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Research paper

Surface detection errors cause overestimation of the modulus in nanoindentation on soft materials

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ABSTRACT

The accuracy of mechanical properties from depth-sensing indentation, or nanoindentation, depends on the accuracy of the displacement measurement used to calculate these properties. Here, current nanoindentation techniques and analysis methods for accurate displacement measurements are reviewed. First, the ability of a commercial instrument to sense the surface of soft materials is examined. Second, methods of sample surface detection are reviewed. Finally, a case of overestimation of the elastic modulus of a compliant material using nanoindentation with incorrect displacement values is presented.

1. Introduction

1.1. *Nanoindentation of soft materials*

The application of nanoindentation to soft tissues and biomaterials has increased over recent years. The lateral spatial resolution of nanoindentation allows local testing of mechanical properties in tissues that is not possible using macroscale techniques. For example, Leong and Morgan (2008) used nanoindentation to describe local changes in the mineralization across a rat fracture callus. Ferguson et al. (2003) applied the same technique to human articular calcified cartilage and subchrondral bone from normal and osteoarthritic patients. Still, there are several unique challenges associated with applying nanoindentation to soft materials. Major sources of difficulty include evaluating the appropriateness of mechanical models (Cheng et al., 2000, 2005), defining suitable calibration materials (Bushby and Jennett, 2001; Klapperich et al., 2001; Odegard et al., 2005; Oyen, 2005), characterizing and maintaining sample hydration (Bembey et al., 2006), and eliminating other sources of experimental error in the measurements (Cao et al., 2005).

Viscoelastic modeling of nanoindentation has improved understanding of the mechanical properties of polymers and tissues, replacing the fully elastic modeling used for relatively hard materials. Oyen (2005) examined nanoindentation creep experiments in polymers following ramp loading. Cheng et al., derived a solution for standard linear solid materials indented with both a flat-punch (Cheng et al., 2000) and spherical tip (Cheng et al., 2005) geometry. Fujisawa, et al., studied the viscoelasticity of polymethylmethacrylate (PMMA) by quantifying the strain dependence of the elastic modulus from nanoindentation (Fujisawa and Swain, 2006).

Dynamic nanoindentation is a newer method to describe time-dependent mechanical properties of polymers. White, et al., obtained good agreement for the storage and

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loss modulus from dynamic nanoindentation and rheometry for epoxy, polydimethylsiloxane (PDMS), and polymethylmethacrylate (PMMA) (White et al., 2005). Dynamic nanoindentation was used to distinguish small differences in the storage moduli of eight engineering polymers with different material densities (Odegard et al., 2005). Bouaita, et al. tested several polyolefins using a dynamic stiffness measurement method where an AC signal is applied on top of the DC actuation of a standard nanoindentation test (Bouaita et al., 2006).

1.2. *Special considerations for soft samples*

Many soft materials also require hydrated testing because of the significant effect of swelling on the mechanical properties of biological samples and polymeric hydrogels. By adjusting the percentage of ethanol in the storage solution for bone samples, Bembey, et al., showed that the mechanical properties of the bone changed with the level of hydration (Bembey et al., 2006). Hydration history can also affect the mechanical properties of hydrogels as shown in Thomas et al. (2004). Even hard tissues, like cementum, show markedly different properties when tested in dry and hydrated states (Ho et al., 2004). Carillo, et al. also showed hydrated polydimethylsiloxane (PDMS) has a smaller pull-off force than dry PDMS (Carrillo et al., 2005, 2006).

Adhesive behavior further complicates the determination of the surface location in the Z-direction (Ebenstein and Wahl, 2006; Wahl et al., 2006). The Z-direction and Z-displacement referred to in this text represent the movement and position of the tip in the direction of the indentation. Adhesion models, such as Johnson–Kendall–Roberts (JKR), can be successfully applied to both quasistatic (Ebenstein and Wahl, 2006) and dynamic (Ebenstein and Wahl, 2006; Wahl et al., 2006) nanoindentation experiments to accurately determine elastic modulus values that demonstrate this "jump into contact" behavior. In a prototypical indent, the indenter tip senses a negative load as it approaches the sample such that a minimum force is seen on the loading curve prior to the applied load increasing. The minimum force on the loading curve is then defined as the "jump into contact" and zero displacement position.

1.3. *Importance of zero point determination*

Here, we focus on one source of experimental error in nanoindentation measurements. The accurate determination of surface position is essential to the accurate measurement of mechanical properties. With nanoindentation of soft materials, it is often necessary to modify the standard testing protocols for stiff materials to accurately calculate the surface position (VanLandingham et al., 2001; Hayes et al., 2004). Even in stiff materials, surface location corrections are sometimes needed. For example, Chudoba, et al. noted indents with a 20 nN preload force on fused silica, silicon, or sapphire required offsetting the zero displacement position (Chudoba et al., 1999) to obtain the correct elastic modulus values. Fischer-Cripps also raised the issue of zero displacement determination in his excellent, critical review of nanoindentation (Fischer-Cripps, 2006).

Our laboratory has most experience with quasistatic nanoindentation on the Hysitron TriboIndenter, so the discussion centers on these methods. However, the testing protocols and principles discussed are relevant and can be adapted to other instrumentation. Other important methods for detecting the surface include monitoring the change in stiffness (Li and Bhushan, 2002) and phase-shift measurements to identify the surface location during dynamic testing (Ebenstein and Wahl, 2006). Li, et al. reviewed the use of continuous stiffness measurements on MEMS devices (Li and Bhushan, 2002). The continuous stiffness measuring technique eliminates many of the problems with sensing the surface, but in fragile materials can cause nanofatigue from cyclic loading. In dynamic testing, very high frequency tests must be avoided with polymer samples to avoid local melting or softening (Bouaita et al., 2006).

2. Experimental techniques

2.1. *Machine limits*

Nanoindentation was initially developed to study stiff materials, such as metals and ceramics (Cheng et al., 2004). When using the TriboIndenter (Hysitron, Inc, Minneapolis, MN) microscope to locate an indent position, the instrument moves the tip to the sample surface and applies a preload force for preset time periods to allow the piezo scanner thermal drift to settle and to measure the electronic drift prior to the start of the experiment. In a typical test, the tip is held on the surface with a preload force for 5 – 80 s while the software performs feedback sensing and drift correction. Carillo, et al. studied the effect of preload forces on pull-off forces on polydimethylsiloxane (PDMS). The pull-off force is the minimum force on the unloading curve or the force required to separate the tip from an adhesive sample surface (Ouyang et al., 2001). Using a preload force as high as the test load produced the most repeatable response, possibly because the creep reached equilibrium prior to testing (Carrillo et al., 2005, 2006). While these pre-indentation steps reduce error for indents in some materials, the minimum preload force of 100 nN can cause the tip to travel several microns below the surface before the start of data collection. This is a particularly important issue for the Hysitron TriboIndenter that has a maximum displacement of 5 microns.

For soft materials, the instrument limit of displacement is often reached resulting in the immediate termination of the experiment. Even when instrumentation limits are higher, the data from indents with displacements greater than 10% of the total starting sample thickness should be ignored in order to avoid substrate effects (Tsui and Pharr, 1999). Recent work indicates that indents with 10% thickness displacements may still have substrate effects in softer materials. Finite element analysis of nanoindentation of two elastic layers indicates that layer thickness and tip geometry affect the displacement at which substrate effects are seen (Clifford and Seah, 2006). Further, indents with a maximum load below 10 μN have significant error due to the sampling speed and drift in the transducer (VanLandingham et al.,

2001). For most experiments described below, both tip area and Z-displacement were maximized to achieve these loads between 10 and 100 μN.

2.2. Tip selection and sample preparation

One improvement for the nanoindentation of soft materials is the use of the flat punch and conospherical tips, as opposed to the sharper Berkovich tip, to create larger contact areas (Cheng et al., 2005). Using a larger tip helps to avoid sample fracture during indentation, which would make calculation of material properties significantly more challenging. The larger contact area also ensures smaller displacements at a given load to prevent permanent deformation of the sample. With the softest samples (low kPa) range, larger tips make testing possible by avoiding the maximum displacement limit of the indenter. Here, a sapphire, flat punch tip with a 50 micron diameter (Hysitron, Minneapolis, MN) was used to indent poly-2-hydroxyethyl methacrylate (pHEMA) and PuraMatrix-collagen hydrogels.

Some drawbacks of choosing the flat punch tip were greater sample size and lower spatial resolution. An advantage of the flat punch tip was its length compared to conospherical tips. Using the longer, flat punch tip facilitated submerged testing of the hydrogels. Briefly, each hydrogel sample was kept in a fully submerged state prior to testing to prevent partial dehydration. The samples were then glued to the top of a glass slide with a small droplet of cyanoacrylate. A magnetic atomic force microscopy disc (Ted Pella, Redding, CA) was glued to the bottom of the slide directly beneath the sample to keep the slide in position on the magnetic sample stage. The sample was then encircled with a hydrophobic barrier pen, ImmEdge (Vector Laboratories, Burlingame, CA). The small circle was carefully filled with water to ensure complete submersion during testing.

2.3. PuraMatrix-collagen hydrogels

PuraMatrix is a self assembling, patented 16-mer peptide that forms a 99.5%–99.9% water content gel in the presence of salt solutions including Dulbecco's Modified Eagle's Medium (DMEM) (Zhang, 2002). PuraMatrix-collagen gels were synthesized using the protocol below. To prepare the collagen solution, type I collagen from bovine Achilles tendon (Sigma-Aldrich) was dispersed in 0.05 M acetic acid. Collagen was diluted to achieve a final concentration of 0.5% w/v. The collagen solution was homogenized for approximately 5 min until no large fibrils were present. The solution was then ultrasonicated for 3 h at 4 °C and used within 24 h. PuraMatrix-collagen hydrogels were then synthesized by adding 50 μL PuraMatrix, 50 μL of 0.5% w/v collagen solution, and 150 μL DMEM to a well in a 96-well plate and incubating at 37 °C for 90 min.

2.4. Surface detection

In order to maximize the Z-displacement during the test and accurately determine the surface position, the standard nanoindentation procedures for hard materials must be modified (Hayes et al., 2004). One method to accurately define the surface position of soft materials is to perform displacement controlled experiments as described in Cao et al. (2005). A schematic of this method is shown in Fig. 1. Instead of using the software-controlled optics method to locate the surface, the tip was manually moved into position above the sample. The Z location of the surface was then found by monitoring the tare value while lowering the nanoindenter tip on the finest motor speed. The tip was then raised approximately one micron above the sample surface until the tare value returned to zero. From this starting position above the gel, a manual displacement controlled indent was performed. With this method, the thermal drift was significantly reduced allowing for much longer hold periods, up to 10 min. Previously, hold times were limited to 20 s because of the large thermal drift that occurs after each large lateral movement of the tip between the microscope and indenter positions (Zhou and Komvopoulos, 2006).

2.5. Curve fitting procedures

The Oliver–Pharr method and the Maxwell–Wiechert model were used to analyze the data as described previously (Kaufman et al., 2008). First, the unloading curve was fit to a power law curve using the Oliver–Pharr method to calculate the unloading stiffness, S. The indents were performed with a constant area flat punch tip, so the reduced modulus, Er, was equal to the unloading stiffness divided by the tip diameter. Second, the Maxwell–Wiechert viscoelastic model was fit the hold period stress relaxation curve. The Maxwell–Wiechert model is an expanded form of the standard linear solid (SLS) model. They are equivalent when only Maxwell element is used. The stress relaxation curves were fit with $j = 1, 2$, and 3, but little or no improvement in fitting was seen with the addition of a third Maxwell element. The $j = 2$ model was selected for further analysis. The three spring constants from this curve fit were then added to calculate the instantaneous modulus.

3. Overestimation of the elastic modulus with incorrect surface detection

Recently, we have looked at several soft hydrated polymers in our lab (Kaufman et al., 2008). Here we present the results from indenting two soft hydrogels in which the tip was intentionally positioned below the surface of the gel before the beginning of the test. Because of the low-modulus and high water content of the PuraMatrix-collagen hydrogels, large starting depths (greater than 200 microns) could be used without damaging the nanoindenter tip. The zero displacement position for a single indent is shown in Fig. 2. An example of two indents illustrate the problem of zero displacement determination in a pHEMA hydrogel is shown in Fig. 2. The dashed line shows a test that began below the hydrogel surface. The solid line shows a test that began in the water above the hydrogel. The calculated reduced moduli from the Oliver–Pharr method are shown in the legend. The large difference in value at the same position demonstrates how large a factor surface detection can play in the calculated modulus values.

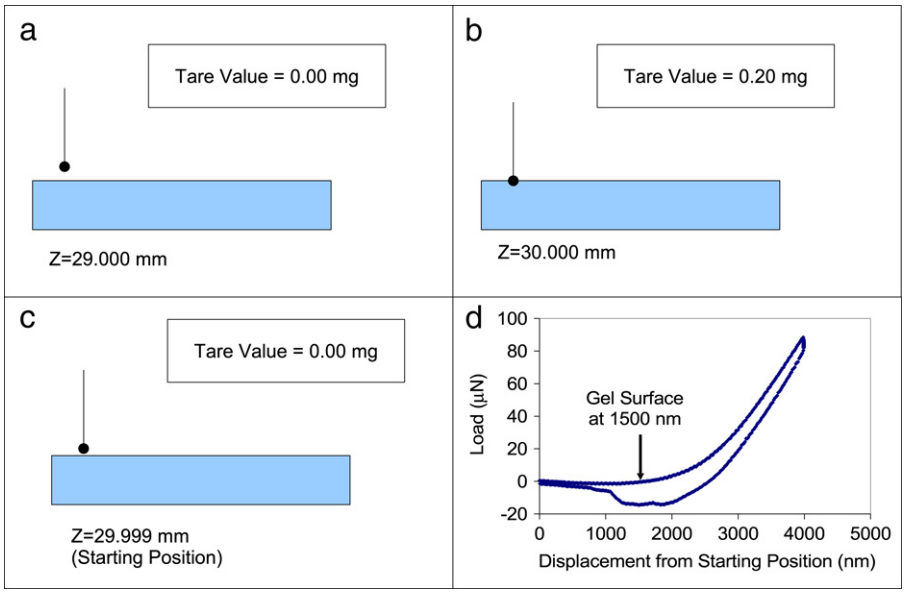

Fig. 1 – An illustration of the modified nanoindentation protocol. (a) The tip was lowered very close to sample surface. Note that tare value was set to zero. The tare value is proportional to the load measured by the transducer. (b) The tip was lowered until tare value increased. The Z-position of tip was recorded. (c) The tip was raised approximately 1 micron above the position with increased tare value (b) until the tare value returned to zero. (d) An indent in a polyHEMA hydrogel showing the tip engaging the sample at 1500 nm below the starting position. For the Hysitron TriboIndenter, larger, more positive Z position values correspond to the tip nearing the sample surface.

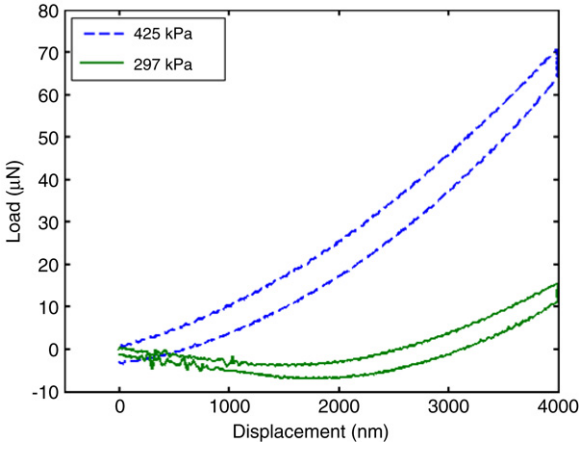

Fig. 2 – Two examples of indents at the same position in the same pHEMA hydrogel. The first indent (dashed blue line) began with the tip below the sample surface. The second indent (green solid line) began in the water above the sample. Note the large error (43%) in the reduced modulus values from the Oliver–Pharr curve fitting method.

Hydrogels composed of PuraMatrix (3DM, Inc., Cambridge, MA) and collagen have similar chemical and physical properties to native extracellular matrix (Zhang, 2002; Ellis-Behnke et al., 2006) making them well suited for tissue engineering applications, in particular for mimicking the cellular microenvironment of soft tissues. Mechanical testing of these artificial extracellular matrices poses several challenges. In a research setting, the hydrogels are often made in small amounts and are difficult to test using traditional methods due to difficulties gripping the samples and maintaining proper hydration. These very soft hydrogels must be tested while hydrated to maintain their mechanical properties.

PuraMatrix-collagen hydrogels were indented at a single position at increasing starting depths. From these depth dependence experiments, the reduced modulus and instantaneous modulus both increased linearly with starting depth into the sample as shown in Fig. 3. In Bembey, et al. fitting the creep curve performed better than fitting the unloading curve of the same indent (Bembey et al., 2006). Using both curve fitting methods showed that both elastic and viscoelastic models have a strong dependence on the starting displacement.

We believe that depth dependence was due to testing of a compressed sample. Since a flat punch tip was used, the tip geometry cannot be the source of this effect. Particularly in the case of hydrogels, we have observed that the preload on the sample can have a significant effect on the properties measured. This effect may be sample dependent. In Simha, et al. decreasing indenter tip size caused a monotonic increase in elastic modulus for cartilage samples, but not for urethane rubber (Simha et al., 2007).

4. Summary

Nanoindentation experiments on soft materials must be designed to ensure that the point of zero displacement is accurately determined. Tests starting below the sample surface can cause an overestimation of elastic modulus using both the Oliver–Pharr and Maxwell–Wiechert fitting methods. It is likely that tip-sample interactions and sample stiffness

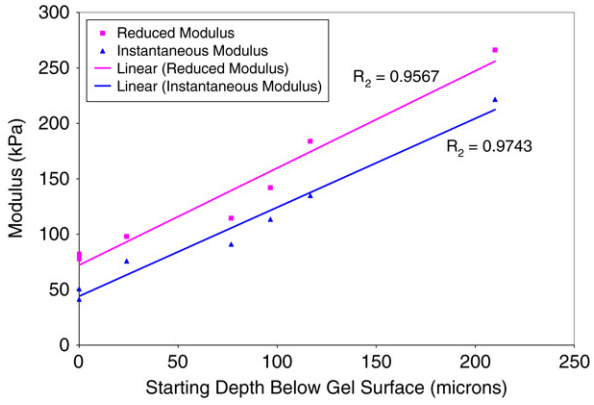

Fig. 3 – The effect of starting depth on the calculated modulus of PuraMatrix-Collagen gels. The reduced modulus was calculated from an Oliver–Pharr fit to the unloading curve. The instantaneous modulus was calculated from a Maxwell–Wiechert fit to the stress relaxation curve.

both affect whether or not there is significant creep into the sample during the preload period. In the design of soft material indentation experiments, care must be taken to minimize pre-indentation creep and accurately determine the zero displacement position. As illustrated here, errors due to surface detection can be quite large. At the largest starting depth, the calculated modulus is almost 5 times the actual value. Using a manual indentation method for new samples is one effective method to accurately determine the zero displacement position.

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