Effect of Local Tissue-Aging on Water Compartments of Human Cortical Bone *via* Raman Spectroscopy

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INTRODUCTION: ~20% of cortical bone volume is composed of water, which can be divided into structural (molecular) water, tightly bound water, partially bound water, and free water compartments [1]. Both age-related increases in free water and decreases in bound water play a significant role in reduction of bone strength and toughness [1]. Furthermore, water can be bound with the mineral phase or organic matrix (*i.e.*, collagen and non-collagenous proteins) or at the interface. At the ultrastructural level, AFM [2] and *in silico* studies [3] have identified that interfacial mechanical behavior between the phases is heavily dependent on bound water content in bone. Despite the significance of water on bone mechanical behavior, there is little information on local changes in water content associated with mineral or organic constituents as well as underlying ultrastructural mechanisms leading to those changes observed in bulk tissue. However, current methods to investigate water content in bone (*i.e.*, MRI, NMR) are limited for differentiating local microheterogeneity and for differentiating water content bound to mineral and collagen. To this end, the purpose of our study was to develop Raman spectroscopy techniques in order to investigate the tissue age-related changes in mineral and collagen water compartments of bone with concurrent Raman properties of other bone constituents.

METHODS: Cortical bone cubes (~300mg) were prepared from the diaphysis of 6 middle-aged, female cadaveric femurs. The bone samples underwent a sequential dehydration process where corresponding weights and Raman spectra were obtained from the samples for the following conditions: 1) wet, 2) oven-dried at 35°C for 48hrs, and 3) ethanol treatment + vacuum dried for 48hrs. For visualization, one sample was further treated with d_2O for 48hr to remove all bound water. Gravimetric water loss was calculated as shown: $\%\,Water\,Weight\,loss = 100(Wwet - W_{dry})/Wwet$. Raman spectra (800-4000 cm^{-1}) were taken from 6 secondary osteons and 6 interstitial tissues in the bone sample from each donor using a 532nm laser. The spectra for each tissue type were averaged, baselined with a 7th order linear baseline, and smoothed with a 2nd order Savitzky-Golay polynomial filter. Peak intensities were collected from mineral (PO_4 at 960 cm^{-1}) and organic phase (CH_2 at 2940 cm^{-1}) using a custom MatLAB script. Furthermore, the intensities of the collagen and mineral-associated water peaks (3277 and 3567cm^{-1}, respectively) were extracted from the OH stretching peak in terms of 1) peak intensities and 2) deconvolution in LabSpec6 software. Given that the Raman peaks of both mineral and organic phases used for normalization were also changing with tissue age, mineral and organic water were also evaluated *via* normalized intensity (ex: $3277_{sample}/3277_{mean}$). Other Raman properties of bone (collagen helical status, matrix maturity, mineral/matrix ratio, mineral crystallinity, glycosaminoglycan (GAG)/mineral content, and hydroxyproline/proline ratio) were taken to investigate concurrent changes with those in water compartments. Raman water measurements were bulk corrected ($Bulk\,Raman\,Water = \%Int * Int\,Raman + \%Osteon * Osteon\,Raman$) and correlated with volumetric changes in water (assessed with gravimetric % water weight loss) *via* Pearson regression. Paired-*t* tests were used to identify differences between tissue sites at 95% significance. Then, water compartments were correlated with Raman properties of bone using Pearson Regression.

RESULTS: Based on **Tables 1 & 2**, both the 3277/mineral and 3277/mean water were significantly correlated with bulk water loss. Furthermore, the normalized 3277 (bound and structural water/mineral) was significantly reduced with tissue age and negatively correlated with mineral/matrix ratio. The intensity of 3567 (mineral bound and structural water) was similarly reduced with tissue age, although not correlated with bulk tissue water. No other changes in water compartments were occurring with tissue aging. However, correlation analysis in **Table 2** reveals collagen helical status and matrix maturity to be highly associated with most water compartments, especially loosely bound water.

DISCUSSION: The 3277/mineral water may be the most suitable peak for representing volumetric changes in water content in local bone tissues since it was most correlated with bulk gravimetric water loss. This peak was also strongly associated with the ratio of organic to mineral content (*i.e.*, mineral/matrix), suggesting that volumetric changes in bound and structural water may be primarily due to tissue age-induced changes in the volume of each constituent. On the other hand, tissue-aged differences of the 3567 peak were not correlated with bulk tissue water. This may be because the 3277 is the predominant water lost during the dehydration process, whereas the current setup may not be sensitive enough to detect small changes in mineral-associated water. Furthermore, another reason for this is probably that 3567 peak may mostly represent tightly bound or structural water, which would be difficult to remove using our sequential dehydration process. Independent of tissue age, most of the loosely and tightly bound water compartments were highly correlated with the quality of organic material (*i.e.*, collagen helical status, matrix maturity). This suggests that loss of bound water in local bone tissue may stem from the quality of organic material coupled with changes in the amount of organic material relative to mineral phase. Lastly, 3277 total water was negatively associated with mineral/matrix ratio, further supporting that organic associated water is dependent on organic content in bone.

SIGNIFICANCE/CLINICAL RELEVENCE: Despite water compartments playing a critical role on bone toughness, there is little information of underlying mechanisms resulting in adverse changes to water content occurring during aging. This study developed and verified the efficacy of a non-destructive, Raman spectroscopy method to detect the local, tissue age-related changes in the different water compartments and concurrent associations with other bone constituents that may lead to bulk tissue impairment with age.

Table 1. Effect of local tissue age on water compartments of bone and correlation between peaks with bulk gravimetric water loss

Tissue age changes of water compartments N=6/group		Total Water	Loosely Bound water	Tightly Bound and Structural Water	Bulk corrected correlation with gravimetric water loss
Mineral water	3567/mineral	NS	NS	Reduced with tissue age (p = 0.046)	NS
	3567/organic	NS	NS	NS	NS
	3567/mean	NS	NS	NS	NS
Organic water	3277/mineral	NS	NS	Reduced with tissue age (p = 0.009)	R= 0.72, p <0.001
	3277/organic	NS	NS P=0.07	NS	NS
	3277/mean	NS	NS	NS	R= 0.70, p =0.001

Table 2. Correlation between water compartments and Raman properties of bone

		Total Water	Loosely Bound water	Tightly Bound and Structural Water
Mineral water	3567/mineral	• Collagen helical status (R=0.77, p<0.01) • Matrix maturity (R=0.69, p<0.01)	• Collagen helical status (R=0.89, p<0.01) • Matrix maturity (R=0.88, p<0.01)	• Collagen helical status (R=0.78, p<0.01) • Matrix maturity (R=0.71, p<0.01)
	3567/organic	NS	• Collagen helical status (R=0.82, p<0.01) • Matrix maturity (R=0.87, p<0.01)	• Collagen helical status (R=0.67, p<0.05) • Matrix maturity (R=0.60, p<0.05)
	3567/mean	NS	• Collagen helical status (R=0.90, p<0.01) • Matrix maturity (R=0.89, p<0.01)	• Collagen helical status (R=0.71, p<0.01) • Matrix maturity (R=0.68, p<0.01)
Organic water	3277/mineral	NS	• Collagen helical status (R=0.72, p<0.01) • Matrix maturity (R=0.60, p<0.05)	• Mineral/Matrix (R= -0.78, p<0.01) • GAG/mineral (R= 0.71, p<0.01)
	3277/organic	Mineral/Matrix (R= -0.63, p<0.05)	• Collagen helical status (R=0.82, p<0.01) • Matrix maturity (R=0.83, p<0.01) • GAG/mineral (R= -0.70, p<0.05)	NS
	3277/mean	NS	• Collagen helical status (R=0.82, p<0.01) • Matrix maturity (R= 0.72, p<0.05)	• Collagen helical status (R=0.61, p<0.05)

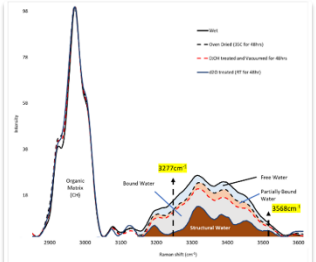

Figure 1. Sequential dehydration reveals different water compartments of bone for the 3277 (organic) and 3567 (mineral) associated peaks.