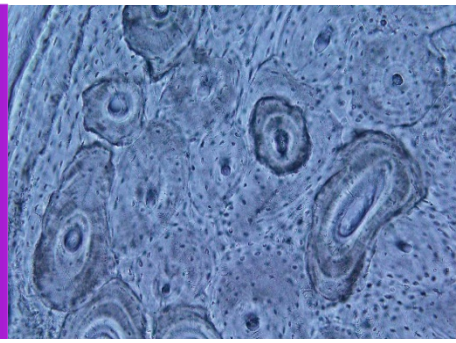
KLA INSTRUMENTS

Measuring the Mechanical Properties of Bone by Instrumented Indentation



Introduction

The adaptive ability of living tissue implies a reciprocal relationship between physical properties and function. In other words, properties affect function, but function can also affect properties by means of adaptation. The ability to measure physical properties is critical to understanding this interrelatedness. Thus, the purpose of this work was to use instrumented indentation to measure elasticity and strength of bone at the level of individual osteons, quantified through elastic modulus (E) and hardness (H), respectively. This work has been published in more detail elsewhere[1] and builds upon previous efforts to use instrumented indentation to characterize bone[2-5], dentin[6] and enamel[7].

Osteons are the primary structural units of compact bone. A polished cross-section of an osteon is shown in Figure 1. An individual osteon is roughly cylindrical; it is several millimeters long and has a diameter of about 200µm. Each osteon is comprised of a Haversian canal and concentric layers of lamellar bone. The Haversian canal runs through the axis of each osteon and contains small blood vessels which deliver nutrients to individual bone cells (osteocytes)[8]. In living bone, new osteons are generated to replace older osteons; this process is called bone remodeling. As osteons age, they become increasingly mineralized. Thus, the goal of this work was to measure the mechanical properties of osteons with varying degrees of mineralization. We hypothesized that the older (more mineralized) osteons would have higher elastic modulus and hardness. These types of measurements are necessary for understanding the relationship between mineralization, properties, and function in compact bone.

Experimental Method

Specimen Source

Bone samples were obtained from five skeletally mature male dogs. In order to highlight newly formed osteons, two doses of Calcein (Sigma-Aldrich, St. Louis, MO) were administered to the dogs intravenously at 17 days and 3 days prior to euthanasia.

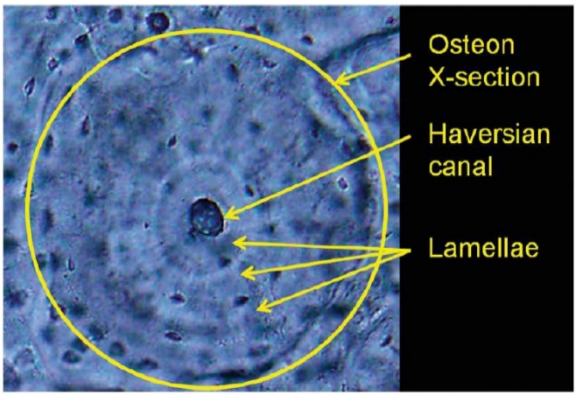


Figure 1. Polished cross-section of bone highlighting an individual osteon and its parts.

Calcein is a fluorescent dye that preferentially labels newly forming bone surfaces. The femurs were harvested immediately after euthanasia and frozen in saline soaked gauze at -20°C.

Specimen Preparation

Two mid-femoral cross sections, each about 3mm thick, were obtained from each dog, for a total of 10 samples. The test surfaces were lightly polished according to the following procedure. The bone block was glued into the well of a custom-made polycarbonate specimen holder. The mounting was verified on a certified level stage to ensure parallelism. The sectioned specimens were wet-polished on a rotary wheel (Ecomet, Buehler, Lake Bluff, IL) at 120rpm with 2,400 grit SiC papers. Additional polishing was done on a napless cloth (OP-Chem, Struers A/S, Rodovre, Denmark) with diluted 0.3µm and 0.05µm alumina oxide pastes (Micropolish C alpha Alumina, Buehler, Lake Bluff, IL). The specimens were sonicated for two minutes. After polishing, surface roughness was less than 30nm.

Test Site Selection

Next, labeled and unlabeled osteons were identified and their coordinates mapped under an epifluorescent microscope (Olympus BX 51, Tokyo, Japan). This identification was essential,

because labeled osteons cannot be identified under the optics of the indenter system. Multiple perpendicular lines were scribed into the surface of the polished bone specimen with a surgical blade. The exact location (x, y coordinates) of the central Haversian canal of the labeled osteon relative to two orthogonal scribe lines was measured in microns using a linear microscope eyepiece of the epifluorescent microscope. In addition, photomicrographs aided in documenting the unique cross sectional morphology of each labeled osteon site and its neighboring osteons and other structures such as blood vessels.

After the bone slice was mounted in the KLA Nano Indenter® G200, the photographic map produced from the epifluorescent microscope was referenced. A specific labeled osteon was located using the intersection of the nearest two perpendicular lines from the osteon. The sample was moved so that the intersection of the two perpendicular lines was visible in the indenter optics. From this position, the sample was moved so that the labeled osteon was visible. The location of the labeled osteon, in the reference frame of the indenter, was recorded. The photographic map confirmed the specific osteon of interest and a neighboring unlabeled osteon, as shown in Figure 2.

Each osteon was targeted using 5 to 6 indents located at approximately half the distance between the central canal and the outer border. These indent locations were programmed into the software to run the tests sequentially without further user interaction. A total of 610 indents were made on 147 osteons (labeled = 35, unlabeled = 112).

Testing

During testing, a hydration system, was used to keep the specimens moist for the entire test. The hydration fluid was a mixture of distilled water and 0.5mg/ml of gentamicin sulphate (Sigma Chemical Company, St. Louis, MO).

Each indentation test consisted of the following segments:

1. The indenter approaches the test surface until contact is sensed.
2. The indenter is pressed into contact with the test material at a rate of 10nm/s to a peak depth of 500nm. During this pressing, a small oscillation is superimposed on the quasi-static loading by means of the continuous stiffness measurement (CSM) option. The amplitude of the oscillating force, F_O, was continuously adjusted in order to maintain the amplitude of the resulting displacement oscillation at z_O = 2nm.

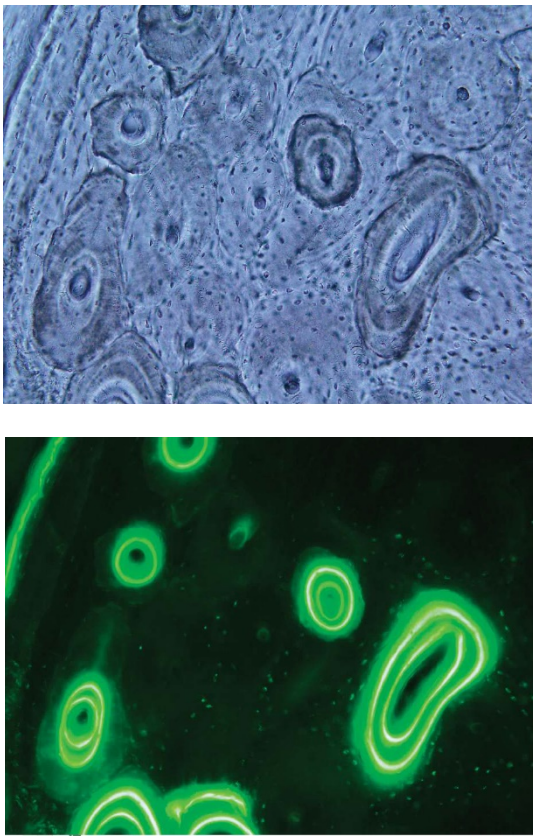


Figure 2. Complementary pair of typical images used to identify osteons for testing; top image is bright-field and bottom image is epifluorescent. In the bottom image, the young osteons glow (i.e., are "labeled") due to the calcein injections administered prior to euthanasia.

3. The force on the indenter is held constant for a dwell time of 30s.
4. The indenter is withdrawn from the sample completely, and the sample is moved into position for the next test.

Post-Test Analysis

Contact stiffness was calculated as a continuous function of penetration depth from the amplitude ratio F_O/z_O, measured during test Segment 1. Elastic modulus (E) and hardness (H) were calculated from this continuous measure of contact stiffness; this analysis is described in detail elsewhere[9,10]. In order to report scalar properties of each type of osteon (labeled and unlabeled), CSM results for E and H were taken at the maximum displacement of 500nm.

Results

The results are summarized in Table 1 and plotted in Figure 3 and Figure 4. Statistically significant differences were found between labeled and unlabeled osteons. The average elastic

modulus of unlabeled (older) osteons was 40% higher than that of labeled (newer) osteons and the hardness of unlabeled osteons was 66% higher than that of labeled osteons. The difference in mechanical properties is directly related to the degree of mineralization.

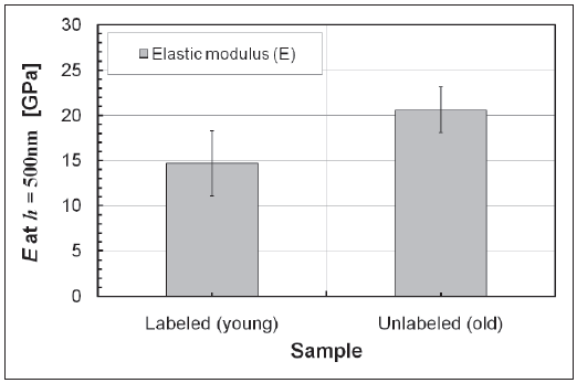


Figure 3. Comparison of elastic modulus for labeled and unlabeled osteons. Difference is directly related to the degree of mineralization.

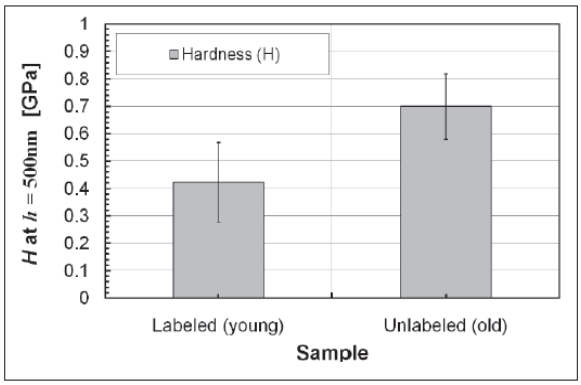


Figure 4. Comparison of hardness for labeled and unlabeled osteons. Difference is directly related to the degree of mineralization.

Both hardness and elastic modulus were higher for unlabeled osteons, but hardness was more so. The ratio H/E was 2.8% for labeled (newer) osteons and 3.4% for unlabeled (older) osteons. These results imply that mineralized osteons will be more resistant to permanent deformation than newer osteons. This finding verifies previous observations that excessive remodeling, stimulated by micro-cracks, increases the likelihood of stress fracture[11]. Phenomena that result in poor mineralization tend to make bone more vulnerable to excessive deformation and fracture (osteomalacia)[12]. However, the characterization of bone should not be oversimplified. As a composite, it is likely that the mechanical properties of compact bone are determined by complex interactions between osteons

Table 1. Summary of results.

Osteon Type	E ± σ(E), GPa	H ± σ(H), GPa	E/H
Labeled (young)	14.7 ± 3.58	0.422 ± 0.146	2.87%
Unlabeled (old)	20.6 ± 2.53	0.700 ± 0.120	3.40%

of differing properties, not simply by a volume-weighted average.

Conclusions

A KLA Nano Indenter G200 was used to measure the mechanical properties of individual osteons of a femur bone. This study confirms that new and old osteons have significantly different mechanical properties and that those properties are directly related to the degree of mineralization. The testing techniques developed in this work, especially those related to imaging and hydration, should be useful in the testing of other biological tissues.

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KLA Corporation
One Technology Drive
Milpitas, CA 95035
Printed in the USA
Rev 3 2020-10-05