The Phosphate/Amide I ratio is Reduced by *in vitro* Glycation and may Correlate with Fracture Toughness

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Introduction: Advanced glycation end products (AGEs) form when reducing sugars react with proteins. In bone AGEs can form in type I collagen which results in non-enzymatically derived crosslinks. While enzymatic crosslinks play an important role in strengthening the collagen matrix, non-enzymatic crosslinks are believed to reduce toughness. AGEs accumulate in bone over time and play an important role in reducing bone quality particularly in aging and diabetic patients who accumulate AGEs more rapidly due to increases in circulating glucose. Non-enzymatic glycation of bone can be modeled experimentally by soaking samples in a sugar solution which allows decades worth of AGE accumulation to occur in a short time. AGEs are primarily measured using fluorescence measurements or high performance liquid chromatography (HPLC). Spectroscopic techniques have been developed to determine enzymatic crosslinking maturity by detecting perturbations in collagen structure in the Amide I region and it may be possible to detect similar changes caused by AGEs. We hypothesized that the formation of AGEs in collagen would perturb the Amide I band of Raman spectra causing changes to the mineral to matrix ratio (MMR) which would correlate with AGE-induced mechanical changes in an *in vitro* ribose soaking experiment. If changes due to non-enzymatic glycation can be detected in the Amide I band, Raman spectroscopic techniques could be developed to assess the presence of AGEs in a non-destructive and widely available manner.

Methods: Five femurs were harvested from male hounds from a previous IACUC approved study. From the mid-diaphysis, six beams ~1.4 x 4 x 24 mm were sectioned from each bone. Two beams from each sample were randomly assigned to one of three groups. One of those beams was sanded to 1.4 x 2 x 20 mm for fracture toughness testing while the other was used for Raman spectroscopy and Reference Point Indentation (RPI). All beams were soaked for 14 consecutive days at 37°C in solutions containing 1% Pen-Strep, 1.3mM $CaCl_2$ and either no ribose (Control), 0.2M ribose (Low), or 0.6M ribose (High) in Hank's Balanced Salt Solution with solutions changed every other day. After soaking, a notch was started in the sanded beam with a diamond wire sectioning saw and then sharpened by hand with a razor using a 1μm diamond suspension. Notched beams were submerged in fluid and loaded in displacement control to 0.03mm, unloaded to 0.015mm, held for 10s, then cycled until failure with a 0.05mm load, a 0.02 unload, and a 10s hold. J-R curves were calculated using ASTM E1820-5a to obtain initiation stress intensity factor (K_{Ic}) and maximum stress intensity factor (K_{max}). Raman spectra were acquired at five points along the length of the second beam using a LabRAM HR 800 with a 660nm laser focused to a spot size of ~10μm. After baseline correction, OriginPro 8.6 was used to calculate MMR as the area of the PO_4^{3-} ν1 peak over the area of the Amide I band. Following Raman spectroscopy, co-localized RPI was performed at each Raman location using 10 cycles of a 5N force at 2Hz. One-way ANOVA tested mean differences between samples. Pearson product-moment correlation coefficients were calculated between MMR and parameters from RPI and fracture toughness. All values are presented as mean ± standard deviation and all statistics were carried out using SAS 9.4.

Results: Raman spectroscopy and RPI were not performed on one sample from the Low group. Data were not available for one Control sample and K_{max} was excluded for one High sample. Neither K_{Ic} nor K_{max} were significantly different between groups (Control: 6.59 ± 0.42, 13.55 ± 1.38 MPa√m; Low: 6.19 ± 1.98, 14.80 ± 2.00 MPa√m; High: 6.84 ± 1.18, 15.25 ± 2.35 MPa√m). MMR was significantly different between groups (p=0.039). Tukey HSD post-hoc tests revealed that Control (2.45 ± 0.37) was significantly greater than High (1.85 ± 0.20) while Low was intermediate (2.18 ± 0.37) but not significantly different. No significant differences were observed with RPI. A weak positive correlation was observed between average creep indentation increase (CID) and MMR (R^2=0.079, p=0.0185) but no other RPI measurements were correlated with MMR. Two influential points, determined by a Cook's distance > 4/n, were excluded from the regression K_{Ic} to MMR. A mild trend was observed between K_{Ic} and MMR but the fit did not reach significance (R^2=0.334, p=0.0628).

Discussion: Because samples were all from the same 5 animals and randomized into groups, any differences between groups arose from the soaking in solutions of different concentrations of ribose. AGEs were not measured to confirm the expected dose-dependent increase, but noticeable browning occurred in the High group which was less pronounced in the Low group and not present in Control. The soaking protocol and ribose concentrations were chosen based on previous literature showing increases in AGEs. Therefore, we are confident changes noted here are due to the presence of AGEs and the resulting non-enzymatic crosslinks. Because soaking was performed in appropriately buffered solutions, decreased MMR in the High group relative to Control are expected to occur due to glycation of collagen rather than changes in mineral content. We suspect that perturbations in collagen structure due to the presence of non-enzymatic crosslinks are causing the differences in the area of the Amide I band between groups. Given the changes in MMR with glycation, future studies investigating models where AGEs are likely present should be cautious in their interpretation of MMR if it is not supported by other measures of mineralization. The lack of significant differences between groups for RPI and fracture toughness parameters may be due to the small sample size (n=4-5 per group) and biological variations associated with mechanical techniques. However, the sample size was adequate to assess correlations between Raman and RPI due to the co-localized measurements in each sample (n=70). The positive correlation between CID and MMR was expected given AGEs have been shown to reduce creep behavior and since MMR is decreased by AGEs. However, the correlation is weak which is likely due to the overall small non-significant effect in CID compared to its variation. The correlation between MMR and initiation toughness similarly suggests that as AGEs reduce MMR, K_{Ic} decreases which is known to occur with glycation. While the correlation did not reach significance (p=0.063), the trend is compelling given the small sample size (n=11) and the use of Raman data from an adjacent beam from the same sample rather than the beam used to measure K_{Ic}. In conclusion, MMR changes in response to *in vitro* glycation and these changes are correlated to CID and possibly to K_{Ic}. Deconvolution of the Amide I region into sub-peaks to determine which peak(s) are altered in the presence of AGEs is an important next step to developing a spectroscopic technique that can assess the presence of AGEs and is recommended in future work.

Significance: Correlations were performed between Raman spectroscopy, Reference Point Indentation, and fracture toughness measurements to evaluate the ability of perturbations in the Amide I band to explain glycation-induced changes in tissue mechanics. Non-enzymatic glycation is an important determinant of bone quality especially in aging and diabetic patients and understanding the specific roles composition and microscale mechanics play in determining how non-enzymatic glycation affects fracture toughness may lead to new therapeutic targets.