Compositional Differences Among Undamaged, Strained, and Failed Regions of Bone Using Raman Spectroscopy

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Introduction: Understanding compositional changes that occur when bone fails may help predict fracture risk. Compositional differences that arise among failed, strained, and undamaged regions of bone can be determined using Raman spectroscopy and double-notch specimens. Double-notch specimens were first introduced to biomechanics in order to determine whether fracture in bone is strain- or stress-controlled [1,2]. A double-notch specimen is a rectangular beam that has identical, rounded notches milled equidistant from each end. When subjected to a four-point bend test, maximum strains occur at the roots of the notches, and eventually the bone fractures at one of the notches. Because both notches experience the same force, when one notch breaks, the other is ‘frozen’ in the state directly preceding fracture. Spectra can be taken around the roots of strained and failed notches and progressively farther away from them to measure changes in tissue spectra that occur prior to and after bone failure. Vibrational spectroscopy has been previously used to measure the state of collagen crosslinking in FTIR [3] by calculating amide I (collagen carbonyl C=O stretch) band area ratios (1690 cm⁻¹/1660 cm⁻¹). Because amide I is moderately intense in the Raman spectrum of bone tissue, the FTIR metrics can be used. We have successfully used amide I to map damage in fractured specimens by Raman microscopy [4].

Materials and Methods: Specimen Preparation: Fresh-frozen equine third metacarpals from three female thoroughbreds were used; one horse died of a fracture of the left forelimb fetlock and death information on the other two horses was unknown. Rounded notches (depth ~ 0.6-0.7 mm) were created using a 0.75 mm dental cutting disk (SummaDisk, Shofu Corp.) mounted on a CNC machine. The depths of both notches were made as similar as possible to ensure comparable stress-strain fields at the notch tips. Symmetric four-point bending tests (inner loading span S ~ 5 mm, sample width W ~ 2 mm) were conducted on the double-notch specimens. The 12 specimens remained hydrated throughout the preparation and testing processes. Raman Measurements: An epi-illumination Raman microspectroscopic system with a line-focused laser beam was used to determine the chemical composition of the double-notch specimens. Briefly, a 785-nm diode laser (Invictus, Kaiser Optical Systems) was focused onto the specimen through a 20x/0.75 NIR-coated objective (delivered laser power ~ 100 mW). Raman scatter was collected (integration time 200 sec) through the same objective and focused onto the entrance slit of a NIR-optimized spectrograph (Kaiser Optical Systems) fitted with a back-illuminated deep-depletion CCD detector (Andor Technologies). The line focus enabled the simultaneous collection of 126 spectra (one for each row of pixels on the CCD detector). Spectra collected along a line in this way are called a transect. Of the 126 spectra in the transect, the central 64 were retained because they contained a higher signal/noise ratio. In the strained regions (unbroken notch), 10 transects were acquired parallel to the base of the notch, beginning at the base and moving progressively farther away from it (see Figure 1). In the failed regions (broken notch), transects were acquired parallel to the crack at 10 regions along its length. During all Raman measurements, specimens were kept hydrated in PBS buffer.

Results: Damage maps based on crosslink ratios (1690 cm⁻¹/1660 cm⁻¹) were calculated for the central 64 spectra of each transect. It has been shown that the 1690 cm⁻¹ component of the amide I envelope increases when collagen crosslinks are broken. Thus, a higher ratio indicates a greater strain on the bone. The transects from the strained region of bone were plotted as a function of distance away from the notch base. To smooth the effects of local irregularities in the tissue and make trends more apparent, the data was averaged over a quarter of the distance measured. A pattern emerged, into which 10 of the 11 specimens could be divided.

Discussion: Raman spectroscopy can measure strain-induced (or stress-induced) collagen crosslink distortion across a specimen. As expected, the maximum distortion occurs on the side of the notch nearest the site of fracture, but with local irregularities across the notched regions. Importantly, the measurements are made on fresh, hydrated tissue, eliminating artifacts that can arise during the fixing and embedding process. In spectroscopic measurements, these include interference from bands of the embedding medium, and distortion of the data by the dehydration that accompanies tissue fixation.

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