Nanoindentation of biological materials

Nanoindentation has recently emerged as a powerful tool for measuring nano- and microscale mechanical properties in tissues and other biomaterials. This technique has been used to measure the mechanical properties of microstructural features in bone and teeth, investigate variations in mechanical properties with changes in tissue organization or composition in mineralized and soft tissues, and map mechanical properties spatially in complex biomaterials. Continuing advancements in indentation data analysis will increase the method's utility in the characterization of biomaterials.

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The mechanical characterization of tissues and other biological materials is of utmost importance in clinical medicine and the field of biomaterials. However, there is a paucity of information regarding the role of tissue mechanics in disease progression, tissue repair, and remodeling mechanisms associated with medical treatments. Tissues are a challenging class of materials as they are composed of hierarchical structures with important features down to the nanometer or micrometer scale. A technique that can probe mechanical properties at these scales has the potential to address numerous questions that are relevant to the medical community.

Nanoindentation, also known as instrumented or depth-sensing indentation, involves the application of a controlled load to the surface to induce local surface deformation. Load and displacement are monitored during the loading and unloading, and properties such as hardness and reduced modulus are calculated from the unloading curves using well-established equations based on elastic contact theory[1]. With a typical working force range of 1 μN to 500 mN and displacement range of 1 nm to 20 μm[1], this technique bridges the gap between atomic force microscopy (AFM) and macroscale mechanical testing.

Indentation with millimeter-sized probes (or larger) has been used for the characterization of cartilage and other soft tissues for many decades[2-11]. Applications have included mapping of cartilage mechanical properties within joints and comparisons of healthy and diseased cartilage[3,5-9]. Similarly, hardness and microhardness testing (e.g. Vickers and Knoop indentation)[12] have been used to investigate the mechanical properties of teeth and bones[13-16]. Nanoindentation improves upon the spatial, force, and displacement resolutions of these traditional techniques to provide a powerful tool for the characterization of tissues and other biomaterials with submicron resolution.

Because of its small probe size, nanoindentation can be used to measure local material properties in small, thin, and heterogeneous samples. This allows testing of tissue specimens that are unsuitable for

ISSN:1748 0132

traditional mechanical testing techniques, including biopsy specimens or tissue samples from small animal models. Nanoindentation is also useful for measuring mechanical properties of microstructural features within bulk samples, characterizing the properties of individual constituents within composite or heterogeneous samples, or mapping mechanical properties across a sample surface. As a result, nanoindentation is emerging as a valuable mechanical testing technique for biomaterials.

Mechanical property measurements

In commercial nanoindenters, displacement is typically monitored by capacitance or inductance, while force actuation is provided through electrostatic force generation, magnetic coils, or expansion of a piezoelectric element[1,17]. A schematic of a nanoindenter system using a three-plate capacitor for displacement sensing is shown in Fig. 1. The tip is mounted directly onto the middle plate of the capacitor and a load is applied to move the tip into the sample. Load and displacement are monitored continuously during the indentation process, resulting in a load-displacement curve, as shown in Fig. 2a. The interaction between the tip and the sample during the indentation process is illustrated in Fig. 2b.

A large portion of the current biomaterials nanoindentation literature focuses on the measurement of material properties such as Young's modulus or elastic modulus (E) and indentation hardness (H) from indentation data. Doerner and Nix[18] published the first comprehensive experimental and analytical approach to a generalized form of nanoindentation analysis from load-displacement data for nonrigid indenters of all geometries. Oliver and Pharr[19] further generalized their approach, resulting in the widely used compliance method for indentation analysis. Modulus can also be extracted from load-displacement curves following the method of Field and Swain[20]. These methods have been thoroughly reviewed, along with limitations and necessary instrument calibrations, in many recent publications[1,17,21-24]. While E and H are the most commonly reported nanoindentation parameters, they are not always the most relevant properties for all materials characterization. In recent years, great advances have been made in expanding analysis techniques to include the measurement of more complex material parameters such as loss modulus, storage

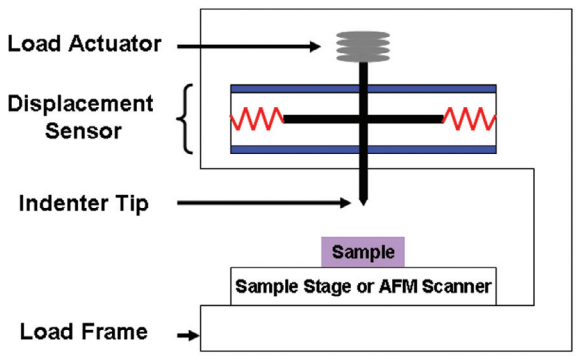

Fig. 1 Schematic of a nanoindenter system.

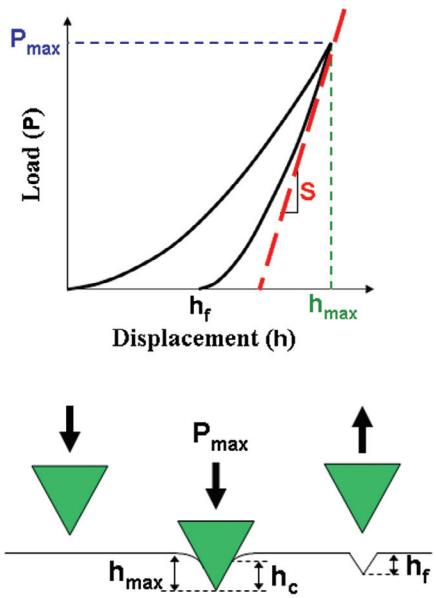

Fig. 2 Schematic of (a) a typical load-displacement curve and (b) the indentation process. P_{max} = maximum load applied; h_{max} = penetration depth; h_c = contact depth (the height of the contact between the tip and the sample); h_f = final depth; S = unloading stiffness.

modulus, and creep compliance functions[1,17,25,26]. This review will focus on the compliance method, but will also present some advances in indentation analysis that are relevant to biomaterials characterization.

The compliance method

Consider the load-displacement curve shown in Fig. 2a. This is representative of the data collected through quasi-static indentation in metals and ceramics. Using the compliance method[19,23], the hardness H and reduced modulus E_r (the combined modulus of the tip and the sample) can be determined directly from analysis of load-displacement data using the relationships[19]:

$E_r = S/2(\sqrt{\pi}/\sqrt{A})$ and $H = P_{max}/A$

where S is the initial unloading stiffness (the slope of the unloading curve dP/dh, evaluated at the maximum load), P_{max} is the maximum load, and A is the projected contact area between the indenter tip and the sample at maximum load. The reduced modulus is related to the Young's modulus E by the relationship:

$1/E_r = (1 - \upsilon_i^2)/E_i + (1 - \upsilon_s^2)/E_s$

where subscript i refers to the indenter material, subscript s refers to the substrate material, and υ is Poisson's ratio. The indenter material properties are usually known (E_i = 1141 GPa, υ_i = 0.07 for diamond, a common tip material), so the Young's modulus of a material can be calculated from the reduced modulus if the Poisson's ratio of the sample material is known. If the Poisson's ratio of the sample is not known, the plane strain modulus $E' = E/(1 - \upsilon^2)$ can be reported.

As illustrated in Fig. 2a, P_{max} and S can be determined by analysis of the load-displacement data. The projected contact area at peak load A is

most often determined by a premeasured tip area function. This shape function can be measured directly by AFM or scanning electron microscopy (SEM)[27,28], or calibrated by indenting in a sample material of known modulus (e.g. fused silica)[19].

Adaptations for indentation of polymeric biomaterials and tissues

The compliance method is based on the assumption of elastic, isotropic sample materials and negligible adhesion[19,23]. Polymeric biomaterials and tissues often exhibit viscoelastic or time-dependent behavior[29] and may be subject to adhesive interactions between the tip and the sample[30]. These characteristics can lead to errors in modulus determination using the compliance method. Indentation methods that account for the effects of viscoelasticity and adhesion, as well as tip selection, hydration, and surface preparation considerations for biomaterials analysis, are examined below.

Viscoelasticity

Many polymers and biomaterials exhibit time-dependent or viscoelastic behavior. The most readily observed effect of viscoelasticity on indentation is creep, or a sinking of the tip into the sample under a constant load. In cases where creep behavior dominates the elastic response of the material, a 'nose' is observed on the indentation unloading curve, as illustrated in Fig. 3a[27,31]. A thorough discussion of this nose phenomenon was presented by Briscoe *et al.*[27]. In short, it was found that when loading is followed by unloading without a hold at peak load (Fig. 3a), displacement increases slightly in the initial portion of the unloading phase, because the creep rate of the material is initially higher than the imposed unloading rate. This phenomenon results in a negative and changing slope in the initial unloading region (Fig. 3a), making it impossible to use the compliance method to measure modulus. To eliminate this nose, Briscoe and colleagues incorporated a 10 s hold period at peak load into their applied load functions in order to allow the material to approach equilibrium prior to unloading[27]. The effect of this hold period on load-displacement data is illustrated in Fig. 3b. This same approach has been used in many indentation studies of biomaterials, with hold periods ranging from 3 s[32] to 120 s[33,34] to allow the creep rate to dissipate prior to unloading[35-38]. An appropriate hold time should be selected based on the creep and unloading rates used in the experiment[39,40].

More recently, Ngan *et al.*[41-43] and Cheng *et al.*[44] have proposed additional corrections for analysis of viscoelastic materials using trapezoidal load functions (Fig. 3b) to ensure that creep does not affect the modulus calculation. These numerical corrections to data analysis are based on the creep rate just before unloading and the initial unloading rate (both of which can be determined from data collected during indentation)[41-44]. These new methods have been applied to recent studies of bone[45] and teeth[13], revealing substantial errors in

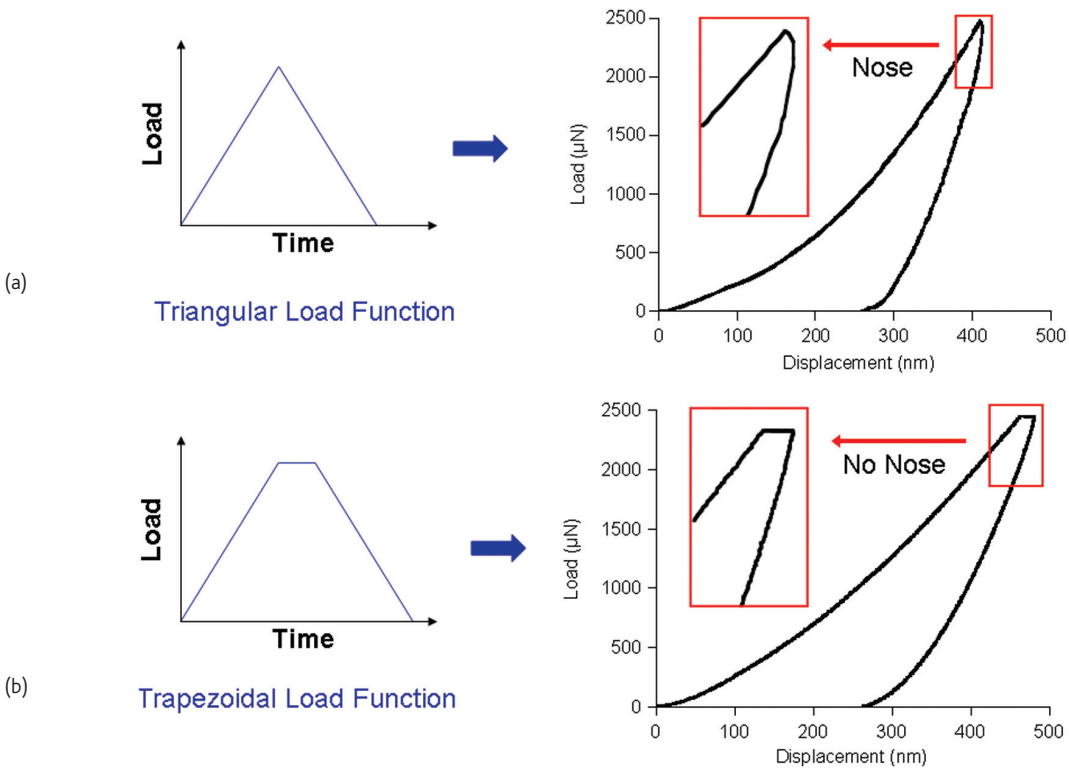

Fig. 3 (a) Illustration of the nose effect resulting from indenting a viscoelastic material with a triangular load profile. (b) Illustration of the elimination of the nose effect by indenting a viscoelastic material with a trapezoidal load profile.

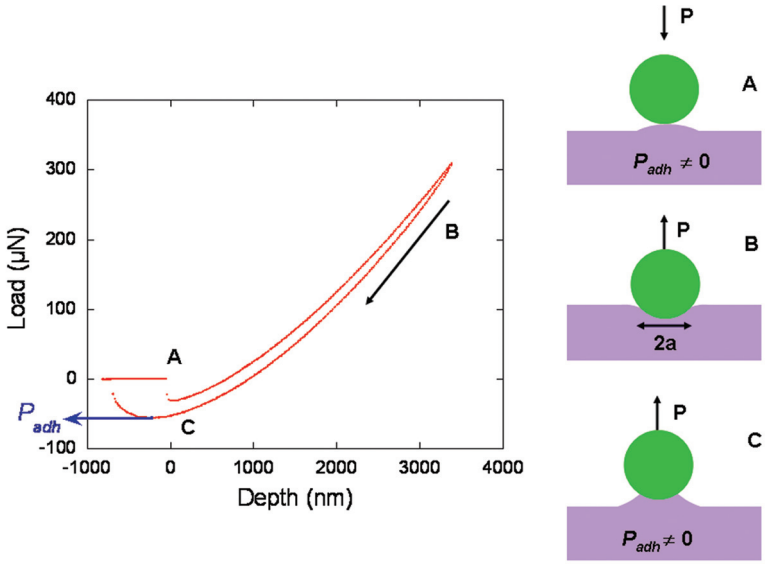

*Fig. 4 Force curve and schematic representing the interaction between the indenter tip and a poly(dimethyl) siloxane sample. P_{adh} = maximum adhesive (negative) force during unloading. **A** marks the snap-to contact point, where the tip first contacts the sample. **B** marks the unloading curve, where the tip is being withdrawn from the sample. **C** marks the pull-off point, where the tip and sample are separating; notice that the indenter tip is above the undeformed height of the sample at separation in this example.*

modulus calculations when the corrections were not used. However, if the unloading rate is high enough relative to the creep rate, the effects of the corrections will be negligible.

More rigorous nanoindentation methods that model the time-dependent behavior rather than allowing it to dissipate have also been developed for viscoelastic materials[1,17,25,26,46-49]. These include analysis of load-displacement data using creep compliance functions[25,26], three element standard solid models[47,48], and viscous-elastic-plastic models[49]. In addition, many commercial nanoindenters have a dynamic testing option, where sinusoidal loads can be applied to measure storage and loss moduli as a function of loading frequency[1,17]. These methods provide alternatives to quasi-static indentation with a trapezoidal load function, allowing direct measurement of time-dependent properties rather than measuring an equilibrium modulus. Viscoelastic results have been obtained for nanoindentation studies of assorted tissues, including bone[40], demineralized dentin[50], arteries[51], and skin[52]. Since many tissues, especially hydrated soft tissues, exhibit time-dependent behavior under physiological conditions, explicit viscoelastic (or poroelastic or biphasic) analysis techniques are likely to be central to future studies of biomaterials. However, since these techniques have not yet been broadly applied to the characterization of biomaterials, the majority of examples in this review will focus on quasi-static indentation.

Adhesion

Adhesion between the tip and the sample can interfere with measurements of modulus using the compliance method in polymeric and soft tissue samples[30,31,53,54]. Adhesion is observed in a load-displacement curve as a region of negative load during unloading. Recent studies of soft polymers (silicones) have demonstrated that the compliance method overestimates the sample modulus when there is significant tip-sample adhesion[30,54]. For indents performed with a spherical tip, contact mechanics methods based on the Johnson-Kendall-Roberts (JKR)[55] adhesion model have been shown to provide a more accurate measurement of sample modulus[30,54,56]. More complex models combine JKR and viscoelastic analyses[56-59]. The JKR approach can best be utilized by starting the indent out of contact and capturing a full force curve as the tip approaches, indents, and retracts from the sample[56,58,59]. A typical force curve is shown in Fig. 4. These force curve methods have not yet been applied to many nanoindentation studies of biomaterials, but are widely used in AFM[60,61].

Tip selection

Indenter tips are typically made out of very stiff materials, such as diamond or sapphire, so that the compliance of the sample will be greater than that of the tip. For nanoindentation of metals and ceramics, three-sided pyramidal tips that come to a sharp point, such as the Berkovich or the cube corner tip, are commonly used. These tip geometries have also been used to indent many mineralized tissues and glassy polymers[17,27,32,36,38,62-66]. However, for indenting soft polymers and tissues, a spherical tip is commonly used to minimize plastic deformation and stress concentrations and avoid damaging the sample[20,31,37,67-72]. A third option occasionally used in the study of viscoelastic materials is the cylindrical flat punch[47,50,51,67]. A flat tip has the advantage of a constant, known contact area as a function of depth, but has high stress concentration at the contact perimeter.

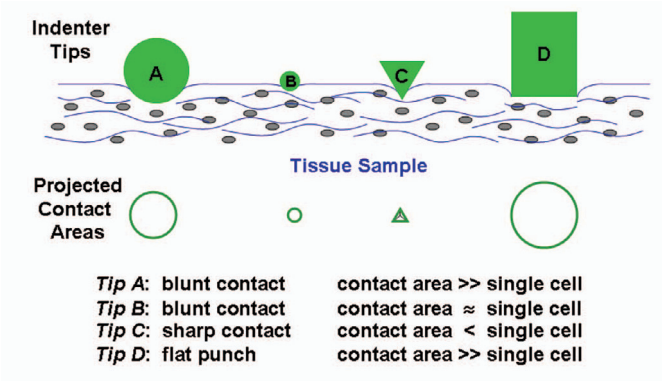

Fig. 5 Schematic illustrating tip selection for indentation of a tissue sample. Comparison of contact sizes during indentation using (A) a large diameter spherical tip, (B) a small diameter spherical tip, (C) a Berkovich tip, and (D) a cylindrical flat punch.

In addition to selecting a tip geometry, the dimensions of the tip may also be important. For example, if the goal is to measure tissue-level properties in a soft tissue, a large diameter spherical tip (> 50 μm) will be necessary so that the contact area will be much greater than the diameter of an individual cell (~8 μm) or fiber (as illustrated in Fig. 5)[37]. In contrast, when measuring the mechanical properties of microstructural features in bone or teeth, a Berkovich tip is used to maximize the spatial resolution and allow measurement of mechanical properties at precise locations within the highly mineralized samples.

Sample hydration

Despite the fact that most biological materials are naturally hydrated, much of the early work in bone indentation was performed on dehydrated samples embedded in epoxy or resin to facilitate sample preparation[38,62,73]. Studies of bone specimens report increases in indentation moduli of 11% to 28% after dehydration[34,74-76], with further increases after embedding[75,76]. A study of fully demineralized dentin (more comparable to a soft tissue) shows increases in moduli of four orders of magnitude upon dehydration, and samples that are still three times stiffer than the original specimens even after rehydration[50]. A study of carious dentin (with reduced mineral content)[77] also shows substantial increases in modulus (100 fold) after dehydration[78]. These studies demonstrate the importance not only of testing hydrated specimens but also of avoiding prolonged dehydration during sample preparation.

Other studies illustrate the importance of selecting an appropriate hydrating fluid. For example, Habelitz *et al.*[79] demonstrate that storage of dentin and enamel specimens in Hank's balanced salt solution (HBSS) maintains mechanical properties, while storage in deionized water or $CaCl_2$ solution results in a decrease in modulus and hardness that can be attributed to demineralization of the surface[79].

Testing a sample submerged in a fluid cell is one option that has been used for hydrated testing of tissues[34,50,51,74,75,79-81], but data acquisition and analysis are complicated by meniscus forces acting on the shaft of the indenter tip[34,82], and specialized tips with long shafts are required. Alternatively, tissue samples can be submerged prior to testing and removed from the fluid just before indentation[52,71], a valid technique if indents are performed over a short time period before dehydration can occur. Other solutions include hydrating samples from the edges using gauze[70], foam[37,83], or specialized irrigation systems[62,84], or applying several drops of fluid to the surface prior to indentation[35].

Surface preparation

The method of surface preparation can also affect mechanical properties because surface roughness can have a significant impact on the measurement of modulus using nanoindentation[31,68,85]. To minimize errors arising from surface roughness, mineralized tissues are often microtomed or polished (usually down to a 0.05 μm particle size) prior to indentation[36,86,87]. It has been suggested that indent depths then be chosen such that the surface roughness (R_a) is less than 10% of the total penetration depth[32]. Comparisons of microtomed and polished bone and tooth surfaces have revealed that the microtomed surfaces show lower surface roughness[86,87]. For soft tissues, cryomicrotoming may be an option for reducing surface roughness.

Biomaterials applications

While nanoindentation is still a fairly young technique in the field of biomaterials, it has proven useful in allowing measurement of mechanical properties that are nearly impossible to assess through bulk testing. Applications include measurement of mechanical properties of microstructural features in biomaterials and mapping of mechanical properties in tissues with complex microstructures. Nanoindentation can serve as a complementary characterization tool to other techniques that assess composition or structure with high spatial resolution, such as Raman spectroscopy, magnetic resonance imaging, micro-computed tomography, histology, or infrared spectroscopy. In this regard, nanoindentation has played a pivotal role in defining structure-property relationships for tissues and their constituents. While this review will not present a full review of all the biomaterials characterization that has been performed using nanoindentation, it will provide several examples to illustrate the wide range of biological, biomedical, and materials design questions that can be addressed through nanomechanical testing.

Mineralized tissues

Mineralized tissues have been extensively studied using nanoindentation, and some of this work has been reviewed recently by Kinney *et al.*[13] (dentin) and Haque *et al.*[88] (bone). Localized testing in bone specimens has been used to compare the mechanical properties of lamellar versus interlamellar bone[38,62,84,85,89], individual layers of osteonal bone[33], and trabecular versus cortical bone[36,38,62,63,73,84]. The fine spatial resolution required for mechanical testing of microstructural features in bone is illustrated in Fig. 6. Indentation has

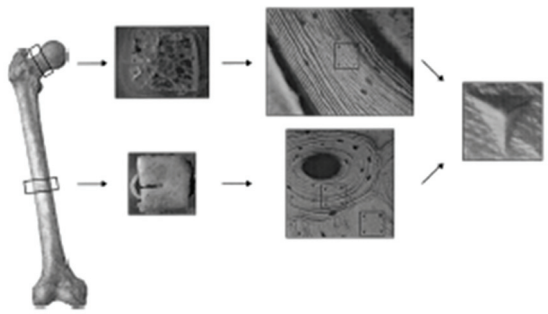

Fig. 6 Nanoindentation in microstructural features of bone, illustrating groups of indents in 30 μm by 30 μm boxes placed in trabecular bone (top) and in osteons and interstitial regions of cortical bone (bottom). (Reprinted with permission from[62]. © 1999 Elsevier Ltd.)

also been used to compare the bone tissue properties in healthy bones with those of diseased and genetically modified small animal models (including mice, rats, and zebrafish)[90-95]. Together, these studies aid in understanding the role of local tissue properties and hierarchical structure in macroscale fracture and deformation mechanisms of bone.

In teeth, a primary focus has been the mapping of mechanical properties across the dentin-enamel junction to study how the less mineralized dentin transitions to the more mineralized (and higher modulus) enamel[66,96,97]. An example of this work is shown in Fig. 7. Nanomechanical mapping has also been coupled with chemical mapping in dental tissues, demonstrating that enamel and crown intertubular dentin are both graded materials with decreased modulus, hardness, and mineral content near the dentin-enamel junction[98,99]. Nanoindentation has also been used to compare mechanical properties at the microstructural and tissue level, including enamel prisms versus sheaths[100], peritubular versus intertubular dentin[81,101,102], and dentin versus enamel[103]. Other studies have included characterization of the cementum-dentin junction[104-106], evaluation of the mechanical, chemical, and structural changes in dentin following formation of dental caries[35,77,78,80], and investigation of the effects of various solutions that cause demineralization and remineralization of dental tissues[107-109].

Additional applications to mineralized tissues aid in understanding wound healing, tissue engineering, and disease processes. These include

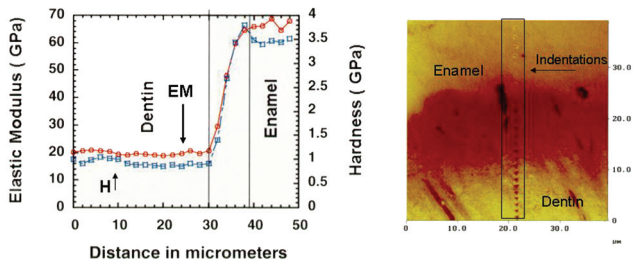

Fig. 7 Nanoindentation mapping of the dentin-enamel junction in a tooth specimen. AFM image of indents through the dentin-enamel junction and plots of the modulus and hardness values measured from the indents. (Reprinted with permission from[66]. © 2001 Wiley.)

investigations of spatial correlations between mineral content and modulus in calcified cartilage and subchondral bone[110,111], mechanical property measurements of bone mineral formed by cultured osteoblasts[112], characterization of new bone laid down on tissue-engineered scaffolds by bone cells[113], and measurement of mechanical properties of arterial calcifications[83] and hypomineralized enamel[114].

Soft tissues

In more recent years, indentation has been used to measure mechanical properties of a variety of soft tissues. Nanoindentation has proven to be a valuable tool for cartilage characterization because of its ability to measure mechanical properties *in situ* without disrupting the complex microstructure (as illustrated in Fig. 8). Preparation of cartilage plugs for nanoindentation is shown in Fig. 9. Indentation (typically with tip diameters on the order of millimeters) has long been used for mapping of viscoelastic or biphasic mechanical properties in cartilage[2-6,8-10]. The smaller probe sizes in nanoindentation allow mapping of mechanical properties in cartilage from smaller animal

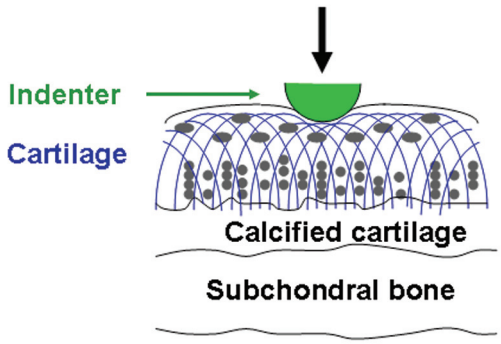

Fig. 8 Schematic of spherical indentation of a cartilage surface in situ.

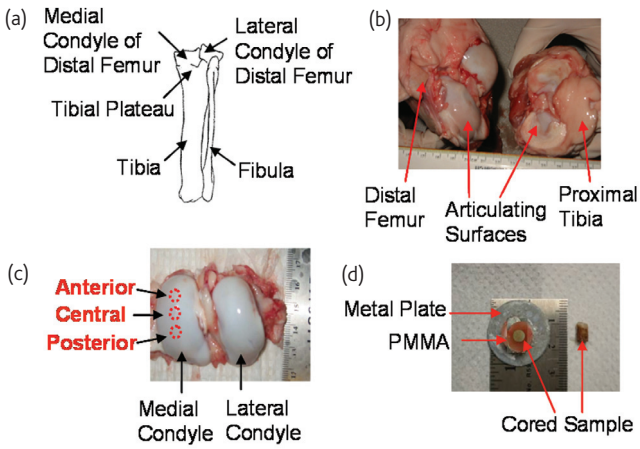

Fig. 9 Illustration of sample preparation for nanoindentation of cartilage plugs from a porcine femoral condyle. (a) Schematic of long bones. (b) Dissected joint. (c) Femoral condyle with core sites labeled. (d) Example core before and after potting in poly(methyl methacrylate) (PMMA) for indentation testing. Whole joints from small animals can also be mounted in a similar fashion.

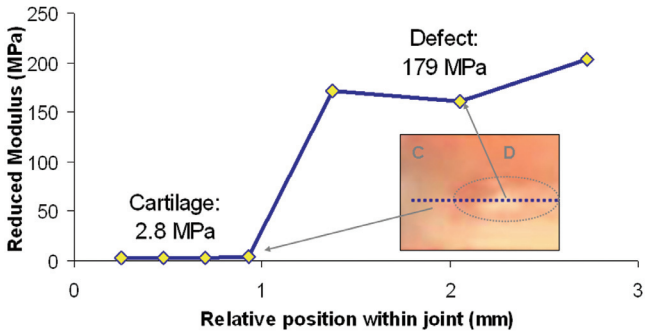

Fig. 10 Mechanical property mapping through a cartilage defect in a rabbit knee joint using nanoindentation. The 2 mm x 7 mm defect was created by scraping the cartilage from the subchondral bone using a curet.

models and smaller joints[70,71]. For example, nanoindentation has been used to measure functional mechanical properties of repair cartilage in a rabbit knee[70], and to correlate changes in mechanical parameters in specific regions of rabbit finger joint cartilage with structural parameters such as cell density and thickness of the superficial zone[71]. Fig. 10 illustrates the ability of indentation to detect changes in mechanical properties associated with small cartilage defects. The application of biphasic models to nanoindentation data analysis will enhance future cartilage studies.

Other applications of nanoindentation to soft tissue characterization include the correlation of the modulus of diseased artery tissue with the mineral content in the tissue (the degree of calcification)[83], measurement of the fracture toughness of cartilage[115], and characterization of the viscoelastic properties of healthy arteries[51], demineralized dentin[50], and the stratum corneum layer of skin[52].

Other biomaterials applications

An increasing interest in biomimetics – the design of materials based on natural biological structures – has led to the nanomechanical characterization of acellular biomaterials. In this regard, nanoindentation has been used in conjunction with computational modeling to determine the mechanical properties of the aragonite blocks and interfacial proteins that make up the complex microstructure of nacre, the fracture resistant mother-of-pearl that lines mollusk shells[116,117]. Other biomimetics applications have included characterization of structure-property relationships in specific regions of insect cuticle (exoskeleton)[118,119], mapping the variation in modulus and hardness across the 200-600 μm diameter sponge spicule[120], investigation of correlations between hardness and local chemical composition in polychaete jaws[121], and measurement of anisotropic mechanical properties in spider silk fibers[122]. By understanding the micro- and nanoscale structural, chemical, and mechanical properties of natural biomaterials, engineers and materials scientists hope to learn how to design new composite materials with enhanced materials properties at the macroscale.

Conclusions

Nanoindentation has proven itself to be a powerful technique for the measurement of mechanical properties in diverse biomaterials ranging from mineralized tissues to soft tissues. Its utility stems from the ability to measure mechanical properties of small or heterogeneous samples nondestructively, as well as microstructural features in complex biomaterials. This makes quantitative assessment of mechanical behavior possible for materials that were previously unattainable. This technique is particularly effective for characterizing biomaterials that are hierarchical in structure, allowing measurement of properties at small length scales that can be used to better understand or model macroscale behavior. By selecting appropriate tip geometries and testing protocols, a variety of mechanical properties can be measured. Nanoindentation will become even more powerful as improvements are made to existing analysis methods. nt

Acknowledgments

D. M. E. was a National Research Council Postdoctoral Associate at the US Naval Research Laboratory during preparation of this manuscript. The authors would like to thank Jove Graham, Shikha Gupta, and Cheng Li for their useful comments on the text. The authors gratefully acknowledge Phillipe Zysset for providing Fig. 6, Grayson Marshall for Fig. 7, and Cheng Li for Fig. 9.

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