ($\beta = 0.23, p = 0.043$)	

^a β is the standardized coefficient for each independent covariate in the linear model