3D bone composition and fibers orientation at the crack site to understand bone fracture mechanisms

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INTRODUCTION: Currently the most accurate measure of bone fragility is determined by its toughness. Studies have shown that bone develops most of its toughness during the process of crack growth. Measuring and visualizing the propagation of the cracks from a notch provides insight into how bone fractures. Studies have examined crack propagation in cortical bone using environmental scanning electron microscopes (ESEM), synchrotron computed tomography CT, high-resolution optical microscope and digital cameras [1]. These studies found that healthy bone naturally shields itself from fracturing, generating deflections, splitting and bridging during crack propagation. These toughening mechanisms are able to increase the bone toughness up to 4-6 times during the actual crack growth [2]. In bones with osteogenesis imperfecta (OI), also known as brittle bone disease, we have found that the crack propagates quickly across the bone, in an almost straight path. The complex hierarchical structure of bone, composed primarily of mineral and collagen, dictates its mechanical properties and resistance to fracture [3]. Thus OI bone changes in composition and structure might explain some of the observed alterations of bone mechanical properties, but it is still unknown *how* and *why* they specifically influence the crack progress. Toughening mechanisms such as crack deflection are created by the interaction of the growing cracks with the bone structure at the micrometer length scale (i.e. intracortical porosity, organized collagen lamellar structure and mineralization heterogeneity). However, how cracks in bone relates to its local environment is not clear yet. Here, we directly relate the changes in the crack path to bone tissue structure and composition at its microscale. Specifically, we use 3D Raman spectroscopy to measure localized variations in bone composition and Second Harmonic Generation (SHG) microscopy to examine the structural organization of collagen fibers in models of healthy and brittle bones.

METHODS: Femurs of 14-week-old WT and *oim* mice (B6C3Fe-a/a-Col1a2^{oim}, N=2/group) were micro-notched and fractured in controlled 3-point-bending to measure fracture toughness. Following fracture, the specimens were embedded in polymethyl-methacrylate, and the blocks were sliced and polished to expose the stable crack growth site. Visualization of 3D bone composition at the crack sites was obtained acquiring Raman microspectrography (WiTec Confocal Raman microscope alpha300R) at 633 nm monochromatic laser (18 mW power, 50X magnification, 3 cm^{-1} spectral resolution, integration time per voxel set to 0.4s) and spatial resolution of 1 x 1 x 2.5 μm. Spectra postprocessing and analysis was conducted using the Project 5 software and custom-built Matlab code, and spatial mappings of bone mineral-to-matrix ratio, carbonate-to-phosphate ratio and crystallinity were plotted around the crack path. Bone composition was analyzed in subsequential 5 um thick regions from the crack path. We compared results of our new 3D Raman microspectrography analysis of bone to conventional single-point Raman spectroscopy analysis (40 acquisitions in 5 local points) with the same instrument and the same spectral setting to assess the ability of single-point Raman spectroscopy to effectively represent the volumetric composition in WT and *oim* bones. Second harmonic generation microscopy images of the collagen fibers inside the bone tissue in the region surrounding the crack path were acquired (Prairie Technologies Ultima IV Multiphoton Microscope ,Bruker) in backwards-emitted mode at an excitation wavelength of 920 nm using a 40X magnification and 0.8 N.A. water immersed objective lenses. Quantitative analysis of fiber orientation and organization around the crack path and their interaction with it were quantified using our recently developed FiberO software [4]. ANOVA and Tukey *post-hoc* tests were used (SPSS) to test for statistical significance.

RESULTS: In a general comparison between *oim* vs. WT bones, Raman microspectrography showed that *oim* tissue displayed higher mineralization and diminished carbonate substitution. Furthermore, the SHG images revealed reduced collagen content and organization in *oim* bones, when opposed to healthy counterparts. Notably, volumetric Raman microspectrography demonstrated superior specificity in distinguishing between experimental groups compared to traditional single-point spectra, that therefore do not fully represent bone composition. When looking specifically at the bone region around the crack path, bone mineralization was lower in the first region next to the crack, particularly in *oim* specimens. Collagen fibers were mostly oriented in the directionality of the crack path in WT bones.

DISCUSSION: 3D Raman microspectrography, enabling simultaneous in-plane analysis at different depths, revealed several important compositional variations between the experimental groups, in agreement with previous literature [1,3]. Reduced carbonate substitution in OI may be attributed to more immature bone tissue found in OI due to increased bone turnover, while mineralization has long been proved to be higher in OI [3]. The spatial distribution of changes in composition might have an impact on bone's ability to withstand fracture and crack propagation in OI. The reduced mineralization at the crack interface might have provided a localized point of failure, but it could also possibly be attributed to after-fracture alterations in composition. In agreement with previous studies [5], SHG images of OI bones showed lower collagen content in a less organized matrix. Additionally, the SHG microscopy analysis of the fibers orientation and organization at the crack site, and the crack directionality show a relation between them: the presence of deflections in WT bone fracture surfaces are located in agreement with adjacent collagen fibers orientation in the tissue. The disoriented and disorganized fibers in OI instead relate to a flat crack path.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding the formation of crack deflection and other bone toughening mechanisms in relation to tissue microstructure in healthy bone, and their absent or alteration in brittle bones, can inform us on what to target in bone for the development of effective treatments aimed to rescue bone fragility.

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