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Nanostructure in the trabecular bone of postmenopausal women: Mechanical and chemical analysis

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ABSTRACT

The possibility of diagnosis and prediction of multiple disorders in trabecular bone through nano-biomechanics and chemical analysis are summarized. Improvements to the understating of the compositional contributors of bone mineral and organic components to mechanical competence are crucial. Viscoelastic properties and Raman characterization have been used to evaluate possible alterations of the trabecular bone associated with aging, disease, or injury. In this study, the trabecular bone of postmenopausal women has been analyzed throughout. (a) Nanomechanical characterization, by using nano-DMA: complex modulus, tan δ, loss modulus (E'), and storage modulus (E'); and (b) Raman analysis: relative presence of minerals, carbonate-to-phosphate ratio (both from the mineral components), the crosslinking and nature/secondary structure of collagen (both from the organic components). Complementary nano-morphological studies were done assessing roughness (SRa) and collagen fibrils width, on this trabecular bone. A general idea of the behavior of the viscoelastic performance can be obtained by the Tan δ (E''/E'), that achieved 0.98 GPa of damping. 249 nm and 0.898 μm of SRa roughness and fibrils width were obtained, respectively. The relative presence of minerals, the carbonate-to-phosphate ratio, the crosslinking and the nature/secondary structure of collagen, between 700 and 1700 cm^{-1}, were also obtained, in order to propose a study protocol for trabecular bone characterization.

Introduction

Bone has a hierarchical structure from molecular to the macroscopic level. As a natural composite material, bone primarily comprises water, a hard mineral phase (mainly hydroxyapatite crystals), which controls the stiffness of the bone, and a more compliant collagenous matrix (90% type I collagen), accountable of strength and toughness [1]. Collagen adds plasticity and ductility to the bone [2]. According to its structure, there are two types of bone tissue: trabecular and cortical. Trabecular bone is a highly porous structure that fills the proximal and distal ends of all bones (e.g. femur) and is also present as a filler in other bones (e.g. vertebral bodies and both Maxillary bones), providing structural support and flexibility [3]. Many studies indicate that the tissue mineral distribution plays an important role in determining bone properties at the macro level. However, macro-level analyses cannot determine the detailed mechanical behavior of bone at the tissue level where microcracks initiate and propagate [4]. Material properties of trabecular bone are influenced not only by the architecture and connectivity of individual trabeculae, but also by the properties at the molecular levels. At ultrastructural levels, the collagen molecules and crystals of hydroxyapatite are assembled into microfibrils. These fibrils are again assembled into fibers with thickness about 3–5 μm [3]. To relate the overall mechanical properties to its microstructure, it is necessary to measure the properties of individual trabeculae. The typical length of a trabecula is 1–2 mm with diameter around 100 μm. This makes assessment of mechanical properties at the level of single trabeculae quite a challenging task especially due to its inherent anisotropy and asymmetry of the micro-samples [3].

The inner structure of the trabecular bone is a result of structural optimization provided by remodeling processes. Most of the remodeling process takes place on the surface of the trabecular structure; the interstitial bone (bone tissue in the middle part of the trabeculae) is often excluded from remodeling [5]. This phenomenon has a consequence in highly nonuniform distribution of mineral content; thus, the interstitial bone has a larger mineral content compared to the bone on the surface [3].

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Accumulation of microdamage in bone, as a function of the rate of production and rate of repair, underlies the development of stress fractures, increasing fragility associated with age and osteoporosis [6]. Bone derives its mechanical behavior from the collective contributions of collagen molecules and nanocrystals of hydroxyapatite and their structural arrangements over length scales ranging from the nano- to the microscale [7]. Hence, measurements on the nanoscale may add new aspects to the inter-individual biological variations and require precise measurement protocols [1].

AFM nano-indentation is a suitable method for the determination of the viscoelasticity of hard tissues at the nanoscale [8]. Viscoelastic materials dissipate energy when loaded. In bone, this is important for its fracture resistance under dynamic or impact loading [2]. Viscoelasticity is the type of behavior attributed to materials that exhibit both elastic and viscous qualities under deformation. Viscoelastic materials, as bone or dentine deform according to a combination of these properties and, as such, exhibit time-dependent strain [9]. The complex modulus, as a measure of the resistance of a material to dynamic deformation [10], can be decomposed into storage (elastic) and loss (damping) modulus components [11]. The storage modulus E' (also called dynamic stiffness) characterizes the ability to store energy by the sample during a cycle of loading [9], which is then available for elastic recoil. The storage modulus is the measure of the sample's elastic behavior. Any resulting phase lag between the force applied and the displacement is related to a loss of energy known as the loss modulus or damping E''. The ratio of the loss to the storage is the tan delta (δ) and is often called damping [9]. Not only proper, precise, and reliable assessment of mechanical properties but morphometric characterizations of trabecular bone have both biological and clinical importance [12].

Raman spectroscopy is an analytical and nondestructive technique able to measure the molecular composition by providing a spectrum that contains information regarding all the chemical bonds present within the sample [13] for investigating molecular species. The Raman peak intensity is proportional to the number of molecules within the volume of the scanned area [14]. It furnishes biochemical specificity because it is based on spectral peaks specific to the biochemical and structural properties of hard tissues mineralization [15,16–18]. This technique is sensitive to differences in mineral and organic compositions that can be used to identify damaged, damage-susceptible, or restored areas. The main advantages of Raman spectroscopy include its application to fresh tissue and higher spatial resolution, with sampling volumes of 1 μm^3 or less [19]. Combining these technologies takes advantage of their synergies for characterizing tissues and for providing objective nanomechanical and biochemical information at ultrastructural levels [19].

At present, studies addressing the association between the tissue composition and viscoelastic properties in human trabecular bone are lacking. Thereby, the viscoelastic properties and the molecular composition of human trabecular bone using nanoindentation and Raman microspectroscopy will be assessed. The goal of the present study was to propose a study protocol on trabecular bone, based on nanoindentation and Raman characterization of the specimens, in order to study degenerative bone diseases and to evaluate and develop successful therapies.

Material and methods

Femoral neck biopsy specimens

Bone specimens from study subjects were retrieved from the base of the femoral neck of postmenopausal women diagnosed with hip fracture, at the time of total hip replacement. Patients signed a consent form for undergoing the surgical interventions as well as a femoral neck biopsy. All protocols were approved by the Research and Ethics Committees of our Institution, prior to obtaining the biopsy samples. These samples were then kept immersed in a phosphate-buffered saline (PBS) solution (pH 7.4) and stored frozen at –20°C [20]. The biopsy specimens were processed in methylmethacrylate [1].

Nano-DMA analysis and atomic force microscopy analysis (AFM) imaging

To prepare the specimens for such measurements, the methylmethacrylate-embedded biopsy specimens that were used to generate sections, were further polished with different diamond pastes, from 10 to 0.1 μm in diameter, to obtain smooth surfaces. Measurements were performed on randomly selected femoral specimens and ~4–5 different trabeculae from each sample [21].

Three sections of methylmethacrylate-embedded bone were submitted to nano-DMA and AFM analysis. Property mappings were conducted using a HysitronTi 950 nanoindenter (Hysitron, Inc., Minneapolis, MN) equipped with nano-DMA III, a commercial nano-DMA package. The nanoindenter was a Berkovich (three-sided pyramidal) diamond indenter tip (tip radius ~20 nm). The nanoindenter tip was calibrated against a fused quartz sample using a quasistatic force setpoint of 5 μN to maintain contact between the tip and the sample surface. A dynamic (oscillatory) force of 5 μN was superimposed on the quasistatic signal at a frequency of 200 Hz. Based on a calibration-reduced modulus value of 1.1400E + 03 N/mm^2 for the fused quartz, the best-fit spherical radius approximation for tip was found to be 150 nm, for the selected nano-DMA scanning parameters. Trabecular struts near the center of the biopsy were chosen from the optical image for testing. Modulus mapping of our samples was conducted by imposing a quasistatic force setpoint, F_q = 5 μN, to which it was superimposed a sinusoidal force of amplitude F_A = 1.8 μN and frequency f = 200 Hz. The resulting displacement (deformation) at the site of indentation was monitored as a function of time. Data from regions approximately 30 × 30 μm in size were collected using a scanning frequency of 0.2 Hz. Each scan resulted in a 256 × 256 pixel data array. Specimens were scanned in a hydrated state.

Under steady conditions (application of a quasistatic force) the indentation modulus of the tested sample, E, can be obtained by application of different models that relate the indentation force, F, and depth, D [22,23].

Roughness assessments and fibril diameter

An atomic force microscope (AFM Nanoscope V, Digital Instruments, Veeco Metrology group, Santa Barbara, CA, USA) was employed in this study for topography analysis. The imaging process was undertaken inside a wet cell in a fully hydrated state, using the tapping mode, with a calibrated vertical-engaged piezo-scanner (Digital Instrument, Santa Barbara, CA, USA). A 10-nm-radius silicon nitride tip (Veeco) was attached to the end of an oscillating cantilever that came into intermittent contact with the surface at the lowest point of the oscillation. Changes in vertical position of the AFM tip at resonance frequencies near 330 kHz provided the height of the images registered as bright and dark regions. 30 × 30 μm digital images were recorded with a slow scan rate (0.1 Hz). For each image, five randomized boxes (3 × 3 μm) were created for examination of the surface roughness of the trabecular bone. Nanoroughness (Ra, in nanometers) was measured with proprietary software (Nanoscope Software, version V7).

Five phase images and five three-dimensional (3D) digital images were captured for each specimen. Assembled in a single user interface, NanoScopeAnalysis.Ink software served as a semi-automatic analysis tool capable of measuring several geometrical properties (length, volume, and angles). Collagen fibril diameter was determined by section analysis using data that had been modified only by plane fitting. Five fibrils were analyzed from each image. Measurements were corrected for tip broadening by the equation $e = 2r$, where e is the error in the horizontal dimension and r is the tip's radius [24].