Ultrastructural Changes during the Fatigue of Bone

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Repetitive mechanical loading of bone can lead to ultrastructural-level damage, which can lead to fracture if not repaired. Skeletal fractures result not only from a loss in bone mass, but also because of alterations in tissue quality. Therefore, it is important to also delineate how changes in tissue ultrastructure affect the mechanistic response of bone to its physical environment. In this overview, factors affecting tissue quality, in particular fatigue resistance, are reviewed, followed by examples of recent work that has identified ultrastructural and compositional changes that occur in bone during fatigue.

INTRODUCTION

Bone is an exquisitely designed material that has a composite nature at multiple levels of dimensional scale (Figure 1).[1] The skeletal system serves three main functions: force transmission during locomotion, metabolic regulation, and protection of inner organs. Two of these functions are clearly mechanical. Therefore, understanding the factors contributing to the mechanical competence of bone is critical medically, as well as from a biomimetics standpoint of using nature's design criteria for synthesis of manmade materials.

At the macroscopic level, there are two general types of bone—cortical and cancellous (trabecular). Cortical bone, which forms the outer shell, is dense with only microscopic channels and comprises approximately 80% of the skeletal mass. Cancellous bone is found in the interior, and is more porous, consisting of a network of rods and struts. The density and mechanical properties of cortical bone are greater than cancellous bone at the global level, but it is not clear whether there are differences in structure and function at the tissue level. At the microscopic level, bone consists of organic and inorganic phases. The organic phase is approximately 90% collagen and the inorganic phase is a nonstoichiometric, carbonated apatite. At the ultrastructural level, elegant interactions between the organic and inorganic phases (and water) can be observed,[2] and it is these interactions that significantly contribute to the mechanical properties of bone.

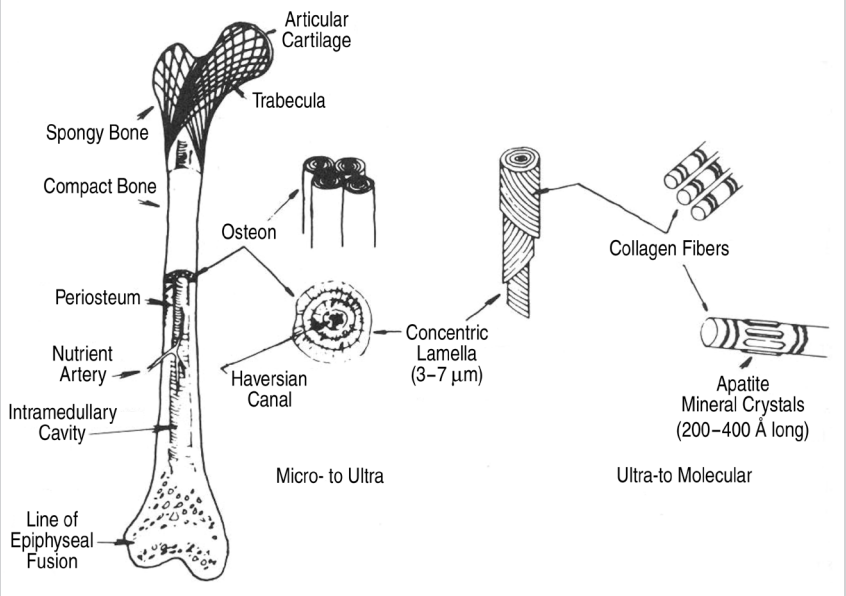

Figure 1. The hierarchical levels of structural properties in a human long bone.

Humans subject their bones to approximately 2 million loading cycles a year. Factoring in the contribution of muscle forces, the average load on the proximal femur from normal walking can be 4–5X body weight, and with running and jumping, peak forces can be as high as 10X body weight. Therefore, the functional requirements for the skeletal system are demanding, and the mechanical design requirements are significant. Nonetheless, these requirements have been met with a minimal amount of mass. Such an efficient design results from elegant "processing" strategies used by nature that are currently beyond the capabilities of manmade composites. For example, the organic and inorganic phases are interdigitated at the nano-scale through a self-assembly process in which mineral crystals nucleate onto a protein (collagen) template, and the preferential growth of the crystals in specific directions is mediated by proteins.[3] In addition to the anisotropy arising from orientation differences at multiple length scales, tissues are functionally graded (i.e., structure and mechanical properties are spatially nonuniform in response to differing mechanical demands in different locations). Bones undergo a continual turnover process to remodel older parts of the skeleton and also adapt locally,

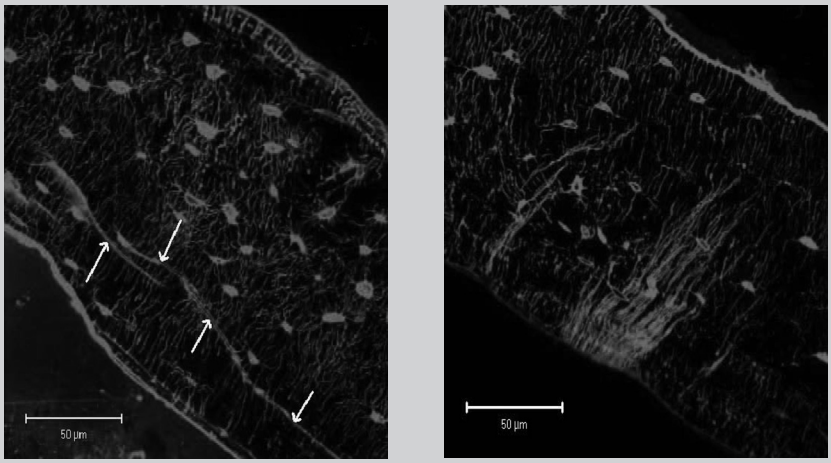

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Figure 2. Confocal images of murine cortical bone subjected to fatigue under four-point bending. In (a) the compressive region, long linear microcracks are present (indicated by arrows); in (b) the tensile region, there is a cloudy network of tiny cracks classified as diffuse damage (60X).[7]

in response to changes in their physical environment, resulting in the ability to repair local regions of the tissue.[4,5] Collectively, therefore, the skeletal system has achieved what much of the materials science community has been striving for for decades: an array of processing strategies (organic template-mediated synthesis of organic/inorganic hybrids, self assembly, intelligence, functional grading, transformation toughening, and ion exchange) to achieve function in a material subjected to significant design demands.

STRUCTURE-FUNCTION RELATIONS IN BONE

The processing-composition-structure-property synergy that characterizes materials science also holds for investigating the properties of tissue. One difference, however, is that in manmade materials such as metals, processing parameters required to control composition, grain size, anisotropy, texture, orientation, impurities, and porosity have been identified. Therefore, parameters known to influence physical and mechanical properties can be well controlled, leading to quality control in materials and products. With tissues, however, processing is the result of evolution and, on a shorter time scale, the exogenous effects of aging, disease, and alterations in physical influences. Therefore, the mechanical properties of tissues are subject to significantly more variance, and characterizing the mechanical properties of tissues is more difficult. In addition, because of the temporal (adaptive changes due to remodeling) and spatial inhomogeneity (compositional and mechanical gradients, even on a scale of 100 µm), bulk mechanical testing results in significantly more averaging of microstructure influences than with manmade materials, making it more difficult to identify mechanisms contributing to failure.

Repetitive mechanical loading of bone can lead to ultrastructural-level damage.[5–7] Even though younger people are more active and subject to loading of greater magnitude and duration, they typically have a greater capacity to withstand the functional demands placed on the skeletal system, and damage is repaired by remodeling. However, with age, pathologic conditions, and/or intense exercise regimens, the compensatory mechanisms needed to maintain the mechanical stability of skeletal tissue become impaired. As a result, ultrastructural-level damage, which may no longer be repaired as efficiently, can play a role in bone fracture.[5–7] Distributions of sub-threshold microcracks, which range in length up to 300 µm (Figure 2), may ultimately contribute to property degradation and fatigue fracture in cortical and trabecular bone.[6]

It is generally accepted that skeletal fractures result not only from a loss in bone mass, but also because of alterations in tissue quality.[5] In other words, bone mass alone is not a significant predictor of skeletal fragility, and predisposition to fracture is a function of tissue quality as well as quantity. Therefore, it is important to also delineate changes in tissue quality and how changes in tissue ultrastructure affect the mechanistic response of bone to its physical environment.

In engineering composite materials, it is well known that composition has a significant influence on mechanical properties as well as on the spatial and temporal progression of damage accumulation. By analogy, bone may be viewed as a composite (at multiple levels of hierarchy), and its composition as well

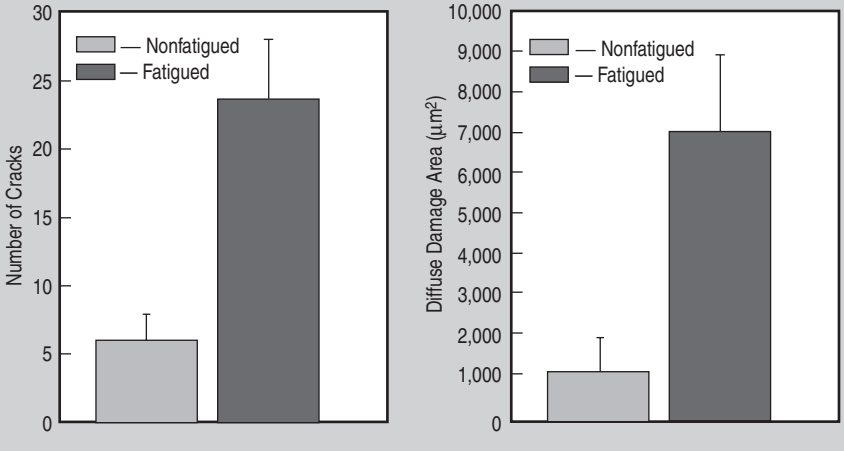

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Figure 3. The (a) number of microcracks ($p < 0.001$) and (b) diffuse damage areas ($p < 0.015$) significantly increase with fatigue.[15]

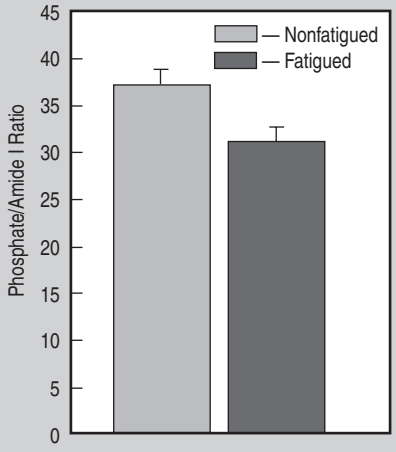
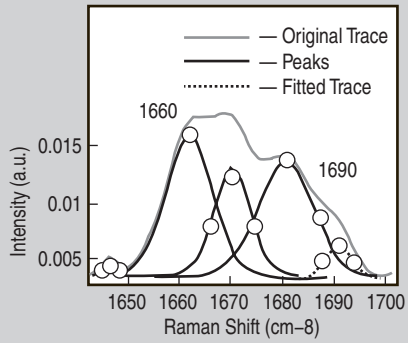


Figure 4. The mean mineral-to-matrix ratio in fatigued (damaged) and control bones. Fatigue leads to a statistically significant reduction in phosphate to amide I ratio ($p < 0.024$).



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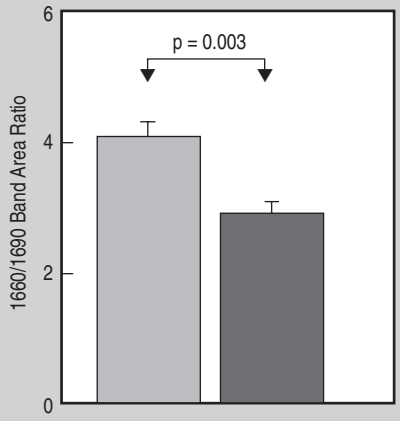

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Figure 5. (a) The region of Raman spectra associated with amide I. Umbrella graph shows the relative intensity as a function wave number of the integrated amide I band region (1,600–1,700 cm^{-1}) and the deconvoluted sub-bands. The 1,660/1,690 band area ratio is used as a metric for inter-fibril cross-link density and degree of cross linking. (b) Following fatigue loading, there is a statistically significant reduction in the ratio of non-reducible to reducible cross links, indicating that cross link ruptures occur during fatigue.

as architecture (e.g., mineral crystallite size, shape, and orientation, and specific mineral-to-mineral and mineral-to-matrix ratios; collagen fiber organization and orientation; and bonding between the mineral and collagen phases, among other parameters) also dictate mechanical performance.[7–11] Compositional and ultrastructural parameters of bone are acknowledged to be significant modulators of whole bone properties and local fatigue-induced damage.[7,10,12–15]

The mineral composition of bone is not constant, however. As bone advances from the initial stages of formation to full maturity, the degrees of lattice substitution and mineralization vary. Mineral composition also varies spatially and with the chronological age of humans and animals, as well as with disease.[16] For example, in fully mineralized bone, the calcium phosphate lattice contains substantial amounts of carbonate and small amounts of monohydrogen phosphate. However, newly deposited mineral contains less carbonate and a larger relative amount of monohydrogen phosphate. There may also be highly disordered (amorphous) calcium phosphates present in varying amounts. Changes in compositional parameters are therefore likely contributors to the mechanical properties of bone.

In addition to the effect of mineral composition on bone quality, the organic matrix plays just as critical of a role. Normal collagen assembles into triple helices, which are bound together in fibrils by cross links formed by a sequence of reactions that begins with hydroxylation of lysine.[17] There are several types of cross links, but the ones that appear to be most important for mechanical strength of mature bone are pyridinoline cross links that join several bundles of fibrils together. While some of the detail of cross linking remains uncertain, it is known that factors that perturb normal cross linking will weaken bone and modulate bone quality.[18] For example, increases in non-reducible (immature) cross links (pyrrolic, pyridinium, and

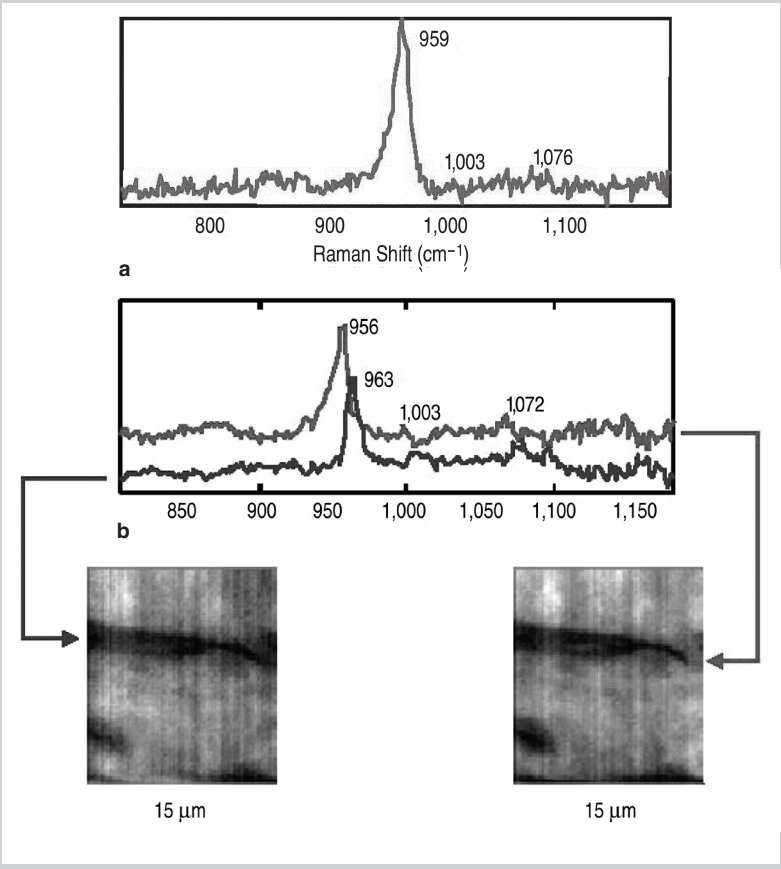

Figure 6. The Raman spectra for (a) undamaged and (b) microcracked regions of cortical bone. Factor analysis revealed an additional high-frequency phosphate species in cracked regions. Score images reveal the spatial distribution of these two mineral factors.[14]

non-enzymatic glycation-mediated cross links) lead to alterations in mechanical properties and may therefore be associated with skeletal fragility.[19]

MECHANICAL INFLUENCES AND COMPOSITION IN BONE

Using the combined strategies of detecting mechanically induced small-scale damage in bone via acoustic emission, along with Raman spectroscopic analyses of composition on the same dimensional scale as damage, the authors have identified local compositional changes associated with deformation and damage in bone.[14,15,20–22] Significant changes in Raman spectra occur when bone is deformed under mechanical loading, providing evidence that the spectroscopic changes are caused by previously unidentified ultrastructural changes in bone.[14,15,20–22]

In bones subjected to fatigue loading in bending, linear microcracks are the predominant form of damage in compressive regions, whereas in tensile regions, there exist cloudy networks of tiny cracks that are classified as diffuse damage (Figure 2). Fatigue loading of bones results in significant increases in the number of cracks and diffuse damage (Figure 3).

Bone responds to fatigue loading by undergoing ultrastructural damage. This damage has been identified in both the mineral and matrix phases as shifts in mineral and matrix Raman spectral bands, which include changes in frequency of the phosphate P-O stretch (~960 cm^{-1}), B-type carbonate symmetric stretch (~1,070 cm^{-1}), and collagen amide I envelope (~1,660 cm^{-1}). These shifts are indicative of stress-induced changes in mineral unit cell parameters and matrix collagen secondary structures.[14,20,22]

Fatigue loading leads to a significant decrease in phosphate-to-amide I ratio (e.g., Figure 4). The amide I band, reflective of collagen, changes shape following fatigue loading and damage accumulation because of a more prominent high-frequency component. The creation of higher-frequency shoulders and resultant change in band shape are indicative of a larger amount of disordered secondary structure in amide I, likely due to rupture of cross links. The two peaks at 1,660 cm^{-1} and 1,690 cm^{-1} underlying the amide I band (Figure 5a) are indicative of changes in collagen cross linking, with the area ratio between them (1,660/1,690) corresponding to the non-reducible/reducible crosslink ratio. This ratio decreases with fatigue (Figure 5b), meaning that a higher percentage of ruptured cross links is present. Raman data therefore show that there are significant changes in tissue quality associated with fatigue damage, and identify the physical nature of damage at the ultrastructural level.

Multivariate analyses detect local compositional changes that may be lost with spatial averaging of univariate metrics in whole bone cross sections. By applying principle component analyses to local domains (~30×30 μm) near and at distances from microcracks, compositional changes were detected associated with damage.[14] In undamaged regions of bone, the PO_4^{3-} ν_1 band is found at 959 cm^{-1}, as expected for carbonated apatite (Figure 6a). However, in regions of microcracks, an additional high-frequency component of the PO_4^{3-} ν_1 band is observed at 963 cm^{-1} (Figure 6b), corresponding to a more stoichiometric, less carbonated mineral species and suggestive of a decrease in carbonate-to-phosphate ratio. Broadening of the PO_4^{3-} ν^1 peak is also observed and attributed to local fatigue-induced amorphization, which is also consistent with a reduced carbonate-to-phosphate ratio, because this ratio serves as a metric for crystallinity.

Transmission-electron-microscopy (TEM) analyses demonstrate that undamaged bones display well-aligned and uniformly distributed collagen fibrils, mostly parallel to the bone axis (Figure 7a, indicated by arrow). In fatigue-damaged bones, however (Figure 7b), the aligned arrangement of collagen

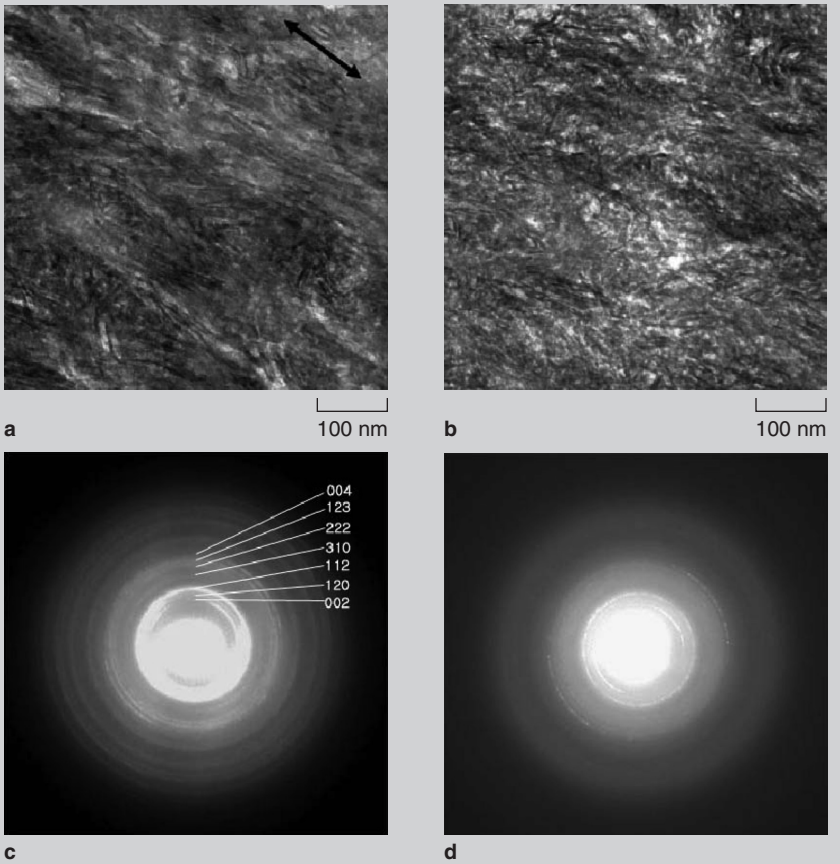

Figure 7. The ultrastructure of mouse bone (a) before and (b) after fatigue. The non-fatigued bones display well-aligned and uniformly distributed collagen fibrils mostly parallel to the long axis of the bone (indicated by the arrow in [a]). In the fatigue-damaged bones, the aligned arrangement of collagen fibrils becomes impaired and fibrils appear to be loosened. The (c) selected area diffraction pattern from control bones exhibit mostly discrete sharp rings, suggesting a significant fraction of mineral apatites are crystalline. The (d) pattern from the fatigue-damaged bones exhibits a small number of weak discrete rings and halos, suggesting that a significant fraction of crystalline mineral apatites became amorphous after fatigue.[15]

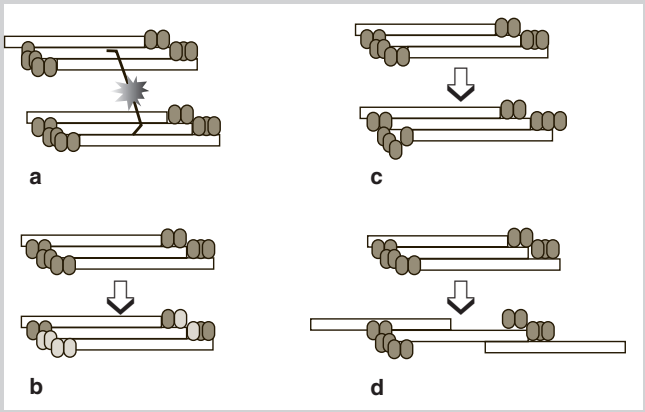

Figure 8. A schematic of ultrastructural failure mechanisms in bone: (a) high and low frequency shoulders on the amide bands indicate distortion of intrafibrillar cross links; (b) high- and low-frequency shoulders on PO_4^{3-} band indicates carbonate substitution; (c) changes in center of gravity of phosphate and carbonate bands with pressure indicate ion motion in the elastic regime, leading to (d) lattice distortion and crystal plate slippage upon yielding.[14]

fibrils becomes impaired and loosened. Damage is manifested by nonuniformly distributed and shorter collagen fibrils, which may result from rearrangement and fragmentation of collagen fibrils in reaction to the applied load. At a higher magnification, high-resolution TEM (HRTEM) images of undamaged bones display tablet-like crystals with lattice fringes. In the damaged bones, crystalline lattice fringes are much less frequently observed and small pieces of particles adjacent to each other were observed. Based on HRTEM images and the presence of diffuse halos in the diffraction patterns (Figure 7 c,d), some apatite crystals are broken into small pieces and the crystallite size decreases or a fraction of crystalline mineral apatites became amorphous during fatigue. The observation of amorphization represents morphological validation of the Raman finding of PO_4 peak broadening. The load-carrying capacity of mineral apatites deteriorates following amorphization and fragmentation, which likely trigger fatigue-induced microcracking of bone.

CONCLUSIONS

Skeletal integrity can become compromised with excessive mechanical loading and is dependent upon intrinsic factors, such as the composition of the mineral and matrix phases. It is therefore important to define relationships between composition and biomechanical competence of bone. Coupling of Raman spectroscopy with mechanical loading has identified mechanisms of ultrastructural deformation in bone at smaller scales than previously understood (Figure 8). Mineral and matrix phases of bone respond to mechanical loading by undergoing measurable changes in band position and symmetry (Figure 8a,b), as well as creation of distinct subspecies (Figure 8b). The incipient mechanisms of failure are ion motion (Figure 8c), ultimately resulting in lattice distortion and plate slippage (Figure 8d). The importance of these findings is that identification of specific constituents within the bone lattice that deform preferentially can significantly impact how drugs are designed and targeted.

ACKNOWLEDGEMENT

Parts of the work reviewed were supported by U.S. Department of Defense/ U.S. Army DAMD17-030100556.

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