Published in final edited form as:

J Biomed Mater Res A. 2006 May ; 77(2): 426–435.

Effects of surface roughness and maximum load on the mechanical properties of cancellous bone measured by nanoindentation

Eve Donnelly¹, Shefford P. Baker², Adele L. Boskey^{3,4,5}, and Marjolein C.H. van der Meulen^{1,3}

¹Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, New York

²Department of Materials Science and Engineering, Cornell University, Ithaca, New York

³Musculoskeletal Integrity Program, Hospital for Special Surgery, New York, New York

⁴Department of Biochemistry, Weill Medical College of Cornell University, New York, New York

⁵Graduate Program in Physiology, Biophysics, and Systems Biology, Weill Medical College of Cornell University, New York, New York

Abstract

The effects of two key experimental parameters on the measured nanomechanical properties of lamellar and interlamellar tissue were examined in dehydrated rabbit cancellous bone. An anhydrous sample preparation protocol was developed to maintain surface integrity and produce RMS surface roughnesses ~ 10 nm ($5 \times 5\text{-}\mu\text{m}^2$ area). The effects of surface roughness and maximum nanoindentation load on the measured mechanical properties were examined in two samples of differing surface roughness using maximum loads ranging from 250 to 3000 μN . As the ratio of indentation depth to surface roughness decreased below $\sim 3:1$, the variability in material properties increased substantially. At low loads, the indentation modulus of the lamellar bone was $\sim 20\%$ greater than that of the interlamellar bone, while at high loads the measured properties of both layers converged to an intermediate value. Relatively shallow indentations made on smooth surfaces revealed significant differences in the properties of lamellar and interlamellar bone that support microstructural observations that lamellar bone is more mineralized than interlamellar bone.

Keywords

cancellous bone; nanoindentation; mechanical properties; surface roughness; sample preparation

INTRODUCTION

Skeletal function depends critically on bone structural integrity, which is governed by tissue apparent density, architecture, and material properties. Although the effect of apparent density on structural behavior is well-studied, and microcomputed tomography imaging has lately enabled investigation of the relationship between microarchitecture and structural properties, ¹ relatively little is known about the local material properties and the effects of their variation on the structural behavior of bone. Reductions in bone apparent density and modification of the tissue material properties are major components of diseases such as osteoporosis.²⁻⁵

Mechanical characterization of bone at the microstructural level can provide additional insight into the origins of skeletal fragility seen in osteoporosis.

Nanoindentation is increasingly being used to assess the mechanical properties of lamellar bone. However, material property data obtained from such studies are often characterized by considerable variability, with maximum standard deviations ranging from 40 to 60% for measurements made within a single specimen.^{6,7} Although these relatively large standard deviations undoubtedly partially reflect natural material inhomogeneity, experimental artifacts may also contribute to the observed variabilities. Most nanomechanical studies of lamellar bone have not reported sample surface roughnesses and have used deep indentations relative to lamellar dimensions.⁸⁻¹⁰ To probe intralamellar properties, indentations must be sufficiently shallow that the corresponding sampled volumes lie within individual lamellae yet adequately deep to avoid a substantial influence of surface roughness on the measured mechanical properties. Therefore, minimization of sample surface roughness is crucial for making small indentations and measuring the properties of individual lamellae. Additionally, nearly all previous nanoindentation studies of bone have used aqueous specimen preparation techniques to obtain smooth sample surfaces. Problematically, aqueous solutions are known to cause demineralization¹¹ and solid phase transformations¹² in bone mineral. These effects are of particular concern with a surface-sensitive technique such as nanoindentation. Thus, a chemically appropriate preparation technique that produces smooth surfaces in bone specimens, as well as careful selection of indentation size, is needed to ensure nanomechanical property measurements unaffected by artifacts related to surface roughness or demineralization.

The layered microstructure of cancellous bone is thought to comprise lamellae consisting of highly oriented mineralized collagen fibers and less-oriented interlamellar regions,¹³ although the details of the sublamellar-scale microstructure are still under debate.¹⁴⁻¹⁶ In this study, nanoindentation was used to assess the mechanical properties of lamellar and inter-lamellar tissue in representative rabbit cancellous bone. The objectives were (1) to develop an anhydrous preparation technique for bone nanoindentation specimens that produces smooth surfaces suitable for making indentations sufficiently small to probe individual lamellae and (2) to isolate the effects of indentation load and surface roughness on the measured mechanical properties of lamellar and interlamellar trabecular tissue using nanoindentation. We hypothesized that (a) as the maximum indentation load increases, the hardness and modulus values obtained from indentations in lamellar and interlamellar bone would converge to a single composite values, and (b) as the indentation depth relative to the surface roughness decreases, the variability of the measured mechanical properties would increase.

MATERIALS AND METHODS

Specimen preparation

One cancellous bone cube ($4 \times 4 \times 4 \text{ mm}^3$) was excised from each left distal lateral femoral condyle of two skeletally mature male New Zealand white rabbits with no known skeletal pathologies. The cubes were cut with a low-speed diamond saw (Buehler, Lake Bluff, IL), dehydrated in 100% ethyl alcohol and propylene oxide, embedded in Spurr's resin, and mounted on an atomic force microscope (AFM) stub with epoxy. All grinding and polishing were performed with a semiautomatic polisher (Allied High Tech Products, Rancho Dominguez, CA) at 10 RPM with a 20 g sample load. The specimens were ground on silicon carbide paper (400, 600, 800, and 1200 grit) using ethylene glycol as a lubricant and polished in a series of slurries of aluminum oxide powder in ethylene glycol on neoprene cloth (Buehler, Lake Bluff, IL). To generate samples of differing surface roughness, one sample ("smooth") underwent polishing with the full series of aluminum oxide slurries (3, 1, 0.3, and $0.05 \mu\text{m}$ particle size), while one sample ("rough") was polished through the $3 \mu\text{m}$ slurry, but did not

undergo the final three polishing steps. At each step, the samples were polished until a thickness of material equal to three times the previous abrasive particle size was removed and then ultrasonically cleaned in 100% isopropyl alcohol for 60 s. The specimens were stored in a desiccator prior to testing.

Contact atomic force microscopy was used to characterize the surface topography of the trabeculae selected for nanoindentation. No effort was made to select trabeculae of a particular orientation; the trabeculae characterized here obliquely intersected the sectioned surface. The local roughness was measured from several $5 \times 5 \mu\text{m}^2$ scans over a total area of $100 \times 100 \mu\text{m}^2$. The final RMS roughnesses of the smooth and rough samples were 7–15 and 33–35 nm, respectively. The characteristic peak-and-valley topography that arises from the difference in hardness between the hard lamellar bone and the soft interlamellar bone¹⁷ and has previously been associated with polished sections of lamellar bone^{7,8} was also observed in the specimens in this study. Previous nanoindentation studies of lamellar bone treated these components as compositionally homogeneous layers that differed in primary collagen fiber orientations and termed the peaks and valleys thick (hard) and thin (soft) lamellae, respectively.^{7,8,18} However, here we have adopted the terminology of Boyde and Hobdell, who described the broad ($\sim 5 \mu\text{m}$ width) peaks as lamellae composed of aligned, mineralized collagen fibers and the narrower ($<1 \mu\text{m}$ width) valleys as interlamellar transition zones containing less oriented, mineralized collagen and more polysaccharides.^{13,17} Thus, the structures described in some previous nanoindentation studies as thick and thin lamellae are here termed lamellar and interlamellar regions, respectively.

Nanoindentation

A scanning nanoindenter, that is, a combined AFM (Digital Instruments, Santa Barbara, CA) and nanoindenter (Hysitron, Minneapolis, MN) system,¹⁹ was employed to image the sample topography in the scanning mode and to obtain force–displacement data in the nanoindentation mode. Indentations were made with a Berkovich diamond indenter with a tip radius of ~ 120 nm. Before testing, the tip shape was characterized using the method proposed by Oliver and Pharr.²⁰ A series of indentations was made in a fused silica calibration specimen ($E = 72$ GPa), and the measured contact stiffnesses and known specimen modulus were used to establish the relationship between tip contact depth and contact area.

Prior to each test, the indentation site was imaged with multiple scans of decreasing size to minimize lateral drift of the transducer. First, a $15 \times 15\text{-}\mu\text{m}^2$ surface topography scan was performed to select the indentation site. The indenter tip was then positioned at the site of interest and held there for 60 s. Finally, the position was checked with a $7 \times 7 \mu\text{m}^2$ scan, corrected as necessary, and held at the indentation site for another 30 s before indenting. A contact load of $1 \mu\text{N}$ and a scanning frequency of 0.6 Hz were used for all the scans. This procedure was crucial for accurate placement of indentations. The indenter tip shape was considerably more acute than the peak and valley topography, even in the rough specimen (Fig. 1). Although these global features were not expected to influence the measurements, at shallow contact depths the local features that were much more acute than the tip were expected to contribute to artificially small calculated contact areas and correspondingly lower hardness and modulus values.

Two types of indentation experiments were performed to investigate the effect of indentation depth on the measured mechanical properties: single-load and multiple-load. In the single-load experiments, the tip was loaded into the sample at a rate of $50 \mu\text{N/s}$, held at a maximum load of 500 or 3000 μN for 10 s, and unloaded at $50 \mu\text{N/s}$ [Fig. 2(a)]. The two maximum loads were chosen such that the resulting contact depths of 108–161 nm for the 500- μN (“shallow”) indentations and 329–527 nm for the 3000- μN (“deep”) indentations were approximately ten times the respective surface roughnesses of the smooth and rough samples. Indentations with

contact depths an order of magnitude larger than local surface roughness are thought to be sufficiently deep to avoid a strong effect of roughness on the measured properties.²¹ One trabecula in each sample was selected for mechanical characterization. In each sample, five indentations at each maximum load were made along the centers of three lamellae, and five indentations at each maximum load were made along three adjacent interlamellar regions (Fig. 3). The distance between shallow indentations was $\sim 3 \mu\text{m}$, and the distance between deep indentations was $\sim 9 \mu\text{m}$. In each specimen, a total of 60 single-load indentations were made over an area of $15 \times 140 \mu\text{m}^2$.

Multiple-load indentation experiments were also performed on three lamellar and three interlamellar regions in the same trabeculae probed in the single-load experiments. Each of these indentation tests comprised five sequential load/unload cycles at $50 \mu\text{N/s}$ with five increasing maximum loads of 250, 500, 1000, 2000, and 3000 μN [Fig. 2(b)], which resulted in contact depths of ~ 70 , 110, 180, 270, and 340 nm, respectively. These tests enabled us to examine the effect of indentation load on the measured mechanical properties at a fixed location within the tissue. Five multiple-load indentations were made in three lamellar and three interlamellar structures with a minimum spacing between indentations of $\sim 9 \mu\text{m}$. In each specimen, a total of 30 multiple-load indentations were made over an area of $15 \times 110 \mu\text{m}^2$. Because each multiple-load indentation test produced a load-displacement curve for each of the five load/unload cycles, 150 hardness and modulus measurements were made on each sample.

In both types of indentation experiments, the hardness H and reduced modulus E_r , which includes contributions from both the sample and the indenter tip, were calculated from the unloading portion of the load-displacement curve using the Oliver-Pharr method.²⁰ The hardness was calculated as the average pressure under load, $H = P_{\text{max}}/A_c$, where P_{max} is the maximum load, and A_c is the contact area. The indentation modulus E_i was then calculated via the following relation:

$$E_i = \left(\frac{1}{E_r} - \frac{1 - \nu_{\text{tip}}^2}{E_{\text{tip}}} \right) = \frac{E_s}{1 - \nu_s^2}$$

where the Poisson ratio and Young's modulus of the tip are $\nu_{\text{tip}} = 0.07$ and $E_{\text{tip}} = 1440 \text{ GPa}$, and E_s and ν_s are the elastic properties of the sample.

For each sample, the means and standard deviations of the lamellar and interlamellar properties were calculated. Because the variation in properties between regions of the same type (lamellar or interlamellar) was small compared to the differences in properties between regions of different types, the data for the three lamellar areas and three interlamellar areas were pooled to obtain mean lamellar and interlamellar properties, respectively, in both the single- and multiple-load tests. The variability of the pooled data was characterized using the coefficient of variation (COV).

RESULTS

Single-load indentations

For the shallow indentations in the smooth sample, the lamellar bone was stiffer than the interlamellar bone, with indentation moduli of 26.6 ± 2.27 (mean $\pm$ standard deviation) and $20.3 \pm 2.09 \text{ GPa}$, respectively. These differences in mechanical properties diminished for the deep indentations; the moduli of the lamellar and interlamellar regions were indistinguishable for the 3000 μN indentations [Fig. 4(a)]. The indentation modulus of the lamellae decreased with increasing load, while load had little effect on the measured indentation modulus of the interlamellar regions. Similar trends were observed in the rough specimen, although the

indentation modulus of both the lamellar and the interlamellar bone decreased with increasing load [Fig. 4(b)]. However, regardless of sample roughness, the indentation moduli derived from the large indentations in lamellar and interlamellar regions were essentially equal. In the smooth sample for the large indentations, both the lamellar and interlamellar regions had indentation moduli of ~20 GPa [Fig. 4(a)]; the corresponding value in the rough sample was somewhat lower, ~16 GPa [Fig. 4(b)]. In the smooth sample, the lamellar and interlamellar hardness ranged from 0.99 ± 0.14 and 0.95 ± 0.11 GPa, respectively, for the small indentations to 0.83 ± 0.06 and 0.82 ± 0.09 GPa, respectively, for the large indentations. The measured hardness showed the same trends with load observed for the indentation moduli, although the lamellar and interlamellar hardnesses were not as markedly distinct.

Generally, the variability of the indentation moduli was greater in the rough sample than in the smooth sample. For the shallow indentations, the increase in surface roughness had little effect on variability of the lamellar moduli, but the COV of the interlamellar moduli increased from 0.10 in the smooth sample to 0.18 in the rough sample (Table I). For the small indentations on the smooth sample, the COV of the indentation moduli ranged from 0.08 to 0.10, with the maximum variability occurring for the shallow indentations in both the lamellar and interlamellar tissue. In the smooth sample, indentation depth did not substantially affect the variability in the mechanical properties.

Multiple-load indentations

Similar trends in the effect of load on indentation modulus were observed in the multiple-load experiments. At low loads, the modulus of lamellar bone was considerably greater than that of interlamellar bone in both samples. The lamellar and interlamellar moduli converged with increasing load to ~26 and 20 GPa in the smooth and rough samples, respectively. In both the samples, the lamellar modulus decreased and the interlamellar modulus remained constant over the 250–3000 μ N load range [Fig. 5(a,b)]. The lamellar and interlamellar hardness in the smooth sample ranged from 1.47 ± 0.16 and 1.31 ± 0.14 GPa, respectively, for the smallest indentations to 1.09 ± 0.09 and 1.18 ± 0.13 GPa, respectively, for the largest indentations. As in the case of the single-load indentations, the trends in hardness mirrored those observed for the indentation modulus, although the differences between the lamellar and interlamellar hardnesses were not as great as the differences in modulus.

Surface roughness and indentation depth had little effect on the variability of the data, with the exception of the shallowest (250- μ N) indentations on the rough sample. Most of the COVs were ~0.10 at all but the smallest loads in the rough sample, whereas the COVs of the moduli derived from the 250- μ N indentations on the rough sample were markedly greater than the equivalent COVs in the smooth sample. This effect was especially pronounced in the lamellar tissue, in which the COV for the 250- μ N indentations was twice the corresponding COV values for the other four loads (Fig. 6).

DISCUSSION

A key goal of this study was to assess the effects of indentation load and surface roughness on the measured mechanical properties of lamellar and interlamellar bone. In both the single-load and multiple-load experiments, the indentation modulus of the lamellar bone was 20–30% greater than that of the interlamellar bone at relatively low loads, while the measured moduli of these tissues were nearly identical at high loads. In the single-load experiments, the lamellar modulus decreased substantially with increasing load in both samples; however, the interlamellar modulus remained constant in the smooth sample but decreased with increasing load in the rough sample. The multiple-load tests clarified the origin of these differing responses of the mechanical properties to increasing load.

In the multiple-load experiments, the lamellar modulus decreased monotonically with increasing load, while the interlamellar modulus remained constant in both samples. The observed decrease in lamellar modulus with increasing load suggests that the shallowest indentations in the relatively wide lamellae ($\sim 3\text{--}6\ \mu\text{m}$) primarily probed stiff lamellar material, but as the indentation depth increased, the sampled volume beneath the indenter tip included neighboring compliant interlamellar material. In contrast, the insensitivity of the interlamellar modulus to increasing load indicates that the indentation response of the relatively narrow interlamellar regions ($<1\ \mu\text{m}$) was influenced by the adjacent lamellae, even at the lowest load investigated. The indentation moduli of both tissues converged with increasing load. Estimates of the volumes of material sampled by the indentations support this argument; the lateral dimensions of the sampled volumes beneath the indenter tip, estimated to be approximately seven times the contact depth,²² ranged from an average of $\sim 500\ \text{nm}$ for the $250\text{-}\mu\text{N}$ tests to $\sim 2400\ \text{nm}$ for the $3000\text{-}\mu\text{N}$ tests. The sampling volumes of the smaller indentations lay within individual lamellae, but those of the larger indentations approached lamellar dimensions and likely sampled material from neighboring layers.

Finally, the consistent behavior of the lamellar and interlamellar moduli with increasing load in both the samples in the multiple-load tests suggests that spatial variation in material properties may explain the somewhat anomalous decrease in interlamellar modulus with increasing load observed in the rough specimen in the single-load tests. Because the order of the shallow and deep indentations was not randomized in the single-load tests, the deep indentations may have been placed in a naturally more compliant area of the interlamellar regions. The multiple-load tests therefore provided complementary information on the effects of load on the measured indentation modulus while controlling for spatial variation in material properties, which may have obscured these effects in the single-load experiments.

While the multiple-load indentation tests helped to elucidate the effects of load on the measured mechanical properties, the shallow and deep single-load indentation tests provided the most appropriate measures of the local and composite properties, respectively. Because the multiple-load experiments contained no hold segments to allow for creep before unloading, the unloading contact stiffness and indentation modulus are likely artificially high.²³ In fact, the composite indentation moduli derived from the $3000\text{-}\mu\text{N}$ multiple-load indentations were 20–30% greater than those from the corresponding $3000\text{-}\mu\text{N}$ single-load indentations. Consequently, the properties derived from the single-load tests best represent the absolute tissue properties.

Both large-scale roughness, such as the undulating topography of polished lamellar bone, and small-scale asperities can affect the mechanical properties measured by nanoindentation. Therefore, a smooth, flat surface is necessary to achieve the well-defined contact geometry that underlies the Oliver-Pharr data analysis model. In this investigation, the effects of the large-scale surface corrugations were minimized by creating a relatively flat sample surface on which the “wavelength” of the peaks and valleys was large relative to the radius of the indenter tip (Fig. 1). Thus, this study addressed primarily the effects of the small-scale ($\sim\text{nm}$) roughness. The effect of small-scale surface roughness on the variability of the mechanical properties measured in this study was most pronounced in the case of the $250\text{-}\mu\text{N}$ indentations made on the rough sample in the multiple-load experiments (Fig. 6). In those tests, the COV of the interlamellar modulus was $\sim 25\%$ greater than the COVs at higher loads, and the COV of the stiffer lamellar tissue, in which the contact depths were smaller, was $\sim 100\%$ greater. In tests at higher loads, however, small-scale roughness had little effect on the variability of the measured properties. The marked increase in the COV of the $250\text{-}\mu\text{N}$ indentations relative to those of the higher-load tests in the rough sample suggests that roughness effects may be small at contact-depth-to-RMS roughness ratios (h_c/R_{RMS}) greater than or equal to the range of 2.8–4.3 observed in the $500\text{-}\mu\text{N}$ tests but may begin to dominate the measured mechanical

properties at h_c/R_{RMS} values less than or equal to the range of 1.7–2.8 observed in the 250- μ N tests.

The sample preparation methodology developed here substantially reduces the roughness and improves the reproducibility of the surface finish compared to methods used previously. Our anhydrous technique yields the smooth surfaces necessary for making indentations sufficiently shallow to probe individual lamellae. The use of a semiautomatic polisher produces smoother and more uniform surfaces than are typically achievable with manual polishing. The sample preparation method used in this investigation reliably produces surfaces with RMS roughnesses of ~ 10 nm over a $5 \times 5\text{-}\mu\text{m}^2$ area. In contrast, RMS roughnesses of ~ 60 nm were achieved in samples prepared with careful manual polishing through $0.05\text{-}\mu\text{m}$ particle size, the method used in nearly all the previous nanoindentation studies of bone. The range of RMS roughness produced by manual polishing was 18–150 nm, while the range achieved using the current technique was 7–25 nm. Therefore, the sample surface roughness cannot be inferred from the smallest abrasive particle size used in polishing and must be measured directly. The final surface roughness of a sample can be highly scale-dependent and is influenced by a number of factors, including abrasive size and composition, polishing speed and force, and amount and composition of lubricant. With two exceptions,^{24,25} surface roughness has not been reported in the bone nanoindentation literature. As shown in this study, the measured mechanical properties of bone are sensitive to surface roughness; therefore, quantification of roughness is essential.

Although aqueous preparation techniques have long been known to modify the microstructure of bone tissue, all previous nanoindentation studies have employed them. Because aqueous solvents alter the microstructure of the bone mineral by dissolution,¹¹ resulting in mineral loss, reprecipitation, and phase transformations,^{12,26} their use in nanoindentation specimen preparation can be expected to change the measured mechanical properties. Although we did not directly examine the effect of aqueous preparation on the mechanical properties of bone, in studies of other mineralized tissues, the nanomechanical hardness and indentation modulus of dentin and enamel decreased significantly in human teeth stored in deionized water for one day.²⁷ Considerable demineralization of bone tissue may occur even during the relatively short times required for polishing. For example, surface demineralization was evident in bovine vertebral bone specimens rinsed with water and characterized by atomic force microscopy.²⁸ In addition, thin sections ($600\text{ }\text{\AA}$) of undecalcified avian embryonic bone demineralized completely in 6.5–8 min of exposure to distilled water in a microtome collecting-trough.¹¹ As the total polishing and ultrasonic cleaning time for a bone nanoindentation specimen is $\sim 20\text{--}30$ min, the thickness of the demineralized layer of bone in an aqueously prepared sample may be on the order of the contact depth of a 500- μ N indentation.

The specimen preparation technique used in this study was adapted in part from an ethylene glycol-based anhydrous preparation method developed for undecalcified bone sections for transmission electron microscopy (TEM).²⁹ In TEM studies of undecalcified embryonic chick bone, the location, structure, and chemistry of the bone mineral in samples processed with ethylene glycol were unchanged relative to dry, untreated controls. The organic components of the cells and extracellular matrix were also preserved. In contrast, phase changes and considerable decreases in the electron density of the bone mineral were observed in aqueously processed specimens.^{26,29} Thus, the specimen preparation method developed in this study achieves the smooth surfaces necessary for making small indentations while avoiding the potential microstructural modifications associated with aqueous preparation.

The generalizability of the results obtained in this investigation is affected by two factors. First, the measurements presented here were made in dehydrated samples and may not be representative of *in vivo* values. In previous nanoindentation experiments, increased modulus

values have been reported for dehydrated samples ranging from 10%³⁰ to 75%⁷ over hydrated samples; yet the relative differences in lamellar and interlamellar mechanical properties have been shown to be preserved in dehydrated bone.⁷ In the current investigation, dehydration and embedding of the cancellous specimens were essential to provide support for the trabeculae during testing and to achieve the uniform surface roughnesses required for this study. Second, despite the high reproducibility of the nanoindentation measurements in this study, the mechanical properties were measured in only two samples. However, the primary purpose of this study was to evaluate nanoindentation methodology as applied to bone tissue, not to definitively characterize the nanomechanical properties of lamellar bone. The relatively laborious nanoindentation techniques described here allow approximately three indentations per hour and preclude characterization of a large number of samples. However, eliminating the natural interspecimen variability in the properties of bone has enabled us largely to isolate the contribution of experimental parameters to variability in the measured properties.

The observed differences in lamellar and interlamellar properties are consistent with the work of Boyde and Hobdell, who postulated that the interlamellar bone was poorly mineralized,¹³ assuming that a relationship between mineral content and modulus extends to the microscale in bone tissue. Our findings are inconsistent with Ascenzi et al.'s suggestion that the interlamellar areas were hypermineralized.³¹ In addition, in quantitative backscattered electron studies (BSE) of lamellar bone,^{32,33} the interlamellar areas appeared darker than the lamellar areas, suggesting that the interlamellar regions contained less mineral than the lamellar regions. However, the undulations of the lamellar bone surface complicate interpretation of the BSE image contrast,^{33,34} and the relative contributions of topography and mineral content variations to interlamellar contrast are unknown.³² Thus, definitive data on the relative mineral content of lamellar and interlamellar material remain elusive.

To our knowledge, the only previously reported nanoindentation measurements of individual lamellae were those of Hengsberger et al.^{7,24} Hengsberger et al. used loads ranging from 200 to 5000 μN to produce indentation depths of about 100–530 nm in single-load indentation tests of dehydrated and hydrated human femoral trabeculae.⁷ For small indentions, both hardness and modulus were greater in the lamellar than in the interlamellar material, although the effects of surface roughness on the measured properties were not quantified. The hardness and modulus generally decreased with increasing load in the lamellae, but were approximately constant with load in the interlamellar regions. Hengsberger and coworkers attributed the variations in properties with depth to intrinsic spatial variability of tissue properties and to preferential damage accumulation in the lamellae. To control for intrinsic tissue heterogeneity in our examination of the depth-dependence of tissue properties, we employed multiple-load indentations to eliminate a key source of variability in the data and clarify the results of the previous work. We saw no indication of the lamellar cracking reported by Hengsberger et al. No cracks were visible in AFM scans of indentations in lamellar or interlamellar regions, and the force–displacement data of the multiple-load indentations showed no sudden increases in compliance that might be indicative of cracking. However, this finding does not preclude diffuse microcrack formation far from the indentation site, which may have contributed to the decrease in lamellar properties with load observed in the present study. Although different species and anatomic locations were examined in the present study and that of Hengsberger and coworkers, the general agreement between the trends observed in these investigations demonstrates the importance of such model studies to understanding the mechanical response of lamellar bone tissue.

The variability of the measured material properties in this study was substantially less than those of previous studies. In the single-load indentations in the smooth sample, the maximum COV was 0.10, compared to corresponding values of approximately 0.4–0.6 in previous studies.^{6,7} This variability can be minimized by making indentations whose sampling volumes

lie within the microstructural features of interest, which, in turn, requires a smooth sample surface. The multiple-load indentations used in this study allowed us to examine the depth-dependence of lamellar and interlamellar properties while controlling for the effects of spatial variation in tissue properties, which have confounded interpretation of the results of previous nanoindentation studies.⁷ In addition, because the measured material properties are sensitive to the position of the indenter tip relative to the micro structural features, consistent positioning of the tip in the center of the lamellar or interlamellar regions is important. The use of a scanning nanoindenter and implementation of a drift-reducing multiple-scanning protocol helped to ensure accurate placement of indentations. Thus, the variability in material properties due to experimental artifacts can be substantially reduced by minimizing sample surface roughness, probing relatively small indentation volumes, and careful lateral positioning of the indenter tip.

Examining the effects of key experimental parameters on the measured tissue properties enabled better understanding of the “true” lamellar-level properties without artifacts produced by surface roughness and aqueous preparation. More extensive testing will determine whether the trends observed in this study persist in other species, anatomic locations, and disease states. The experimental methodologies developed here allow accurate measurement of lamellar-level properties with minimal variability. This ability to detect relatively small differences in tissue properties may provide insights into the effects of mineralization defects and matrix abnormalities on the local material properties of bone tissue.

Acknowledgements

The authors thank Ben Bourne and Dr. Stephen Doty for assistance with sectioning and embedding and Mick Thomas, John Sinnott, and Everett Ramer for advice on surface preparation. Equipment support was provided by Hysitron, Inc. The staff and shared facilities of the Cornell Center for Materials Research (NSF DMR 0089992) were instrumental to this research.

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Figure 1.

Comparison of the Berkovich indenter tip used in this study and the sample topography. The topography shown is taken from an AFM line scan made perpendicular to the lamellae in the rough sample. The Berkovich diamond indenter tip is represented by the profile of a tip with circular cross section with projected contact area at each contact depth equivalent to that of the real pyramidal indenter. This comparison is *to scale*; note different units on *x*- and *y*-axes.

Figure 2.

Force *versus* time profiles for (a) the single-load indentation experiments, where $P_{\text{max}} = 500$ or $3000 \mu\text{N}$ and (b) the multiple-load indentation experiments.

Figure 3. Scanning nanoindenter images of selected shallow and deep indentations in adjacent lamellar (lighter areas) and interlamellar (darker areas) material in (a) the rough sample and (b) the smooth sample. Indentations are outlined.

Figure 4. Average lamellar and interlamellar indentation moduli from the single-load indentation experiments in (a) the smooth sample and (b) the rough sample. Each bar shows the average of 15 indentations, and error bars indicate standard deviations. The lamellar moduli were 20 – 30% greater than the interlamellar moduli at the lower indentation load, but the moduli were equal at the higher load in both the samples.

Figure 5.

Average lamellar and interlamellar indentation moduli from the multiple-load indentation experiments in (a) the smooth sample and (b) the rough sample. Each bar shows the average of 15 indentations, and error bars indicate standard deviations. The lamellar modulus was distinctly greater than the interlamellar modulus at low indentation loads, but the moduli converged with increasing load in both the samples.

Figure 6. COVs of the indentation moduli from multiple-load experiments in (a) the smooth sample and (b) the rough sample. Although the variability was constant with load in the smooth sample, the COVs of the moduli obtained from the shallowest (250 μN) indentations on the rough sample were 25–100% greater than those obtained from deeper indentations.

Table 1.

COVs of Lamellar and Interlamellar Indentation Modulus for the Single-Load Experiments in the Smooth and Rough Specimens

	Smooth		Rough	
	500 μ N	3000 μ N	500 μ N	3000 μ N
Lamellar	0.10	0.08	0.09	0.14
Interlamellar	0.10	0.10	0.18	0.15