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RAMAN AND MECHANICAL PROPERTIES CORRELATE AT WHOLE BONE- AND TISSUE- LEVELS IN A GENETIC MOUSE MODEL

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Abstract

The fracture resistance of bone arises from the composition, orientation, and distribution of the primary constituents at each hierarchical level of organization. Therefore, to establish the relevance of Raman Spectroscopy (RS) in identifying differences between strong or tough bone and weak or brittle bone, we investigated whether Raman-derived properties could explain the variance in biomechanical properties at both the whole bone and the tissue-level, and do so independently of traditional measurements of mineralization. We harvested femurs from wild-type mice and mice lacking matrix metalloproteinase 2 because the mutant mice have a known reduction in mineralization. Next, RS quantified compositional properties directly from the intact diaphysis followed by micro-computed tomography to quantify mineralization density (Ct.TMD). Correlations were then tested for significance between these properties and the biomechanical properties as determined by the three point bending test on the same femurs. Harvested tibia were embedded in plastic, sectioned transversely, and polished in order to acquire average Raman properties per specimen that were then correlated with average nanoindentation properties per specimen. Dividing the ν_1 phosphate by the proline peak intensity provided the strongest correlation between the mineral-to-collagen ratio and the biomechanical properties (whole bone modulus, strength and post-yield deflection plus nanoindentation modulus). Moreover, the linear combination of ν_1 phosphate/proline and Ct.TMD provided the best explanation of the variance in

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CONFLICT OF INTEREST STATEMENT

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strength between the genotypes, and it alone was the best explanatory variable for brittleness. Causal relationships between Raman and fracture resistance need to be investigated, but Raman has the potential to assess fracture risk.

Keywords

Raman; Bone Quality; Nanoindentation; micro-CT; Fracture Risk

INTRODUCTION

Osteoporosis and aging increase the likelihood of bone fracture (Kanis et al., 2001; Lewiecki, 2004), and given the costs and co-morbidity associated with bone fractures, this increase is a major health concern in the US (Ray et al., 1997) as well as other countries (Levy et al., 2002; Lippuner et al., 2005; Sambrook et al., 2002). While low areal bone mineral density (BMD) as determined by dual-energy X-ray absorptiometry (DXA) can be an important risk factor for fracture susceptibility, age alone significantly and independently contributes to fracture risk (Johnell et al., 2005). Complicating the assessment of fracture risk is the fact that there are multiple factors contributing to the structure and quality of bone as reviewed by others (Martin, 1993; Nyman et al., 2005; van der Meulen et al., 2001) and identified in the early mechanical testing studies of cadaveric tissue dating back to the 1950's (Evans and Lebow, 1951), 1960's (Ascenzi and Bonucci, 1967; Sedlin and Hirsch, 1966; Weaver and Chalmers, 1966), and 1970's (Currey, 1979; Evans and Vincentelli, 1974; Smith and Smith, 1976). A survey of recent studies is provided in Table 1. Moreover, the effects of aging and disease on these determinants involve changes in bone composition, organization, and distribution at varying length scales within the hierarchical arrangement of collagen, mineral, and water into bone.

One promising technique for assessing the contribution of composition to fracture resistance is Raman spectroscopy (RS). Raman spectra provide quantitative information on both mineral and organic matrix composition including crystallinity, the degree to which carbonate replaces phosphate ions in the mineral lattice, and the ratio of immature-to-mature collagen crosslinks (albeit the latter is not fully validated); and does so with little interference from water (Carden and Morris, 2000). As such, RS has been used to identify compositional differences in mouse models of bone fragility, namely osteopontin-deficient mice (OPN^{-/-}) vs. wild-type controls (OPN^{+/+}) (Kavukcuoglu et al., 2007) and senescence accelerated aging mice (SAMP6) vs. control mice (SAMR1) (Silva et al., 2006). Additional studies involving both animal and human cadaveric tissue reported the sensitivity of RS to detect age-related changes in the compositional properties in bone (Ager et al., 2005; Akkus et al., 2004; Yerramshetty et al., 2006). In fact, RS has been found to discriminate between bone excised from women with a hip fracture and bone from age-matched women without a fracture (McCreadie et al., 2006). Although most work using RS for the characterization of compositional properties of bone has been done *ex vivo*, recent advances in a fiber optic probe based technique known as spatially offset Raman spectroscopy (SORS) allow for *in vivo* analysis (Matousek et al., 2006; Schulmerich et al., 2008; Schulmerich et al., 2009; Schulmerich et al., 2006).

Prior to the application of SORS to clinical assessment of fracture risk, an intermediate step is to establish the ability of RS to provide quantitative characteristics that relate to the biomechanical properties of bone. Currently, there are only a few studies that investigated correlations between RS and biomechanical properties of bone (Akkus et al., 2004; Yerramshetty and Akkus, 2008), and no studies have related Raman properties to biomechanical properties acquired at two different length scales. Documenting these

relationships advances not only the development of RS for assessing the fracture resistance of bone, but also the more general application of RS for understanding factors that influence bone strength.

We investigated whether RS-derived compositional properties correlated with the biomechanical properties at both whole bone-level and tissue-level using a genetic mouse model with a known compositional phenotype. Specifically, bones were collected from mice lacking matrix metalloproteinase 2 (*Mmp2*^{-/-}) and wild-type mice (*Mmp2*^{+/+}) because *Mmp2*^{-/-} mice have a reduced BMD (Inoue et al., 2006). In addition, MMP-2 is a member of a family of enzymes that degrades a broad range of extracellular matrix components, namely type I collagen, (Krane and Inada, 2008) and so there was a potential that a difference in the matrix, not just mineralization, could be contributing to potential differences in the biomechanical properties.

MATERIALS AND METHODS

Specimen preparation

Nine male and 11 female *Mmp2*^{-/-} mice (n = 20) along with 8 male and 8 female wild-type mice (n = 16), all on an FVB background, were sacrificed at the age of 16 weeks following an IACUC approved protocol. The right femora and tibia were harvested removing the surrounding soft tissue. The femurs were stored in phosphate buffered saline (PBS) at -20 °C prior to analysis; whereas, the tibia were dehydrated in series of increasing concentrations of ethanol and then embedded in polymethylmethacrylate (PMMA) (Erben, 1997).

Raman spectroscopy

Prior to Raman Spectroscopy, the intact mouse femora were thawed and left at room temperature in air for ~30 min. to partially dehydrate the tissue before the acquisition of Raman spectra. The embedded tibiae were sectioned at the mid-shaft using a metallurgical saw. Next, the exposed surface of the cortical bone was ground on successive grits of silicon carbide paper followed by polishing with an alumina suspension on a synthetic cloth. These specimens were analyzed in a dry state.

Raman spectra were then either acquired from the intact diaphysis of the femora or the cross-section of the tibial cortex using a confocal Raman microscope (Renishaw, Ramascope Mark III) with a 785nm laser diode source (Innovative Photonic Solutions). A 50x objective focused the light to a 5 µm spot just below the surface of the bone tissue. The Raman scattered (30 s exposure time) light was collected by a spectrograph with a spectral resolution of 1 cm⁻¹. For each femur, five spectra were collected along the diaphysis of the intact bone and were averaged per bone. For each tibia, 9 spectra were collected from the cortex (3 per side). Background fluorescence in the spectra was subtracted by a modified polynomial fitting algorithm (Lieber and Mahadevan-Jansen, 2003).

Figure 1 shows a representative Raman spectrum from the diaphysis of femur. Peak intensities were recorded for Amide I (1665 cm⁻¹), Amide III (1250 cm⁻¹), B-type carbonate (1070 cm⁻¹), proline (856 cm⁻¹) and the ν₁ phosphate (960 cm⁻¹) using custom developed Matlab program (Matlab, Mathworks). The mineral-to-matrix ratio was calculated based on the intensity ratio of the ν₁ phosphate peak to either the amide I, amide III, or proline peak intensity. The ratio of the carbonate peak to the ν₁ phosphate peak gave the degree of carbonate substitution in the lattice structure of the apatite. Mineral crystallinity was assessed by taking the inverse of the ν₁ phosphate peak width at half-max intensity (ν₁FWHM⁻¹).

Micro-computed tomography (μ CT)

Micro-computed tomography (μ CT) assessed the structure of the diaphysis of the femur. In addition, it provided the mineralization density of the cortical bone tissue (Ct.TMD) in mg of hydroxyapatite (HA) per cubic centimeter since the μ CT scanner is calibrated to known densities of a HA phantom. At an isotropic, 12 μ m voxel size, we scanned (μ CT40, Scanco Medical; Switzerland) the region spanning 1.2 mm at the mid-point of the diaphysis acquiring 500 projections through 180°. After reconstruction, the bone tissue was segmented from air or soft tissue using a threshold of 410 per thousand (or 763.2 mgHA/cm³), a Gaussian noise filter of 0.8, and support of 2. Using the Scanco evaluation software (mid-shaft script), we quantified the average principal moment of inertia for bending about the minor axis ($I_{\min}$) among the cross-sectional slices as well as the average distance between the centroid and the outer most material element in the direction of loading ($c_{\min}$). These structural indices coincide with axis of bending in the three-point (3pt) bending test and are used to estimate the material properties of bone.

Biomechanical testing

Whole bone testing provided estimates of strength and toughness, which are directly related to the risk of fracture. In genetic mouse models, flexural testing of the long bones is commonly used to calculate the biomechanical properties of whole bones (Schrieffer et al., 2005). Using the Dynamight 8841 (Instron, Canton, OH), a servo-hydraulic, bench-top material testing system, the 3pt bending tests were performed on the same right femurs that were analyzed by Raman spectroscopy and μ CT. Following 2 hours of thawing in physiologic saline (PBS), all bones were tested under hydrated conditions. Each bone was placed on the lower support points of a 3pt bend fixture with the anterior side down (*i.e.*, bending about the medial-lateral plane). The span between the lower supports was 7 mm, and the load rate was 3.0 mm/min. Force from an appropriately sized load cell (100 N) and displacement from the LVDT were recorded at 20 Hz during the load-to-failure test.

The estimated material properties – bending modulus (E) and bending strength (σ_b) – were determined for the mid-diaphysis of the femur using beam theory that accounts for the effect of structural geometry on force and displacement values (Turner and Burr, 1993). To characterize brittleness of the bone, we also measured the post-yield deflection (PYD), which was the displacement at failure minus the displacement at yielding (Fig 2).

Nanoindentation

We also compared the RS-derived properties to nanoindentation-derived properties as determined for the tibial cortex to demonstrate whether Raman can explain potential differences in biomechanical properties at the tissue-level. The same specimens of dry bone analyzed by RS were subsequently mounted in a nanoindentation system (MTS XP). A Berkovich diamond tip (a pyramidal-shaped indenter with a center-line-to-face angle of 65.3 degrees) was then driven into the surface of the bone section using the following scheme: 1) load at a constant loading rate chosen to reach a maximum depth of 1 μ m in 10 s, 2) hold at $P_{\max}$ for 10 s (in order to minimize the effects of viscoelastic deformation), 3) unload at the same rate used in the first loading step to 10% of $P_{\max}$, 4) hold the indenter on the surface for 60 s in order to establish thermal drift, and 5) completely remove the indenter from the bone surface. Recording the slope (S) from the initial (upper) portion of the unloading force-displacement curve, the peak force ($P_{\max}$), and the peak displacement ($h_{\max}$), the nanoindentation modulus (E_{ni}) and hardness (H_{ni}) of the tissue at the point of indentation (0.25 μ m resolution) were calculated per the method described by Oliver and Pharr (Oliver and Pharr, 2004; Oliver and Pharr, 1992). Nine indents were performed throughout the cortex; and as with RS analysis, the average values per specimen were used in the statistical analysis.

Statistical Analysis—Pearson product moment correlation was performed to determine whether the compositional parameters (Raman and μ CT) were correlated with whole bone biomechanical properties (Table 2). Backward stepwise regression was applied to each biomechanical property with $\nu_1\text{PO}_4/\text{Proline}$, $\text{Carb}/\nu_1\text{PO}_4$, $\nu_1\text{FWHM}^{-1}$, and Ct.TMD as the initial explanatory variables in order to determine whether the Raman properties could provide independent and significant contributions to the biomechanical properties with respect μ CT-derived BMD. Note that of the mineral-to-collagen ratio calculations $\nu_1\text{PO}_4/\text{Proline}$ provided the strongest correlation with strength. Interaction terms were not included, and so initially, the explanatory variable with the lowest contribution (highest p-value) to the explanation in the variance of a given biomechanical property was removed. This was repeated until all variables in the model were statistically significant. The Pearson analysis was repeated to determine whether the RS properties correlated with the nanoindentation properties (Table 4). All statistical analyses were performed using SigmaStat V3.5 (Systat Software, San Jose, CA). Statistical significance was considered at a p-value less than 0.05.

RESULTS

Whole bone-level

All 3 Raman peak ratios quantifying the mineral-to-matrix ratio were directly proportional to the bending modulus and strength of the whole femur, but the traditional calculation of ν_1 phosphate peak intensity divided by the Amide I peak intensity provided the weakest correlation (Table 1). The μ CT-derived measurement of mineralization density (Ct.TMD) explained the variance in strength to greater extent than did $\nu_1\text{Phos}/\text{Proline}$, the Raman measurement having the strongest correlations with the biomechanical properties of bone. All the measurements of mineralization were inversely correlated with post-yield deflection, a measure of brittleness, with $\nu_1\text{Phos}/\text{Proline}$ having the strongest correlation with PYD. As expected, the mineral-to-matrix ratios all correlated with Ct.TMD, but the association between $\nu_1\text{Phos}/\text{Amide I}$ and Ct.TMD was the weakest (Table 2). The correlations between Raman-derived crystallinity and the biomechanical properties of bone followed the same trends as did the mineral-to-matrix measurements; whereas the measurement of the substitutions of carbonate for phosphate ($\text{Carb}/\nu_1\text{PO}_4$) in the mineral did not correlate with any of the biomechanical properties (Table 2).

Even though Ct.TMD had the strongest correlation with strength, the stepwise regression indicated that $\nu_1\text{Phos}/\text{Proline}$ and Ct.TMD are both independent and significant contributors to strength (Table 3). Carbonate substitutions and crystallinity were not. That is, the linear combination of the mineral-to-matrix ratio and mineralization density explained 77.1% (adjusted R^2) of the variance in whole bone bending strength, whereas, Ct.TMD alone explained 75.0% of the variance and $\nu_1\text{Phos}/\text{Proline}$ alone explained 65.6% (Fig. 3). Even though bone mineral density has a strong correlation with modulus, two Raman properties, $\nu_1\text{PO}_4/\text{Proline}$ and $\nu_1\text{FWHM}^{-1}$ accounted for more of the difference in the bone strength between the genotypes than did the other compositional properties, according to the stepwise regression (Table 3). Lastly, of all the compositional properties included into the statistical model – $\nu_1\text{PO}_4/\text{Proline}$, $\text{Carb}/\nu_1\text{PO}_4$, $\nu_1\text{FWHM}^{-1}$, and Ct.TMD – the mineral-to-matrix ratio alone accounted for the greatest amount of the variance in PYD.

Tissue-level

As was the case for the biomechanical properties at the whole bone-level, the Raman-derived measurements correlated with the modulus and hardness at the tissue-level. Specifically, $\nu_1\text{PO}_4/\text{Proline}$ and $\nu_1\text{PO}_4/\text{Amide III}$ were directly correlated with nanoindentation modulus (E_{ni}) and hardness (H_{ni}); whereas, $\text{Carb}/\nu_1\text{PO}_4$ was inversely correlated with these properties. Interestingly, the traditional method of calculating the

mineral-to-matrix ratio as $\nu_1\text{PO}_4/\text{Amide I}$ did not correlate with the tissue-level properties. Neither did the Raman measurement of crystallinity.

DISCUSSION

Aside from the recent introduction of the *in vivo* microindentation test of human bone (Diez-Perez et al.), traditional measurements of the biomechanical properties of bone is a destructive process, and thus, surrogates – such as DXA or peripheral QCT (pQCT) – are necessary to provide quantitative characteristics related to fracture resistance. Even though the direct relationships between DXA-derived areal BMD and strength (Stromsoe et al., 1995) and between pQCT-derived structure and strength (Alho et al., 1988) are well established, these diagnostic tools do not necessarily identify individuals at a high risk of fracture. This is partly due to the fact that a fracture event is not entirely explained by low bone strength as failure also involves toughening mechanisms (i.e., dissipation of energy) to which the plastic nature of the tissue (i.e., collagen) is important. Therefore, to successfully advance toward a more complete assessment of fracture risk, new diagnostic tools need to provide quantitative characteristics of mineral and collagen that relate to both bone strength and toughness. Presently, Raman measurements acquired from intact, albeit excised, mouse femurs correlated with whole bone strength and post-yield deflection, the latter being a measure of bone brittleness. Moreover, in relation to μCT -derived BMD, certain RS properties were independent explanatory variables of the variance in these biomechanical properties of bone.

The known increase in strength with an increase in mineralization (Keller et al., 1990) was observed in the present application of RS to the bones of a genetic mouse model in which phenotypic differences for the *Mmp2*^{-/-} mice included both reduced BMD and bone strength. In effect, the reduction in both mineral-to-collagen ratio and mineralization density with the loss of the MMP-2 explained, for the most part, the lower whole bone bending strength for the *Mmp2*^{-/-} mice relative to the wild-type mice (Table 2 and Fig. 3). In humans, Raman could conceivably detect a low mineral-to-collagen ratio as an indicator of low bone strength since the causal relation has been established in demineralization experiments (Broz et al., 1995; Shah et al., 1995). Nonetheless, in a prior application of RS to bones from aging rats, there was an inverse relationship between the mineral-to-collagen ratio, as measured by $\nu_1\text{Phos}/\text{Amide I}$ from tissue sections, and bending strength, as determined by 3pt bending of intact femurs and an estimate of the moment of inertia from a stained cross-section of the bone (Akkus et al., 2004). The age-related decrease in bone strength appeared to occur in spite of the age-related increase in the mineral-to-collagen ratio suggesting that other changes in the bone matrix affected the strength in this rodent model. For example, there were also age-related changes in crystallinity and type-B carbonate content.

To date, causal relationships have not been established for the effect of mineral structure and maturity on bone strength, but there are suppositions that mineral maturation (more crystalline) increases microstrains within the matrix (Baig et al., 1999) and that the creation of vacancies upon ion substitutions (carbonate) for phosphate groups also unfavorably affects local strain environment in the tissue (Akkus et al., 2004). Nonetheless, in the present study with only one age group, $\text{Carb}/\nu_1\text{PO}_4$ did not correlate with the biomechanical properties, and $\nu_1\text{FWHM}^{-1}$, the measure of crystallinity, was actually directly or positively related to bending strength and modulus. Thus, the interpretation of the correlations between RS and biomechanical properties of bone should be understood in the context of the factors affecting the bone tissue (e.g., aging in rats vs. deletion of a gene in mice).

There was an inverse relationship between the mineral-to-collagen ratio and post-yield deflection because the weaker *Mmp2*^{-/-} femurs with the lower Ct.TMD elongated before breaking to a greater extent than did wild-type femurs. This fits the general understanding of how mineralization influences the contribution of collagen to deformation in that decreasing the mineralized volume fraction increases the failure strain of bone (Broz et al., 1995). In another context, the brittle bones of the SAMP6 mice had a lower collagen-to-mineral ratio (νCH_3 area/ $\nu_1\text{PO}_4$ area) than the appropriate control SAMR1 (Silva et al., 2006), thus suggesting that Raman can also detect differences in collagen that relate to the toughness of bone. Interestingly, the inverse relationship between crystallinity and PYD in the present mouse model is similar to the observed inverse relationship of mean crystallinity, as determined by RS, and the fracture strain of human cortical bone (Yerramshetty and Akkus, 2008). Unlike the causal connection between collagen and bone toughness (Jonas et al., 1993; Mitchell et al., 2004; Wang et al., 2001), exactly how crystallinity influences bone brittleness is unknown, though presumably the maturation of the crystal affects conformation of the collagen molecules and fibrils.

The RS properties such as $\nu_1\text{PO}_4$ /Proline approach the predictive ability of μCT for bone strength (Fig. 3) and exceed that of μCT for brittleness (Table 1). Relative to mechanical testing though, the application of Raman to bone is relatively new with studies first appearing in 1990 (Walters et al., 1990) and 1995 (Rehman et al., 1995). Thus, there is still considerable potential to refine the experimental methods and analysis techniques used in RS to assess specific aspects of bone integrity in a non-destructive manner. For example, future work could validate the quantification of the immature-to-mature collagen crosslink ratio using sub-peaks in the Amide I band, as has been done for FTIRM (Paschalis et al., 2001) or analyze the polarization sensitivity of collagen peaks like Amide I to determine the orientation of collagen fibrils, thus potentially providing additional factors to incorporate into a more developed RS based optical model for understanding differences in the mechanical properties of bone. Moreover, optimizing the collection of the inelastic light from bone would increase the signal-to-noise ratio of the collagen peaks. In addition, this would assist in resolving overlapping bands in the spectra, such as carbonate and ν_3 phosphate (Awonusi et al., 2007), thereby increasing the precision to which Raman can assess compositional properties.

In addition to the ability of RS to help explain differences in the biomechanical properties of the whole bone between the genotypes (Fig. 3), RS also explained the differences in the tissue-level modulus and hardness (Table 4) as determined by nanoindentation. However, these correlations were not as strong as those for the whole bone properties. There are several possible explanations for the weak correlations at the tissue-level: 1) the sites of the Raman measurements were not co-localized with sites of the nanoindentation measurements; 2) the embedding media can influence the Raman spectra (Yeni et al., 2006), and it likely adds noise to the spectra, thereby increasing the variance of the peak ratio calculations; 3) a certain degree of heterogeneity exists at the tissue-level, and perhaps the number of sites was not sufficient to capture the true mean of each property for a given bone specimen.

In another context, Donnelley and colleagues (Donnelly et al., 2010) found stronger tissue-level correlations than the present study between E_{ni} and $\nu_1\text{PO}_4$ /Amide I ($r_{xy}=0.735$) and between H_{ni} and Carb/ $\nu_1\text{PO}_4$ ($r_{xy}=0.787$). In this particular study, the animals were administered chelating fluorochromes to label mineralization fronts, and thus, regions of known tissue age could be distinguished in cross-sections of bone post-mortem. With this method, the range in the mineral-to-collagen ratio (2 – 14) and nanoindentation modulus (5 GPa – 35 GPa) of bone tissue from rats was substantially wider than that of the phenotypic differences between *Mmp2*^{-/-} and wild-type mice. In both studies, lower mineral-to-

collagen explained the lower tissue-level modulus and hardness. There was a discrepancy however in the relationships between nanoindentation properties and the type B carbonate content. As previously mentioned, context is important to the interpretation of how RS-derived properties relate to mechanical properties of bone. In all likelihood, the differences in the mineral-to-collagen ratio dictated the differences in E_{ni} and H_{ni} between young rat bone tissue and aged rat bone tissue as well as between *Mmp2*^{-/-} bone tissue and wild-type bone tissue. Nonetheless, strong correlations are not expected since tissue modulus is not entirely dependent on mineralization status with collagen fibril orientation being a contributor as well (Hofmann et al., 2006).

In a genetic mouse model, Raman spectroscopy provided quantitative characteristics that helped explain differences in biomechanical properties acquired at the whole bone and tissue-level. Moreover, the RS-derived mineral-to-matrix ratio was not necessarily a surrogate to mineralization density, as determined by μ CT. This Raman property was an independent and significant explanatory variable of the differences for both whole bone strength and post-yield deflection between the genotypes. Thus, RS has the potential of being developed into a clinical tool for assessing fracture risk. However, there are several areas of research that would further establish it as a unique diagnostic tool: 1) inclusion of non-mineral properties through validated quantification of collagen content and crosslinking status, two important determinants of bone toughness and 2) mechanistic understanding of how Raman properties such as crystallinity affect the mechanical properties of bone, especially in relation to the aging effects on bone tissue. There is considerable amount of information in the Raman spectra of bone (Fig. 1), and to date, only a few peak ratios and areas have been explored. At this point, Amide I is not recommended in the calculation of the mineral-to-collagen ratio due not only to the low correlation between $\nu_1\text{PO}_4/\text{Amide I}$ and strength (Table 2) but also to the fact that Amide I intensity depends on collagen orientation (Kazanci et al., 2006; Kazanci et al., 2007) (i.e., $\nu_1\text{PO}_4/\text{Amide I}$ is not a pure compositional measurement). In its current application, RS provides possible explanations for differences in bone strength and toughness. Ultimately, this work furthers the understanding of the relationship between the compositional properties of bone measured by RS and the biomechanical properties of bone, and is an important step to realizing the potential for non-invasive RS as a tool for the assessment of fracture risk in patients.

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Figure 1.

A representative Raman spectrum collected from intact bone. Peaks calculated in the present study were labeled as Amide I (1665 cm⁻¹), B-type carbonate (1070 cm⁻¹), proline (856 cm⁻¹) and the phosphate v₁ (960 cm⁻¹) and v₄ (588 cm⁻¹).

Figure 2. Force-displacement curve from three-point bending biomechanical test. Stiffness (δ), ultimate load (P_u), the yield load (P_y), the post-yield deflection (PYD), and work-to-fracture (W_f) generated by the testing are labeled on the graph.

Figure 3.

Both mineral-to-collagen (left) and mineralization density (right) explain the variance in bending strength of mouse bones with lower strength of the MMP-2 deficient being strongly related to a reduction in bone density. Although the relationship between strength and mineral-to-collagen ratio may be different between the genotypes, Raman can identify weak bone from strong bone.

Table 1

A number of age-related changes in bone tissue at different length scales relate to a decrease in fracture resistance

Length Scale (μm)	Bone tissue characteristic		Material Property	Reference
Bulk	Hypermineralization	↑	Impact strength	↓ (Currey et al., 1996)
100	Osteons per area	↑	Tensile strength	↓ (Evans, 1976)
100	Percentage osteons	↑	Resist. to crack prop.	↓ (Yeni et al., 1997)
10-50	Porosity	↑	Post-yield toughness	↓ (McCalden et al., 1993)
10-50	Porosity	↑	Resist. to crack prop.	↓ (Yeni et al., 1998)
10-150	Porosity	↑	Crack propagation toughness	↓ (Nalla et al., 2004)
1-5	Microdamage	↑	Work-to-fracture	↓ (Zioupou, 2001)
1-5	Microdamage	↑	Resist. to crack prop.	↓ (Norman et al., 1998)
0.1	Collagen integrity	↓	Toughness	↓ (Zioupou et al., 1999)
0.1	Collagen crosslinks	↑	Fracture toughness	↓ (Wang et al., 2002)
0.1	Collagen content	↓	Post-yield energy diss.	↓ (Nyman et al., 2007)

There were statistically significant correlations ($p < 0.05$ for each r_{xy} value) between the Raman and whole-bone mechanical properties (femur). The highest correlation coefficient for each compositional vs. mechanical property comparison is bolded

Table 2

Description	Property	Modulus	Strength	PYD	Ct.TMD
Mineral-to-matrix ratio	$\nu_1\text{PO}_4/\text{Proline}$	0.827	0.810	- 0.756	0.810
	$\nu_1\text{PO}_4/\text{Amide III}$	0.747	0.706	-0.718	0.700
	$\nu_1\text{PO}_4/\text{Amide I}$	0.496	0.360	-0.494	0.342
Type B carbonate substitution	$\text{Carb}/\nu_1\text{PO}_4$	N.S.	N.S.	N.S.	N.S.
Crystallinity	$\nu_1\text{FWHM}^{-1}$	0.801	0.630	-0.668	0.674
Mineralization density	Ct.TMD	0.747	0.866	-0.692	1

Table 3

The best linear combination of explanatory variables (Raman- and μ CT-derived) for selected biomechanical properties of whole bone were determined from stepwise regression that gave the highest R^2 value with only statistically significant coefficients (a_i)

	Intercept ^a		$\nu_1\text{PO}_4/\text{Proline}$		$\nu_1\text{FWHM}^{-1}$		Ct.TMD	
	a_0	p-value	a_1	p-value	a_2	p-value	a_3	R^2_{adj}
Strength	-371	<0.001	5.35	0.029	NI	NI	0.33	<0.001 0.771
Modulus	-49.1	<0.001	0.623	<0.001	1030	0.003	NI	NI 0.736
PYD	1.247	<0.001	-0.0548	<0.001	NI	NI	NI	NI 0.558

^aRepresents an unmeasured variable.

^bExplanatory variables were not included (NI) when their contribution (i.e., coefficient) was not significant ($p>0.05$).

Table 4

There were statistically significant correlations ($p < 0.05$ for each r_{xy} value) between the Raman and tissue-level mechanical properties (tibia). The highest correlation coefficient for each compositional vs. mechanical property comparison is bolded

Description	Property	E_{ni}	H_{ni}
Mineral-to-matrix ratio	$\nu_1\text{PO}_4/\text{Proline}$	0.565	0.567
	$\nu_1\text{PO}_4/\text{Amide III}$	0.530	0.535
	$\nu_1\text{PO}_4/\text{Amide I}$	N.S.	N.S.
Type B carbonate substitution	Carb/ $\nu_1\text{PO}_4$	-0.365	-0.365
Crystallinity	$\nu_1\text{FWHM}^{-1}$	N.S.	N.S.