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Nanomechanical mapping of the osteochondral interface with contact resonance force microscopy and nanoindentation ☆

Sara E. Campbell [a,*], Virginia L. Ferguson [b], Donna C. Hurley [a]

[a] Materials Reliability Division, National Institute of Standards and Technology, Boulder, CO 80305, USA
[b] Department of Mechanical Engineering, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

The bone–cartilage, or osteochondral, interface resists remarkably high shear stresses and rarely fails, yet its mechanical characteristics are largely unknown. A complete understanding of this hierarchical system requires mechanical-property information at the length scales of both the interface and the connecting tissues. Here, we combined nanoindentation and atomic force microscopy (AFM) methods to investigate the multiscale mechanical properties across the osteochondral region. The nanoindentation modulus M ranged from that of the subchondral bone ($M = 22.8 \pm 1.8$ GPa) to that of hyaline articular cartilage embedded in PMMA ($M = 5.7 \pm 1.0$ GPa) across a narrow transition region <5 μm wide. Contact resonance force microscopy (CR-FM), which measures the frequency and quality factor of the AFM cantilever's vibrational resonance in contact mode, was used to determine the relative storage modulus and loss tangent of the osteochondral interface. With better spatial resolution than nanoindentation, CR-FM measurements indicated an even narrower interface width of 2.3 ± 1.2 μm. Furthermore, CR-FM revealed a 24% increase in the viscoelastic loss tangent from the articular calcified cartilage into the PMMA-embedded hyaline articular cartilage. Quantitative backscattered electron imaging provided complementary measurement of mineral content. Our results provide insight into the multiscale functionality of the osteochondral interface that will advance understanding of disease states such as osteoarthritis and aid in the development of biomimetic interfaces.

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1. Introduction

Within the articular joint, forces are transmitted across the mechanically dissimilar layers of tissue that comprise the osteochondral region: rigid subchondral bone (SCB), a thin (∼50 μm to 100s μm) layer of articular calcified cartilage (ACC), and compliant hyaline articular cartilage (HAC). In vivo loading of the osteochondral region generates high stresses [1,2]. In particular, a stress concentration exists at the leading edge of mineralization, or the tidemark interface, between the mineralized ACC and the unmineralized HAC. Collagen fibers that traverse this interface perpendicularly are thought to dissipate and resist shear stresses [3]. The mineral within the ACC has long been thought to vary in mineral volume fraction to functionally grade properties from the SCB to the HAC [4]. Mineralization of the SCB and ACC often increases with age and altered loading conditions, and likely plays a key role in the development of disease states such as osteoarthritis [5–7]. However, our limited understanding of load transmission and mechanical properties across the osteochondral region restricts our ability both to understand disease progression and to engineer replacement materials [8].

Past investigations of the osteochondral region have focused primarily on bulk techniques [9,10] that test the combined mechanical response of several tissues (i.e. SCB, ACC and HAC). More recently, nanoindentation studies have probed the mechanical properties of these individual tissues [6,11–13]. However, the leading tidemark interface between ACC and HAC has been studied much less [11], primarily due to the micrometer-scale spatial resolution constraints of nanoindentation. Furthermore, to our knowledge, no studies have examined the spatial distribution of viscoelastic properties within the osteochondral region.

Atomic force microscopy (AFM) methods can provide nanometer-scale mechanical property measurements on a wide array of materials [14–16]. Here, we demonstrate contact resonance force microscopy (CR-FM) [17,18], an AFM method for quantitative mapping of viscoelastic properties across the tidemark interface. The results are compared to complementary information about microscale mechanical properties obtained by nanoindentation and to mineral content obtained by quantitative backscatter electron microscopy (qBSE) imaging. Our results provide new insight into multiscale mechanical properties of the osteochondral region that

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* Corresponding author. Tel.: +1 303 497 6570.
E-mail address: sara.campbell@nist.gov (S.E. Campbell).

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will help elucidate its functionality in transmitting loads and anchoring HAC to SCB.

2. Materials and methods

2.1. Sample

A femoral head from a New Zealand white rabbit approximately 6 months old was dehydrated in a series of ethanol solutions and embedded in poly(methyl methacrylate) (PMMA). The sample was collected under full Institutional Animal Use and Care Committee approval at the University of Colorado at Boulder. The femoral head was cut in half through the coronal plane with a low-speed saw. A small sample was removed from the anterior section and faced using an ultramicrotome. Use of an ultramicrotome has been shown to minimize the contribution of surface roughness to the measurement of modulus with nanoindentation [19]. The resulting surface had a root mean square roughness of ~13 nm for a 20 μm × 20 μm region, as measured by tapping mode AFM.

2.2. Nanoindentation

Nanoindentation tests were performed with a commercial instrument (TriboIndenter, Hysitron, Minneapolis, MN). A nanoindentation test array comprising 30 indents × 8 indents was placed traversing the osteochondral region from the SCB across ACC and into HAC. Indentation tests were performed with a ramp-and-hold method, with constant loading and unloading rates of 100 μN s^{-1}. A 30 s hold at maximum load F_{max} = 500 μN was used to minimize the contribution of creep to the unloading curve [20]. Tests were run with a Berkovich indenter tip calibrated with a fused-silica reference standard. The reduced modulus E^R was measured from the slope of the unloading curve (at maximum load) by using a power law fit to 20–95% of the unloading curve with the Oliver–Pharr method [21]. Here, we report the indentation modulus M, which eliminates the need to assume a Poisson's ratio for the sample. M is given by

$$E^R = \left[\frac{1}{M} + \frac{1-\nu_t^2}{E_t}\right]^{-1} \quad (1)$$

where E_t = 1140 GPa is the Young's modulus and ν_t = 0.07 is the Poisson's ratio, respectively, of the diamond indenter tip. Indent sites were located in both scanning electron microscope and light microscope images for classification as falling on SCB, ACC or HAC. Test sites within 5 μm of the leading tidemark interface between ACC and HAC were not included in the determination of the average modulus value for each tissue type (see below). Any test sites falling within 5 μm of a crack or void was discarded from the analysis.

The variables required to determine the minimal spacing between indent sites are listed in Table 1. The use of a fixed maximum load in the nanoindentation measurements resulted in different maximum values of the contact depth h_c between and within different tissue types. The range of values for h_c measured on each tissue type, as listed in Table 1, are a direct result of tissue mechanical heterogeneity. The critical dimension of interest, namely the contact radius a, can be calculated for a Berkovich tip from the contact depth h_c by

$$a = h_c \tan\theta \quad (2)$$

where θ = 70.3° is the half-included angle of the Berkovich tip. Assuming sphere–plane Hertzian contact mechanics, the stress field is maximal directly beneath the indenter tip and rapidly decreases with increasing depth z into the sample and radial distance r away from the contact [22]. Near the surface (z = 0), at r = 1.5a the stress decreases to 10% of the maximum value at r = 0 [22,23]. Therefore, the minimum lateral spacing between indentations should be at least 3a in order to avoid the influence of neighboring indents. The values of a and 3a listed in Table 1 were calculated for the largest h_c value in each tissue type. An experimental test spacing Δx = 5 μm was chosen based on the value of 3a calculated for HAC, which was the largest of the two materials.

Table 1
Mechanical testing experimental parameters.

Experimental parameters	Nanoindentation		CR-FM	
	ACC	HAC	ACC	HAC
F_{max} (μN)	500	500	0.4	0.4
Δx (μm)	5	5	0.1–0.5	0.1–0.5
h_c (nm)	120–230	200–450	2.5	3.7
a (μm)	0.64	1.26	0.011	0.014
3a (μm)	1.92	3.78	0.033	0.042

Values for the maximum load F_{max} and test spacing Δx used in nanoindentation and CR-FM experimental parameters. Also shown are the corresponding calculated values of contact depth h_c, contact radius a at maximum depth and approximate lateral width 3a of the elastic zone, as explained in Section 2.

2.3. AFM

AFM measurements were performed with a commercial atomic force microscope (MFP-3D, Asylum Research, Santa Barbara, CA) equipped with a specialized cantilever holder containing a damped, high-frequency piezoelectric actuator. Cantilevers were glued directly onto the cantilever holder with fast-setting epoxy. All experiments used rectangular cantilevers with an average manufacturer-specified spring constant k_c = 13.5 N m^{-1} and free resonance frequency $f_1^{free} = 127\ kHz$ for the first flexural eigenmode. Experimental values of k_c for each cantilever were determined with the thermal noise method [24]. CR-FM measurements utilized the second flexural eigenmode with an experimentally measured free resonance frequency $f_2^{free} = 755\ kHz$.

Originally developed for quantitative elastic-property imaging of relatively stiff materials [25,26], CR-FM techniques have been recently advanced to allow mapping of viscoelastic properties on more compliant materials [27–29]. Viscoelastic CR-FM involves measuring the frequency f^{CR} and quality factor Q^{CR} of the AFM cantilever's vibrational resonance while the tip is in contact with the sample. With the use of a point mapping procedure [29], f^{CR} and Q^{CR} are determined at each point in an image.

Detailed explanations of viscoelastic CR-FM analysis are provided elsewhere [27–29]. Briefly, the resonance of the cantilever is analyzed with a distributed-mass Euler–Bernoulli beam model. A Kelvin–Voigt element is included to model the response of the tip–sample contact. The element consists of a spring of stiffness k in parallel with a dashpot with damping σ located near the end of the cantilever. With this model for the cantilever dynamics, the normalized tip–sample contact stiffness $\alpha = k/k_c$ and damping coefficient $\beta \propto \sigma$ are determined from the experimental values of f^{CR} and Q^{CR}. Complete mathematical derivations of α and β are too lengthy to include here. Explicit equations to calculate α and β are given in Ref. [29]. Application of sphere–plane Hertzian contact mechanics allows calculation of the reduced storage modulus E'^R of the unknown sample, as given by [27–29]

$$E'^R = E'^R_{cal}(\alpha/\alpha_{cal})^{3/2} \quad (3)$$

This relation relies on calibration values E'^R_{cal} and α_{cal} [29]. We ultimately report values for the elastic storage modulus M' calculated from

$$E'^R = \left[\frac{1}{M'} + \frac{1}{M_{tip}}\right]^{-1} \quad (4)$$