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Publication Date

2011

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A Hierarchical Approach to Characterizing the Fracture Behavior of Bone utilizing Synchrotron Radiation

By

Holly Dana Barth

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Engineering – Materials Science and Engineering

and the Designated Emphasis

in Nanoscale Science and Engineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Robert Ritchie, Chair

Professor Ronald Gronsky

Professor David Attwood

Fall 2011

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Bone utilizing Synchrotron Radiation

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by Holly Dana Barth

Abstract

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Bone has a complex hierarchical microstructure that spans from the nanoscale of the collagen molecules to the macroscopic physiological scale. In order to assess bone's risk of fracture with issues such as aging, disease, or irradiation it is critical to have a clear mechanistic framework which analyzes bone's deformation and fracture behavior at each structurally significant length scale. In this context, this present study seeks to characterize the fracture properties of bone by applying a hierarchical approach. Accordingly, this study utilizes x-ray microtomography from synchrotron radiation in order to identify the crack-resistant extrinsic mechanisms in different types of cortical bone. It was found that bone, which is a highly anisotropic material, displays toughening through crack deflection, out of plane twist and crack bridging. Next this study utilizes *in situ* tensile tests with x-ray scattering to investigate the submicron deformation in bone; at this length scale bone toughens intrinsically through plasticity mechanisms such as fibrillar sliding. Lastly, this study uses this mechanistic framework to evaluate the effects of irradiation on the fracture properties of bone. Here it was found that bone exposed to high doses of irradiation, greater than 70 kGy ($\text{Gy}=\text{J/Kg}$), leads to a severe progressive dose dependent degradation in strength, ductility, and toughness which is attributed to a change

in the crack path (extrinsic effect) and a degradation of the collagen integrity from altered collagen cross-link (intrinsic effect). Overall, the goal of this work is to outline a framework that can be applied to future studies investigating the effects of disease and aging on bone.

Dedication

This dissertation is dedicated to my supportive and loving family. Thank you to my parents, Peter and Ilene, and my sister Rachel, for always being there for me through countless obstacles! I hope I can repay you all one day for your endless kindness and compassion. Om mani padme hum.

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List of Symbols and Abbreviations

2D — two dimensional

3D — three dimensional

α — mass attenuation coefficient (cm^2/g)

α_1 — alpha - one

α_2 — alpha - two

a — crack length

AGEs — advanced glycation end-products

Al_2O_3 — aluminum oxide

ALS — Advanced Light Source

ASTM — American Society for Testing and Materials

A_{pl} — plastic area under force *vs.* displacement curve

b — uncracked ligament length ($W-a$)

B — thickness of sample

BL — beamline

BM — bending magnet

BMD — bone mineral density

c — collagen phase

C — carbon

$^{\circ}\text{C}$ — degrees Celsius

cc — cubic centimeter

CCD — charged coupled device

cm — centimeter

CT — computed tomography

d — diameter of sample

d_{hkl} — Bragg hkl plane spacing
 $\dot{d}$ — dose rate
 DESY — Deutsches Elektronen-Synchrotron
 DHLNL — divalent dehydrodihydroxynorleucine
 E — elastic modulus
 E_ν — energy
 E_0 — monochromtatic photon energy
 E_c — critical photon energy
 E_p — energy density
 $\Delta E/E$ — The monochromaticity or energy resolution
 ϵ_F — fibrillar strain
 ϵ_T — tissue strain
 ϵ_M — mineral strain
 EDTA — ethylenediaminetetraacetic acid
 el — elastic
 ESF — edge spread function
 eSEM — environment scanning electron microscope
 Φ — flux
 Ψ — radiation flux density
 f — focal length
 Fig. -- figure
 FTIR — Fourier Transform Infrared
 hr — hour
 γ — gamma
 g — gram
 G — strain-energy release rate

Gy — gray
 H — hydrogen
 HAP — hydroxyapatite mineral phase
 HASYLAB — Hamburger Synchrotronstrahlungslabor
 HBSS — Hanks' Balance Salt Solution
 HCl — hydrogen chloride
 HFD — high fat diet
 HR — pCT - high resolution peripheral quantitative computed tomography
 I — intensity
 ID — insertion device
 IR — infrared
 J — joule
 J — nonlinear strain-energy release rate
 K_I — mode I stress-intensity factor
 K_0 — initiation toughness
 keV — kilo electron volt
 kg — kilogram
 K_{JC} — toughness ($\text{MPa}\sqrt{\text{m}}$)
 kV — kilovolt
 kVp — peak kilo voltage
 λ — X-ray wavelength
 L — linear attenuation measured
 LBNL — Lawrence Berkeley National Laboratory
 LSF — line spread function
 M — measured linear attenuation
 μ — linear attenuation coefficient (cm^{-1})
 μm — micrometer

mm — millimeter
 MTF — modulation transfer function
 M — mineral phase
 N — number of elements in a detector
 N — nitrogen
 n — refractive index
 NA — numerical aperture
 nm — nanometer
 O — oxygen
 π — pi
 $p(r, \theta)$ — projection function
 ρ_i — density of material i
 Pa — Pascal
 pl — plastic
 PMMA — poly methyl methacrylate
 pyr — pyridinoline
 $r_{x,y,z}$ — position
 R-curve — resistance curve
 rpm — revolutions per minute
 σ_y — flow stress
 s — seconds
 S — span
 Si — silicon
 SLS — Swiss Light Source
 SR μ T — X-ray micro-tomography using synchrotron radiation
 S(E) — energy spectrum
 SE(B) — single edged notch bend sample

SAXS/WAXD — small- and wide-angle x-ray scattering/diffraction

θ — angle

UV — ultraviolet

ν — frequency (s^{-1})

v — voxel size

vBMD — volumetric bone mineral density

V_M —volume fraction of mineral phase

W — width of sample

w_i — mass fraction of material i

Y8a — tyrosine side chains

Z — atomic number

Acknowledgments

This work was supported by the National Institute of Health (NIH/NIDCR) under grant no. 5R01 DE015633 to the Lawrence Berkeley National Laboratory (LBNL); support for S.Y.T. and T.A. was additionally provided by NIH grants no. R01DE019284 and F32AR059497. I acknowledge the use of the two x-ray synchrotron beam lines 7.3.3 (SAXS/WAXD) and 8.3.2 (micro-tomography) at the Advanced Light Source at the Lawrence Berkeley National Laboratory (LBNL), which is supported by the Office of Science of the U.S. Department of Energy under contract no. DE-AC02-05CH11231. I would also wish to thank Drs. Tony Tomsia, Maximilien Launey, Joel Ager, Hans Bechtel, Alex Hexemer, Andrew Tauschera, Eric Schaible, Howard Padmore and Alastair MacDowell at LBNL for their considerable help, Professor Tony Keaveny and Mike Jekir, of the Mechanical Engineering Department at the University of California, Berkeley, Simon Tang and Tamera Alliston at the Department of Orthopaedic Surgery, University of California, San Francisco, and Elizabeth Zimmermann, Kurt Koester, Joseph Lemberg and Brian Panganiban of the Materials Science and Engineering Department at University of California, Berkeley, for all of their countless hours of assistance.

Chapter 1:

Introduction

1.1. Motivation

The mechanical properties and fracture behavior of mineralized tissue, such as bone, deteriorate due to aging, disease, or irradiation; indeed these factors are found to lead to a significant increase in susceptibility to bone fracture. In the past bone fracture has been attributed to issues of *bone quantity* where the loss in bone mass or bone-mineral density (BMD) is used as a predictor of fracture risk. However, there is mounting evidence that BMD alone cannot be the sole predictor for a bone fracture. For example work by Hui et al. reported a roughly ten fold increase in fracture risk with aging in a sample group in which the BMD alone remained practically unchanged [1]. Consequently, it is now apparent that BMD alone may not be the primary factor responsible for increased bone fractures in the elderly [2, 3], and that issues associated with *bone quality* may also be important when evaluating age, disease or irradiation related fracture. Though there has been detailed work that evaluates the risk of fracture in bone [3-9] it is only recently that there is a renewed interest in how biological factors from aging, disease or irradiation can alter the mechanical properties of bone, particularly the fracture resistance in the context of *bone quality* issues.

Bone quality refers to characteristics of the bone structure, comprised primarily of type I collagen and hydroxyapatite nanocrystals, which can influence bone's strength and toughness irrespective of BMD. With regard to bone structure, the apatite crystals play an important role in bone's stiffness or bone quantity, while the collagen component of the bone matrix plays a vital role in the bone quality, specifically the energy that the bone can absorb prior to failure. In this context it is important to study the microstructural mechanisms that play a role in the fracture of bone in order to characterize the effects that factors such as aging, disease or irradiation have on bone's fracture behavior.

Bone has a complex hierarchical microstructure, with characteristic features from the molecular to the macroscopic length scales, therefore it is necessary to analyze the contributions to the toughness at nearly each size-scale, and standardize such methodology. This study begins by characterizing the fracture toughness of biomaterials, at the micron length scale, by three dimensionally imaging the interaction between the crack path and the microstructure. Here the need for a more detailed mechanistic framework develops to explain changes in the mechanical properties and fracture toughness of bone with factors, such as

aging, disease or irradiation. This study addresses this by presenting a complete hierarchical approach that characterizes the relevant deformation mechanism on each length scale of bone, focusing on two specific case studies, irradiation and aging. In the future the mechanistic framework developed here can be applied to many other studies concerning the analysis of bone fracture in order to evaluate the impact of a wide range of factors that can affect the mechanical performance of cortical bone.

1.2. Structure of Bone

Cortical bone is the hard outer layer of bone that is designed to resist fracture. It is a unique and highly complex biomaterial to study due to its ability to be strong, tough, and lightweight. The internal structure of bone is hierarchical in nature and it spans many length scales [9-11], from molecular to macroscopic size scales. The basic structure of bone is an organic matrix comprised of ~90% collagen and a mineral phase consisting of calcium phosphate-based apatite mineral. At the molecular level, bone is made up of fibrous polymer type I collagen (up to 15 μm in length, and 50-70 nm in diameter) bound and impregnated with hard carbonated apatite nanocrystals (tens of nm in length and width, 2-3 nm in thickness) [9] that reinforce it. These crystals are mostly flat and are arranged mostly parallel to each other and to the long axis of the collagen fibrils in the bone composite in a regularly repeating staggered arrangement [12]. Type I collagen, the primary type found in bone, is also hierarchical in nature. The collagen is composed of a triple helix of peptide chains, specifically two $\alpha 1$ chains and one $\alpha 2$ chain, each of which are ~1000 residues long. The molecules are staggered by 67 nm and covalently cross-linked between lysine residues, the intra and intermolecular cross-links provide for the tensile strength [13, 14]. These mineralized collagen fibrils are further organized at the microscopic lengthscale into lamellar structure with roughly orthogonal orientations of adjacent lamellae (3-7 μm thickness). Within the lamellar structure are secondary osteons (200-300 μm diameter) [15], which are bone cylinders that contain a central longitudinal tubular cavity (Haversian canal) housing blood vessels and nerves. These large vascular channels are surrounded by circumferential lamellar rings. The interface between the osteons and the primary lamella is called the cement line region (1-5 μm in thickness), which is a poorly organized highly mineralized region.

1.3. Fracture of Bone

The mechanical behavior of bone is a function of the multi-dimensional hierarchical nature of its structure [16-19]; indeed, bone derives its resistance to

fracture from a multitude of deformation and toughening mechanisms at its' many length scales, ranging from the nanoscale structure of its protein molecules to the macroscopic physiological scale of tubular bone structure [20, 21] (Figure 1). Specifically, bone derives its toughness and hence its ability to resist fracture from both *intrinsic* and *extrinsic* mechanisms.

Intrinsic toughening mechanisms, principally at sub-micron dimensions, operate ahead of the crack tip to generate resistance to microstructural damage. The most prominent mechanism is that of plastic deformation which provides a way of blunting the crack tip through the formation of "plastic" zones. These intrinsic mechanisms include the molecular uncoiling of the tropocollagen molecules at the nanoscale [10], and at slightly coarser scales the sliding of mineralized collagen fibrils [22, 23].

At the length scales of 10 to 100's μm , conversely, the primary toughening mechanisms in bone are extrinsic. *Extrinsic* toughening mechanisms operate primarily in the wake of the crack tip to inhibit cracking by "shielding" the crack from the applied driving force [10, 24, 25]. Whereas intrinsic toughening mechanisms are effective in inhibiting both the initiation and growth of cracks, extrinsic mechanisms are only effective in inhibiting crack growth [10]. These mechanisms affect solely the growth of cracks most notably in bone by crack deflection and twisting and bridging of the crack by fibrils or intact regions of bone matrix in the crack wake, processes that are motivated by the presence of microcracks. A central feature for extrinsic toughening is the specific nature of the crack path which is controlled by the direction of the applied forces and the nature of the microstructure, in particular the hyper-mineralized interfaces of the osteons (cement lines), which provide microstructurally 'weak', and hence preferred, paths for cracking. Microcracks most often form at these cement lines and are thus primarily aligned with the osteonal orientation, *i.e.*, along the long axis of the bone, with a typical spacing of $\sim 10\text{-}100\text{s } \mu\text{m}$ [26-30]. Indeed, $\sim 99\%$ of all microcracks in bone are aligned at an angle of less than 25° with respect to the osteons [31]. It is because of the orientation dependence of the microcracking, and as well as the crack arrest and deflection as the crack encounters the cement lines [26, 32], that the fracture properties of bone are highly anisotropic, with the toughness in the transverse orientation being far higher than in the longitudinal orientations, *i.e.*, bone is easier to split than to break.

A better mechanistic understanding of bone will allow for a better evaluation of therapeutic treatments aimed at increasing bone strength with age and disease. This work seeks to outline a method for investigating bones hierarchical

structure in terms of its extrinsic and intrinsic mechanisms and apply this framework to explore the effects of many factors such as radiation damage, aging, etc. on cortical bone. Specifically, this framework will use nonlinear elastic fracture mechanics measurements to quantify macroscopic bone toughness, coupled with post-testing three-dimensional synchrotron x-ray computed microtomography of the microscopic crack paths, next small- and wide-angle x-ray scattering/diffraction (SAXS/WAXD) of *in situ* uniaxial tensile tests are employed to examine the effectiveness of fibrillar sliding as a nanoscale deformation mechanism in bone (by measuring the strain partitioning between the mineral and collagen phases), and lastly, the use of deep UV Raman and Fourier Transform Infrared (FTIR) spectroscopies and a fluorometric assay to characterize the damage to the collagen fibrils.

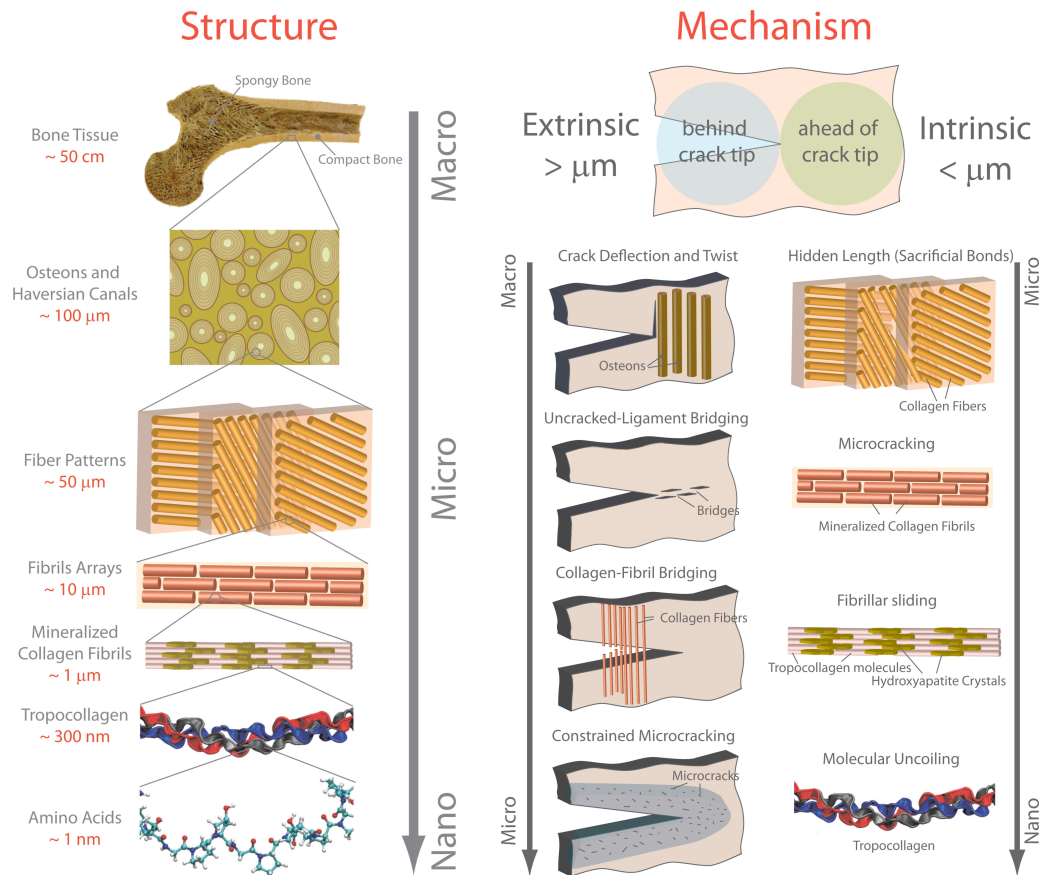


Figure 1-1: The structure of bone showing the seven levels of hierarchy with the prevailing toughening mechanisms. At the smallest level at the scale of the tropocollagen molecules and mineralized collagen fibrils, (intrinsic) toughening, *i.e.*, plasticity, is achieved via mechanisms of molecular uncoiling and intermolecular sliding of molecules. At coarser levels at the scale of the fibril arrays, microcracking and fibrillar sliding act as plasticity mechanisms and contribute to the intrinsic toughness. At micrometer dimensions, the breaking of sacrificial bonds at the interfaces of fibril arrays contributes to increased energy dissipation, together with crack bridging by collagen fibrils. At the largest length-scales in the range of 10s to 100s μm, the primary sources of toughening are extrinsic and result from extensive crack deflection and crack bridging by uncracked ligaments, both mechanisms that are motivated by the occurrence of microcracking. (Adapted from Ref. [20]).

1.4. Research Overview

The multidimensional hierarchical nature of bone must be explored in order to fully understand its mechanical deformation and fracture behavior; indeed, these mechanical properties are derived from very different length scales. This is especially true with respect to resistance to fracture where a suite of physical toughening mechanisms are activated at varying levels within bone. Specifically, bone derives its toughness and hence its ability to resist fracture from both intrinsic and extrinsic mechanisms. In the research presented here a hierarchical approach is utilized to explore bone's resistance to fracture at each of these size scales and apply it to the study of irradiation and aging. This exploration is done in stages, each chapter investigates a different layer of bone's complex structure. Chapter 2 presents the characterization technique synchrotron radiation microtomography (SR μ T) and its application to explain bone's fracture behavior. Chapter 3 delves into the characterization of the macroscopic to microscopic properties of bone and how this can be applied to assessing damage to human cortical bone due to irradiation. Chapter 4 explains the detailed characterization of bone at the nanoscale and applies these techniques to the investigation of irradiation damage to human cortical bone. Chapter 5 discusses the orientation affects on the mechanical properties of cortical bone, as well as analyzes the macro- to micro-scale mechanisms in bone that change with aging. This is done by utilizing the technique SR μ T and resistance curve testing to look at the failure of bone in the transverse or breaking orientation. Chapter 6 discusses future work in which the mechanistic framework laid out here can be applied to the study of aging and diseased bone. Each chapter will have an introduction and background for the presented study, followed by a description of the different techniques and experiments performed, the results obtained and a discussion of the results and will end with a brief conclusion.

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Chapter 2:

Three-Dimensional Visualization of Crack Propagation in Biological and Bio-inspired Materials using X-ray Micro-Tomography

2.1. Introduction

This chapter highlights recent advancements in x-ray micro-tomography using synchrotron radiation (SR μ T) as applied to bio- and bio-inspired materials, specifically advancements made in characterizing the fracture behavior of these materials by three dimensionally imaging the interaction between the crack path and microstructure. Third generation synchrotron radiation facilities perform three dimensional imaging using a tomography setup at one of the highest spatial resolutions ($\sim 1 \mu\text{m}$) and contrast sensitivity in order to visualize the contrasting properties of the microstructure. In absorption mode SR μ T provides information on the linear attenuation of a material, which is sensitive to the materials atomic composition and density at a spatial resolution that is on the order of features necessary for observing crack propagation. Hard biomaterials, such as cortical bone, are designed to act as structural support; therefore their fracture properties are of great physiological relevance. The deterioration of bone is both a *quality* (e.g., strength and fracture resistance) and *quantity* (e.g., bone mineral density) issue when evaluating fracture risks. The main focus of this chapter is to identify the uses of SR μ T in characterizing both the *quality* and *quantity* of biomaterials and bio-inspired materials in three dimensions. Here we examine the role that different microstructures of materials, namely human cortical bone, mouse bone, antler, and bio-inspired composite, play in affecting the material's fracture properties by using the non-destructive three dimensional imaging technique, x-ray tomography, to image the microstructure at a relevant spatial resolution, and how this technique can be applied in the analysis of disease and aging studies.

Imaging is an important technique used to visualize a structure; however three-dimensional imaging is necessary in order to optimize microstructural investigation. In the past 3D investigation of samples was done by serial sectioning the specimen; however sectioning can be extremely destructive and introduce flaws into the material. Synchrotron radiation micro-tomography (SR μ T) is a technique, which utilizes the high flux and brightness of a third generation synchrotron facility, that can characterize and image the three dimensional internal structure of specimens non-destructively in the field of materials science, biology, and medical imaging. This is an especially useful

technique when visualizing the phenomenon of crack propagation in materials, since air from voids formed from the crack and bone components produce high contrast, which is extremely relevant in investigating the fracture properties of biological and bio-inspired materials since the toughness of these materials is designed to change and increase with crack extension. Indeed these natural materials are designed so that their mechanical properties are determined by their adapted structure. The biomaterials and bio-inspired materials reviewed here are all similarly designed as a hierarchical composite material comprised of an organic phase, in bone the organic phase is collagenous protein molecules, and an inorganic phase, in bone the inorganic phase is mineralized hydroxyapatite nanoparticles. This composite like make-up allows the material to be mechanistically both tough and strong, indeed these biomaterials found in nature are known to combine readily available compounds that typically exhibit poor macroscale mechanical properties to produce composites such as bone, nacre or dentin, that are all much stronger and tougher than could be expected from the simple mixture of the individual constituents [1, 2].

This chapter will have a major emphasis on bone, since bone has a similar structure to other mineralized musculoskeletal tissue and can be used as a model for identifying toughening mechanisms in other hierarchical materials. In quantifying the fracture risk in biomaterials such as bone it is important to take into account both the bone quantity, in terms of bone mineral density, and bone quality, which is loosely defined as the characteristics of the bone matrix nano- and microstructure that can influence mechanical properties [3]. Bone is an adaptive material that has an intimate structure-function relationship. Toughness has been thought of as the ability of a material to dissipate deformation energy without the propagation of a crack. However, fracture in biomaterials, like dentin and bone, is actually a mutual competition between extrinsic toughening mechanisms and intrinsic damage mechanisms acting ahead of the crack tip to promote crack growth. Extrinsic toughening mechanisms act in the wake of a growing crack to 'shield' it from the applied stresses. Bone exhibits several of its most important crack resistant qualities at the micrometer length-scale (10-100s μm) which are observable using SR μ T. In cortical bones extrinsic toughening mechanisms are identified as crack bridging, 90 degree crack deflection, and out-of-plane crack twist. These mechanisms are activated as a crack propagates throughout the specimen, specifically when the crack interacts with the microstructure.

Applications for SR μ T range from medical to materials science to engineering. Therefore a major emphasis has been placed on developing these devices at

synchrotrons all over the world. This chapter describes x-ray tomography as a technique to non-destructively characterize the microstructure of a material in three dimensions at a micrometer resolution. First a brief description of the physics and the mathematical analysis behind the technique is presented, also addressed here is the comparison between laboratory based computed tomography systems and x-ray microtomography systems. This is followed by several examples of the technique, with a particular focus on the use of SR μ T to characterize the fracture properties and fracture resistance, with respect to both bone quantity and bone quality, of biomaterials [4-6] and bio-inspired hybrid [7] materials.

2. 2. Tomography

2.2.1. Background of Tomography

Computed tomography is defined as the reconstruction of an image from its projections taken at varying angles. The word ‘tomography’ comes from the greek word *tomos* meaning slice and *graphin* meaning to write. A brief history of tomography starts with the discovery of x-rays. In 1895 x-rays were discovered by Roentgen and his first use of them was to image his wife’s hand, which demonstrated the ability of x-rays to interact with biomaterials and produce projections [8]. Since then non-destructive imaging has proven a powerful tool in many fields. Though the discovery of x-rays made it possible to make two-dimensional radiographic projections it was not until the advent of computers that three-dimensional imaging became feasible. In 1917 Johann Radon [9] conceived the mathematical solution to the problem, called the radon transform also known as the sinogram. In 1964 Allan Cormack developed the initial algorithms to derive the mass distribution inside an object from its radiographic projection images taken at different angles [10, 11]. Based on this idea, in 1971, Godfrey Hounsfield built the first computed tomography scanners utilizing all the previous discoveries into a system that would be termed: computerized tomography [12]. In 1979 Cormack and Hounsfield received the Nobel Prize for their contributions to medical imaging. Then in the 1980s Grodzin purposed the use of synchrotron radiation as an x-ray source to produce high intensity monochromatic x-rays for tomography, which brought significant enhancements to imaging [13-15].

2.2.2. Tomography Overview

Tomography refers to the cross sectional imaging of an object from either transmission or reflection data collected by illuminating the object from many

different directions [16]. Synchrotron radiation micro-tomography (SR μ T) is based on the same principles used in conventional and medical computed-tomography (CT) scanners [17]. The aim of the reconstruction is to retrieve the attenuation distribution from the projected data [18]. As the object rotates, each angle creates a series of projections. After all projections are acquired they are subdivided by taking all the projections for a single thin slice (thickness determined by the depth of field of the camera/lens set up) of the object. All the projections for each slice are then ordered into an image called a sinogram. It represents the projection of the attenuation at a single slice on the camera at every angle (fig. 1). To account for noise in the data it is important to filter the data by performing smoothing and ring removal operations to the sinogram [19]. Usually the ring artifacts are due to a non-linear response of some of the pixels on the charge coupled device (CCD) detector and normalization problems due to beam drift. Also artifacts in the image could be caused by a non-perfect rotation of the sample during scanning. Once the data has been transformed into the frequency domain and filtered to smooth out the statistical noise the data can then be transformed back into the spatial domain by back projection, which converts the camera bin data from the filtered sinogram back along the same lines from where the photon was emitted. In order for an image to have high resolution in three dimensions it necessary that the tomography setup have a sufficient number of photons for good statistics, and a detector that has sufficient spatial resolution so that the system can distinguish between neighboring photon x-ray paths.

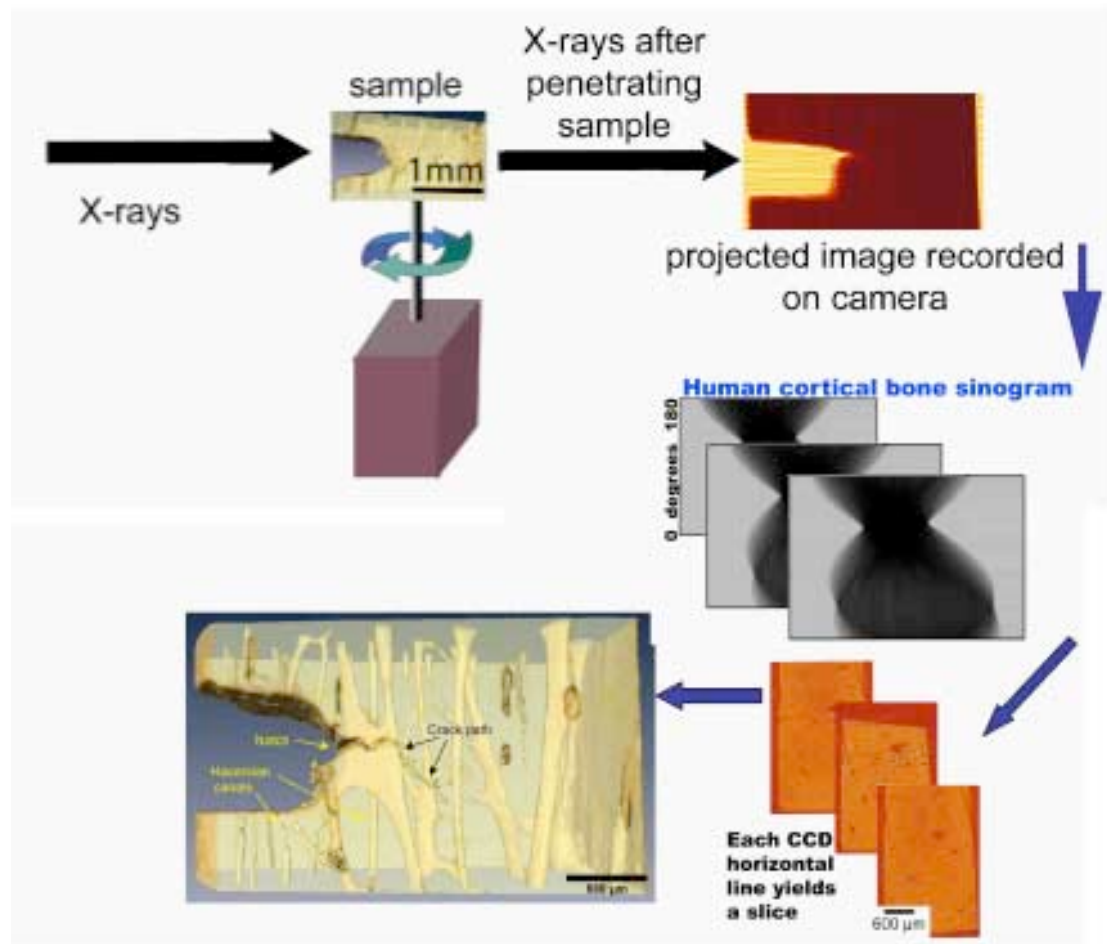


Figure 2-1: Reconstruction process. First the projected images are recorded onto the CCD one for each angle. After all projections are acquired they are subdivided by taking all the projections for a single thin slice (thickness determined by the depth of field of the camera/lens set up) of the object. All the projections for each slice are then ordered into an image called a sinogram. It represents the projection of the attenuation at a single slice on the camera at every angle. Then the sinogram is put through math transforms and the 2D reconstructed image is produced, which can be compiled into a 3D image.

2.2.3. Tomography Reconstruction Algorithm

For characterizing x-ray absorption it is worth noting that the absorption of light as it passes through a material is a logarithmic function of the absorptivity of the material, and the distance through which the light must travel. The true absorptivity of a material depends on the number and type of atoms. In general, a higher Z atom absorbs more than a lower Z atom at the same wavelength¹. The x-ray attenuation coefficient, μ , at a point $r_{x,y,z}$ in a sample is determined from a finite set of x-ray attenuation measurements, also called the projection data, which is taken at different angles (number of angles changes depending on the lens system and the desired resolution). The intensity, I , is recorded onto a position sensitive detector with values that represent the number of photons transmitted through an object at a given angle and a fixed position $r_{x,y,z}$. Each projection represents a nearly independent measurement of the object. The recorded projection is directly related to the materials microstructure and is given by the Beer Lambert law:

$$I = \int S(Ev) \left[\exp - \int \mu(x, y, z, Ev) dl \right] dE \quad (1)$$

where $S(Ev)$ is the energy spectrum of the x-ray source, which in our cases is monochromatic synchrotron radiation, and $\mu(x, y, z, Ev)$ is the energy-dependant attenuation coefficient at a single point (x, y, z) in the sample at a specific x-ray energy selected to maximize the signal to noise on the detector. The line integral is taken along a straight path, dl , through the sample. The line integral represents the total attenuation suffered by a beam of x-rays as it travels in a straight line through the object. Then a coordinate transform $x \cos \theta + y \sin \theta = r$ is performed to get the measurements of the attenuation through the sample as a function of angle. Next step is to rewrite the line integral using a delta function. Now the projection function, $p(r, \theta)$, is defined as:

$$p(r, \theta) = \ln \left(\frac{I_0}{I} \right) = \iint \mu(x, y, z, E_0) \delta(x \cos \theta + y \sin \theta - r) dx dy \quad (2)$$

Where I_0 is the initial x-ray intensity prior to penetrating the object and E_0 is the selected monochromatic photon energy. The equation (2) then reduces into the familiar form of the radon transform or sinogram:

$$p(r, \theta) = \ln \left(\frac{I_0}{I} \right) = \int \mu(x, y, z, E_0) dl \quad (3)$$

¹ Tables on the x-ray absorption coefficients can be found [20] and online [21, 22]

The mathematical problem is to numerically invert the integral in Eq. 3 in order to solve for the attenuation coefficient, $\mu(x, y, z, E_0)$. This is done using reconstruction algorithms. The two most common methods for reconstruction are the algebraic method, which consists of solving a linear system of equations of which the unknowns are the values of the μ coefficients within the sample. The other method commonly used today is the analytical methods, which relies on the filtered back projection algorithm [23], which is based on the projection slice theorem, stating that the Fourier transform of the real projection is the slice in 2D Fourier space. For the setup the sample rotates around one axis, however it is not sampled uniformly by the x-rays along a section so in order to correct for this the back projection must be filtered. All the reconstructed images presented in this chapter were produced using filtered back projection algorithms, which is the faster method.

2.2.4. Spatial Resolution

Synchrotron radiation micro-tomography provides volumetric information on the sample in a non-destructive way. In order to get the most detailed information out of the reconstructed images it is important to have high spatial resolution. For large features, $\sim 10\text{s } \mu\text{m}$, there is no x-ray diffraction effects from the collimated light, however for smaller features, 100 nm or $1\mu\text{m}$, there will be diffraction effects. Therefore, for large enough features the image spatial resolution is determined by the defect of focus, numerical aperture, and spherical aberration arising from the thickness of the scintillator and the substrate [24]. The image system is shown in figure 2. A nearly parallel x-ray beam that is partially absorbed by the scintillator screen generates a visible light image. The light image (object plane of the optical system) is relayed to the charged coupled device (CCD) (image plane). Exact reconstruction requires that the sample stay within the field of view of the visible light detector for every angle that a projection is recorded. If the sample has a diameter d and the detector has N elements then the minimum number of voxels that contain physical information is $v = d/N$. It is possible to scan samples larger than the field of view, termed local tomography, but the quality of the reconstructed image can deteriorate due to the introduction of artifacts [25], however fast reconstruction algorithms are being developed to deal with reconstruction artifacts [26]. The Nyquist limit [18] defines the rotation increment consistent with a given voxel size. The contrast is determined by the x-ray intensity flux and the exposure time that will determine the total number of photons incident on the detector per image pixel. The

detector has a saturation limit for the amount of photons that the detector can collect. Dynamic range is the range of intensities spanning from where the x-rays go through air and are not attenuated by the sample to the highest detectable attenuating spot through the sample.

The modulation transfer function (MTF) of an imaging system is used to describe a systems spatial resolution. The MTF is defined to be the modulus of the Fourier transform of a system's line spread function (LSF) also known as impulse response function. The LSF response of the detector can be recorded by investigating its Edge Spread Function (ESF). Before performing a Fourier transform of a LSF, it must be multiplied by a window function having the property of reducing the leading and trailing parts of the LSF to zero while preserving the important central portion.

Another type of imaging, besides absorption tomography, that can be obtained with a synchrotron x-ray source is phase contrast tomography [27, 28]. During phase contrast imaging there is additional radiographs of the sample that are superimposed on the attenuation contrast. This contrast is due to the large spatial coherence of the third generation synchrotron, which introduces Fresnel diffraction fringes at internal interfaces of the sample [29]. The size of the phase contrast effect is based on the sample to detector distance. Most x-ray tomography setups, even in absorption mode, have small sample to detector distances on the order of a mm or a cm, however even though there are small phase contrast effects at these distances one can still obtain the absorption coefficient using the classical filtered back projection algorithm, since the phase contrast is limited to the internal interfaces within the sample and to the first approximation the attenuation can still be retrieved [29]. When the sample to detector distance becomes greater than a few cm the diffraction fringes produced become larger than the interface thickness and special reconstruction algorithms must be used. Phase contrast tomography should be used with weakly attenuating samples and heterogeneous materials showing phases with very similar attenuation coefficients [30, 31].

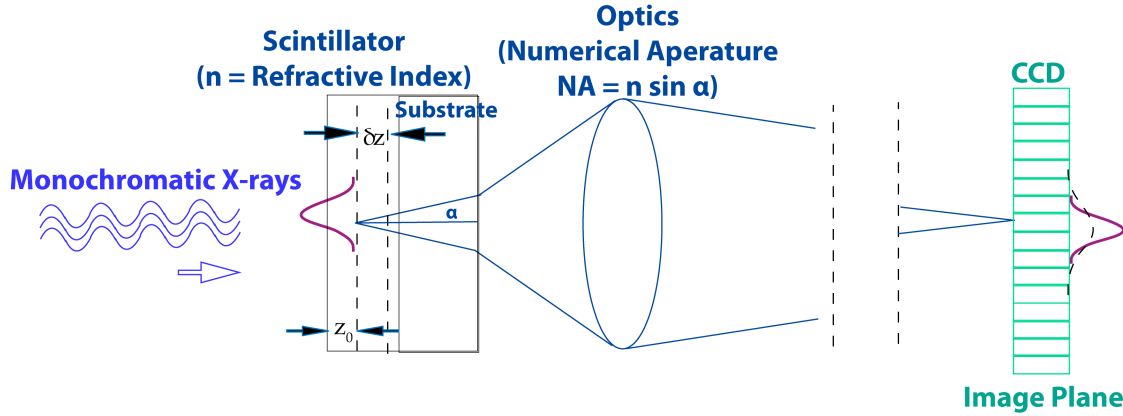


Figure 2-2: Scintillator setup. A nearly parallel x-ray beam is partially absorbed by the scintillator screen generates an identical visible light image. An image plane z_0 is focuses onto the CCD (solid curve). An image plan $z_0 + \delta z$ is out of focus at the CCD [24].

2.2.5. Tomography Beamline Specifics

In order to obtain x-rays at a synchrotron radiation facility, electrons in a storage ring are accelerated by a magnetic field, then synchrotron radiation is emitted into a small solid angle directed tangentially to the electron orbit, this method of obtaining x-rays dramatically increases the source brightness (photons per unit solid angle) [32]. Indeed, the emitted light is orders of magnitudes brighter than that emitted by a conventional x-ray source making it ideal for high resolution computed tomography. Synchrotron radiation micro-tomography is a technique used to characterize and map the three dimensional variation in absorption within the microstructure of materials with high contrast. This is useful when studying the physical and chemical composition of biological tissue because different material features and phases often have different x-ray absorption properties, so we can easily identify the different features from these images. Through the use of filtered back projection algorithms the tomography system is able to take two-dimensional radiographic projections and turn it into three-dimensional information about the material. Tomography beamline setups at third generation synchrotron facilities allow for a spatial resolution that can be scaled to a micron and in some cases even submicron resolution. Tomography

setups for 3D imaging can be found at most of the third generation synchrotron facilities e.g., 2-BM at the Advanced Photon Source in the United States, BL-47XU at SPRing-8 in Japan, TOMCAT beamline in Switzerland [33], Jeep beamline at Diamond in England, DORIS at HASYLAB in France, beamlines ID15, ID17, ID22, BM05 and ID19 at the European Synchrotron radiation Facility in France etc. All of the tomographic reconstructed images shown in this chapter were taken at beamline 8.3.2 [34] at the Advanced Light Source (ALS) [35] at Lawrence Berkeley National Laboratory (LBNL) [36], which is representative of what can be achieved at other synchrotron facilities. The schematic is shown in figure 3. The setup is similar to standard tomography procedures [16]. The tomography beamline is off a super bend magnet source and has an energy range from soft x-rays to hard x-rays (6-46 keV) with a critical photon energy (E_c) of 12 keV. The beamline consists of a pair of monochromators used to focus the wavelength to a specified energy. The two types of monochromators in place at beamline 8.3.2 are multilayers or Si 111 crystals. The multilayer monochromator has a wide band pass with an energy resolution ($\Delta E/E$) of $\sim 1\%$ and the Si 111 crystal monochromator has a small band pass with an energy resolution ($\Delta E/E$) of $\sim 1/7000$. The x-ray source of the tomography beamline of the Advanced Light Source is a 6 Tesla super bend magnet, with a local field of 4.37 Tesla for this source point, with a ring current of 500 mA and a ring energy of 1.9 GeV [37]. The calculated photon flux at the exit of the x-ray source is 10^5 hv/sec/ μm^2 [34].

To achieve high spatial resolution without damaging the camera the tomography systems is comprised of a scintillator, used to convert x-rays to visible light, and diffraction-limited objectives, to magnify the image onto a charge-coupled device (CCD). The spatial resolution of the detector changes for different scintillator thickness and objectives. For a low numerical aperture the optical system is limited by diffraction and the scintillator thickness does not affect the performance, for high numerical apertures the modulation transfer function depends on the amount of defocus. At the highest magnification, at the tomography beamline at the ALS, a modulation transfer function of 9% is achieved which corresponds to a spatial resolution of 1.6 μm . The detector is a Cooke PCO4000, which is a low-noise fast readout CCD camera. The beamsize on the sample is 35 mm x 5 mm. Overall the performance of the optically based detector is restricted by the scintillation properties, optical light transfer, and CCD granularity. The x-ray energy is selected based on the absorption of the sample being scanned in order to optimize the signal-to-noise of the image. Also the sample size is limited by the size of the CCD and lens system used to record the attenuated images. The sample is placed on a rotating stage and the samples axis is perpendicular to the incoming x-rays. Images are obtained by scanning 2-

dimensional radiographs every quarter degree from 0-180 degrees and then these radiographic projections are put through a filter back projection algorithm to obtain the 3-dimensional information about the sample (fig. 1). For each sample the reconstructed images are obtained using the software Octopus [38] and the three dimensional visualization and segmentation was obtained using Avizo™ software [39].

Synchrotron radiation produces a much higher flux than conventional tabletop computed tomography (CT) systems, which gives synchrotron radiation CT setups the advantage of having much better image statistics due its higher flux. Laboratory CTs use a micro-focus source a few micrometers wide, which delivers the x-rays in a cone beam rather than a parallel beam found in synchrotron sources. Because of the methods to produce them, laboratory x-ray tomography systems have a polychromatic source and also require the sample to go through 360 degree rotation for full reconstruction. In contrast, x-ray tomography has the benefit of being off a super bend source, which produces extremely high flux photons that can be tuned to a specific x-ray wavelength and energy with a monochromator, as well as produce a collimated beam that only requires 180 degree rotation. This allows for the images to have higher signal-to-noise ratio and higher spatial resolution. This is necessary when deciphering what phases are present inside a sample, since a monochromatic beam will have one set value for the mass attenuation coefficient, μ/ρ , while a polychromatic beam, often found in tabletop CT systems, will produce a range for the mass attenuation coefficient making it difficult to distinguish and the polychromatic source will produce beam hardening effects, which are artifacts that correspond to low x-ray energies being arrested as they enter the attenuating sample. Beam hardening can lead to geometric nonuniformities in signal and a nonlinear relationship between attenuation and material density. However, since daily access to synchrotron facilities is not always easy the use of a tabletop CT has become very common. Turnkey microCT systems have become routine and recently nanoCT systems have started being offered with a spatial resolution of less than 1 μm (μCT 50 (nanoCT), SCANCO Medical microCT systems). Now turnkey microCT systems are more widely offered for laboratory use and each machine can vary based on what specifications are needed, such as spatial resolution, sample size, and temperature. Recently, the high resolution peripheral quantitative computed tomography (HR-pQCT) has become clinically available as a CT system that can scan human body parts and provide non-invasive measure of 3-dimensional bone geometry and micro-architecture with great detail. In combination with microarchitectural finite element models HR-pQCT can be used to determine bone strength using a strain based failure criterion [40, 41]. The HR-pQCT scanner (a

prototype of the XtremeCT, Scanco medical AG, Bruttisellen, Switzerland) provides a nominal resolution of 89 μm in plane and 93 μm slice thickness. The x-ray tube operates at 60 kVp. This system is designed for in vivo situations in living subjects. A limitation of the system is that it only offers imaging of a relatively small part of the bone. Studies are still investigating whether this method is the best for predicting fracture and what area of the bone would be the best to scan using HR-pQCT.

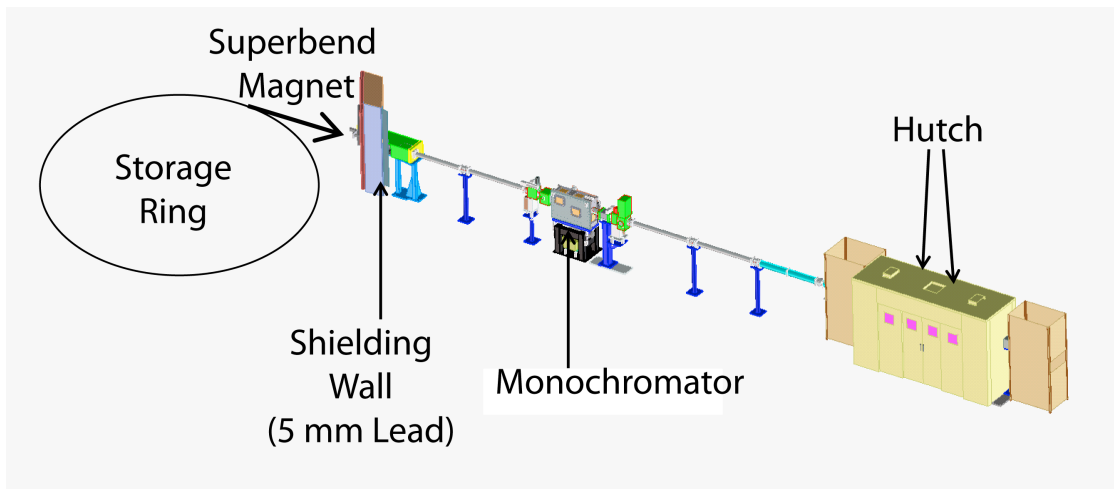


Figure 2-3: Tomography Beamline setup at 8.3.2 at the Advanced Light Source. The tomography beamline is off a superbend source and has an energy range from soft x-rays to hard x-rays (6-46 keV). The beamline consists of a pair of monochromators used to focus the wavelength to a specified energy. The two types of monochromators in place at beamline 8.3.2 are multilayers or Si 111 crystals.

2.3. Applications

2.3.1. Phase Volume Fractions

Synchrotron radiation micro-tomography (SR μ T) gives quantitative information on volumetric bone mineral density (vBMD), porosity,

microstructure, and crack path. The tomographic process creates a distinct representation of an object by penetrating the object with monochromatic x-rays. The value of any pixel in the reconstructed image is equal to the linear attenuation coefficient, $\mu = \alpha_b \rho_b$, where α_b is the mass attenuation coefficient for x-ray absorption within the pixel (in cm^2/g) and ρ_b is the density of the material in the pixel (in g/cm^3). Note that the product ($\alpha_b \rho_b$) has units of cm^{-1} , which is the linear attenuation coefficient, μ . As mentioned earlier the linear attenuation coefficient is a function of both the chemical composition and the density.

To calculate the volume fraction of any phase in the material, for this case we use bone as our object, we can approximate the system as a two-phase mixture comprised of hydroxyapatite and collagen. From the definition of the mass attenuation coefficient, we can write α for the mixture as:

$$\alpha_b = \omega_M \alpha_M + \omega_c \alpha_c \quad (4)$$

Where w_i is the mass fraction of material i , where M is the mineral and c is the collagen phase respectively. Now, let's multiply both sides of the equation by the density of the bone:

$$\alpha_b \rho_b = L = V_M \rho_M \alpha_M + (1 - V_M) \rho_c \alpha_c \quad (5)$$

The left hand side of equation 5 ($\alpha_b \rho_b$) is precisely what is measured in the tomography system, so we will call this L , for linear attenuation measured from the bimodal distribution of the mineral. Also, the weight fraction times the density of the structure is the same as the volume fraction times the density of the constituent. We can rewrite the equation (for a two phase mixture) as:

$$V_M = \left(\frac{L - \mu_c}{\mu_M - \mu_c} \right) \quad (6)$$

Equation 6 allows us to quantitatively estimate the volume fraction of the hydroxyapatite (HAP) in different samples. The μ_c and μ_M both vary with energy and are known. This allows us to do comparative studies on bone quantity by solving for the volume fraction and the mineral concentration of the specimen, which is just V_M multiplied by the density of hydroxyapatite. For HAP, we can approximate ρ_M as 3.15g/cc, and for collagen we can approximate ρ_c as 1.25g/cc. Because of the large atomic number difference between HAP and collagen, errors in collagen density are insignificant. Therefore, to gain information on the vBMD

the histograms of the reconstructed volumes are bimodal distributions with a peak that is representative of the void space and a peak representative of the bone. The attenuation coefficient of each pixel in the reconstructed image is directly related to the mineral concentration of the bone.

2.3.2. Examples of Materials Applications

Synchrotron radiation micro-tomography (SR μ T) has been applied to many fields of materials and engineering problems, especially at a spatial resolution reaching the micrometer range which allows for three-dimensional representative volumes of heterogeneous materials with sufficient microscopic detail to predict macroscopic properties. All of the studies discussed here were carried out in conventional absorption mode. In absorption mode the sample is placed as close as possible to the detector to avoid phase effects due to propagation from sample to detector. In absorption mode the contrast recorded on the charge coupled device, CCD, is representative of the difference between the linear attenuation coefficients. It is important to calibrate the resulting photon statistics, because if the transmission is too high the resulting contrast between elements will be too low and vice versa. A good trade off is at a transmission of ~10% [42].

The following examples show the capabilities of the x-ray micro-tomography technique to answer new questions that arise from materials science and medicine in regards to structure and fracture properties in different types of bone and composites. Bone material is a hierarchical structure made primarily of collagenous protein molecules, mineral carbonated apatite nanoparticles, and water [43]. Bone's response to an applied load is based on these varying structures; indeed cortical bone's response to mechanical loading is inherently three-dimensional at the different length scales. To understand bone's mechanical behavior it is important to first understand the relationship between the hierarchical structural and its function [1, 44-46]. At the micron length scale many mechanical properties of bone can be observed. The studies presented here look at this relationship for mouse bone, human cortical bone, and antler at the micrometer scale.

Bone is an adaptive material that is designed for different functional requirements depending on its location in the body. The mineral component of bone's structure is related to its stiffness², so an increase in the mineral density

² Stiffness is related to the elastic modulus and defines the force required to produce corresponding elastic deformation.

increases the stiffness. Each type of bone discussed in this chapter has adapted to a different composition of mineral and collagen based on its function. Antler is 40% mineral (one of the lowest) due to its need to have high toughness in order to absorb energy while fighting, while human cortical bone is ~60% mineralized and bone structure is designed to have both high toughness and strength³. Different bones have different mechanical properties depending on the growth, structure and adaptation, all of which are interconnected to serve a specific function [47, 48] and can be resolved in absorption mode with an optimal energy range of 15 keV to 22 keV using synchrotron radiation micro-tomography (SR μ T). In the examples discussed here, SR μ T is used to examine damage in bone with varying degrees of mineral that were subject to mechanical loading; qualitatively one is able to observe the crack growth and the network of osteons throughout the different biomaterials.

Anisotropy of Bone

Human cortical bone is identified as a secondary bone meaning that it is the product of the resorption of previously existing bone tissue and the deposition of new bone in its place. This process results in secondary osteons, known as Haversian canals, which is surrounded by a thin mineralized region called a cement line. The Haversian canals have a preferred orientation with respect to the long axis of the bone. The entry of a crack into the osteon is blocked by the cement line and by concentric lamellae of mineralized collagen fibers that are packed in an alternating loose and dense pattern and are oriented in various directions. On the length-scale that the resolution of the tomography setup is capable of achieving, ~1 μ m, cortical bone consists of overlapping parallel osteons. A large number of osteons per unit volume of bone limits the propagation of cracking because they obstruct the passage of a crack as it navigates between the many osteons.

Due to the orientation of the osteonal system bone is highly anisotropic, its fracture behavior will change when the direction of the load applied changes with respect to the long axis of the bone [49, 50]. SR μ T is used to identify the toughening mechanism in the various orientations. Figure 4 shows tomographic images of crack propagation and microstructure in human cortical bone for the

³ Strength, defined by the yield stress at the onset of permanent deformation or maximum strength at the peak load before fracture, is a measure of the force/unit area that the bone can withstand. The fracture toughness measures the resistance to fracture of a material.

two extreme orientations, longitudinal and transverse. Specifically, it was shown here (Fig. 4) that when the direction of maximum stress is in the same direction as the osteons that crack bridging is the predominate mechanism and when the maximum stress is perpendicular to the osteons crack deflection and out of plane twist are the predominate mechanisms.

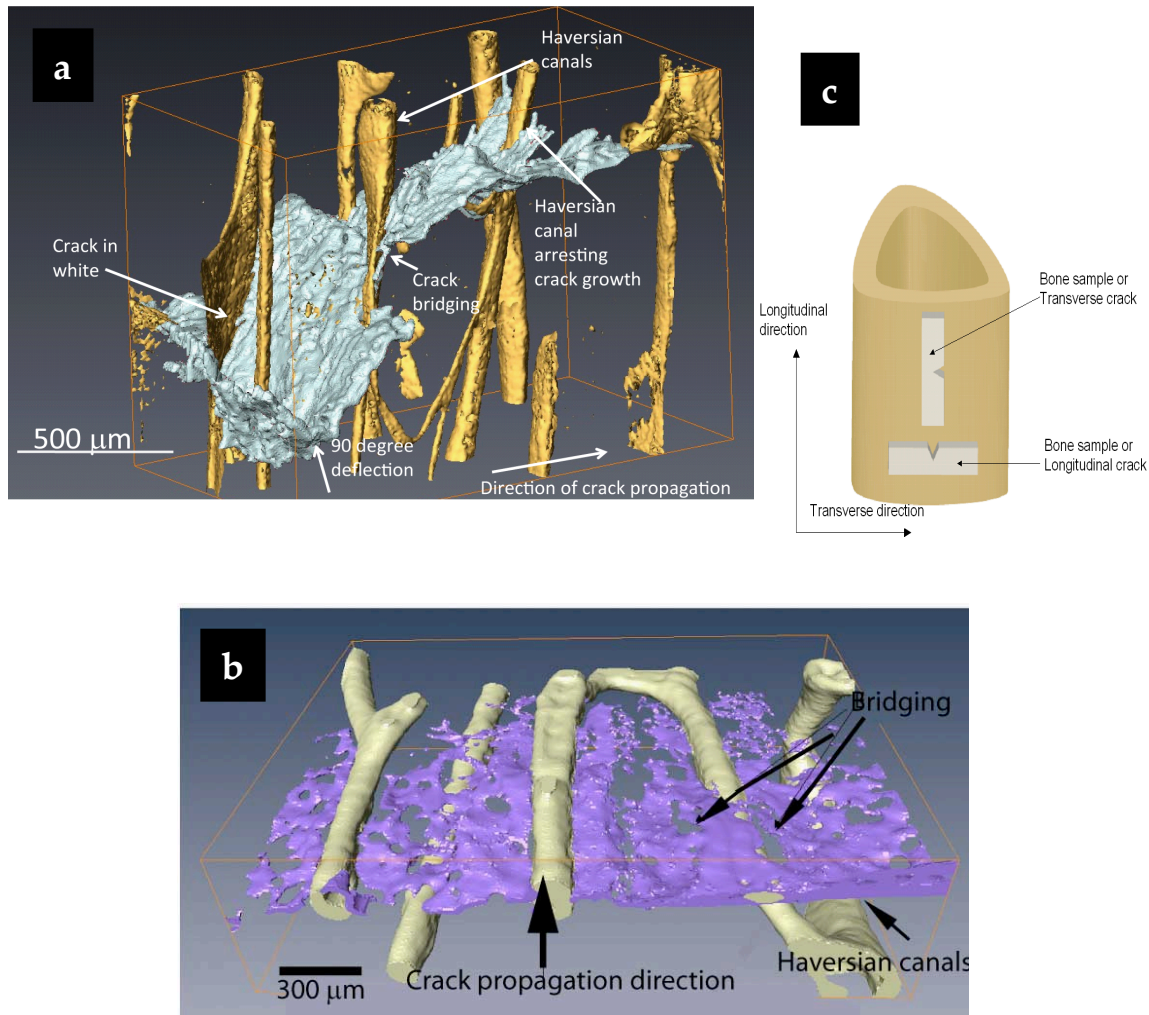


Figure 2-4: Tomographic images showing *transverse* and *longitudinal* orientation. Synchrotron radiation micro-tomography images, taken at the ALS beamline 832 at 18 keV with .25 degree turn with a spatial resolution of 1.6 μm per pixel, showing the dominant mechanisms of (a) crack deflection and twisting in the transverse orientation and (b) crack bridging in the longitudinal orientation. In this three-dimensional reconstruction of the sample the Haversian canals are segmented out in yellow and the crack is segmented in white for transverse and purple for longitudinal. (C) Schematic showing the orientation of the samples within the cortical wall.

Aging in Bone

Several recent studies have used synchrotron radiation micro-tomography (SR μ T) to assess effects of aging on the fracture toughness properties in human cortical bone in both the transverse (breaking) [51] and longitudinal (splitting) [6] orientations. Bone exhibits rising crack-growth resistance (R-curve) behavior with crack extension and extrinsic toughening is responsible for the rising R-curve behavior [52, 53], therefore the toughness for the different age groups can be evaluated by visualizing how the crack path interacts with the microstructure. Nalla et al. [6] examined toughening mechanisms in the longitudinal orientation of bone by quantifying their crack growth resistance curves along with visualizing these mechanisms using SR μ T. The two mechanisms identified for the longitudinal orientation are micro-cracking and crack bridging. Both mechanisms are designed to absorb energy that would otherwise be used to extend the crack. Bridging was identified in both the young and the aged cortical bone [6]. Crack bridging is the formation of uncracked ligaments in the crack wake; these are intact regions, often tens of micrometers in size, that form along the crack path, either by the non-uniform advance of the crack front and/or by the imperfect linking of microcracks, that initiate ahead of the crack tip, with the main crack. Both the crack initiation and crack growth toughness are determined to significantly deteriorate with age in the longitudinal orientation

It was found that due to the anisotropy in bone, which alters the prominent toughening mechanisms, that aging decreased the fracture toughness in the longitudinal orientation (by an order of magnitude) much more significantly than in the transverse orientation (~14%). The primary toughening mechanism in the transverse orientation was found to be crack deflection and out of plane twist. For the transverse orientation, the reduced osteonal spacing due to more remodeling that comes with aging should result in more frequent crack defections as the growing crack encounters the cement lines, and this is what is observed [54] (figure 5). At first sight, this might be expected to increase the toughness, but the result of these more frequent crack deflections is that the extent of the individual “delaminations” along the cement lines (nominally perpendicular to the main crack path) becomes smaller so that the overall degree of crack-path meandering is actually lessened. Thus, the aging-related reduction in osteonal spacing leads to shorter crack-path excursions away from the plane of maximum tensile stress and the overall decrease in crack-path tortuosity, which in turn results in a smaller influence of aging on the fracture toughness in this transverse orientation. These findings are similar to that found with x-ray irradiation bone [55] which will be addressed in more detail in the next two

chapters. Briefly, the findings for irradiated bone, greater than 70 kGy (1Gy=1J/kg), show similarities to aging bone in the transverse orientation [51], irradiated bones (greater than 70 kGy) are subject to an increasing incident of crack deflection, principally along the cement lines, but the deflections are smaller and results in generally less tortuous crack path.

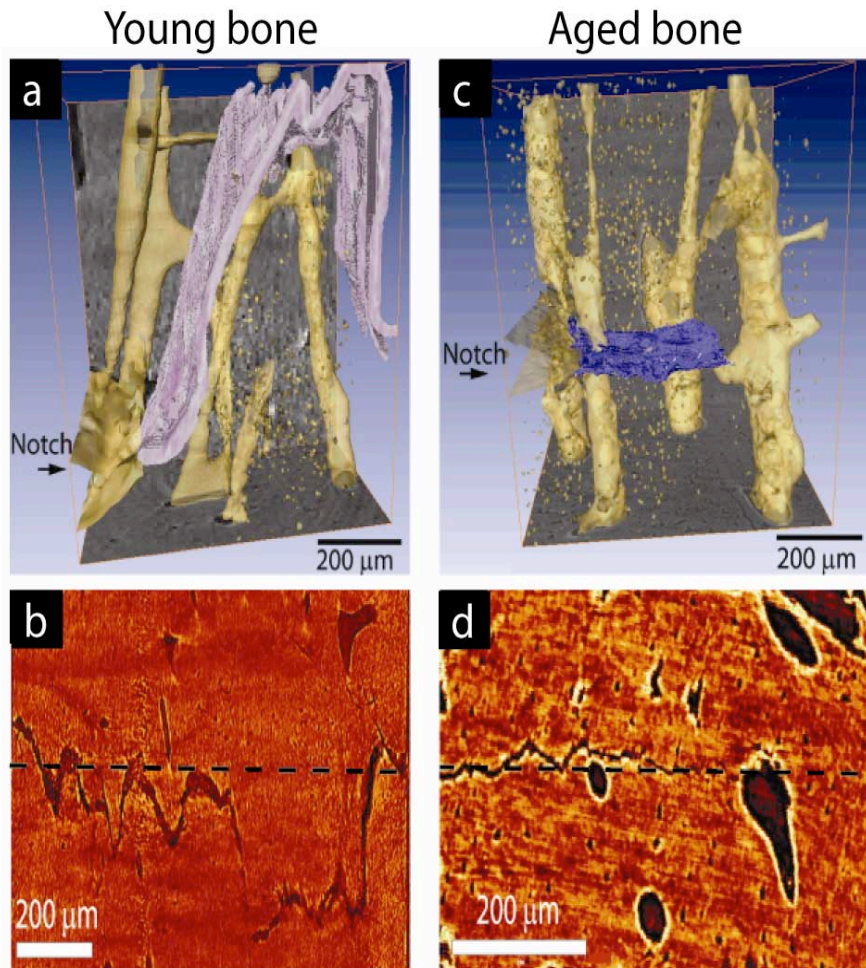


Figure 2-5: Synchrotron radiation micro-tomography of crack paths. 3-D tomographic images of (a) *Young* and (c) *aged* showing the crack path in purple and the Haversian canals in yellow/brown, and (b) and (d) 2-D tomographs of the paths from the back face of the sample. The crack deflects on encountering the osteons; such crack deflection and crack twisting is the prime extrinsic toughening mechanism in bone in the transverse orientation. Note, however, that the frequency of such deflections is increased whereas their severity is decreased with aging, resulting in less meandering crack paths in aged bone. The tomographic imaging took place at Advanced Light Source with an energy of 20 keV and a resolution of 1.8 $\mu\text{m}/\text{pixel}$.

Obesity

Another use of SR μ T is to evaluate the volumetric bone mineral density (vBMD) for comparative bone studies that study the effects of disease and other factors on bone. One such study looked at the changes in mechanical properties of cortical bone in high-fat diet (HFD) induced obesity by evaluating changes to both the bone quality and bone quantity [16]. Obesity is associated with greater bone mineral content that might be expected to protect against fracture. However, sometimes the incident of fracture is higher in overweight and obese children and adolescence. This study measured bending strength, modulus, and toughness for each of the 4 groups (adult HFD, young HFD, adult normal diet, and young normal diet). The results showed that even though the bone size increased in the high fat diet that the vBMD did not increase, and the strength, bending stiffness, and toughness are all reduced. This suggests that obesity leads to a reduction in bone quality despite an increase in overall bone quantity indicating that both bone quality and bone quantity play important compensatory roles in determining fracture risks.

Antler bone

Unlike human cortical bone, elk antler is predominately composed of primary osteons that contain vascular channels surrounded by concentric lamellae that are surrounded by hyper-mineralized regions. Antler in the transverse orientation is found to be one of the toughest biological materials, since it is highly adapted to its functions, which is to put on display and for fighting and is shed every winter. Therefore, the antler is designed to have low stiffness compared to skeletal bone, but is designed to undergo high impact loading and large bending moments without fracture. Antler is similar to cortical bone, since its' resistance to fracture is achieved extrinsically via crack growth by combining ninety degree crack deflection, out of plane twist, and crack bridging, both toughening mechanisms results from the occurrence of microcracking [5, 56]. The ability of the bone to form microcracks is essential for many toughening mechanisms at the micrometer length scale such as crack bridging and crack deflection. Antler bone has been reported to exhibit rising R-curves [57, 58].

Here synchrotron radiation micro-tomography, tuned to a monochromatic energy of 15 keV and a spatial resolution of 1.6 $\mu\text{m}/\text{pixel}$, is used to examine the extrinsic mechanisms in the compact bone of elk antler with different orientation [4]. Figure 6a shows mechanistically that in the transverse orientation the crack deflects by as much as 90 degrees and there is major twist in the transverse (breaking) orientation. As microcracks predominates along the weaker interfaces

of the osteon interfaces, the largest microcracks form along the long axis of the bone since this is nominally orthogonal to the fracture direction in the transverse orientation the degree of toughening can be large ($K_{IC} \sim 20 \text{ MPa}\sqrt{\text{m}}$ [4]) due to major deflections and twists, resulting surfaces are consequently very rough. Conversely in the longitudinal orientation, as seen in figure 6b, the major interfacial microcracks are now mainly parallel to the fracture direction. The formation of cracks parallel or ahead of the main crack allows for intact regions in between, resulting in uncracked ligament bridging [5, 56] that carry the load. Crack paths are much more planar and have little crack deflection resulting in a much smoother fracture surface. The crack bridging does supply some amount of toughness ($K_{IC} \sim 4\text{-}5 \text{ MPa}\sqrt{\text{m}}$ [4]). Antler has the highest strain to failure of the entire bone family, with an ultimate tensile strain of $\sim 12\%$ which is six times higher than the ultimate tensile strain of human cortical bone ($\sim 2\%$).

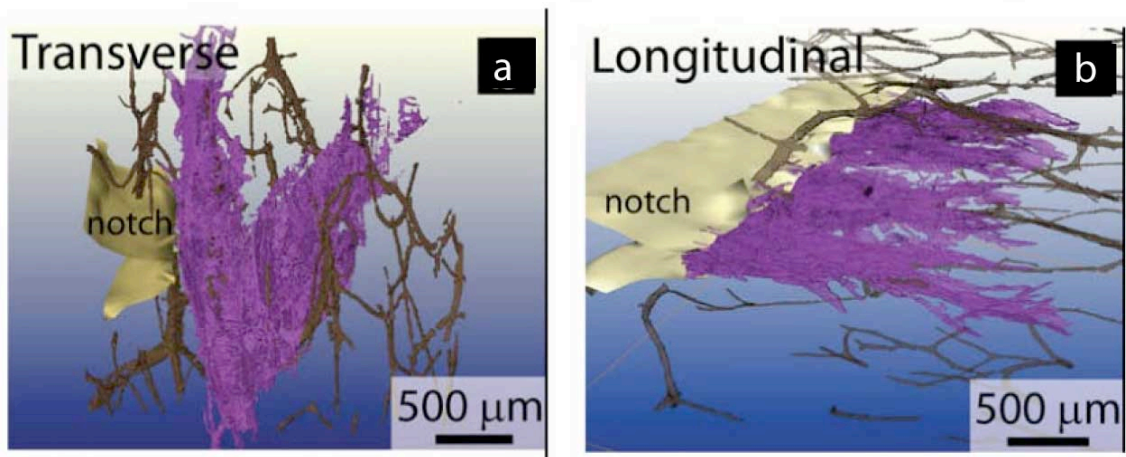


Figure 2-6: Synchrotron radiation micro-tomography images of the crack path in both the (a) transverse and (b) longitudinal orientations of antler compact bone. In the transverse (breaking) orientation (a) the crack undergoes severe deflections as it interacts with the osteons and lamellar interfaces. In the longitudinal (splitting) orientation (b) the crack is very planar with little evidence of deflection. The crack path is in purple and the Haversian canals are in brown and the notch is in white. The antler samples were scanned at an energy of 15 keV and a spatial resolution of $1.6 \mu\text{m}/\text{pixel}$.

Nature-Inspired Hybrid Materials

The challenge to existing structural materials is to develop new materials that are stronger, tougher, and lightweight. This challenge can only be met through understanding the relationship between materials architecture and mechanical response, spanning the structural parameters acting at multiple length scales. This delicate balance of light, tough, and strong is found in nature. Natural materials create their fracture resistance or toughness primarily by extrinsic toughening [59] which shields the advancing crack from the applied load. The extrinsic toughening mechanisms can be visualized at the micrometer scale. These mechanisms generate a crack resistant curve behavior where the fracture resistance actually increases with crack extension. These types of materials develop their toughening mainly during crack growth and not crack initiation.

A recent study applies a hierarchical design, similar to what is found in nature, to conventional compounds such as alumina and poly methyl methacrylate (PMMA) [7]. The concept in this study is to design a material that mimics the intricacy of the hierarchical structure of natural composites, like cortical bone in an attempt to replicate natural composites unique fracture behavior [60, 61]. The distribution of phases in a material is of interest for a wide range of issues and applications. Consider the three planes of the 2D slice image shown in figure 7 we can observe the multiphase particulate composite made up of PMMA and Alumina. The reason we can distinguish between these two phases is the variation in x-ray absorption at a monochromatic energy of 15 keV. This variation manifests itself through the different grayscale intensities in figure 7. The image has a magnification that produces 4.45 micrometer cubic voxels.

The hybrid material displayed nearly 300 times higher⁴ toughness than either constituent. To characterize the toughening mechanisms for this hybrid material synchrotron radiation micro-tomography was performed on the fractured alumina and PMMA composite samples. In figure 7b the crack path and the dispersed damage have been segmented out to allow for the visualization of damage throughout the structure. The toughness demonstrated here far surpasses what can be expected using the “rule of mixture”, for an ~80% Al₂O₃-PMMA material this study achieved a K_{IC} critical fracture toughness (K_{IC}) greater than 30 MPa m^{1/2} at a tensile strength of ~200 MPa, where the critical fracture

⁴ In energy terms

toughness, measurements were performed using nonlinear-elastic fracture mechanics methods, specifically involving the J -integral⁵ and the analysis is explained in more detail in Ch. 3. The tomographic images identify that the extrinsic mechanisms in this composite are similar to what have been identified in biomaterials, such as bone and dentin.

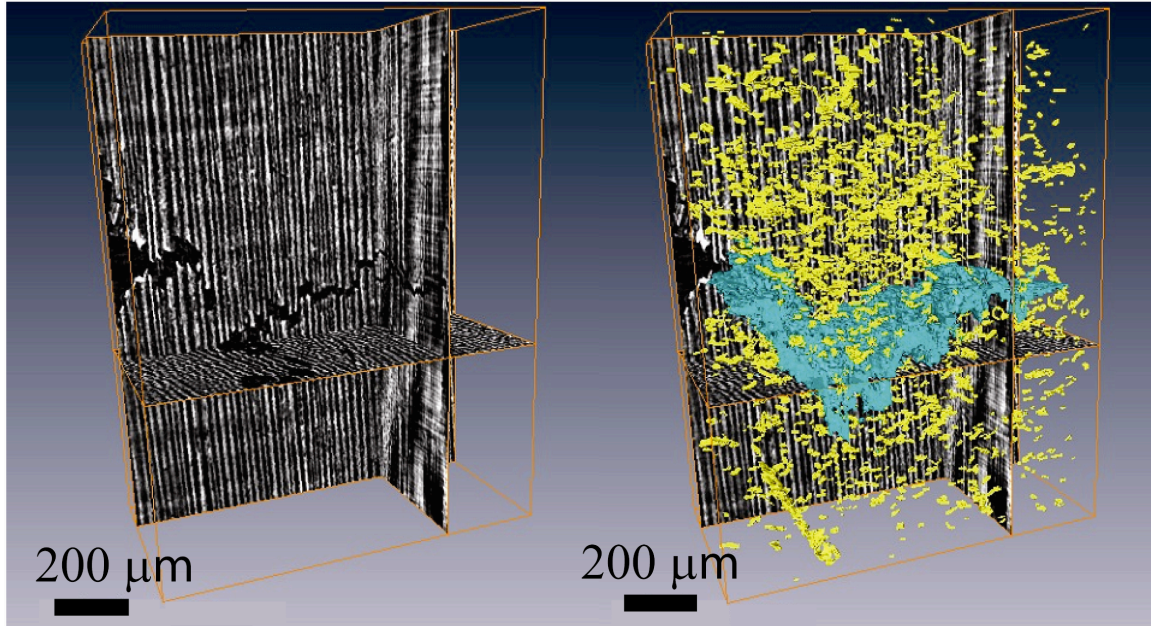


Figure 2-7: Nature-inspired hybrid material. Synchrotron X-ray tomography image of crack propagation from a sharpened notch (on the left-hand side) during an R curve test of a lamellar Al₂O₃-PMMA composite, showing (a) the three planes of 2-D reconstructed images and (b) the wide distribution of damage (in yellow) spread over several millimeters surrounding the deflected crack path (in blue). Crack propagation is nominally from left to right. The Al₂O₃-PMMA composite sample was imaged at 15 keV with a voxel size of 4.45 μm.

2.4. Conclusion

⁶ J is the nonlinear strain-energy release rate, *i.e.*, the rate of change in potential energy for a unit increase in crack area in a nonlinear elastic solid. It is the nonlinear-elastic equivalent of the strain-energy release rate G . It characterizes the stress and displacement fields at a crack tip in such a solid, and as such can be used to define the onset of fracture there.

Synchrotron radiation micro-tomography (SR μ T) has grown in popularity as an important imaging technique for many scientific and medical applications. Fracture resistance is a multi-scale process with each level of structural hierarchy adapted to optimal toughness. These studies show that macroscopic toughness from crack bridging and crack deflection plays a critical role at large length scale in the materials fracture resistance. The extrinsic mechanisms such as crack deflection, twist, and bridging are all highly dependent on structure and orientation. In many mineralized musculoskeletal tissue the osteons are the major structural component that controls the toughness, which represents a length scale that is several hundred micrometers in size. The process of major crack deflection and twist is one of the most potent sources of toughening in these biomaterials by diverting the cracks course to change from the plane of maximum stress. All of the three dimensional images of crack propagation in antler and bone demonstrate crack twisting at angles up to 90 degrees in addition to out-of-plane deflections. In fracture, crack trajectories result from a competition between the direction of maximum mechanical driving force (maximum G or $K_{II}=0$) and the path of weakest microstructural resistance [49, 62]. In the longitudinal orientation the preferred mechanical and microstructural paths are nominally in the same direction, while in the transverse orientation the maximum driving force is oriented ahead of the crack tip while the weakest path is oriented perpendicular to the crack tip resulting in different dominate toughening mechanisms. In conclusion, SR μ T is a powerful tool to indentify and characterize the toughening mechanisms in biomaterials and bio inspired materials, however the fracture behavior of these materials, like bone, is still not fully understood. To fully utilize this technique higher resolution is necessary in order to see the nucleation of the cracks in the biological structure. In summary, x-ray tomography can provide information that is critical to qualitatively assess and predict bone fracture.

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Chapter 3:

Characterizing the Effects of X-ray Irradiation on the Macro- to Micro-scale Deformation and Fracture Behavior in Human Cortical Bone

3.1. Introduction and Background

3.1.1 Summary

In situ mechanical testing coupled with imaging using high-energy synchrotron x-ray diffraction or tomography is gaining in popularity as a technique to investigate micrometer and even sub-micrometer deformation and fracture mechanisms in mineralized tissues, such as bone and teeth. However, the role of irradiation in affecting the nature and properties of the tissue is not always taken into account. Accordingly, the effects of x-ray synchrotron-source irradiation on the mechanistic aspects of deformation and fracture in human cortical bone are examined here. Specifically, the strength, ductility and fracture resistance (both work-of-fracture and resistance-curve fracture toughness) of human femoral bone in the transverse (breaking) orientation are evaluated following exposures to 0.05, 25, 35, 70, 210 and 630 kGrays (kGy, Gy=J/kg) irradiation.

This chapter shows that x-ray irradiation doses ranging from 35 kGy to 630 kGy deleteriously affect the toughening mechanisms, by primarily suppressing the intrinsic “plasticity” mechanisms at the sub-micrometer level, as characterized by the changes in the collagen environment which will be investigated in the next chapter, as well as altering the extrinsic mechanisms by increasing the amount of crack deflection and decreasing the crack amplitude which is investigated in this chapter. Our results show a major progressive loss in the mechanical integrity of the bone, specifically in its strength, post-yield ductility (strain) and toughness, with increasing levels of irradiation, to an extent that calls into question the use of *in situ* x-ray radiation to study the mechanical properties of biological tissue such as bone.

3.1.2. Radiation Overview

Human bone and tissue can be exposed to a wide range of radiation levels for medical and scientific reasons. At the low dose end, the average radiation from

an abdominal x-ray is ~1.4 mGy and at the high dose end (>10 kGy), gamma irradiation is commonly used to terminally sterilize allograft tissues and bones [1], and has been proven to be a very potent sterilization agent with the ability to effectively penetrate tissue. Medically, bone allografts, a common replacement with orthopedic surgeons for the reconstruction of bone, are invariably sterilized with gamma radiation to minimize the spread of disease [2]. The optimum dosage, however, is somewhat controversial as too much irradiation can cause damage to the collagen matrices [3-6]. Tissue banks typically use between 10 and 35 kGy [5, 7] although 70 kGy is needed in order to sterilize against radiation-resistant viruses. However, even a “standard dose” of ~35 kGy irradiation can have an effect on the mechanical integrity of bone and tissue. For doses up to 35 kGy, several studies [8-12] have reported a significant effect on the post-yield properties of cortical bone, in particular significant reductions in plastic properties such as ultimate strength and work-of-fracture, but little effect on the elastic properties. Conversely, other studies on bones and tendons [13-15] have claimed no significant reduction in biomechanical properties after similar doses.

The effect of irradiation is also a concern with scientific studies that investigate the properties of bone and tissue using computed micro-tomography (SR μ CT) with synchrotron radiation and desktop x-ray sources [16]. Surprisingly, this issue is often ignored but has become of importance as *in situ* mechanical testing with high-energy synchrotron x-ray tomography imaging [17, 18] is now commonly used to identify micrometer and even sub-micrometer deformation and fracture mechanisms in mineralized tissues. Typical *in situ* x-ray synchrotron tomography experiments involve the irradiation of, for example, bone samples at a rate of ~100 Gy/s (see appendix for calculations). The key factors which result in high radiation doses are the high flux density and the exposure time that can be around 30 minutes per data set; this leads to typical radiation doses ranging as high as 1 MGy [17, 18]. The problem here is that scientific observations and measurements on bone exposed to such radiation doses may negate the validity of the results as the structure and properties of the bone is no longer reflective of the human condition.

3.1.3. Identifying Extrinsic Mechanisms

As discussed in chapter 1, The stiffness, strength and toughness properties of bone develop from its’ multi-scaled, hierarchical structure [19-22], which spans from nanometer to macroscopic dimensions [23, 24]. This is especially true with respect to resistance to fracture where a suite of physical toughening

mechanisms can be defined which are activated at varying length-scales. Bone derives its toughness from both intrinsic and extrinsic mechanisms. Intrinsic mechanisms can be classified as “plasticity” mechanisms which operate principally at sub-micrometer length-scales to promote intrinsic toughness, involving molecular uncoiling of collagen molecules and fibrillar sliding. Conversely, extrinsic toughening mechanisms operate primarily in the wake of the crack tip to inhibit cracking by “shielding” the crack from the applied driving force [24-26] via such mechanisms as crack deflection, twist and crack bridging.

In this chapter the macroscopic properties in bone are characterized by quantifying changes to the strength, toughness and elastic properties with increased irradiation doses. This allows for an overview of the affects that x-ray irradiation has on the deformation and fracture properties of human cortical bone; specifically by performing mechanical testing and fracture resistance measurements and then identifying the extrinsic mechanisms via *ex situ* tomography. This will provide micron-scale mechanistic information on how the crack path interacts with the bone-matrix structure which will be useful in characterizing the change to the extrinsic mechanisms in bone with irradiation.

3.2. Experimental Methods

3.2.1. Materials

Bone test samples were taken from the midsection of a frozen human cadaveric femoral cortical bone (male, aged 48 years old). For bend tests, the 49 samples were divided into seven groups based on irradiation exposure: one control group (unirradiated), one group irradiated at a low dose typical of radiation therapy (50 Gy), three groups irradiated at varying doses that are typical of doses received during sterilization (25, 35, 70 kGy, respectively), and two groups irradiated at the typical dose received during one to three x-ray tomography scans (210, 630 kGy). X-ray irradiation was performed at the Advanced Light Source (ALS) synchrotron facility at the Lawrence Berkeley National Laboratory on a super-bend source. The dose rate at the ALS at the energy level of 20 keV is 110 Gy/s [27]. During irradiation, all of the samples were kept hydrated by wrapping the sample in a wet paper towel.

The samples for each of the seven irradiation groups were further divided into two groups for mechanical testing, three samples were used for three-point bending tests and the remaining four were used for crack resistance-curve (R-

curves) measurements;¹ test methods were in general accordance with ASTM standards [28]. Bend samples were machined along the long axis of the bone into 10-mm long rectangle bars (thickness $B = 1.5\text{-}2.0$ mm, width $W = 3\text{-}4$ mm), which following micro-notching were used for R-curve measurements (as single-edge-notch bend, SE(B), samples). Micro-notching was performed by first using a low-speed diamond saw to cut an initial notch then sharpened by repeatedly sliding a razor blade over it while continuous irrigation with a 1- μm diamond slurry; this yielded a consistent micro-notch root radius of $\sim 3\text{-}5$ μm . Using this technique sharp stress concentrators with initial crack length of $a \approx 1.5\text{-}2$ mm ($a/W \approx 0.5$) were produced. The notch was positioned so that the nominal crack growth was perpendicular to the long axis of the bone (transverse orientation). Prior to testing, all of the samples were wet polished in a 0.05 μm diamond suspension to a final polish before being immersed in ambient Hanks' Balance Salt Solution (HBSS) for ~ 24 hr prior to testing.

3.2.1. Mechanical Testing

To evaluate changes to the fracture resistance, i.e., toughness, measurements were performed using nonlinear-elastic fracture mechanics methods, specifically involving the J -integral² on human cortical bone; many past studies have used fracture toughness values, mainly critical stress intensity. While the use of a single value of toughness is adequate for many other materials, in the case of bone which exemplifies increased resistance to fracture with crack extension, when this happens it is more useful to use a resistance curve (R- curve) fracture-mechanics approach these methods provide a more realistic description of the contribution to the toughness from the energy consumed in plastic deformation³ prior to, and during, fracture [29-31]. R-curves are necessary when characterizing the fracture resistance of materials that exhibit extrinsic toughening mechanisms,

¹ The crack resistance- or R-curve provides an assessment of the fracture toughness in the presence of subcritical crack growth. It involves measurements of the crack-driving force, *e.g.*, the stress intensity K , strain-energy release rate G or J -integral, as a function of crack extension (Δa). The value of the driving force at $\Delta a \rightarrow 0$ provides a measure of the crack-initiation toughness whereas the slope and/or the maximum value of the R-curve can be used to characterize the crack-growth toughness.

² J is the nonlinear strain-energy release rate, *i.e.*, the rate of change in potential energy for a unit increase in crack area in a nonlinear elastic solid. It is the nonlinear-elastic equivalent of the strain-energy release rate G . It characterizes the stress and displacement fields at a crack tip in such a solid, and as such can be used to define the onset of fracture there.

³ Plastic deformation here is used as a general term to indicate any of the inelastic, non-recoverable deformation mechanisms, such as local collagen fibrillar shearing, viscoplasticity, and microcracking, that are active at various length-scales in bone [23, 24].

such as crack deflection, twist and crack bridging. J -R curves were performed under rehydrated conditions in mode I (tensile-opening) using SE(B) specimens with a crack-growth direction transverse to the long axis of the osteons (transverse orientation). R-curves were measured on the HBSS-saturated specimens *in situ* in a Hitachi S-4300SE/N environmental scanning electron microscope (ESEM) using a Gatan Microtest three-point bending stage. Crack extension was monitored directly in back-scattered electron mode at a pressure of 35 Pa and a 25 kV excitation voltage. Tests were conducted in three-point bending with a span ($S = 6$ mm) to width ($W = 3$ mm) ratio of ~ 2 , in accordance with ASTM Standard E1820-08 [28]. R-curve tests were terminated after ~ 700 μm of crack extension; the samples that had not failed at this stage were stored for subsequent tomography analysis.

Measured R-curve data points were limited to small-scale bridging conditions, where the size of the zone of crack bridges behind the crack tip remained small compared to the in-plane test specimen dimensions. As noted above, the use of the J -integral as the driving force for crack initiation and growth was employed to capture the contribution from inelastic deformation in the evaluation of toughness. The stress intensity at each measured crack length was calculated by measuring the nonlinear strain-energy release rate, J . The value of J was calculated from the applied load and instantaneous crack length according to ASTM standards [28], and was decomposed into its elastic and plastic contributions:

$$J = J_{\text{el}} + J_{\text{pl}}. \quad (1)$$

The elastic contribution J_{el} is based on linear-elastic fracture mechanics:

$$J_{\text{el}} = \frac{K_{\text{I}}^2}{E}, \quad (2)$$

where K_{I} is the mode I stress-intensity factor, and E is the Young's modulus. Using the load-line displacements, the plastic component J_{pl} for a stationary crack in bending is given by:

$$J_{\text{pl}} = \frac{1.9A_{\text{pl}}}{Bb}, \quad (3)$$

where A_{pl} is the plastic area under force *vs.* displacement curve, b is the uncracked ligament length ($W-a$). K -based fracture toughness K_{Jc} values were then back-calculated from the J measurements using the standard J - K equivalence for nominally mode I fracture, specifically that $K_{\text{Jc}} = (J_{\text{c}} E)^{1/2}$.

Values of the Young's modulus E for the unirradiated and irradiated (70 kGy) cortical bone were determined using nano-indentation. A total of 11 indentations were performed using a Triboindenter (Hysitron, Inc.). At each location the reduced modulus, (indicating the elastic properties of the bone extracellular matrix), was determined. The results showed a true elastic modulus of 17 ± 5 GPa with no statistically significant changes between the unirradiated and irradiated groups.

For all fracture toughness tests conducted, conditions for J -dominance, as specified by the active ASTM standard [28], were met, *i.e.*, $b, B \gg 10 (J_c/\sigma_y)$, where σ_y is the flow stress. This latter criterion ensures that the critical J_c (and calculated K_{Ic}) values represent valid fracture toughness values.

Three-point bend tests were performed to generate quantitative stress-strain curves. The strength tests were performed on unnotched bend specimens using a support span S of 7.5 mm and a displacement rate of 10 $\mu\text{m/s}$. From these measurements, the ultimate bending stress and strain were determined at the point of maximum load. In addition, the bending stiffness was calculated from the slope of the stress/strain curves, and the work-of-fracture calculated from the area under these curve divided by twice the cross-sectional area of the fracture surface.

3.2.3. Extrinsic Mechanisms

The microstructure of bone was characterized in 2D using scanning electron microscopy in the back-scattered electron mode with the Hitachi S-4300SE/N ESEM. Synchrotron Radiation x-ray micro-tomography (SR μ T) was employed to visualize in three-dimensions the crack path and distribution of micro-damage *after* R -curve testing. The SR μ T evaluation was performed at the Advanced Light Source synchrotron radiation facility at Lawrence Berkeley National Laboratory; the setup is similar to standard tomography procedures [32] in that samples are rotated in a monochromatic x-ray beam and the transmitted x-rays imaged via a scintillator, magnifying lens and a digital camera to give an effective voxel size in the reconstructed three-dimensional image of 1.8 μm . Hydrated samples were scanned in absorption mode and the reconstructed images were obtained using a filtered back-projection algorithm. In absorption mode, the gray scale values of the reconstructed image are representative of the absorption coefficient. To maximize the signal-to-noise ratio, an energy of 20 keV was selected; this optimizes the interaction between the x-rays and the sample. Two-dimensional images were taken every quarter of a degree between 0 and 180 degrees. The

data sets were then reconstructed using the software Octopus [33] and the three-dimensional visualization was performed using Avizo™ software [34].

3.2. Result

3.2.1. Strength and Fracture Toughness

Bending stress-strain curves, which assess the macroscopic strength and ductility, and fracture toughness R-curves of human cortical bone irradiated at varying degrees of x-ray irradiation (0, 0.05, 25, 35, 70, 210, 630 kGy), are shown in Figure 1. Data is tabulated in table 2. The data shows that for irradiation exposures starting between 35-70 kGy, there is a severe dose-dependent degradation in mechanical properties; no effect could be discerned at 35 kGy and below. Above this level though, there was a complete loss in post-yield deformation (plasticity) coupled with a progressive and very severe reduction in the ultimate (maximum) bending strength, bending strain and toughness, although little change was seen in the bending stiffness. Specifically, compared to unirradiated bone, exposures of 70, 210 and 630 kGy resulted in a respective decrease in the strength of the bone by ~25%, 60% and more than 80%, while a similar trend was observed for the ultimate strain (Fig. 1a), additionally, the work-to-fracture decreased by 70% to almost 100% as the irradiation dose increased from 70 to 630 kGy (Fig. 2a). This is consistent with previous studies that show that γ - and x-irradiation can degrade the plastic, rather than elastic, properties of bone, such as bending strength and toughness [6, 8, 11, 15, 35-37]. Of importance is that although the irradiation doses in question are very large compared to the few second exposures typical of *in situ* x-ray scattering experiments, they are definitely comparable to the irradiation associated typical tomography imaging runs (Table 1).

The corresponding toughness properties, in terms of full $K_R(\Delta a)$ R-curves for physiologically relevant small cracks ($\Delta a < 700 \mu\text{m}$), are plotted in Fig. 1b for all seven irradiation groups. It was observed that even though the stress-strain curves showed a severe loss in the plastic properties of bone with high doses of irradiation, rising R-curve behavior is still exhibited (albeit diminished), even after all plasticity is lost, indicating that some limited degree of extrinsic toughening, *e.g.*, from crack deflection and/or crack bridging, is still active. Nevertheless, the irradiation caused a very severe degradation in the crack-initiation toughness (assessed in terms of the intercept of the R-curve at $\Delta a \rightarrow 0$)

and crack-growth toughness (assessed as both the slope of the R-curve, $dK/d\Delta a$ and the instability toughness value, K_{Jc}). Specifically, over the first 600 μm of (subcritical) crack extension, K_{Jc} fracture toughness values were as much as a factor of five lower after 210 kGy irradiation, *i.e.*, from values of 13.3 $\text{MPa}\sqrt{\text{m}}$ ($J_c \sim 9 \text{ kJ/m}^2$) in unirradiated samples down to 2.7 $\text{MPa}\sqrt{\text{m}}$ ($J_c \sim 0.3 \text{ kJ/m}^2$) in the 210 kGy irradiated samples; similarly, K_{Jc} values were 10.5 $\text{MPa}\sqrt{\text{m}}$ ($J_c \sim 5 \text{ kJ/m}^2$) and 7.4 $\text{MPa}\sqrt{\text{m}}$ ($J_c \sim 3 \text{ kJ/m}^2$), respectively, after 50 Gy and 70 kGy of radiation.

Table 3-1: Dose rates from typical *in situ* small angle x-ray scattering experiments and *in situ* synchrotron tomography experiments.*

Type of <i>in situ</i> experiment	Synchrotron location	Radiation energy (keV)	Flux (photons/s)	Flux density (photons/s/mm ²)	Radiation dose rate (kGy/s)	Typical radiation dose (kGy)
Small-angle x-ray scattering (SAXS)	Hamburger Synchrotronstrahlungslab (HASYLAB), Deutsches Elektronen-Synchrotron (DESY)	8.27	1×10^9	1.67×10^8	2×10^{-4}	~0.04 - 0.12
Wide-angle x-ray scattering (WAXS)	Advanced Photon Source (APS), Argonne National Laboratory	80.7	1×10^{10}	1×10^{12}	0.2	~6 - 30
SAXS, WAXS	Advanced Light Source (ALS), Lawrence Berkeley National Laboratory	10	1.76×10^{12}	2.2×10^{11}	0.5	~20
Tomography (μ XCT)	Advanced Light Source, Lawrence Berkeley National Laboratory	20-80	8.4×10^{13}	2.1×10^{11}	0.12	~1.3 $\times 10^3$
Tomography	Swiss Light Source SLS, Paul Scherrer Institute	20	3×10^{14}	1.36×10^{12}	0.77	~6.0 $\times 10^3$

* Estimation procedures are described in the Appendix.

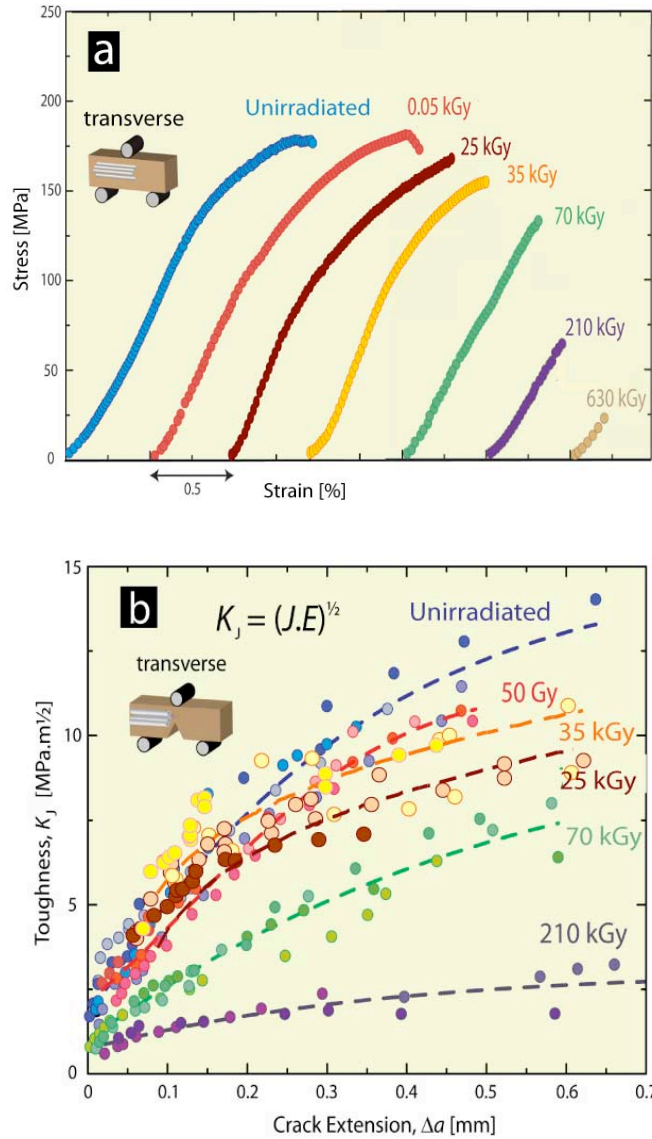


Figure 3-1: Mechanical properties of human cortical bone subjected to varying degrees of x-ray irradiation. (a) Stress-strain curves from three-point bending tests (offset for clarity) for hydrated human cortical bone in the transverse orientation at different irradiation levels. (b) Crack-resistance curves (R-curves) showing resistance to fracture in terms of the stress intensity, K_I , as a function of crack extension, Δa , for human cortical bone in the transverse orientation. K_I fracture toughness values were back-calculated from the J measurements using the J - K equivalence for mode I fracture. The end of the curves indicates the critical toughness values, K_{Ic} , at which complete failure occurred.

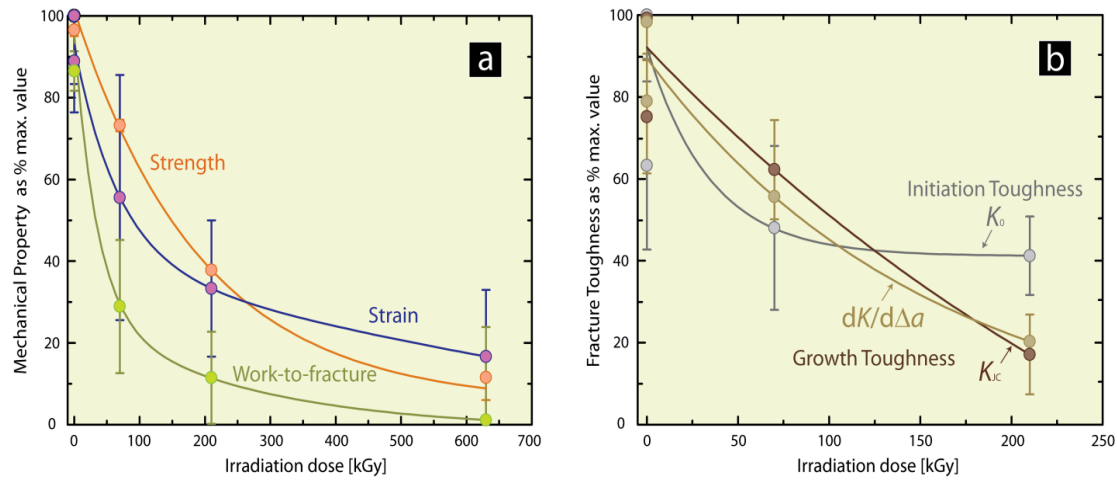


Figure 3-2: Changes in mechanical properties of hydrated human cortical bone with irradiation dose. (a) The effects of irradiation on the ultimate bending stress and strain, and work-to-fracture of hydrated human cortical bone. (b) Corresponding effects of irradiation on the crack-initiation toughness, K_0 , and crack-growth toughness, $dK/d\Delta a$ and K_{IC} . The graphs show that there is a severe and progressive degradation in mechanical properties, specifically in the bending stress/strain and toughness properties, with increase in x-ray irradiation dose. Values plotted are normalized to the highest value for each group (=100%).

3.2.2. Extrinsic Mechanisms

The associated mechanistic sources of toughening in irradiated bone were identified during the *in situ* mechanical tests in the environmental scanning electron microscope on rehydrated samples. This technique provides the opportunity to measure quantitatively the *R*-curve while simultaneously monitoring the evolution of damage mechanisms ahead of the growing crack and the toughening mechanisms that result in its wake; furthermore, how these mechanisms relate to the bone architecture can be imaged in real time (Fig. 3a,d).

Results show that the extrinsic toughening mechanisms in bone, *i.e.*, the crack deflection/twist and crack bridging processes that operate at length-scales above a micrometer [31, 39], are still operative in the irradiated bone; accordingly, all samples displayed subcritical (stable) crack growth over the initial 700 μm of crack extension (Fig. 2b). These mechanisms result from the occurrence of microcracking primarily along the cement lines, *i.e.*, the “weaker” hyper-

mineralized interfaces of the osteons, and to a lesser extent along the lamellar boundaries. The growing cracks deflect by as much as 90 degrees as they encounter these interfaces between the interstitial bone and the osteons, leading to the marked crack deflections and through-thickness twists (Fig. 3) that are the primary source of (extrinsic) toughening in the transverse orientation. Although prior irradiation does not inhibit these mechanisms, SR μ T images show that the frequency of deflection appears to be greater in the irradiated samples (Figs. 3a & d). As this leads to smaller-amplitude crack deflections (Figs. 3b & e, 3c & f), the extent of crack meandering within the bone-matrix is actually lower after irradiation, which is consistent with the crack extending at much lower stress intensities, consistent with the macroscale test data in Fig. 1.

3.3. Discussion

This experimental study highlights a major deleterious effect of x-ray radiation on “bone quality” for human cortical bone. For radiation dosage levels typical of *in situ* x-ray tomography imaging, we report a progressive decrease in both the post-yield mechanical properties (ultimate bending strength and strain) and fracture toughness (work-of-fracture and the crack-initiation and –growth toughness) with increase in irradiation levels from 0.05 to 630 kGy (Figs. 1-3). The effects are not trivial; bending strengths decline by up to ~100% and K_{Ic} fracture toughness values by a factor of five (Table 2). Structurally, the irradiation affects the collagen environment by disrupting the degree of cross-linking, resulting in a total loss in post-yield (plastic) deformation leading to a radical decline in strength and ductility; this loss in intrinsic toughening, coupled with lower extrinsic toughening due to “smoother” crack paths, can be further associated with the large decline in fracture resistance (or toughness).

To understand the origin of this effect, specifically how irradiation can degrade the strength and toughening mechanisms in bone, we note that the fracture resistance of bone is a multiple-scale process with each level of structural hierarchy adapted to provide optimal toughness. Traditionally, toughness has been thought of as the ability of a material to dissipate deformation energy without propagation of a crack. However, fracture is actually the result of a mutual competition of *intrinsic* damage mechanisms ahead of the crack tip that promote cracking and *extrinsic* shielding mechanisms mainly behind the tip that impede it [41, 42]. The influence of irradiation in terms of how it may affect these two mechanistic components is addressed thoroughly in this chapter and chapter 4.

At micro- to macro-scale dimensions, the toughness of cortical bone is dominated by the extrinsic contributions and associated with crack-tip shielding principally from crack bridging and deflection. The main structural feature controlling these mechanisms is the secondary osteons [44], or more precisely their interfaces, the cement lines [45], which act as prime locations for microcracking [31, 46, 47]. In the longitudinal orientations, such microcracking occurs nominally ahead and/or parallel to the main growth crack; the intact regions in between then act as ("uncracked-ligament") bridges which enhance the toughness by carrying load that would otherwise be used to propagate the crack [31, 39]. In the presently tested transverse orientation, microcracking is conversely nominally orthogonal to the path of the main growing crack; the resulting crack arrest and "delamination" along the cement lines as the crack encounters the osteons (see Fig. 3) results in even more potent toughening in the form of crack deflection and twisting [29-31]. Our results show that loss of post-yield (plastic) deformation due to irradiation definitely diminishes this crack-growth toughness (Fig. 1b), although mechanistically, toughening via crack deflection/twist is still prevalent in the irradiated as well as the unirradiated samples (Fig. 3). However, as Fig. 3 illustrates, due to the increased frequency but decreased magnitude of the crack deflections, crack paths in irradiated samples are considerably less tortuous. Because the motion of a crack away from the path of maximum driving force, *e.g.*, the $G_{\max}$ path [31, 46, 47], reduces the local stress intensity actually experienced at the crack tip, such less meandering crack trajectories in irradiated samples would certainly lessen the potency of the crack deflection toughening mechanism, thereby degrading the toughness with respect to unirradiated bone.

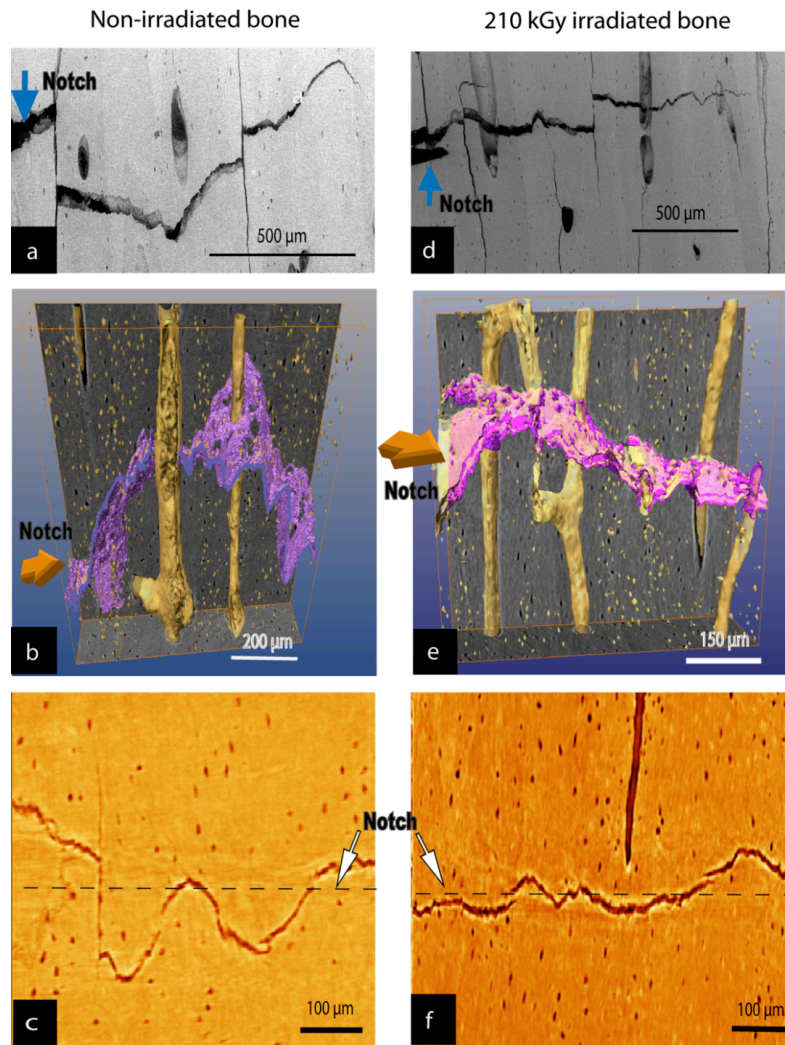


Figure 3-3: Scanning electron microscopy and computed x-ray tomography of crack paths in (a-c) non-irradiated and (d-f) 210 kGy irradiated hydrated human cortical bone (transverse orientation). Images of fracture in the non-irradiated and irradiated bone showing crack paths: (a) & (d) SEM micrographs from side-view perpendicular to the crack plane, (b) & (e) 3-D x-ray tomography images of these paths (notch shown by orange arrow; crack surface is purple/pink; Haversian canals are yellow), and (c) & (f) 2-D tomographs of the paths from the back face of the sample. The crack deflects on encountering the osteons; such crack deflection and crack twisting is the prime extrinsic toughening mechanism in bone in the transverse orientation. Note, however, that the frequency of such deflections is increased whereas their severity is decreased with irradiation, resulting in less meandering crack paths in irradiated bone.

Table 3-2: Mechanical properties of human cortical bone for the varying x-ray irradiation doses**

Irradiation dose (kGy)	Bending stiffness (GPa)	Ultimate bending strength (MPa)	Ultimate bending strain (%)	Work-to-fracture (kJ/m ²)	Crack-initiation toughness, K_0 (MPa√m)	Crack-growth toughness	
						$dK/d\Delta a$ (MPa√m/mm)	K_{Ic} (MPa√m)
0	1.37 (0.51)	166.9 (10.4)	1.6 (0.2)	16.7 (0.8)	1.77 (0.19)	31.8 (3.0)	13.3
0.05	0.81 (0.42)	172.8 (8.5)	1.8 (0.3)	19.3 (0.7)	1.12 (0.23)	23.9 (3.3)	10.5
70	1.43 (0.24)	126.5 (1.8)	1.0 (0.3)	5.58 (0.91)	0.85 (0.17)	19.8 (2.4)	7.4
210	1.20 (0.17)	65.4 (0.01)	0.6 (0.1)	2.22 (0.25)	0.73 (0.07)	5.45 (0.53)	2.7
630	1.53 (0.44)	20.0 (1.1)	0.3 (0.1)	0.22 (0.05)	-	-	-

** Standard deviations in parentheses.

3.4. Conclusion

Many *in situ* mechanical tests require long data acquisition times and use x-rays with extremely high flux density. For biological tissue such as bone, we have shown here that such exposure to high levels of irradiation can cause serious deleterious effects to the collagen which leads to drastic losses in strength, ductility and toughness. It is therefore essential that studies using *in situ* radiation, such as deformation and fracture testing coupled with x-ray diffraction and/or tomography, carefully take this into consideration. For *in situ* high-energy x-ray diffraction studies, the crucial factor is the amount of time that the sample is exposed, since the radiation flux levels are fairly low and generally fall in the “non-damaging” dose range. As such times are typically only a few seconds, irradiation-damage is generally not much of an issue with these techniques. For *in situ* x-ray tomography studies, however, the irradiation levels are much higher and the required exposure times to gain accurate information

about a sample are typically 30 minutes or more, even on a synchrotron. Here the potential for severe tissue damage during the experiment is far more likely. It is therefore imperative to carefully evaluate the irradiation dose level for any *in situ* high energy x-ray study on biological materials to ascertain that the radiation will not lead to severely altered properties of the tissue.

Based on this experimental study which looks at the effects of irradiation by x-rays on the mechanical behavior (macro to micro scale) of hydrated human cortical bone (in the transverse orientation) it is shown that bone exposed to irradiation doses of 70 kGy and above displays a complete absence of post-yield plastic deformation. Mechanistically, this is associated with the diminishing of extrinsic toughening mechanisms. It was shown here that bone subject to prior irradiation exposure in excess of ~70 kGy leads to radical changes in the crack path (Figs. 3d,e,f). Although growing cracks are still subject to such microstructurally-induced deflections, the deflections are far smaller in magnitude (although more frequent) resulting in minimal crack-path deviations and an essentially linear crack trajectory (Figs. 3d,e,f). It is the absence of such highly tortuous crack paths in irradiation-damaged bone, with minimal deviations from the plane of maximum tensile stress, that serves to curtail the high extrinsic toughness which is characteristic of healthy bone in the transverse orientation. This work implies that the results from *in situ* experimental research on biological materials that use a radiation source must be considered with care. Whereas typical irradiation exposures for *in situ* x-ray micro-diffraction studies on bone are generally insignificantly small, the radiation doses examined in the present research are comparable with those typically used for *in situ* synchrotron x-ray tomography studies, which calls into question the veracity of results derived from such work.

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Chapter 4:

Characterizing the Effects of X-ray Irradiation on the Nanoscale Deformation and Fracture Behavior in Human Cortical Bone

4.1. Introduction

4.1.1. Summary

Bone consists of a complex structure of primarily collagen, hydroxyapatite, and water, where each hierarchical level of its structure contributes to its strength, ductility and toughness [1]. These properties, however, are severely degraded by irradiation, which can arise from medical therapy or bone allograft sterilization. Here we use a mechanistic framework to identify the role that irradiation plays in affecting the nature and properties of (hydrated) human cortical bone tissue over a range of characteristic length-scales (from nano- to macro-scales), following x-ray irradiation exposures of 70 kGy. As demonstrated in chapter 3, macroscopically, bone strength, ductility and fracture resistance are shown to progressively degrade with increasing levels of irradiation. In this chapter the sub-micron length-scales are investigated by evaluating the strength properties with *in situ* tensile tests in the synchrotron using small- and wide-angle x-ray scattering/diffraction, where the strain can be simultaneously measured in the macroscopic tissue, collagen fibrils and mineral. Compared to healthy bone, results show that the fibrillar strain is decreased by ~40% following 70 kGy exposures, consistent with significant stiffening of the collagen and degradation to the collagen matrix. We attribute the irradiation-induced deterioration in the mechanical properties of bone to mechanisms at multiple length-scales, specifically at the submicron length scale, loss of plasticity is attributed to suppressed fibrillar sliding, and the loss and damage of collagen at the nano-scales, which, in this chapter, is assessed using UV Raman and Fourier Transform Infrared spectroscopy measurements and a fluorometric assay.

We find that the severe deterioration in the mechanical properties of bone following irradiation results from two classes of mechanisms operating at widely differing length-scales, namely, extrinsically from a transition to more linear (less deflected) crack paths at microscopic dimensions (discussed in the previous chapter) and intrinsically (discussed here) by an almost complete suppression of plasticity (post-yield deformation) at nanoscale dimensions in severely irradiated bone, the latter resulting from a suppression of fibrillar sliding due to altered levels of enzymatic (immature *vs.* mature) and non-enzymatic cross-linking of the collagen at molecular levels. Indeed, molecular and protein conformational

measurements suggest that irradiation alters the distribution of multiple cross-links that subsequently suppress fibrillar sliding.

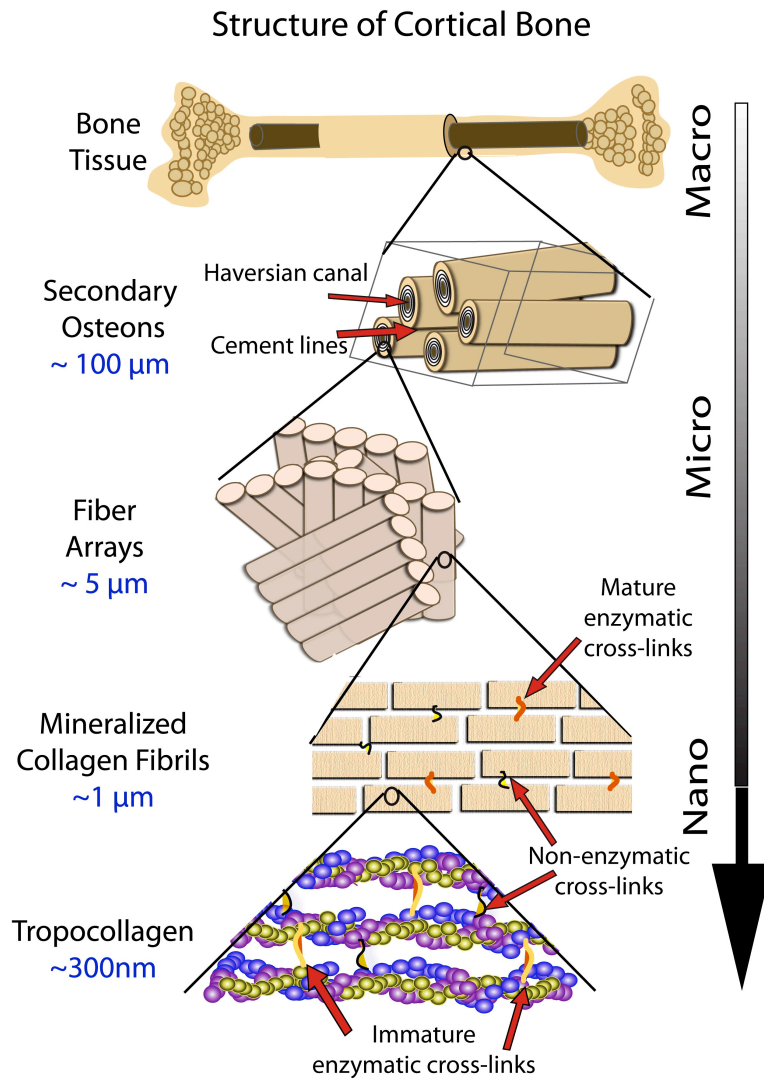


Figure 4-1: The structural hierarchy of bone. At the smallest level, at the scale of the tropocollagen molecules and mineralized collagen fibrils, (intrinsic) toughening is achieved through plasticity, principally via mechanisms of molecular uncoiling and intermolecular sliding of molecules and mineralized collagen fibrils. Cross-links form at these length-scales between the collagen molecules and between the fibrils [2]. At micrometer dimensions, the breaking of sacrificial bonds at the interfaces of fibril arrays contributes to increased energy dissipation, together with crack bridging of microcracks by collagen fibrils. At the largest length-scales in the range of 10s to 100s μm, the primary sources of toughening are extrinsic and result from extensive crack deflection and crack bridging/twisting by uncracked ligaments, both mechanisms that are motivated by the occurrence of microcracking [3, 4].

4.1.2. Structure of Bone

Bone is a natural composite like material with a complex hierarchical structure comprised primarily of collagen, hydroxyapatite and water [1] (Figure 1). The structure of bone is discussed in further detail in chapter 1. Briefly, at the molecular level, it is comprised of polymetric proteins, primarily type I collagen, with hard and stiff mineral nanoparticles of hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) that reinforce it. The mineral particles consist of apatite, which has a hexagonal crystal structure with the c-axis oriented predominately along the fibril direction. The fibril direction is along the tensile direction in the sample. Bone mineral crystals have a thickness of 1.5-4 nm. At the micron-scale these mineralized collagen fibrils are twisted together to form collagen fibers. At even coarser scales on the order of 10 to 100s μm , bone's characteristic structure consists of osteons, which are bone cylinders, $\sim 100 \mu\text{m}$ in diameter, that contain a central, longitudinal, tubular cavity (Haversian canal), blood vessels, and nerves, and which serve as the feature by which bone remodels. Bone's mechanical behavior originates from its multi-dimensional hierarchical nature [1, 5-7], which leads to different deformation mechanisms at each level. Principally at sub-micron dimensions, bone is toughened intrinsically by "plasticity" mechanisms; these include the molecular uncoiling of the tropocollagen molecules at the nanoscale [3], and at slightly coarser scales the sliding of mineralized collagen fibrils [8, 9].

A distinct feature of bone (type I) collagen is its cross-linking chemistry and its molecular packing structure. The intermolecular cross-linking provides the fibrillar collagen matrices with properties such as tensile strength and viscoelasticity [10]. Therefore, disruption to the cross-linking can severely affect bone's mechanical performance. The three types of collagen cross-links found in type I collagen are enzymatic immature (reducible) divalent cross-links (dehydrodi-hydroxynorleucine), enzymatic mature (non-reducible) divalent (pyridinoline and pyrrole) cross-links linking neighboring fibrils, and the non-enzymatic advanced glycation end-products (AGEs) that form both intermolecular and interfibrillar cross-links along the collagen backbone. Cross-links found in biomaterials involve two different mechanisms, one is enzymatically controlled during development and maturation and one is non-enzymatically controlled, known as glycation, following maturation of the tissue. The stabilization of the collagen fibers occurs initially through formation of the divalent intermolecular cross-links based on enzymatically produced lysine aldehydes at the N- and C- terminal domains of the molecule. During new bone formation the collagen molecules need to be aligned within the fibers, which are stabilized initially by the formation of covalent cross-links between neighboring

collagen molecules. Extracellular collagen molecules are aligned relative to their neighbors by a distance of 67 nm [11]. These divalent cross-links mature to form trivalent cross-links capable of linking the microfibrils, therefore forming a network of cross-links through the fiber [12, 13]. In addition to enzymatic cross-links, the adventitious attraction of glucose to collagen results in the formation of non-enzymatic cross-links. Collagen cross-linking formation affects not only the mineralization process but also micro-damage formation. Consequently, collagen cross-linking formation is thought to affect the mechanical properties of bone at a material level [14-16]. In fact, impaired enzymatic cross-linking and/or an increase in non-enzymatic cross-links in bone collagen have been proposed to be a determinant of impaired bone mechanical properties in aging, diseases like osteoporosis, and irradiation.

4.2. Experimental Methods

4.2.1. Materials

Bone test samples were taken from the midsection of a frozen human cadaveric femoral cortical bone (male, aged 48 years old). They were machined into 40 tension specimens for strength and ductility measurements (with *in situ* SAXS/WAXD). All samples were stored in Hanks' Balanced Salt Solution (HBSS) prior to irradiation and/or testing. X-ray irradiation was performed at the Advanced Light Source (ALS) synchrotron facility at the Lawrence Berkeley National Laboratory on a super-bend source. The dose rate at the ALS at the energy level of 20 keV is 110 Gy/s [17]. During irradiation, all of the samples were kept hydrated by wrapping the sample in a wet paper towel.

The 40 samples for the *in situ* tension tests were sectioned using a low-speed saw into ~10 mm long rectangle bars ($B \sim 100\text{-}200\ \mu\text{m}$, $W \sim 1\ \text{mm}$), along the long axis of the bone; the final thickness was achieved by polishing with 800-grit paper. Samples were left to dry in air for roughly 24 hr before 60-grit silicon carbide paper was affixed to the ends of the samples with cyanoacrylate glue to form frictional surfaces to grip during testing. Each sample had four horizontal lines drawn on with marker to act as guides for optical tracking of the tissue strain. The samples for the *in situ* tension tests were divided into two groups: a control group (unirradiated) and a high-dose irradiation group (70 kGy).

4.2.2. Small Angle X-ray Scattering & Wide Angle X-ray Diffraction (partitioning of strain)

X-rays will scatter from the electron clouds of atoms and from nanoparticles and fibrils. The assembly of scatterers with a characteristic size or spacing d have scattered intensity in a well defined direction defined by Bragg's law:

$$n\lambda = 2d_{hkl} \sin \theta$$

This equation describes the relationship between X-ray wavelength λ , Bragg hkl plane spacing d_{hkl} and 2θ , the angle between incident and diffracted beam directions. Index n defines the order of reflection. The collagen in bone has an average period of 67 nm along the fibril axis which produces small angle x-ray peaks. The mineral particles have their own periodicity; indeed the carbonated apatite crystallites which are dispersed in a water-stabilized collagen matrix have a polycrystalline structure that has an Angstrom periodicity that can produce diffraction peaks in the wide angle x-ray diffraction regime. So when the sample is oriented at $\frac{1}{2}$ the Bragg diffraction angle $2\theta_{0002}$ to the direction of the collimated monochromatic x-ray beam the percent shifts in the position of the 0002 peak intensity along the vertical direction will give the mineral strain along the tensile stretch direction. In the transmission geometry used in this study, an area detector is placed normal to the beam transmitted through the sample, and a circular ring of diffracted intensity is recorded where the detector intercepts each cone of diffracted radiation.

For this study strength and ductility measurements are made using uniaxial tension tests under conditions where simultaneous small angle x-ray scattering (SAXS) and wide angle x-ray diffraction (WAXD) patterns could be recorded in real time. For a given strain on the bone tissue, this enabled estimates of the individual strain carried by the fibrillar and mineral phases. Samples were mounted in a load frame at the Advanced Light Source synchrotron facility (Lawrence Berkeley National Laboratory, Berkeley, CA) so that the long axis of the bone was oriented perpendicular to the 10 keV x-rays; a schematic of the experimental setup is shown in Figure 2. A tensile load was applied parallel to the long axis of the femur. The sample was kept hydrated 12 hr prior to the experiment as well as throughout the experiment by means of a hydration cell comprised of a strip of cellophane held to the sample through capillary action with a few drops of HBSS.

A parallel beam of monochromatic x-rays (at an energy of 10 keV and a wavelength of 0.124 nm) with a 1 mm x 0.25 mm ellipsoidal cross-section was directed perpendicular to the applied load. A high speed Pilatus 1M detector was used for collecting SAXS data and a Quantum 4u CCD X-ray detector (Area Detector Systems Corporation) was used for reading WAXD patterns. The sample was mounted into a custom built tensile stage fitted with a 5-kgf load cell (Omega, LC703-10) and a motor-driven displacement stage. All samples were loaded under strain control at a strain rate of 1 $\mu\text{m/s}$, with SAXS/WAXD measurements taken at various points along the stress/strain curves, with an exposure time for the frames of 0.5 s. The number of exposures was regulated in order to keep the radiation dose below 30 kGy so that there would not be any additional damage to the tissue due to the radiation received during *in situ* tensile testing; specifically, the x-ray irradiation was blocked between exposures.

Synchrotron radiation was used to generate the SAXS and WAXD patterns which were analyzed to find percent changes in the fibril and mineral meridional strain along the tensile axis. For the SAXS region of reciprocal space the meridional collagen molecules in the fibrillar have a staggering distance, ~ 67 nm, which leads to a diffraction peak at a q -value of ~ 0.009 $\text{\AA}^{-1}$. The SAXS detector was located at a sample-distance of ~ 4100 mm in order to detect changes in the position of the collagen peak. The WAXD detector was placed ~ 250 mm away from the sample. For the WAXD region of reciprocal space the detector must be oriented to get diffraction from the 0002 peak at a lattice spacing of ~ 0.344 nm. The SAXS and WAXD employed radial integration of the two-dimensional SAXS or WAXD patterns over azimuthal sectors. For this experiment SAXS/WAXD patterns were integrated in a narrow pie shaped sector of 10° angular width and centered along the tensile axis. The analysis software IGORPro (Wavemetrics) and the custom macro NIKA (Jan Ilavsky, Argonne National Laboratory) were used in conjunction to convert the 2-D data to 1-D. The sample-detector distance and the beam center were both calibrated for the SAXS/WAXD data analysis using the silver behenate standard. After integration the peaks around the first order diffraction from the meridional collagen SAXS pattern and around the 0002 diffraction from the apatite WAXD pattern were fit using a Gaussian function and a fourth order polynomial. The peak position was used to find the collagen fibril spacing and the 0002 crystallographic lattice spacing relative to the unstressed state (defined at zero load for each sample).

The tissue strain was determined by measuring percent changes in the distance between the two horizontal lines on either end of the samples using image analysis software (National Instruments Vision Assistant 8.5). This was

achieved using a CCD camera synchronized to take images as the load was being applied.

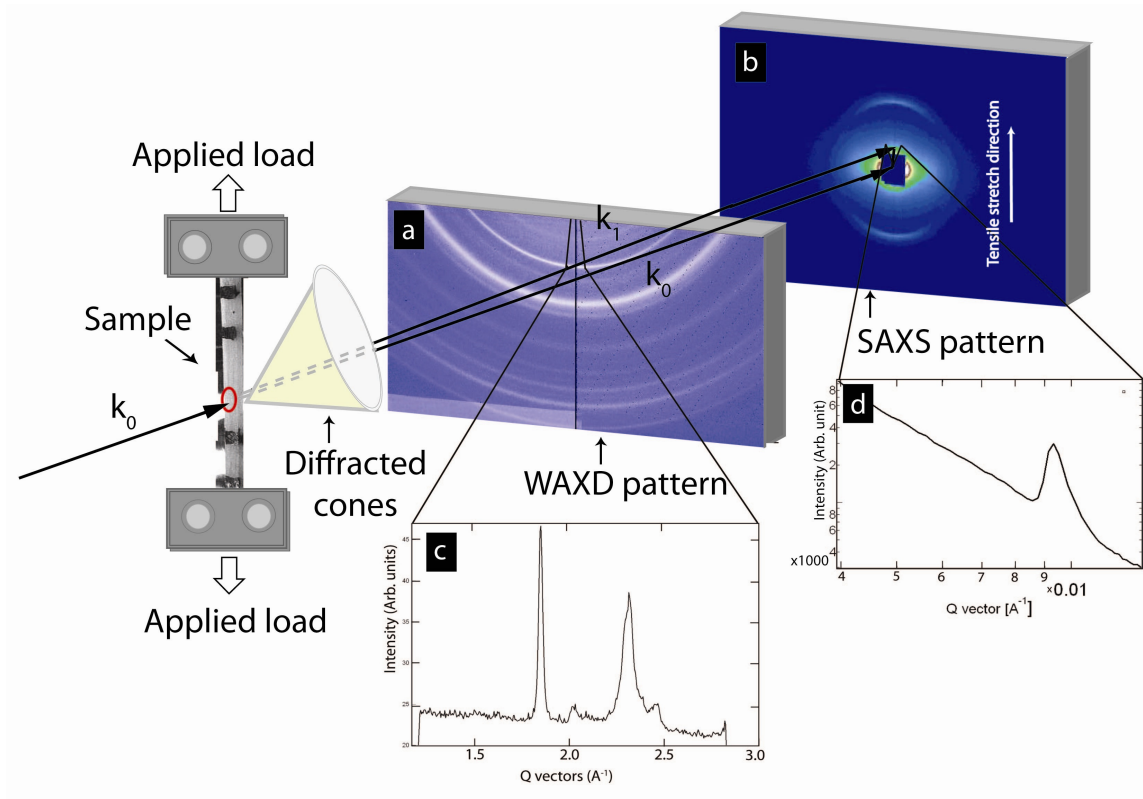


Figure 4-2: Schematic showing the beamline setup for *in situ* tensile testing of bone with real time small-angle x-ray scattering and wide-angle x-ray diffraction (SAXS/WAXD) imaging. The 10 keV x-ray beam penetrates the longitudinally oriented human cortical bone sample perpendicular to the tensile set up. The SAXS detector is positioned to record the meridional spacing in collagen molecules in the fibril. The WAXD detector is positioned to record patterns from the crystallites with *c*-axis along the tensile direction. Tissue strain is determined by the marker lines on the sample (camera not shown in schematic). Images (a) and (b) show WAXD pattern and the SAXS pattern of bone respectively, with the region for azimuthal integration shown in the pie-shaped sector outlined in both. Figures (c) and (d) show the integrated intensity variation over this region. Graph (c) shows a pronounced 0002 diffraction peak for the hydroxyapatite, and graph (d) shows a pronounced third order diffraction peak due to the fibrillar periodicity (67 nm).

4.2.3. Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used in transmission mode to assess the degree of enzymatic cross-linking in the collagen for two groups: healthy (unirradiated) and irradiated (70 kGy) human cortical bone. Test samples from human cortical bone were initially sectioned with a low-speed saw into 10 samples, then further sectioned using a microtome into a final sample size of $\sim 1 \text{ mm} \times 1 \text{ mm} \times 2 \text{ }\mu\text{m}$; thicknesses are required to be 1-5 μm for bone to be transparent to infrared light. To microtome the samples, they must first be preserved to maintain their properties by embedding into a supportive material; since water has strong absorbance in the IR, the tissue was dehydrated with a series of ethanol baths. The bone sections were embedded in Technovit 7100 (Kultzer & Co, Wehrheim, Germany), a liquid containing hydroxyethyl-methacrylate according to manufacturer's instructions. Briefly, Technovit 7100 was admixed with hardener I and II at 300:3:20 v/w/v ratio, as described in [18].

The protein Amide I (peptide bond C=O stretch) undergoes frequency and intensity changes as a result of changes in protein secondary structure [19]. One of the unique capabilities of FTIR spectroscopy is the ability to quantify these changes, specifically by tracking the spatial variation of the collagen components in mineralized tissue. Information on the protein structure can be extracted from broad spectral bands consisting of components of peaks resulting from Amide I modes of various secondary structures by using a spectral resolution technique [20]. For type I collagen in bone the two major components of importance are the enzymatic collagen cross-links: pyridinoline (pyr) and divalent dehydrodihydroxynorleucine (DHLNL). Indeed, the ratio of peak areas of the pyr and DHLNL subbands, at 1660 cm^{-1} and 1690 cm^{-1} respectively, provides a semi-quantitative measure of the cross-linking profile in the collagen matrix; this ratio has been correlated to the relative amounts of enzymatic cross-links, specifically mature non-reducible (interfibrillar) cross-links and immature reducible (intrafibrillar) cross-links (Figure 1). A method has been developed to enable spectral analysis of the amide I band to isolate peaks corresponding these two major type I collagen cross-links, namely pyridinoline (pyr) and DHLNL, [21] using second-derivative spectroscopy. All FTIR spectroscopy data were obtained using a conventional Globar IR source at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, USA). Image spectroscopy consists of a source, a sample handling unit and a focal plane array detector on an IR microscope. The instrument is equipped with a photographic device to capture an image of the area of the tissue being examined. The recorded spectra

were taken in transmission mode with 128 scans and had a resolution of 4 cm^{-1} and a spot size of $\sim 150\ \mu\text{m}$. OMNIC software (Thermo Fischer) was used for data processing.

4.2.4. UV Raman Spectroscopy

As a further measure of the extent of collagen cross-linking, deep-ultraviolet Raman spectroscopy, with a 244-nm excitation source, was used to evaluate irradiation-related changes in the structure of bone at the molecular level [17]. The setup was designed to evaluate irradiation-related changes in the structure of bone at the molecular level [22]. The UV Raman technique eliminates the fluorescence interference found with visible excitation. Also due to resonance effects, the signal strength of some features from the organic phase (particular those associated with the amide moiety formed by the bonds between peptides) are enhanced relative to those from the inorganic phase (*e.g.*, phosphate and carbonate stretching modes). To avoid damage to the organic component of the bone matrix the laser power was kept below 5 mW and the sample was rotated at approximately 45 rpm. The laser was focused to a $\sim 500\ \mu\text{m}$ spot on the surface of the bone samples with an $f/4$ 100 mm fused silica lens. Backscattered light was collected with the same lens, collimated, and directed to the entrance slit of a triple spectrometer optimized for performance in the deep-UV regime. The instrument dispersion used was 2.1 $\text{cm}^{-1}/\text{pixel}$. The instrument resolution was varied between 15 and 30 cm^{-1} by adjusting the slit width of the dispersion stage of the triple spectrometer. These measurements established that the linewidths of the major features in the spectrum were 40 cm^{-1} and higher. Spectra were collected with liquid nitrogen cooled CCD camera. Ten frames of 10 s exposure were collected; comparison of the first and last frames from each set confirmed that there was no sample degradation under laser illumination. For quantitative analysis of this effect, spectra were processed by subtracting a small linear background defined by the signal at 500 and 2000 cm^{-1} and then normalizing to the height of the CH_2 wag peak at 1460 cm^{-1} (this peak does not have a strong resonance enhancement). It is known [17, 22] that the height of the amide I feature at 1650 cm^{-1} is sensitive to the collagen environment, in particular to the extent of cross-linking, increasing, for example, with dehydration or with increasing tissue age.

4.2.5. Accumulation of Advanced Glycation End-Products

Bone collagen is also susceptible to the age-related accumulation of non-enzymatic cross-links resulting from the covalent bonding of oxidizing sugars and free amino groups on the collagen protein including arginine and lysine.

These cross-links are known as advanced glycation end-products (AGEs) and they form both intra- and inter- fibrillar links along the collagen backbone [2]. The AGEs were quantified using a fluorometric assay on both unirradiated bone and bone irradiated at 70 kGy. For each sample a section of the femoral midshaft was demineralized using ethylenediaminetetraacetic acid (EDTA) and then hydrolyzed using 6N HCl for 24 hr at 110°C. AGE content was determined using fluorescence readings taken using a microplate reader at the excitation wavelength of 370 nm and emission wavelength of 440 nm. These readings were standardized to a quinine-sulfate standard and then normalized to the amount of collagen present in each bone sample. The amount of collagen for each sample was determined based on the amount of hydroxyproline, the latter being determined using a chloramine-T colorimetric assay that recorded the absorbance of the hydrolysates against a commercially available hydroxyproline standard at the wavelength of 585 nm [23].

4.3. Results

4.3.1. Small Angle X-ray Scattering & Wide Angle X-ray Diffraction

To further investigate the loss in plasticity with irradiation, hydrated bone samples were loaded in uniaxial tension with simultaneous examination using SAXS/WAXD. For a given strain in the bone tissue sample, the individual strains in the collagen and mineral were measured in human cortical bone exposed to 70 kGy of x-ray irradiation, as compared to unirradiated bone. Results are shown in Figs. 3a,b, respectively, for the variation in individual strains in the collagen fibrils (ϵ_F) and in the mineral (ϵ_M), as a function of the global strain (ϵ_T) applied to the bone tissue. With increasing tissue strain, a linear increase in both the fibrillar and mineral strain is apparent, until yielding occurs at roughly 1% tissue strain (Fig. 3); at this point most of the strain continues to be carried by the collagen, but not in the irradiated bone, which then fractures. With plastic deformation, the fibrillar and mineral strains assume approximately constant values (~1.1% in the collagen, ~0.2% in the mineral), consistent with results in the literature [9]. Following 70 kGy irradiation, however, the fibrillar strains are roughly up to 50% lower for a given tissue strain, and the sample fails catastrophically with little to no evidence of post-yield deformation, indicative of the loss of plasticity in highly irradiated bone. The actual ratios of fibrillar to tissue strain (ϵ_F/ϵ_T) and mineral to tissue strain (ϵ_M/ϵ_T) are plotted for both groups in Fig. 6 to compensate for sample-to-sample variability. The strain carried by the collagen fibrils in the 70 kGy irradiated samples is ~40% of the applied tissue strain, which is considerably lower than in the unirradiated bone where the fibril strain is ~80%

of the tissue strain. The effect is summarized in Figure 7 where at an applied tissue strain of 0.85%, exposure to 70 kGy of irradiation causes a ~40% decrease in the strain carried by the collagen fibrils and a ~20% decrease in mineral strain, as compared to unirradiated bone. The data for the tissue and mineral strain are both binned in regular intervals of tissue strain ($N=20$ for each group), with error bars representing the standard errors of mean.

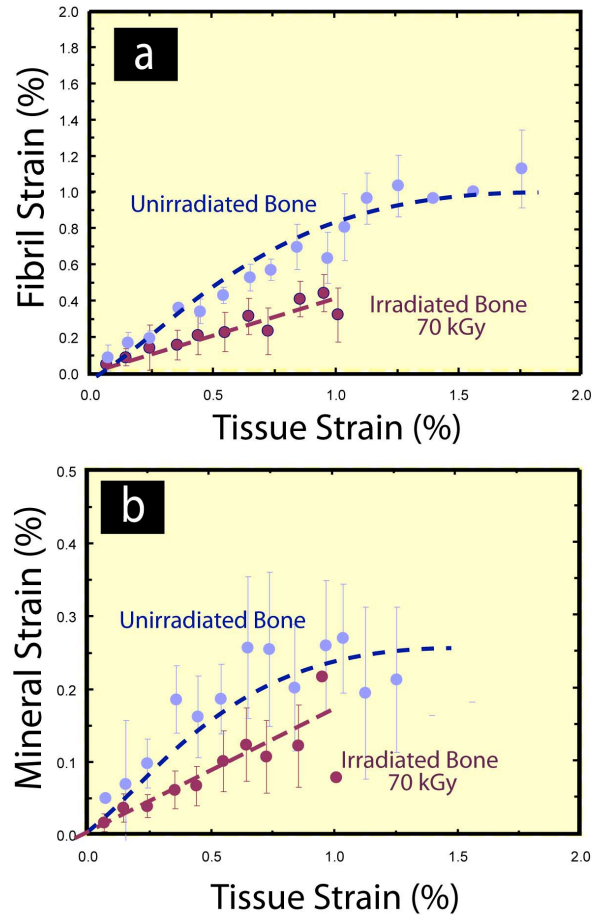


Figure 4-3: Partitioning of strain between the collagen and mineral as a function of the global tissue strain in 70 kGy irradiated *vs.* unirradiated hydrated human cortical bone. Variation of the strain from SAXS/WAXD measurements in the (a) collagen and (b) mineral as a function of the global applied strain in the bone tissue for unirradiated and 70 kGy irradiated bone. The collagen and mineral strain are binned in regular intervals of tissue strain ($N=20$ for each group). Error bars in the graphs are standard deviation of the binned value. Note that for a given strain in the bone tissue the strain in the collagen fibrils is diminished in irradiated *vs.* unirradiated bone, consistent with the progressive loss in macroscopic plasticity in bone with increasing irradiation.

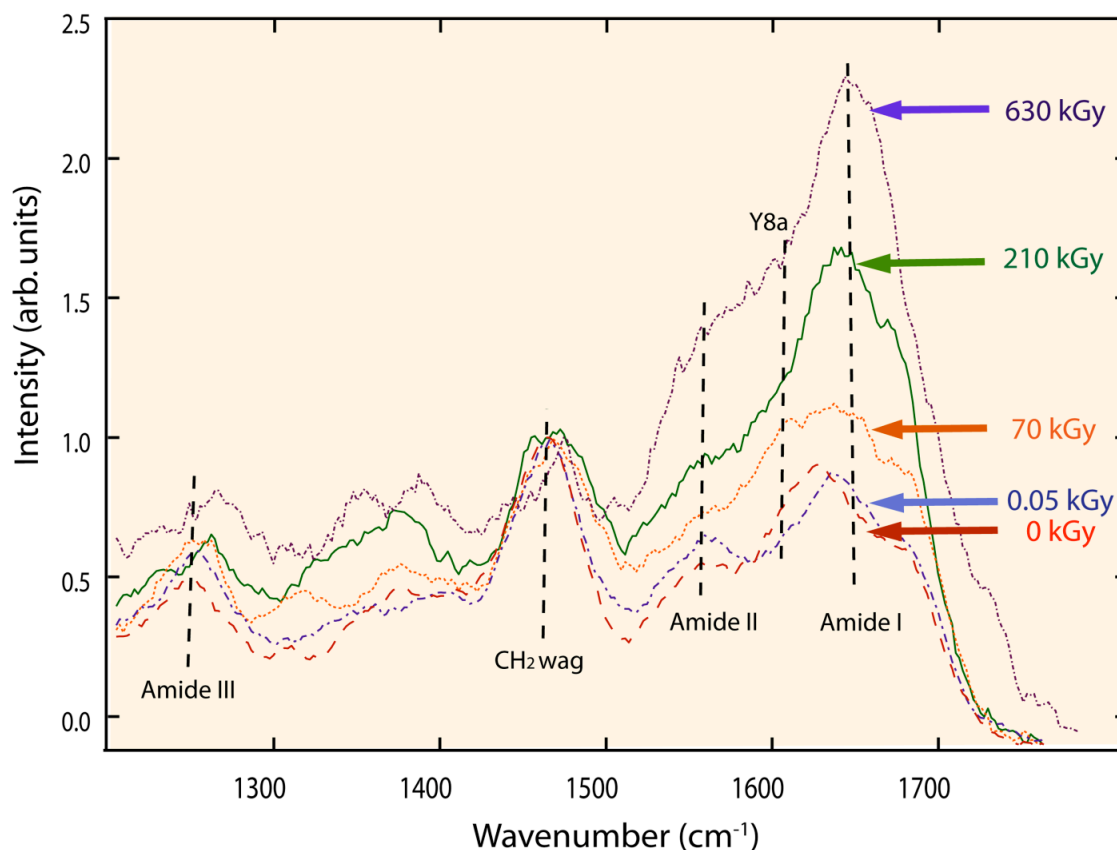


Figure 4-4: UV-Raman spectroscopy of irradiated hydrated human cortical bone. (a) UV-Raman spectra for four different irradiation groups after doses of 0, 0.05, 70, and 210 kGy, showing specifically the large changes in the relative height of the amide I feature compared to the CH₂ wag peak. The amide I (primarily from C=O stretch) peak has previously been a good indicator for observing changes in the protein arrangement since the amide is known to play a role in cross-linking and bonding. The amide I peak height of the peak monotonically increases with irradiation, consistent with an increase in cross-linking in the collagen. Some of the other noticeable organic features for the bone matrix are the CH₂ wag peak (1454-1461 cm⁻¹), amide III (primarily from in-phase combination of NH in-plane bend and CN stretch, 1245-1260 cm⁻¹), and amide II (primarily from out of phase combination of NH in-plane bend and CN stretch).

4.3.2. Quantification of collagen cross-linking

As high doses of irradiation are known to impart severe damage to the collagen matrix in the form of breaking of peptide bonds in the collagen backbone [17, 24, 25], the nature of the collagen environments was examined specifically using a number of techniques.

Results from deep UV-Raman spectra, shown in Figure 4 for (hydrated) unirradiated and all irradiated groups of bone, indicate a very pronounced increase in the Amide I peak after exposure to x-rays, implying severe damage of the collagen with increasing degree of irradiation. The main peaks observed in the control sample are indicated: amide III (from in-phase combination of NH in-plane bend and CN stretch, *c.* 1245–1260 cm^{-1}), CH_2 wag (*c.* 1454–1461 cm^{-1}), amide II (*c.* 1560 cm^{-1}), Y8a (from tyrosine side chains at 1610 cm^{-1}) and amide I (from C=O stretch, *c.* 1626–1656 cm^{-1}). The main change in the spectra of the irradiated samples is a monotonic increase (relative to the CH_2 wag) of the amide I band, as shown in Table 1. We have previously observed increases in the amide I peak with dehydration and increasing age and have attributed them to broadening of the resonance profile for the amide $\pi \rightarrow \pi^*$ transition caused by changes in the intrafibrillar environment of the collagen molecules [22, 26]. Although this indicates significant changes in the collagen environment associated with increased cross-linking, the identification of the specific nature of the cross-links with this technique remains uncertain.

For this reason, in the present study we performed additional Fourier transform infrared (FTIR) spectroscopy on unirradiated and 70 kGy irradiated bone. The protein amide I (peptide bond C=O stretch) mode near 1650 cm^{-1} undergoes frequency and intensity changes as a result of changes in protein secondary structure. FTIR spectroscopy gives quantitative information on the collagen maturity in bone, specifically the ratio of two of the major enzymatic collagen cross-links: specifically, the mature (non-reducible) divalent cross-links (Pyr) and the enzymatic immature (reducible) divalent dehydrodihydroxynorleucine cross-links (deH-DHLNL). Of these components the relative percent area ratio of the two sub-bands at $\sim 1660 \text{ cm}^{-1}$ and $\sim 1690 \text{ cm}^{-1}$ is related to collagen cross linking that are abundant in mineralized tissue (Pyr and deH-DHLNL, respectively) [21]. For our results (Fig. 8), a comparison of the calculated 1660:1690 spectroscopic ratio of these two peaks in the two groups of bone is shown in Fig. 8a. The ratios are calculated using second derivative spectroscopy which locates the position of the underlying bands within the amide I region and then peak fits these sub-bands to obtain information on the

relative percent area contribution of each underlying component for the unirradiated and 70 kGy irradiated groups. The 1660:1690 peak area ratio appears to correspond to the ratio of non-reducible (mature) to reducible (immature) collagen cross-links in bone [21]. The results show that following 70 kGy irradiation, the 1660:1690 ratio decreases by almost two-thirds, indicating that the damaging effects of irradiation affect the mature cross-links such as Pyr to a greater degree than the immature cross-links such as deH-DHLNL.

With respect to the non-enzymatic cross-links, information on the nature of the advanced glycation end-products (AGEs) was obtained fluorimetrically in unirradiated bone and bone subjected to 70 kGy x-ray irradiation. AGEs result from the reactive non-enzymatic glycation between amino residues and sugars that increase the cross-linking of collagen. Results are shown in Figure 5 and indicate that the AGEs increase with irradiation; specifically, there are ~21% more fluorescent cross-links in the irradiated bone.

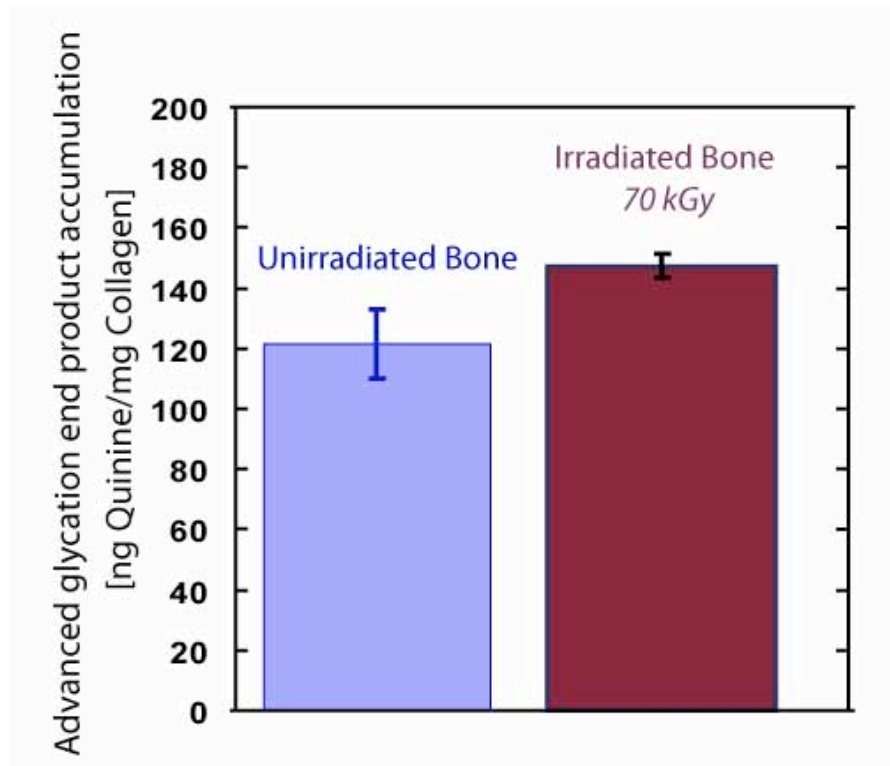


Figure 4-5: The accumulation of advanced glycation end-products (AGEs) was fluorimetrically quantified in the cortex of the femora in unirradiated and 70 kGy irradiated human cortical bone samples. AGEs increase slightly when bone is irradiated with a dose of 70 kGy which increased the concentration of fluorescent (non-enzymatic) cross-links to ~21% more than in the unirradiated bone.

4.4. Discussion

Although irradiation is known to deleteriously degrade the structure and mechanical properties of bone, we show in this work that the effect can be extremely severe; indeed, the tissue can be dramatically and irreversibly embrittled with major losses in strength, ductility and fracture resistance following exposures in excess of 35 kGy. Specifically, compared to healthy (unirradiated) human cortical bone, we find that bone strengths are reduced by ~60% and more than 80% for irradiation doses of 210 and 630 kGy, respectively, with a factor of five loss in fracture toughness at 210 kGy. Additionally, for x-ray irradiation exposures above ~70 kGy, the bone no longer displays evidence of post-yield (“plastic”) deformation, simply failing at its elastic limit (Fig. 3).

Here these effects are explained in terms of how the irradiation affects the intrinsic and extrinsic contributions to the bone toughness, which are developed at widely different structural length-scales. In many respects though, it is the effects of irradiation on the intrinsic contributions to the bone toughness that are more interesting. These contributions arise at much smaller (sub-micron) length-scales and are associated primarily with the generation of “plasticity” in bone from the process of fibrillar sliding, which can occur at both the fibril [9] and fiber [27] level. Within the fibrils non recoverable deformation mechanisms take place, such as sliding at the HAP/tropocollagen interface [8], increased intermolecular cross-linking density [2] and sacrificial bonding [28], these mechanisms constrain molecular stretching and provide the basis for the increased apparent strength of the collagen molecules without catastrophic failure of either of the individual components. The molecular behavior of the protein and mineral phases (fibrillar sliding) within a fibril enables a large amount of dissipative deformation energy once plastic yielding begins in mineralized tissues [9, 29, 30] and other biological materials [31]. At the nano-scale the predominate plasticity mechanisms is represented by this model of load transfer. As is common in most materials, plasticity contributes to the intrinsic toughness by dissipating energy and forming “plastic zones” surrounding crack-like defects that further serve to blunt crack tips, thereby protecting the integrity of the entire structure by reducing the driving force (*i.e.*, stress intensity) for crack propagation.

Changes to the effectiveness of this intrinsic mechanism are largely influenced by the organic matrix. In this regard, damage to the collagen matrix induced by irradiation can be extremely problematic to the biomechanical properties of bone, in particular through the formation of collagen cross-links [32-35] and eventually breakage of the backbone of the collagen molecule. In this

regard, the nature and degree of cross-linking has a major influence on bone function. As noted, three major types of collagen cross-links are found in human cortical bone: enzymatic immature (reducible) divalent cross-links (dehydrodihydroxynorleucine) and mature (non-reducible) divalent (pyridinoline and pyrrole) cross-links linking neighboring fibrils, and glycation or oxidation-induced non-enzymatic cross-links (AGEs) that form both intermolecular and interfibrillar cross-links along the collagen backbone. Intermolecular cross-linking provides the fibrillar matrices with properties such as tensile strength and viscoelasticity [2, 13]. The helix of type I collagen, the primary type found in bone, relays strength in tension; the cross-links keep its helices fastened - if there are too many cross-links collagens ability to absorb energy declines, if there are too few the helix may break apart. The collagen fibers that are formed in type I collagen are initially stabilized by the formation of covalent cross-links between neighboring collagen molecules.

In the current work, macroscopic mechanical tests clearly indicate that a primary aspect of the irradiation-induced loss in fracture resistance can be attributed to a complete loss in post-yielding (plastic) deformation (intrinsic toughness) for radiation exposures of 70 kGy and above. Based on SAXS/WAXD analyses and associated cross-link measurements, we interpret this in terms of a suppression of the prevailing plasticity mechanism in bone of fibrillar sliding, which results principally from a change in the proportion of the three types of collagen cross-linking caused by the irradiation damage.

At the onset of plastic deformation in healthy (unirradiated) bone (Figs. 3& 6), the strain in the mineral becomes roughly constant with increasing tissue strain (Fig. 3b). Initially the load in the bone is carried by the mineral particles through shearing of the collagen matrix [36], but once yielding occurs the mineral and the collagen begin to decouple; a schematic interpretation for the load transfer in bone is shown in Fig. 7. The tensile strain can be divided into two contributions: first from the tensile stretching of the mineralized collagen fibrils (which we studied by examining changes in the 67 nm spacing) and second by shear deformation of the interfibrillar matrix. In contrast, once plastic deformation begins in the irradiated bone, the tissue fails due to degradation of the collagen matrix, which acts to totally eliminate the bone's capacity for plastic deformation; in materials science terms, the bone simply "embrittles" due to irradiation.

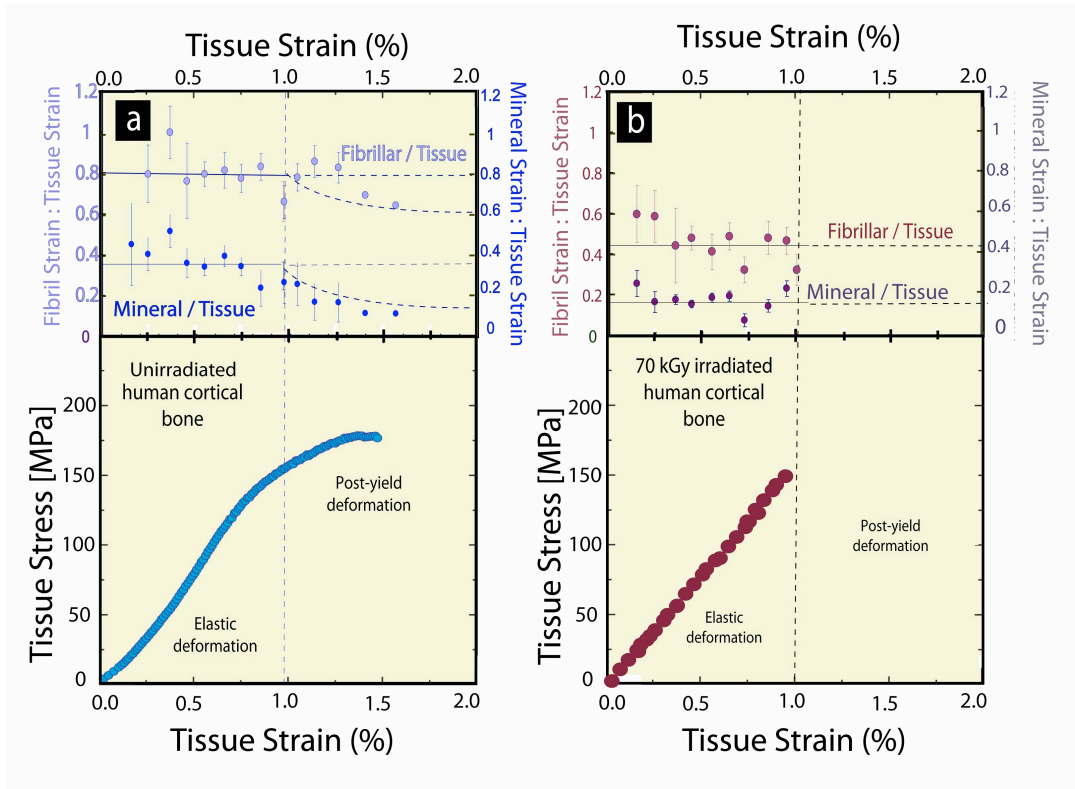


Figure 4-6: Strain in collagen fibril, and mineral in bone as a function of applied strain in the tissue for human cortical bone in the (a) unirradiated and (b) 70 kGy irradiated condition. The upper graphs show the ratios of fibril to tissue strain, ϵ_F/ϵ_T , and mineral to tissue strain, ϵ_M/ϵ_T , for the (a) unirradiated and (b) 70 kGy irradiated bone, averaged from $N=20$ samples for each group. In (a) and (b), the solid lines represent the constant strain ratio expected before yield. In (a), the dotted line represents where the ratio would vary if the fibril and mineral strain remained constant beyond the yield strain. The lower graphs show the corresponding stress-strain curves for the (a) unirradiated and (b) 70 kGy irradiated human cortical bone.

Our spectroscopy and fluorometric measurements (Figs. 4, 5 & 8) strongly suggest that this progressive irradiation-induced embrittlement of bone in the form of the suppression of plasticity from fibrillar sliding and the consequent major losses in toughness initially result from an increase in specific collagen cross-linking which raises the amount of bonds; further irradiation exposures can cause molecular damage due to breaking the peptide backbone. Irradiation exposure leads to the release of free radicals via radiolysis of water molecules in bone which can severely degrade the structural integrity of the fibers in addition to restricting fibrillar sliding mechanisms [24, 25, 37].

Specifically, we observe a significant change in the magnitude of the amide I peak in UV Raman spectra with radiation indicative of a radically changed collagen environment (Fig. 4 and table 1). Indeed, these measurements show that for a very large exposure of 630 kGy, the spectral features were broadened to such an extent that the individual peaks could not be observed, which is likely associated with breaking of peptide bonds in the collagen backbone [24]. Although this change in the height of the amide I peak has been related to collagen cross-linking [17, 22], we obtained further information on the nature of these cross-links using FTIR spectroscopy techniques to measure the vibrational energies of molecules to detect changes to the structure of the collagen. Past studies [38, 39] have used this procedure to examine the effects of gamma irradiation on the mechanical and material properties of tendon, which is primarily comprised of type I collagen organized in parallel arrays, and have reported changes to the cross-linking in collagen with increased exposure to irradiation. The collagen in both tendon and bone is stabilized by interfibrillar and intermolecular cross-links, the primary mature cross-link is hydroxypyridinium; indeed, one study demonstrated [38] a significant decreases in hydroxypyridinium cross-link density with 60 kGy of irradiation. High doses of irradiation may also induce changes in the material properties of tendons by breaking of peptide bonds in the collagen molecules and rupture of the hydrogen bonds, although these changes occur more often in the dry, rather than wet, state.

Table 4-1: UV-Raman spectroscopy data showing the relative intensity (height) of the amide I feature, as compared to the height of the CH₂ wag peak***

Irradiation dose (kGy)	Amide I peak relative to the CH ₂ wag peak
0	0.81 (0.01)
0.05	0.81 (0.12)
70	1.05 (0.11)
210	1.20 (0.15)
630	1.39 (0.15)

*** Standard deviations in parentheses.

For the present FTIR results for unirradiated and 70 kGy irradiated bone shown in Fig. 8, the infrared spectra in the region of 1585-1720 cm⁻¹ are of relevance as they show absorbance bands for the vibrations of protein amide I. Among the underlying bands that make up the amide I spectral peak, two are of particular interest, at ~1660 cm⁻¹ and one at ~1690 cm⁻¹, as it has been shown that during collagen denaturing the relative intensity of the former decreases while the latter increases [11, 21, 40]. Indeed, the ratio of these two bands corresponds to the amount of enzymatic collagen cross-links present, specifically the non-reducible mature Pyr cross-links (interfibrillar) and the reducible immature DHNLN cross-links (intrafibrillar) found in bone [21]. In past studies, the relative areas of 1660:1690 peaks were found to increase with aging in bovine bone [21] and osteoporosis in human bone [40], while the ratio was found to decrease with vitamin B₆-deficient chicken bone [21]. We similarly find here that with 70 kGy of irradiation, the ratio of 1660:1690 peak areas decreases by almost two-thirds, indicative of major changes in cross-linking and bonding in the amide I region. With respect to the enzymatic cross-links in the collagen, the decrease in this ratio with irradiation exposure suggests that there are changes in the cross-link profile with a greater proportion of immature cross-links to the mature cross-links following irradiation [39]; indeed, either an increase in immature cross-links or a reduction in mature cross-links could disrupt the integrity of the mature cross-links, such as covalent hydroxypyridinium, leading to premature failure of the tissue.

Finally, we also find an increase in the concentration of the non-enzymatic (AGEs) cross-links (Fig. 5), which are known to suppress plasticity in older bone [41, 42]. Indeed, we suspect that it is these cross-links that are primarily responsible for the restriction in bone plasticity from fibrillar sliding in irradiated bone. Although the increased accumulation of AGEs in aging bone can be attributed to the longevity of the tissue and age-related changes in biological bone metabolism (including incomplete tissue remodeling and altered glucose metabolism), the mechanism of the apparent increased concentration of AGEs in irradiated bone observed is not known. It may be possible that the non-fluorescent intermediate cross-links that occur during the Amadori rearrangement of sugars [2] is accelerated by irradiation, producing the fluorescent AGEs observed here. It is also important to note that the quantitative AGEs content is normalized with respect to collagen, and any loss of collagen due to the damaging irradiation could also increase the AGEs per amount of collagen. Regardless of the mechanism, however, the increase in AGEs content is consistent with the inhibition of fibrillar sliding and loss of plastic toughening observed in aging bone. Taken together, the harmful effects of bone irradiation can be observed at multiple length scales through the suppression of nano- and micro-scale plasticity mechanisms, and may be the result of alterations in the profile and distributions of the collagen cross-linking.

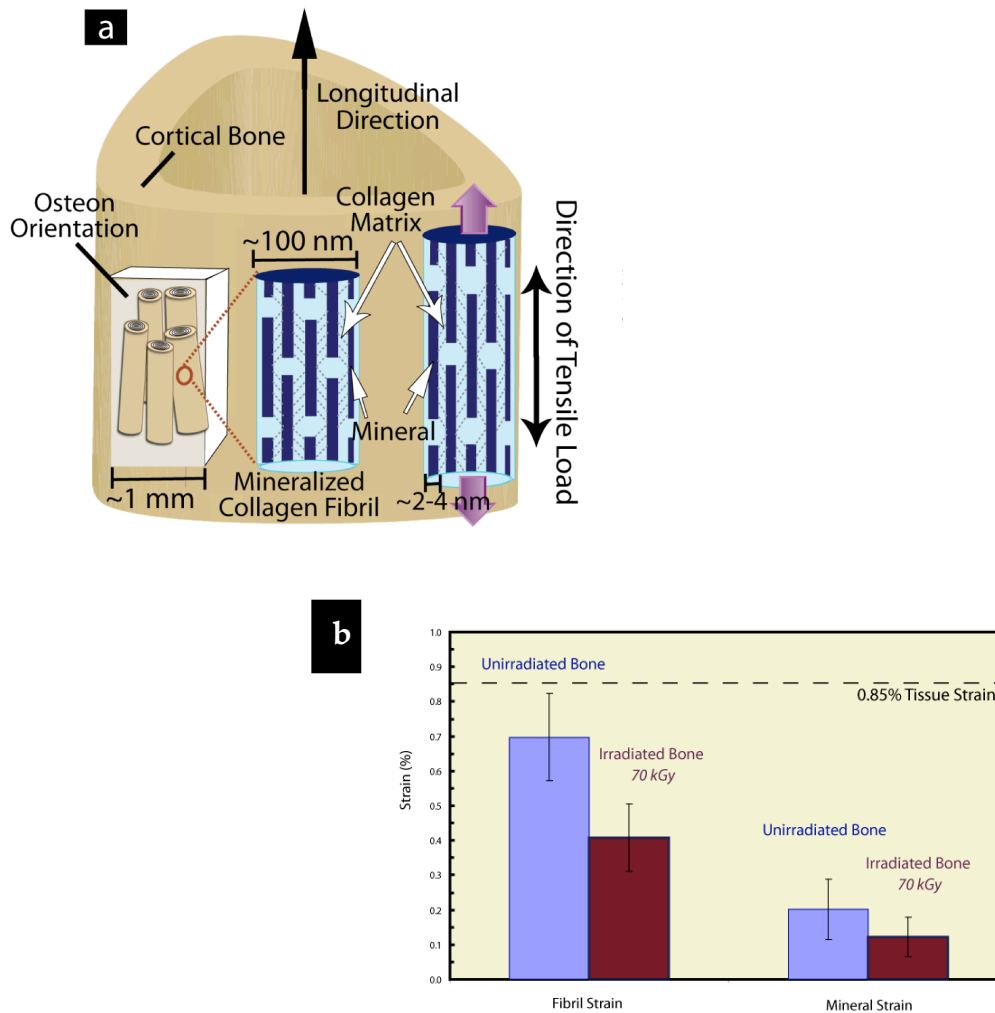


Figure 4-7: (a) Schematic illustration of the sample orientation in relation to the bone-matrix microstructure in human cortical bone (top) and schematic model for bone deformation for the various hierarchical length-scales in response to external tensile load. Strain is simultaneously measured at all three levels of the structural hierarchy (tissue, fibril, and mineral nanoparticles). The mineralized fibrils, which are stiffened with collagen cross-links, deform in tension and transfer the stress between neighboring fibrils by shearing in the thin layer of the matrix. Within each mineralized fibril, the stiff mineral platelets deform in tension and transfer stresses between adjacent platelets by shearing in the interparticle collagen matrix. (Red lines demonstrate shearing qualitatively). (Adapted from ref. [9]) (b) At a fixed tissue strain of 0.85%, the individual strain in the fibrils is 42% less in the 70 kGy irradiated bone than in the healthy unirradiated bone.

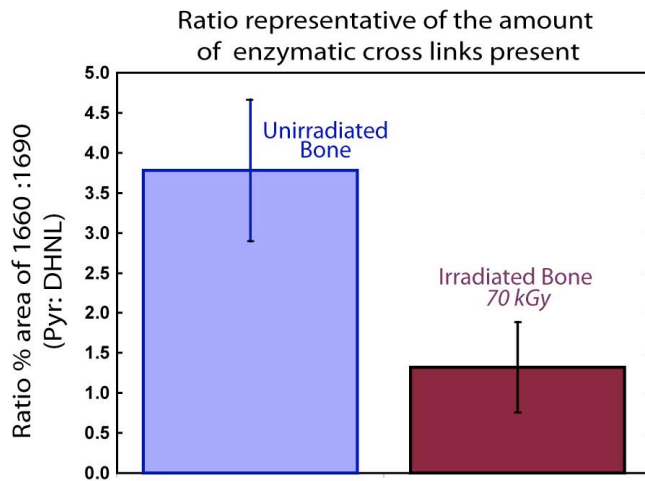
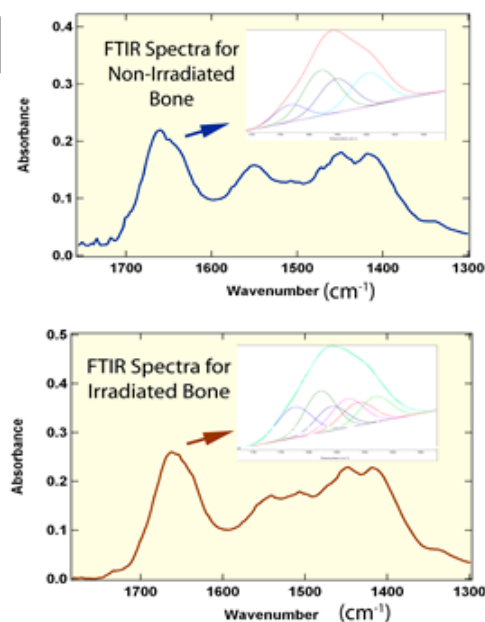
a**b**

Figure 4-8: Fourier transform infrared spectroscopy of human cortical bone in the unirradiated and 70 kGy irradiated condition. (a) Shows a comparison of the calculated spectroscopic ratio of the 1660:1690 cm^{-1} peaks in unirradiated and 70 kGy irradiated samples. The 1660:1690 ratios are calculated through a combination of second derivative spectroscopy to locate the position of the underlying bands within the amide I region and then peak fitting of these subbands to determine the relative percent area of each underlying component. The area 1660:1690 ratio appears to correspond to the ratio of nonreducible/reducible collagen cross-links in bone [21]. Following irradiation of 70 kGy, the 1660:1690 ratio decreases by almost a third. (b) Shows FTIR spectra and the amide I underlying bands for both groups.

4.5. Conclusion

This work has developed a framework for evaluating toughness mechanisms in bone on different length scales. In the last chapter we discussed the nonlinear elastic fracture mechanics measurements of the macroscopic bone toughness, coupled with *in situ* environmental scanning electron microscopy (eSEM) and post-testing three-dimensional synchrotron x-ray computed micro-tomography of the microscopic crack paths, now in this chapter we discuss the use of small- and wide-angle x-ray scattering/diffraction (SAXS/WAXD) of *in situ* uniaxial tensile tests to examine the effectiveness of fibrillar sliding as a nanoscale deformation mechanism in bone (by measuring the strain partitioning between the mineral and collagen phases), and use deep UV Raman and Fourier Transform Infrared (FTIR) spectroscopies and a fluorometric assay to characterize the nature and degree of molecular cross-linking of the collagen.

Based on the experimental study, spanning molecular to macroscopic length-scales, of the effects of synchrotron x-ray irradiation on the structure and mechanical properties of hydrated human cortical bone (in the transverse orientation), the following conclusions can be made:

Healthy cortical bone that is exposed to irradiation doses between 35 kGy and 630 kGy led to a severe progressive degradation in strength, ductility and toughness. The irradiation-induced suppression of bone plasticity, found at a dose of 70 kGy and above, acts to severely limit the intrinsic toughness by macroscopically embrittling the bone tissue. Such irradiation-induced suppression of bone plasticity is consistent with SAXS/WAXD analysis of the partitioning of strain in the bone under load, which indicated that for an applied tissue strain of ~1%, the individual strain carried by the collagen fibrils was some 40% smaller in 70 kGy irradiated bone, as compared to unirradiated bone, indicative of a lack of plasticity in the collagen. SAXS/WAXD studies of unirradiated bone also indicated that at yielding, the individual strains in the mineral and collagen become essentially constant with respect to the applied tissue strain. With elastic straining, the load in the bone is carried by the mineral particles through shearing of the collagen matrix; at yielding though the mineral and the collagen begin to decouple. However, in 70 kGy irradiated bone, the tissue fails catastrophically once the elastic limit is reached. The loss in intrinsic toughness through the suppression of plasticity via the mechanism of fibrillar sliding is attributed to an increased incidence of specific cross-linking of the collagen with irradiation.

Measurements for unirradiated and 70 kGy irradiated bone revealed that there was a ~21% increase in non-enzymatic cross-links, in the form of advanced glycation end-products (AGEs), in the irradiated bone, which would clearly act to restrict fibrillar sliding of the collagen fibrils. With respect to the enzymatic cross-links, we find a decreasing ratio of non-reducible (mature) to reducible (immature) cross-links in the collagen in irradiation-damaged bone. This may be attributed to the increased formation of immature cross-links in irradiated bone, which would progressively disrupt the integrity of the mature cross-links, leading to lower fracture loads. Lastly, at very large exposure of 630 kGy, the distortion of the UV Raman spectra further suggests complete fracture of peptide bonds in the collagen backbone, which would be consistent with the progressively degraded bone strength at high irradiation exposures.

In summary, the decreases in strength, ductility and toughness with irradiation is attributed to (i) changes in crack path (the extrinsic effect), and (ii) a degradation of the collagen integrity from increased collagen cross-linking (the intrinsic effect).

4.6. References

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Chapter 5:

Characterizing the Effects of Aging on the Micro and Macro scale Fracture Behavior in Human Cortical Bone

5.1 Introduction

5.1.1. Summary

The risk of bone fracture increases with age and has become a major health concern today. When evaluating age-related fractures it is extremely vital to understand the relationship between the structure and the mechanical behavior of bone. In the past bone fracture was associated with a decline in bone mineral density; however recent studies have shown that both the structure and properties of bone degrades with age and sometimes the fracture risk can increase even when the BMD stays constant [1]. Therefore, there must be a cohesive and exhaustive method to evaluate the risk of bone fracture that takes into account both bone mineral density and bone quality, in terms of the toughness and strength. The framework for a mechanistic quantitative evaluation has been laid out in chapters 3 and 4. Here this framework is applied to the important issue of aging bone at the micro- to macro-scale.

As already discussed, human cortical bone is a hierarchical mineralized tissue that serves as structural support to the human body and so its fracture properties are of extreme importance. Human bone is an anisotropic structure, indeed it is more difficult to break than it is to split. However, little is known about the fracture toughness and crack properties in the transverse or breaking orientation. In the past the toughness of bone was determined by the crack initiation toughness, however bone exhibits rising crack-growth resistance with crack extension. In this chapter the effects of aging on the fracture toughness of human cortical bone is quantitatively assessed specifically in the transverse (breaking) orientation with fracture experiments.

The results show a 14% age-related decrease in the transverse toughness [2]. This is much smaller than what is reported for the longitudinal or splitting orientation which was shown to be decreased by an order of magnitude [3, 4]. Similar to the findings for x-ray irradiated bone (chapters 3) with aging cracks in the transverse direction are subjected to an increasing incidence of crack

deflection, principally along the cement lines, but the deflections are smaller and result in a generally less tortuous crack path.

5.1.2. Background

When evaluating the fracture behavior of bone it is important to distinguish between the intrinsic “plasticity” toughening mechanisms, which act ahead of the crack tip, and extrinsic mechanisms, which is activated primarily behind the crack tip shielding the crack from the applied driving force. Bone derives its resistance to fracture mainly from extrinsic toughening mechanisms, which are related to the hierarchical structure in bone. Experimentally quantifying these extrinsic toughening mechanisms requires a crack growth approach. In understanding fracture risk in bone it is important to discern which characteristic dimensions in its hierarchical structure is most important to controlling its fracture properties. It has been asserted that the structure at the scale of hundreds of micrometers, i.e. the level of the Haversian canals, is most critical in controlling bone’s fracture toughness [5].

An increased amount of microcracks is known to form with age [6] as well as an increase in density of secondary osteons with age [4]. Fracture mechanics, used in this study, provides a viable method for quantifying the relationships between stresses and strains applied to the bone, the crack or flaw size, and the resistance to fracture from the extrinsic toughening mechanisms. Coupled with examination of these mechanisms relationship to the microstructure, using x-ray tomography, it provides a framework for understanding the failure of bone.

Many studies have observed age-related changes to the mechanical properties of bone showing a significant deterioration in bone toughness with age [7-14]. However, many of these studies have made the assumption that toughness can be represented by a single value representative of the energy used to prevent a crack from causing fracture i.e. the crack initiation toughness. However, the fracture toughness in many biomaterials, for example bone, can be attributed to mechanisms that act at the microscopic length scale to inhibit the growth of small cracks, i.e. the crack-growth toughness; accordingly, characterization of the fracture resistance requires the use of the so-called crack-resistance or R-curve, which defines how the fracture resistance (*e.g.*, K or G) increases with the stable crack extension of small cracks prior to unstable fracture [7, 15].

In the longitudinal orientation the R-curve measurements on human cortical bone have shown a distinct deterioration to the crack-initiation and growth

toughness with aging [3]. However, until this study R-curve measurements in the transverse orientation have not been available. This orientation, which is studied here, is more clinically relevant since it represents the more common breaking orientation in bone. Here, unlike the longitudinal orientations where crack bridging provides for toughening [15-17] the primary microscale contribution to the bone toughness in the transverse orientation arises from crack deflection and twist since the microcracks are now aligned roughly perpendicular to the crack path where they act as “delamination barriers” [17, 18]; which serve to locally arrest growing cracks, mainly at the cement lines, leading to highly tortuous crack paths and consequently an enhanced fracture resistance [16, 17].

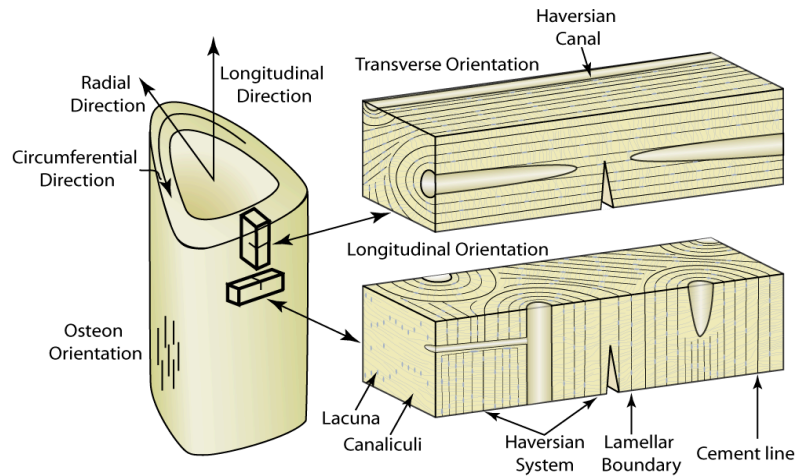


Figure 5-1: Schematic illustrations of the three-point bend samples used for R-curve testing in the transverse and longitudinal (proximal-distal) orientations in relation to the bone-matrix microstructure in human cortical bone (from ref[2]).

5.2. Experimental Methods

5.2.1. Tomography at the Advanced Light Source

X-ray micro-tomography was performed on two specimens each of the *Young* and *Aged* groups at the Advanced Light Source (beamline 8.3.2), at the Lawrence Berkeley National Laboratory; the setup is similar to standard tomography procedures [19] discussed in chapter 2, where the sample is scanned in absorption mode and the reconstructed images are obtained using a filtered back projection algorithm. Each sample is rotated in a monochromatic x-ray beam and the transmitted x-rays are imaged via a scintillator, magnifying lens and a digital camera to give an effective voxel size in the reconstructed three-dimensional image of 1.8 μm . To maximize the signal-to-noise ratio, an input x-ray energy of 20 keV was selected; this optimizes the interaction between the x-rays and the sample. Two-dimensional images were taken every quarter of a degree between 0 and 180 degrees. The data sets were then reconstructed using the software Octopus [20] and the three-dimensional visualization was performed using AvizoTM software [21].

5.2.2. Materials

Test samples from the midsection of frozen cadaveric humeral and tibial cortical bone from six donors were used in this study. The age of these donors, who had no known history of bone-related diseases, were 25, 34, 37, 61, and 69 years old (cause of donor death unrelated to skeletal state); the gender of the donors together with anatomical location are given in Table I. Bone in the 25-37 years old age group is termed *Young* ($N=4$), in the 61 and 69 years old age group *Aged* ($N=5$). Tibiae were used for the 25 and 74 years old donors and humeri were used for the remainder of the donors. All of the donors were male with the exception of the 34 and 69 years old donors.

Bone samples were obtained by sectioning the medial cortices of the mid-diaphyses of the humeri and tibiae using a low speed saw and machined into twelve 1.5-2.2 mm thick, 8 mm long bend samples (width $W = 2$ mm) for R-curve testing. These samples were then notched to form an initial crack of roughly half the sample width, which was then sharpened with a micro-notching technique by polishing with a razor blade irrigated with 1- μm diamond suspension; resulting root radii were consistently ~ 10 μm [29]. The location of the samples along the axis of the bone and their corresponding notching was performed so that they were oriented such that the nominal crack-growth direction for subsequent toughness testing was transverse to the long axis of the humerus, *i.e.*,

they were in the transverse orientation (Fig. 1). All samples were immersed in ambient Hanks' Balanced Salt Solution (HBSS) for 24 hr prior to testing.

5.2.3 Fracture Toughness Measurements

To determine R-curves in the transverse orientation, the small humeri and tibiae samples were loaded in three-point bending in accordance with ASTM E1820-08 [22]; tests were performed in 25°C HBSS at a displacement rate of 0.01 mm/s on an EnduraTec Elf 3200 testing machine (BOSE, Eden Prairie, MN). It has been shown previously that the cracks propagating in the transverse orientation undergo in-plane deflection and through-thickness twisting creating a mixed-mode driving force for crack extension [23, 24]. Additionally, as there are significant inelastic (plasticity) mechanisms in bone which contribute to its intrinsic toughness, we used here a nonlinear-elastic fracture mechanics approach [24-26] and calculate the crack-driving force using the J -integral, where J is the nonlinear strain-energy release rate defined as the rate of change in potential energy in a nonlinear elastic solid for unit increase in crack area [27]. Crack lengths were estimated in terms of the equivalent through-thickness crack of the same compliance. Specifically, to monitor crack extension, measurements of the elastic compliance, C_{LL} , were made during periodic unloading (~10%) every ~25 μm of crack extension during the R-curve test. The relationship between C_{LL} and the equivalent through-thickness crack length, a , was obtained from handbook solutions. Resulting toughness values at each measured crack length were measured in terms of the sum of the elastic, J_{el} , and plastic contributions, J_{pl} , to J [28]:

$$J = J_{el} + J_{pl} \quad (1)$$

The elastic component was determined from:

$$J_{el} = K^2/E \quad (2)$$

where K is the stress intensity and E is Young's modulus. This contribution was quite small, and typically only 5-10% of J_{pl} . The plastic component of J was calculated from:

$$J_{pl} = \frac{2A_{pl}}{Bb} \quad (3)$$

where A_{pl} is the area under the plastic region of the load *vs.* load-point displacement curve, B is the specimen thickness, and b is the (macroscopic) uncracked ligament ($W - a$).

As it is somewhat uncommon to express the toughness of biological materials, such as bone, in terms of J , the toughness results in this paper was expressed in terms of the stress intensity; specifically equivalent (effective) stress intensities were computed from the standard J - K equivalence (mode I) relationship [28]:

$$K_J = \sqrt{J E} \quad (4)$$

The back-calculation of equivalent K -based toughness values requires knowledge of the Young's modulus E . To determine whether the value of E was affected by aging, measurements of the elastic stiffness were made on *Young* and *Aged* cortical bone using nanoindentation. A total of 15 indentations were performed on each of the two groups with a Triboindenter (Hysitron, Inc., Minneapolis, MN). At each location, the reduced modulus of the bone tissue material was determined. Results showed that the *Young* and *Aged* bone had a reduced elastic modulus of 16.99 (+/- 3.8) GPa and 17.11 (+/- 3.5) GPa, respectively, giving a true elastic modulus of $E = 15.70$ (+/- 3.5) GPa for *Young* bone and 15.85 (+/- 3.3) GPa for *Aged* bone. These values were used with Eq. (4) to compute the equivalent K toughness values. The observed lack of an effect of aging on the elastic modulus of bone is consistent with previous studies in the literature [13, 29].

To observe mechanisms, specifically the nature of the crack path in relation to the bone microstructure, three-point bend tests were also conducted on hydrated *Young* and *Aged* bone *in situ* in a Hitachi S-4300SE/N ESEM environmental scanning electron microscope (Hitachi America, Pleasanton, CA) using a Gatan Microtest 2kN three-point bending stage (Gatan, Abington, UK). This technique [24] further permitted measurements of the R-curve while simultaneously imaging the crack path in back-scattered electron mode at 15 kV and a pressure of 35 Pa.

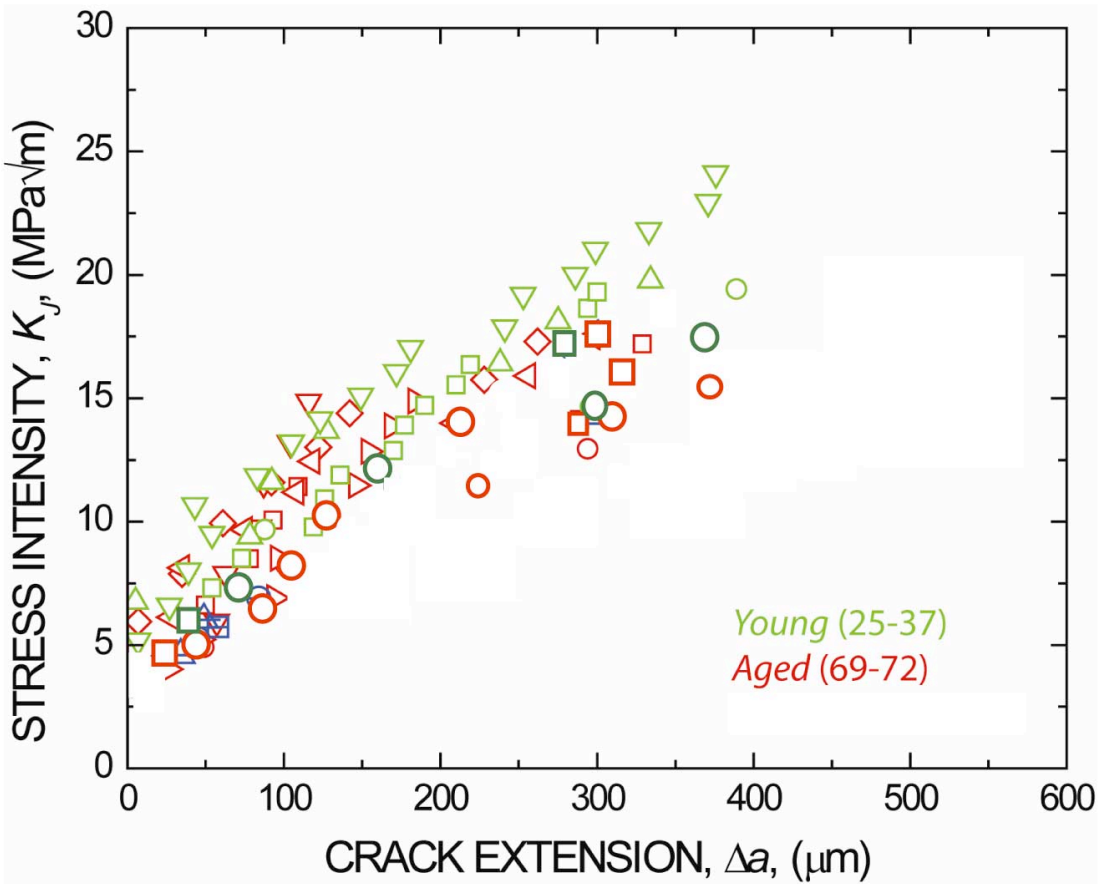


Figure 5-2: Effect of aging on the fracture toughness of human cortical bone in the transverse orientation, showing $K_I(\Delta a)$ resistance-curves for stable *ex vivo* (25°C HBSS) crack extension in human bone in three groups: *Young* (25-37 years), and *Aged* (61-69 years). For the comparison of *Young* vs. *Aged*, note the small average decrease (~6%) in crack-initiation toughness (defined at $\Delta a \rightarrow 0$) compared to the larger (~14%) decrease in crack-growth toughness (slope of the R-curve) with aging. Samples were orientated with the starter notch and the nominal crack-growth direction. (adapted from [2])

5.3. Results

The results of the crack-growth resistance-curve measurements (K_I as a function of crack extension Δa) for the various groups of cortical bone in the transverse orientation are shown in figure 2 [2]. The data shows that both the crack-initiation toughness (determined as $\Delta a \rightarrow 0$) and the crack-growth toughness (slope of the R-curve) decrease with age, although compared to

published results for the longitudinal orientation (Fig. 1) the effect is quite small. The age-related decrease in crack-initiation toughness in the transverse orientation is on the order of ~6%, far smaller than the factor of ~2 reported for longitudinal [3] investigated over a similar age range. The decrease in crack-growth toughness was larger, on the order of ~14%, but this again is far smaller than the almost order of magnitude decrease reported [3] for the longitudinal orientation. Actual values are listed in Table I.

In order to visualize and compare the active extrinsic mechanisms R-curve tests were used to grow cracks in the transverse direction in Young and Aged bone samples during R-curve tests. Results, shown in Fig. 3 based on both *in situ* ESEM and computed x-ray micro-tomographic imaging, indicate extensive crack path meandering which is characteristic of bone fracture in the transverse orientation. On initiating from the sharpened notch, cracks can be seen to deviate away from the path of maximum tensile stress (which is co-planar with the notch plane) and to undergo marked deflections, of 90 degrees or so (in three-dimensions, crack twisting is evident too), on encountering the cement lines of the osteons. Mechanistically, such crack deflection/twist, which is the principal source of microscopic toughening for the transverse orientation [17], was similar in both Young and Aged samples; however, whereas the number of deflections was often increased in the older bone, the deflections were smaller in magnitude and the resulting overall crack paths were less tortuous (Fig. 3).

Table 5-1:
R-Curve Behavior of Human Cortical Bone with Aging (Transverse Orientation)

Donor Information * (R²)	Initiation Toughness <i>K_o</i> (MPa√m)	Growth Toughness (MPa√m/μm)	Coeff. of Determination
Young (25-37 years):			
25MT*	7.28	0.036	0.979
25MT	4.77	0.049	0.989
34FH	6.12	0.032	0.978
37MH	7.27	0.039	0.984
Aged (61-69 years):			
61MH	4.68	0.030	0.969
61MH	5.69	0.034	0.960
69FH	6.63	0.037	0.975
69FH	6.99	0.042	0.668

* The notation reads as follows: Age (years), Sex (M=Male, F=Female), Extremity (H=humerus, T=Tibiae).

* Table adapted from ref [2]

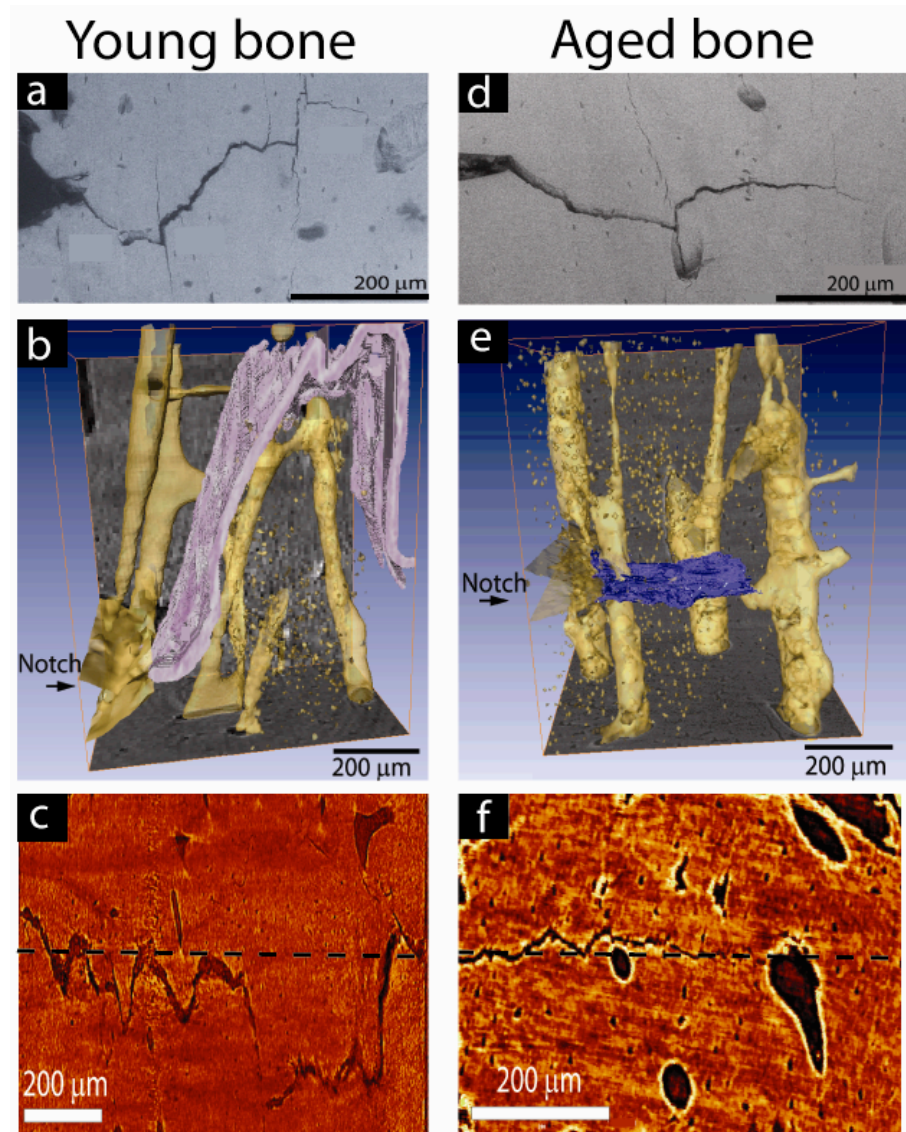


Figure 5-3: Scanning electron microscopy and synchrotron x-ray microtomography of crack paths in (a-c) *Young* and (d-f) *Aged* human cortical bone in the transverse orientation. Images show crack paths: (a) & (d) SEM micrographs from side-view perpendicular to the crack plane, (b) & (e) 3-D x-ray microtomography images of these paths; 3-D crack surface is purple; Haversian canals are yellow brown), and (c) & (f) 2-D tomographs of the paths from the back face of the sample. The crack deflects on encountering the osteons; such crack deflection and crack twisting is the prime extrinsic toughening mechanism in bone in the transverse orientation. Note, however, that the frequency of such deflections is increased whereas their severity is decreased with aging, resulting in less meandering crack paths in aged bone.

5.4. Discussion

The results from this study show that the fracture toughness varies among the two orientations- longitudinal and transverse. It is apparent that cracks in the longitudinal or splitting orientation occur with a lower toughness than the transverse or breaking orientation. A recent study has shown that 99% of all cracks in bone are aligned at an angle of less than 25° with respect to the osteons [40]. It is because of the orientation dependence of the microcracking that bone is much more difficult to *break* than to *split* [27]. Microcracks most often form at these cement lines; they are thus primarily aligned along the long axis of the bone with a typical spacing of $\sim 10\text{-}100\text{ }\mu\text{m}$ [27]. The microcracks serve to arrest growing cracks, cause crack deflection and twist, which all correspond to high toughness.

Extrinsic toughening mechanisms are only activated in the presence of a crack and their potency in resisting crack growth is dependent on the size of the crack. Tomography allows us to image both the size of the crack and the extrinsic mechanisms, in bone in the transverse orientation these mechanisms are identified as crack deflection and crack twist [17]. For the transverse orientation, the reduced osteonal spacing should result in more frequent crack deflections as the growing crack encounters the cement lines, and this is what is observed (Fig. 3). At first sight, this might be expected to increase the toughness, but the result of these more frequent crack deflections is that the extent of the individual “delaminations” along the cement lines (nominally perpendicular to the main crack path) becomes smaller so that the overall degree of crack-path meandering is actually lessened (*c.f.*, Fig. 3c & f). A similar effect has been seen in severely irradiated bone [42]. Thus, the aging-related reduction in osteonal spacing leads to shorter crack-path excursions away from the plane of maximum tensile stress and the overall decrease in crack-path tortuosity, which in turn results in a smaller influence of aging on the fracture toughness in this transverse orientation.

Finally, it should be noted that as most actual bone fractures in humans are rough (multi-orientated) mixed-mode failures, our current observations that the transverse fracture toughness of human cortical bone is only slightly affected by aging is still consistent with the notion of an increased risk of fracture with age due to lower bone quality. Although in medical terms this problem is currently treated solely in terms of the aging-related loss in bone mass, the fracture toughness R-curves and the tomographic images shown in Figs. 2 and 3 of this paper clearly highlight a concurrent and significant loss in the bone's resistance

to fracture. Hopefully future therapies can be developed to treat this aspect of the problem, *i.e.*, that of bone quality, as well as the age-related loss in bone quantity.

5.5. Conclusion

Although the bone toughness is modestly degraded by aging in the transverse orientation, the effect is far smaller than found in the longitudinal orientation. Over the age range of 25 to 72 years the initiation and growth toughness are reduced, respectively, by ~6% and 14%, as compared to corresponding ~15% and 62% reductions in the longitudinal orientation. This is not to say that the effects of aging on the factors that fracture bone are smaller than first thought, as most actual (*in vivo*) bone fractures occur under mixed-mode conditions, *i.e.*, due to the bone geometry and actual physiological loading, they often involve combinations of tensile, compression and shear forces that result in highly complex fractures along both the transverse and longitudinal directions. However, it is of note that mechanistically the nano/microstructural features associated with the aging of human cortical bone have a far greater influence on splitting rather than transverse (breaking) fractures.

With aging, the spacing of these cement lines (associated an increase in osteonal density) will likely be decreased [21]; however, it is uncertain mechanistically whether this will result in a significant change in the incidence of crack deflection, and whether this will significantly affect the crack path and hence the transverse toughness. Accordingly, in this work we specifically examine the measured R-curve fracture toughness and corresponding toughening mechanisms in human cortical bone tested in the transverse orientation as a function of aging to discern whether the transverse toughness is actually affected by age and how mechanistically this could occur.

Mechanistically, whereas the aging-related deterioration in the (extrinsic) toughness in the longitudinal orientation has been associated with an increase in the osteonal density which results in a reduced spacing between the cement lines (the prime location for microcracking) which in turn leads to a diminished toughening role of crack bridging, in the transverse orientation where crack deflection/twist is the prime source of toughening, the smaller spacing of the cement lines in older bone can be seen to cause an increase incidence of crack deflection. However, as these deflections are smaller in magnitude, the resulting overall crack paths are generally less tortuous than in young bone. Consequently, in contrast to the longitudinal toughness, the aging-related effect on the transverse toughness is relatively small.

5.6. References

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Chapter 6: Conclusion and Future Work

6.1. Summary

Human cortical bone serves as the structural load bearing material in the human body. It is well known that bone exhibits a complex hierarchical structure that spans many length scales [1]. This work establishes a framework for characterizing and understanding the mechanical properties and fracture behavior in cortical bone at the size scales of significance. The mechanisms in bone have previously been classified in terms of their intrinsic and extrinsic contributions to bone toughness. At sub-micron size-scales, toughening in bone arises primarily from “plasticity” mechanisms that operate to promote intrinsic toughness, which include molecular uncoiling of collagen molecules and fibrillar sliding of both mineralized collagen fibrils and individual collagen fibers [2-4]. At larger size-scales (~micrometer) toughening in bone arises primarily from extrinsic mechanisms which primarily derives bones resistance to fracture through crack growth, such as crack deflection and twist [5].

The studies presented here investigate both the intrinsic and extrinsic mechanisms for human cortical bone, and apply this in analyzing the effects of irradiation and aging on bone. Specifically, to investigate the macroscopic properties, these studies use resistance curves (ch.3 & ch.5) and x-ray tomography (ch.1, ch.3 & ch.5) to visualize the relationship between the crack path and the microstructure during bone fracture, then to investigate the sub-micron scale properties, (ch.4) *in situ* tensile tests coupled with x-ray scattering experiments are utilized to characterize the mechanisms of fibrillar sliding, and lastly, spectroscopy techniques are used to semi-quantify changes to the cross-linking in bone. This complete hierarchical story, from the molecular to the macroscopic length scales, presented in this work can be applied to better predict fracture.

6.1.2. Conclusion: Effects of Irradiation on Cortical Bone

Understanding the mechanical properties of bone requires probing over multiple dimensions owing to the complex hierarchical structure of bone. The discussed studies [6] have shown a higher than expected effect of prior irradiation on degrading the mechanical properties of human bone. The work presented in chapter 3 and 4 use the mechanistic framework in an experimental study that looks at the effects of synchrotron x-ray irradiation on the structure

and mechanical properties of hydrated human cortical bone in the transverse or 'breaking' orientation.

It was found that when compared to healthy (unirradiated) bone, exposure to irradiation doses between 0.05 and 630 kGy progressively led to a severe degradation in strength, ductility and toughness. At irradiation doses of 70 kGy and above, stress-strain curves displayed a complete absence of post-yield plastic deformation and with increasing irradiation a major reduction in fracture load and toughness. The irradiation-induced loss in fracture resistance of the bone can be attributed to both intrinsic (plasticity) and extrinsic (shielding) contributions to the fracture toughness.

Extrinsically, although unirradiated bone displays extensive deflection and twisting of a growing crack in the transverse orientation as it encounters microcracks, primarily located in the cement lines, fracture in irradiation-damaged bone was characterized by far smaller crack-path deflections, smoother fracture surfaces, and hence much lower fracture toughness values. While the irradiation-induced suppression of bone plasticity acts to severely limit the intrinsic toughness by macroscopically embrittling the bone tissue.

Intrinsically, bone irradiated with a dose of 70 kGy demonstrates a lack of plasticity in the collagen via the suppression of the mechanism fibrillar sliding. In healthy bone the load in the bone is carried by the mineral through shearing of the collagen matrix and at yielding the mineralized collagen fibrils and the matrix decouple, however in irradiated bone when the elastic limit is reached cortical bone fails catastrophically. The loss in intrinsic toughness through the suppression of plasticity via the mechanism of fibrillar sliding is attributed to an increased incidence of specific cross-linking of the collagen with irradiation.

Measurements for unirradiated and 70 kGy irradiated bone revealed that there was a ~21% increase in the concentration of non-enzymatic cross-links, in the form of advanced glycation end-products (AGEs), in the irradiated bone, which would clearly act to restrict fibrillar sliding of the collagen fibrils. With respect to the enzymatic cross-links, we find a decreasing ratio of non-reducible (mature) to reducible (immature) cross-links in the collagen in irradiation-damaged bone. The departure from the healthy distribution of these cross-links would progressively disrupt the integrity of the mature cross-links, leading to lower fracture loads. At very large exposure of 630 kGy, the distortion of the UV Raman spectra further suggests complete fracture of peptide bonds in the

collagen backbone, which would be consistent with the progressively degraded bone strength at high irradiation exposures.

6.2. Future Work

This work investigates the effects of irradiation on bone, besides such radiation-induced embrittlement of bone, similar effects of altered collagen chemistry have been reported to cause increased fragility in bone due to aging [7, 8] and disease [9, 10]. For example, with aging, as shown in ch. 5, there is a significant reduction in the crack-initiation toughness and an almost complete elimination of crack-growth toughness in human cortical bone [7], effects that have similarly been attributed at the sub-micrometer scale to increased cross-linking [11] which suppresses the intrinsic toughening mechanism fibrillar sliding, and at the micrometer scale and above reduces extrinsic toughening from degrading crack-tip shielding [7, 12]. However, the role that each type of cross-linking plays in affecting the biomechanical performance of bone with aging is still controversial.

Collagen plays an important role in the biomechanical performance of bone [13-15]. Disruption in the cross-linking in collagen can increase bones susceptibility to fracture [16-18]. Further studies are needed to clarify the contribution of immature and mature pyridinium and pyrrole cross-links and advanced glycation end products (AGEs) to bone strength, as well as how these cross-links vary with age and disease. Specifically, investigators have been divided among whether enzymatic cross-links change with age, and they are uncertain in the role that enzymatic cross-links play in the deterioration of bone's mechanical properties with age. However, it is commonly accepted that there is an increase of AGEs with aging due to the slow turnover rate of mature collagen, and the accumulation of AGEs has been shown to render the fiber too stiff. This embrittlement of the fibers increases bone's risk of fracture [10, 11, 19-23].

Age related changes to the enzymatic cross-links are still inconclusive, further studies are needed to clarify the contribution of immature and mature pyridium and pyrrole cross-links to bone strength. Therefore, it is critical to study how collagen cross-links change with age. In my proposed future work I plan to investigate the role that cross-linking plays in age related fractures. In this context I plan to perform FTIR spectral analysis on aging human cortical bone. FTIR spectra provide information on all of the biological tissue components, specifically the protein and mineral constituents produce intense structure sensitive IR modes [24]. This technique is important for investigating changes to the collagen cross-linking chemistry as a function of aging and disease.

Mechanistically, another topic worth pursuing is to apply this framework to diseased bone in order to achieve an improved understanding of the effect of different diseases on the mechanical properties of bone. Although only limited tests were performed on diabetic and aged bone, preliminary results do suggest that the degradation effect of this disease on the bone toughness is quite different from the effect of pure aging [25]. Specifically, compared to non-diabetic bone of a similar age, the crack-growth toughness was not that different; however, the crack-initiation toughness was significantly lower by more than 30%. This implies that diabetes does not necessarily affect the larger-scale microstructure of bone that gives rise to extrinsic toughening mechanisms, *i.e.*, bone-matrix structure at micrometer length-scales and above (in particular the secondary osteons), but rather the intrinsic (“plasticity”) mechanisms that are principally affected by structure at sub-micrometer dimensions. However, only limited tests were performed on diabetes and aged bone, so a more extensive study is needed to investigate the effects of diabetes on the mechanical performance of bone.

Overall, the hierarchical approach to characterizing the deformation and fracture behavior of bone, presented in this work, can be applied to many different types of studies, from aging, irradiation or disease. In the future, this analysis will help the evaluation and advancement of therapeutic drug treatments.

6.3. References

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Appendix I

Estimation of radiation exposures

Bone samples in this study were all irradiated from hard x-rays produced from a superbend synchrotron source and double multilayer monochromator combination on beamline 8.3.2 at the Advanced Light Source at the Lawrence Berkeley National Laboratory (LBNL). All of the controls selected for irradiation were selected to mimic a realistic tomography scan. All reported dose values in text follow the same procedural dose calculations.

To estimate the radiation dose absorbed by the bone sample, which is measured in grays ($1 \text{ Gy} \equiv 1 \text{ J kg}^{-1}$), the radiation flux density, ψ (photons/sec/mm²), is computed from the value of the flux, Φ , measured from an ion chamber:

$$\psi = \frac{\Phi}{z} ,$$

where z is the area of the beam at the sample. The flux density is then converted into an energy density E_ρ , using:

$$E_\rho = \psi \times 1.6 \times 10^{-19} \text{ J/eV} \times E ,$$

where E is the energy of the beam in eV.

The transmission, T , of x-rays through a material, of thickness l , is often expressed as:

$$T = \frac{I}{I_0} = e^{-\alpha \rho l}$$

where I and I_0 are the transmitted and incident x-ray intensities respectively, α is the mass attenuation coefficient (cm²/gm) and ρ the density. This is generally referred to as the Beer-Lambert Law. The linear attenuation coefficient μ (cm⁻¹) is also commonly used and is related by: $\mu = \alpha \rho$. The fraction of x-rays absorbed (A) by the sample is given as $(1 - T)$. The dose rate, $\dot{d}$, can then be obtained from:

$$\dot{d} = \frac{A E_\rho}{M}$$

where M is the mass of the bone absorbing the radiation. Given a reasonably uniform distribution for the absorption of x-rays within the sample, the total irradiation dose received during each exposure is then found from the dose rate and the total exposure time, t :

$$\text{Total irradiated dose} = \dot{d} \times t = \frac{A E_\rho}{M} \times t$$