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Nanoscopic Dynamic Mechanical Properties of Intertubular and Peritubular Dentin

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Abstract

An experimental evaluation of intertubular and peritubular dentin was performed using nanoindentation and Dynamic Mechanical Analysis (DMA). The objective of the investigation was to evaluate the differences in dynamic mechanical behavior of these two constituents and to assess if their response is frequency dependent. Specimens of hydrated coronal dentin were evaluated by DMA using single indents over a range in parametric conditions and using scanning probe microscopy. The complex (E^*), storage (E') and loss moduli (E'') of the intertubular and peritubular dentin were evaluated as a function of the dynamic loading frequency and static load in the fully hydrated condition. The mean complex E^* (19.6 GPa) and storage E' (19.2 GPa) moduli of the intertubular dentin were significantly lower than those quantities of peritubular dentin ($E^* = 31.1$ GPa, $p < 0.05$; $E' = 30.3$ GPa, $p < 0.05$). There was no significant influence of dynamic loading frequency on these measures. Though there was no significant difference in the loss modulus (E'') between the two materials ($p > 0.05$), both constituents exhibited a significant increase in E'' with dynamic load frequency and reduction in the quasi-static component of indentation load. The largest difference in dynamic behavior of the two tissues was noted at small quasi-static indentation loads and the highest frequency.

Keywords

Dentin; Dynamic Mechanical Analysis (DMA); Elastic Modulus; Nanoindentation

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INTRODUCTION

Within the last decade there has been a tremendous increase in the application of nanoindentation for characterizing the mechanical behavior of biological materials. These efforts have contributed to greater understanding of their microstructure and properties, and they have facilitated the development of improved/new approaches for testing and analysis. Though research concerning nanoindentation of biological tissues has not reached maturity, reviews of the topic are now available for both hard (i.e. mineralized) [Angker and Swain, 2006; Lewis and Nyman, 2008] and soft tissues [Ebenstein and Pruitt, 2006; Franke et al., 2008]. In fact, Oyen and Taylor [2009] recently assembled a special collection of manuscripts devoted to nanoindentation of biological materials. This special issue introduces some novel applications and addresses concerns regarding the methods of evaluation.

Hard biological tissues are frequently regarded as complex hierarchical composite materials [e.g. Currey, 1982; Weiner and Wagner, 1998; Rho et al., 1998; Kinney et al., 2003a; Balooch et al., 2004], a classification that originates from the arrangements of apatite nanocrystals and the organic matrix (mostly type I collagen). Nanoindentation provides a means of characterizing the properties of isolated components of the microstructure and/or property gradients exhibited from anatomical features of interest. The majority of investigations concerning nanoindentation of hard tissues have examined bone [Lewis and Nyman, 2008]. However, there is a growing number of studies on hard tissues of the tooth including cementum (e.g. Ho et al., 2004a; Ho et al., 2004b; Ho et al., 2009), dentin (e.g. Kinney et al., 1996; Marshall et al., 2001; Habelitz 2002; Angker et al., 2003; Balooch et al., 2004; Brauer et al., 2008; Inoue et al., 2009) and especially enamel (Habelitz et al., 2001; Cuy et al., 2002; Ge et al., 2006; Braly et al., 2007; Zhou et al., 2007; Park et al., 2008; He and Swain, 2008; He and Swain, 2009). A complete review of the studies in this area is beyond the scope of the present investigation. Angker and Swain [2006] have presented an excellent review of the studies involving nanoindentation of dental hard tissues.

In the application of nanoindentation to dentin, there are a few important considerations. Dentin is comprised of approximately 45% mineral, 33% organic material (primarily type I collagen) and 22% water by volume [Ten Cate, 2008]. The dentin tubules, a network of microscopic channels, extend radially outward from the pulp towards the dentin-enamel junction (DEJ) and cementum with density range from 20,000 to 60,000 to per mm² [Pashley, 1989]. The tubules have a diameter ranging from 2.5 μ m (near the pulp) to less than 1 μ m (close to enamel) and are bounded by a highly mineralized cuff of peritubular dentin. The intertubular dentin consists of a collagen fibril mesh oriented essentially perpendicular to the tubules and filled with apatite crystallites [Marshall et al., 1997]. The heterogeneous nature of the microstructure and scale of dominant features makes the indent size and its placement important aspects of the evaluation [Kinney et al., 1996; Inoue et al., 2009]. Nanoindentation has been applied in examining demineralized dentin as well [Balooch et al., 1998; Marshall et al., 2001; Zheng et al., 2003; Angker et al., 2004a; Angker et al., 2005; Hosoya et al., 2008; Pugach et al., 2009]. Collectively these studies have revealed that, 1) the mineral content is critically important to the measured hardness and elastic modulus, and 2) sample hydration can influence the mechanical behavior, especially in evaluations of dentin with low mineral content [Angker et al., 2004b; Bertassoni et al., 2009].

An alternate approach to conventional nanoindentation evaluations that has had only limited application in the characterization of hard tissues is nanoscopic Dynamic Mechanical Analysis (nanoDMA). Balooch et al [2004] explored nanoDMA scanning of dentin and its potential for evaluating the intertubular and peritubular dentin, as well as the junction between these constituents. They found that the nanoDMA enabled characterization of

spatial variations in both the storage and loss moduli (components describing the elastic and viscous behavior), as well as distinction of the junctions between discrete constituents. Nevertheless, that evaluation was conducted under dry conditions and did not consider the potential influence of important indentation parameters on the measurements. As evaluations performed using nanoDMA involve both a quasi-static and dynamic loading component, there are potential contributions of the loading protocol on the responses. Also, previous work has shown that nanoindentation responses of bone are a strong function of loading rate and viscous effects [Fan and Rho, 2003; Bembey et al., 2006; Tang et al., 2007]. To the author's knowledge no investigation has explored the application of nanoDMA to evaluate the parametric dependence in mechanical behavior of dentin under dynamic loading, or the differences between hydrated intertubular and peritubular dentin. Thus, the objectives of the study were to 1) conduct nanoDMA of both intertubular and peritubular dentin under fully hydrated conditions, 2) to distinguish differences in their mechanical behavior and, 3) to identify the dependence of their dynamic mechanical behavior on the primary parameters involved in the evaluations.

MATERIALS AND METHODS

Human third molars were obtained from local clinics in the state of Maryland according to a protocol approved by the institutional review board (IRB) of the University of Maryland Baltimore County. The teeth were placed within a storage bath of Hank's Balanced Salt Solution (HBSS) immediately after extraction to prevent demineralization [Habelitz et al., 2002]. Each tooth was received with indication of patient age and gender only. Four young teeth were selected from patients within the age range of ($18 \leq \text{age} \leq 25$). The teeth were cast in a polyester resin foundation and sectioned using a computer numerical controlled slicing/grinding machine (Chevalier, Smart H818-II) with diamond slicing wheels (#320 mesh abrasives) under copious application of water-based coolant. Primary sections were made perpendicular to the longitudinal axis of the tooth as shown in Figure 1(a) to obtain a single coronal wafer. The wafers were then mounted in cold-cured epoxy (Epofix HQ Resin and Hardener, Struers) according to the manufacturers recommendations. The samples were allowed to harden for approximately 20 hours (maintained within HBSS hydration) before the surfaces were subjected to polishing.

All samples were polished using progressive silicon carbide abrasive paper and diamond particle suspension solutions with cloth polishing disks and water hydration. The process was started with #800 mesh abrasive paper to expose the dentin sample surface from resin residue remaining from the mounting process. Thereafter, #1200, #2400, and #4000 mesh abrasive papers were used successively to polish the surface. Finishing polishing was achieved using $3\mu\text{m}$, and then $0.4\mu\text{m}$ diamond particle suspension solutions, with the corresponding polishing cloth recommended by the manufacture (Struers). A finished sample is shown in Figure 1(b).

Quasi-static indentation and nanoscopic DMA was performed on the prepared dentin surfaces using an instrumented nanoindenter (Hysitron Tribindenter 900, Minneapolis, MN) with a Berkovich diamond tip indenter with a 100 nm tip radius. Individual indentations were made on the intertubular dentin and peritubular cuff as shown in Figure 3. The locations were carefully chosen to centralize the indents within each region of evaluation. For the intertubular dentin, at least $3\mu\text{m}$ of spacing was presented between adjacent indentations and only a single indentation was made on any single peritubular cuff. A small number of quasi-static indentations were made using a maximum load of $200\mu\text{N}$, dwell times of either 2 or 20 seconds and a load/unload rate of $50\mu\text{N/sec}$; these were conducted as a control and to determine the reduced elastic modulus estimated using the conventional approach [Oliver and Pharr, 1992]. Indentations performed for DMA were comprised of a

static load with a superposed dynamic load applied at the peak indentation load. Static indentation loads of 400, 700 and 1000 μN were applied with corresponding dynamic load amplitude of 20 μN . The static indentation load range was chosen such that the effective indentation size at maximum load was limited to the peritubular cuff thickness. At 1000 μN , the indentation depth ranged from 130 nm (peritubular dentin) to 190 nm (intertubular dentin). The frequency of the superposed dynamic load was varied from 2 Hz to 100 Hz at ten different discrete frequencies, including 2, 4, 6, 8, 10, 20, 40, 60, 80 and 100 Hz. For each testing frequency at least three indentations were made on each constituent of four different samples. A total of 120 indentations each were made in characterizing properties of the intertubular dentin and peritubular cuff, resulting in 240 total indentations ($3 \text{ indentations} \times 4 \text{ samples} \times 10 \text{ frequencies} \times 2 \text{ locations} = 240 \text{ indentations}$).

For each indentation the load and displacement signals were used along with knowledge of the indentation area to estimate the storage and loss moduli according to Eqn.'s A3 and A4 (Appendix). The indentation area (A) was estimated from the indentation depth using the tip area function, which was determined according to the Oliver and Pharr [1992] method. That is, a series of indentations were introduced on a fused silica calibration sample (with known elastic modulus) at different indentation loads. The tip area function was generated over indentation depths from 50 nm to 260 nm, which spans the range of the indentations made in the intertubular and peritubular dentin. The machine compliance was calculated from the same data using a conventional method as described by [Odegard et al, 2005]. The storage and loss moduli were used in determining the complex modulus and the $\tan\delta$ parameter according to Eqn.'s A5 and A6 (Appendix), respectively. Statistical analyses of the elastic moduli was performed using a two-way Analysis of Variance (ANOVA) and Tukey's HSD test with significant differences identified at $\alpha = 0.05$.

In addition to performing nanoDMA using discrete indents, a complimentary preliminary evaluation of the dynamic mechanical behavior of intertubular and peritubular dentin was conducted using Scanning Probe Microscopy (SPM). Scanning based nanoDMA was performed using the commercial instrument and indenter as previously described. For scanning evaluations, the indentation process consisted of a 4 μN static indentation load and a dynamic sinusoidal load applied at 100 Hz with amplitude of 50% of the static load. This protocol limits the contact conditions to an elastic indent (comprised of superposed elastic and viscoelastic deformation). The nanoindentation instrument performs the evaluation over a prescribed window of evaluation through 256 horizontal scans which are equidistant over the vertical dimensions of the image space selected. Over each horizontal scan path a total of 256 indents is performed. The assembly of horizontal scans is then used in generating an image of the response with a resolution of 256×256 pixels. For each scan, the contact load and displacement signals were monitored and used in obtaining images of the surface topography and gradient. These signals were also used in calculating the phase angle between the load and displacement, and in generating maps of the complex (E^*), storage (E') and loss (E'') moduli distributions, as well as the $\tan\delta$ distribution. All of the modulus maps obtained utilizing the nanoDMA mode of operation were performed using a scan window of 30 μm by 30 μm .

Due to the potential for changes in the mechanical behavior of dentin as a function of dehydration [Jameson et al., 1993; Kahler et al., 2003; Kruzic et al., 2003; Bajaj et al., 2006; Balooch et al., 2008], both the discrete indent and scanning based nanoDMA evaluations were performed in hydrated conditions. However, due to difficulties encountered in performing scanning evaluations with water droplets and evaporation, an alternate approach was used for achieving sample hydration. All of the samples were stored in HBSS after polishing to maintain the hydration. Prior to the nanoindentation experiments the samples were removed from the hydration bath and the excess HBSS was wiped away. A drop of

99.4% ethylene glycol was applied to the polished sample surface and distributed evenly using a cloth saturated with ethylene glycol. Use of ethylene glycol has been used previously to maintain the hydration of dentin samples after Pashley et al [2007]. Its use prevented the evaporation of water, while also eliminating problems related to the meniscus forces transferred from droplets of fluid to the indenter. Due to the concern that there may be changes in the indentation responses with use of ethylene glycol, separate analyses were performed using the aforementioned indentation approaches on the fused quartz and polycarbonate calibration samples issued by the manufacturer (Hysitron, Inc). For the polycarbonate sample, single indent (N=10) and scanning mode (N=3) nanoDMA were performed with and without the ethylene glycol treatment. Scanning based nanoDMA was performed on the fused silica (N=3) with and without the ethylene glycol. Hydration was achieved by placing a drop of solution on the samples and wiping away excess; they were not presoaked in HBSS due to concern of degradation (polycarbonate). Both the discrete indents and scanning mode evaluations were conducted using the identical indentation parameters applied in the evaluation of dentin. Results obtained for the dry and treated (with ethylene glycol) evaluations were compared using a one-way analysis of variance.

RESULTS

Selected responses for quasi-static indentations performed on intertubular and peritubular dentin using two different hold times are shown in Figure 2. As evident from comparison of Figure 2(a) and 2(b), the intertubular dentin exhibited a lower hardness and elastic modulus than that of the peritubular dentin. However, it is also clear that the indentation hold time is an important factor in the interpretation of mechanical behavior, and that there is an increase in viscous deformation with time. For both the intertubular and peritubular dentin, there is a decrease in the estimated hardness with hold time. The changes in response characteristics resulting from the longer hold period are largest for the intertubular dentin, as expected, from the higher organic and water content and with a lower elastic modulus. However, one aspect of the mechanical response that is not clearly represented by the elastic modulus and hardness is the difference in indentation resistance profiles, which is associated with the time-dependent aspect of deformation.

Results for nanoscopic DMA performed on the calibration samples with and without ethylene glycol treatment are listed in Table 1. The expected reduced elastic modulus for the polycarbonate and fused silica samples is 3.1 GPa and 69.6 GPa, respectively. For both sample materials the complex modulus obtained from nanoDMA without ethylene glycol treatment is consistent with the reported reduced elastic modulus (within 6%). Results for the discrete indentations of the polycarbonate sample are listed in Table 1 in terms of the average and (std dev). Though there was no significant difference in the complex and storage moduli, the loss modulus was significantly lower ($p=0.026$) with use of the ethylene glycol. Results for evaluation of the two calibration samples in scanning mode with and without the ethylene glycol treatment are also listed in Table 1. With ethylene glycol treatment there was a significant ($p<0.05$) reduction in the magnitude of both the storage and loss moduli, which was reflected in the value of the complex modulus as well. However, as expected there was no significant difference in the moduli obtained for the fused silica with and without the ethylene glycol; this material is essentially impermeable.

Discrete indents were made on the intertubular and peritubular dentin (Figure 3) using nanoDMA over the selected range in quasi-static load and dynamic loading frequency. Due to the relatively small feature size of the peritubular cuffs, topographical maps were used to insure that each of the discrete indentations were restricted to the intertubular or peritubular space; results from poorly placed indentations were discarded. The influence of static load magnitude over the evaluation parameter space on the dynamic mechanical behavior is

shown in Figure 4. Specifically, the complex, loss and storage moduli are shown in terms of the quasi-static load in Figures 4(a) through 4(c), respectively, for both the intertubular and peritubular dentin. In addition, the variation in $\tan\delta$ with static indentation load is presented in Figure 4(d). Overall, the complex and storage moduli of the peritubular cuff were significantly larger than those values for the intertubular dentin ($p < 0.001$). However, there was no significant difference in the loss modulus and $\tan\delta$ responses between the intertubular and peritubular dentin, respectively.

The overall average complex modulus (Figure 4(a)) for the peritubular cuff and intertubular dentin was 31.1 GPa and 19.6 GPa, respectively; the average storage modulus for each of these regions was 30.3 GPa and 19.2 GPa, respectively. Both the storage and loss moduli exhibited substantial changes in magnitude over the range in static load and dynamic frequency. Results of the statistical analysis performed on the measured properties are presented in Tables 2 and 3 for the intertubular and peritubular dentin, respectively. As evident in these tables the change in the loss modulus of the intertubular dentin over the range in evaluation parameters was more significant than the change that occurred to the storage modulus. This statement is only partly true for the peritubular dentin. The changes in the loss modulus of the peritubular dentin with frequency were more significant than the changes that occurred over the range in static load.

The loss modulus (Figure 4(b)) and the $\tan\delta$ parameter (Figure 4(d)) for both the peritubular cuff and intertubular dentin decreased with increasing static load. Both of these measures were approximately three times larger at the lowest static load (400 μN) when compared with results obtained at the two larger indentation loads. One observation of note is the difference in variations of the components of mechanical behavior. For both the intertubular and peritubular dentin the coefficient of variation (COV) in the storage and complex moduli increased with the magnitude of quasi-static load. At the largest indentation load (1000 μN) the average COV was approximately 0.25 in the peritubular cuff and 0.2 in the intertubular dentin. However, for the loss modulus and $\tan\delta$ responses the COV decreased with increasing load, and there was no distinct difference between the two constituents. The COV decreased from an average of near 0.4 at 400 μN indentation load to an average of less than 0.2 at the largest load.

Results for the influence of loading frequency on the dynamic mechanical behavior of intertubular and peritubular dentin are presented in Figure 5. The complex, loss, and storage moduli are shown in Figures 5(a) through 5(c), respectively, and the $\tan\delta$ response is shown in Figure 5(d). In general, all four components of the dynamic mechanical behavior underwent an increase in magnitude with loading frequency. When comparing the complex (Figure 5(a)) and storage (Figure 5(c)) moduli for the intertubular and peritubular dentin it is evident that the values obtained for the peritubular dentin are significantly greater. Also, the trends in complex modulus were primarily dominated by the magnitude in storage modulus. Both constituents exhibited similar dependence on the load frequency. For example, the complex modulus for the intertubular dentin increased nearly 20% from approximately 18.5 GPa to 22.0 GPa with increase in dynamic frequency from 2 Hz to 100 Hz. Similarly, the peritubular cuff showed an increase of just over 20% from approximately 29.3 GPa to 35.0 GPa over the same frequency range. Results of the statistical analysis showed that the changes in storage modulus and complex modulus for the intertubular dentin were not significant ($p > 0.05$) (Tables 2 and 3).

The loss moduli (Figure 5(b)) and $\tan\delta$ responses (Figure 5(d)) for the two constituents increased with increasing dynamic loading frequency, with the largest changes occurring from 2 Hz to 60 Hz. The average loss modulus for the intertubular and peritubular dentin increased by approximately 400% from 0.30 GPa to 1.44 GPa and from 0.40 GPa to 1.83

GPa, respectively. Overall, the responses obtained at 400 μN quasi-static load showed the largest extent of viscous behavior (largest loss modulus and $\tan\delta$) and the largest degree of changes with increasing loading frequency. Statistical comparisons showed that the loss moduli and the $\tan\delta$ responses obtained at the higher indentation loads were significantly lower than those values obtained at 400 μN . Also, the increases in the loss modulus and the $\tan\delta$ responses with dynamic load frequency were significant at all indentation loads (Tables 2 and 3).

The results presented in Figures 4 and 5 provide a means of comparing the magnitude of the moduli for the intertubular and peritubular dentin, but they do not convey the individual trends over the entire parametric space evaluated. Thus, property maps were developed to reveal the relative influence of both static load magnitude and dynamic loading frequency on the responses (Figure 6). The complex modulus for the peritubular and intertubular dentin are shown in Figure 6(a) and 6(b), respectively. Similarly, the loss modulus and the storage modulus for the intertubular dentin are shown in Figure 6(c) and 6(d), and Figure 6(e) and 6(f), respectively. As evident from these figures, the largest complex and storage modulus for the peritubular cuff occurred at the largest indentation load and dynamic frequency. Though the intertubular dentin did not exhibit distinct changes in response with either of the loading parameters, the largest complex and storage moduli resulted from indentations with the lowest load and highest frequency. The parametric dependence of viscous behavior (Figure 6(c) and 6(d)) was nearly identical for the two materials; both showed the largest loss modulus at the lowest load and highest dynamic frequency. Unlike the complex and storage moduli, the loss modulus (Figures 6(c) and (d)) and $\tan\delta$ (Figure 6(g) and (h)) responses were influenced substantially by the both static load and loading frequency.

Scanning mode DMA was performed on the polished dentin sections to distinguish if there are differences in properties over a 30 by 30 μm evaluation window, and if the indents were maintained within the elastic range (including elastic and viscoelastic deformation). Two sets of elastic moduli property maps obtained in scanning mode are shown in Figure 7. Figure 7(a) shows the complex, loss and storage moduli, respectively, for a selected section of coronal dentin where the dentinal tubules were sectioned obliquely. A similar set of scans is shown in Figure 7(b) for another section of dentin where the dentinal tubules were sectioned closer to right angles with the long axis of the tubules. In each set of scans the peritubular cuff is clearly demarcated by the higher complex and storage moduli when compared to the intertubular dentin. The cuffs are also quite evident in the maps of the loss modulus. In both scans the peritubular cuff exhibits a higher loss modulus than the surrounding intertubular dentin.

DISCUSSION

Results obtained for the hardness and reduced elastic modulus of the intertubular and peritubular dentin in quasi-static mode (Figure 2) are well within the range of values reported in previous investigations [Angker and Swain, 2006]. The results are also in close agreement with those of Kinney et al., [1996] who evaluated the reduced elastic modulus of fully dehydrated dentin and reported values of 17.7 GPa to 21.1 GPa for the intertubular region and 29.8 GPa (lower limit) for the peritubular cuff. While the agreement serves as a measure of validation, there are unique differences in the indentation response for these two regions as represented by the indentation load history (Figure 2). The differences are not readily identified by the methods of quantification used in the quasi-static approach.

The measures of dynamic mechanical behavior for the intertubular and peritubular dentin (Figure 4 and Figure 5) provide an alternate assessment of the mechanical behavior of dentin; they are the first to be obtained over a range of evaluation parameter space and with

the tissue maintained in the hydrated condition. The storage and complex moduli for both constituents agree well with the values reported for the elastic moduli in the quasi-static mode [Angker and Swain, 2006]. Earlier, Balooch et al., [2004] used nanoDMA to evaluate dehydrated dentin using quasistatic indentation loads of between 1 and 3 μN and at a dynamic loading frequency of 200 Hz. The storage modulus reported for the intertubular and peritubular dentin was 21 GPa and 48 GPa, respectively, which agrees with those values obtained for the hydrated tissue (e.g. Figure 5(c)), particularly when considering the lower dynamic frequency used in this investigation. Though an average loss modulus was not reported by Balooch et al [2004], it appeared to range between 1 and 3 GPa for the intertubular dentin, which is also consistent with the range obtained for results in the hydrated state (Figure 5(d)). However, the loss modulus obtained for peritubular dentin (Figure 5(d)) in the hydrated condition is between 2 to 3 times greater than that reported earlier (i.e. between 0 to 1 GPa for the cuffs). A separate validation performed with the fused silica standard showed no frequency dependence in the loss modulus estimates. Though unexpected, the difference in these results suggests that hydration is also important to the deformation behavior of the peritubular cuff.

Evaluation of the constituents in the hydrated condition was an important objective. A myriad of difficulties were encountered in attempts to perform the testing with a water bath and water droplets. While single indent evaluations could be performed using either form of hydration, scanning mode DMA was not possible due to evaporation of the water over the period of scanning (approx. 20 min) and the consequent changes in meniscus forces on the tip. The scanning evaluations are conducted at very small indentation forces (max = 6 μN). Thus, even small changes in the indenter forces with water evaporation were reflected as gradients in the property maps due to the strong sensitivity of the moduli to static load (Fig. 4). Attempts to reduce the scan period through modifications to the scanning parameters were not successful as they resulted in loss of details in the property maps. As a result, the focus in controlling moisture content changed to reducing the evaporation rate of the hydrating medium.

Application of the ethylene glycol solution served as an effective alternative to maintain sample hydration due to the very low rate of evaporation [Pashely et al., 2007; Ryou et al., 2011a]. This is a novel and relatively simple approach for retaining the moisture of hard tissues during nanoindentation. A calibration was performed to identify effects of the ethylene glycol treatment on the measured properties other than hydration. In scanning mode evaluations of the fused silica there were no significant differences between properties obtained with and without ethylene glycol treatment (Table 1). These scans were performed under load-control and with the same protocol used for evaluating dentin. As such, the depth of indentation in the fused silica was substantially less than for evaluating dentin, thereby increasing the potential influence of surface treatments on the measured responses. There was no influence of the ethylene glycol on the measured properties (Table 1). Also, scanning showed no trend in the moduli over the scan period (20 min). In evaluation of the polycarbonate calibration sample by discrete indents, the differences in moduli with and without hydration were minor and limited to the loss modulus (Table 1). However, there were significant reductions in all three dynamic moduli obtained in scanning mode DMA of the polycarbonate due to the influence of hydration on the polymer chain network. The reduction in moduli of the polycarbonate with hydration is more substantial in scanning mode than evaluation by discrete indentations. This is due to the small indentation depth in scanning and the complete degree of hydration at the surface. Results obtained with the calibration materials provided confidence that application of ethylene glycol did not change the substrate/indenter physical interaction and maintained hydration over the scan period. Nevertheless, further tests should be performed to distinguish how long the substrate hydration may be maintained using an ethylene glycol treatment.

One concern in examination of the results in Figures 4 and 5 is the magnitude of variation in the dynamic moduli for both the peritubular and intertubular dentin. In the majority of conditions the degree of variation (as quantified by the COV) is less than 0.1. However, in some of the parametric conditions the degree of variation exceeds 0.3. In general, the variation increased with a reduction in the magnitude of the quasi-static component of indentation loading. Low loads increase the potential influence of surface roughness to the response and the degree of noise [Donnelly et al., 2006]. Nanoindentation measurements are inherently more susceptible to variability by virtue of the approach and difficulties encountered, but there are a number of potential sources of contribution to the variation. The current study was conducted with four different teeth and despite care used to maintain consistency the location of sectioning was not necessarily the same. The elastic modulus of peritubular dentin is spatially homogenous, but there are intrinsic spatial variations in the elastic modulus of intertubular dentin (increasing from the pulp to the DEJ) [Kinney et al., 1996; Kinney et al., 2003b]. According to Zheng et al., [2005], age does not appear to be an important factor in the elastic modulus of dentin evaluated by nanoindentation, but small variations in mineral content that arise naturally or from the activity of caries or extremely important. There is a decrease in the mineral content from the deep dentin outward, which is important to the macromechanical response [Ryou et al., 2011b]. There are also spatial variations in the alignment of the mineral crystals in the intertubular dentin, as well as a reduction in the crystal size from the DEJ to the pulp space [Märten et al., 2010], which undoubtedly becomes more important in nanomechanical evaluations. All of these factors are potential contributors to the variation in properties and reduce concerns associated with the unique approach.

The dynamic elastic moduli maps obtained in scanning mode (Figure 7) describe the property distribution of the intertubular and peritubular dentin in a manner that is not obtained from discrete single indents. When comparing the properties obtained from the DMA in both modes of loading, there are some differences. Note that while the average complex and storage moduli obtained in the maps for both the intertubular and peritubular dentin agree well with the values obtained with single indents at 100 Hz, the loss modulus for each region does not. Namely, the loss modulus from scanning mode (Figure 7) is between 3 to 4 times higher than those values obtained from the single indentations. This suggests that while scanning mode evaluations may be desired for visualizing property differences and gradients, the viscous behavior should be used for relative comparisons only and at identical scan parameters. It is important to note here that the maximum indentation load in scanning ($4 \mu\text{N}$ (static) + $2 \mu\text{N}$ (dynamic)) = $6 \mu\text{N}$) is nearly two factors of magnitude smaller than the magnitude of static load occurring during the discrete indentations. According to the trends with indentation load in Figures 6(c) and 6(d) it is clear that the apparent loss modulus of both constituents increases with decreasing load. If the loss modulus distinguishes the dissipative (i.e. viscous) behavior, the collective trend indicates that increasing quasi-static load magnitude exhausts the capacity for viscous deformation in both constituents. A similar trend was noted in dynamic fatigue of bovine dentin evaluated under hydration [Arola and Zheng, 2006] where quasi-static loading applied at low rates exhausted the time-dependent deformation and its contribution to fracture energy. In that study, it was believed attributed to the organic component of the intertubular dentin. Results obtained from this nanoscopic evaluation seem to distinguish that both the intertubular and peritubular dentin have the capacity for viscous deformation in the hydrated state.

Both the intertubular and peritubular dentin exhibited a larger loss modulus under low indentation loads. In an evaluation of polymers, a larger loss modulus is indicative of an increase in viscous deformation that results from the specific molecular configuration [Menard, 2008]. For hydrated dentin this behavior could be associated with the constitutive behavior of the organic component (i.e. collagen), the movement of water through the

microstructure under the stressed volume and/or the potential change in hydrogen bonding that results from the water loss. Dentin could be expected to exhibit poroelastic behavior due to its canulated microstructure and water content; it is composed of nearly 50% collagen and water. On the microscale, the largest contributions to porelastic responses would arise from movement of water in the tubules as noted by Kahler et al [2003] during fracture tests conducted with hydrated specimens. But this is not expected to be a large contribution to the responses obtained by nanoDMA. On the nanoscale there is potential for poroelastic behavior to arise from movement of free water about the collagen fibrils. Local variations in moisture that result from the static component of indentation are important to the adjacent collagen peptide chains of the intertubular dentin due to their potential to initiate intra- and inter-chain links around and within the collagen fibrils. On this scale of indentation there is also a potentially greater relative influence from the bound water on the dynamic response. The complexity of this microstructure provides many possibilities for contributing mechanisms and synergistic effects to the loss modulus.

Unlike the intertubular region, the peritubular cuffs possess a comparatively low protein content (13%) and very little water [Gotliv et al., 2006]. Therefore, the intertubular dentin would be expected to exhibit a greater loss modulus or at least a greater relative capacity for dampening (evident in the $\tan\delta$) due to the large collagen content. The peritubular cuffs should exhibit relatively minimal time-dependent variations and/or hydration dependence due to diminished potential contributions from hydrogen bonding. One could speculate that the viscous component of the peritubular cuff responses was related to “squeezing” of water from about the stressed volume surrounding the indentation, and the contribution of viscous deformation of the collagen network in the surrounding intertubular dentin. But due to the aforementioned differences in water and organic content between intertubular and peritubular dentin, this doesn't seem plausible for the peritubular cuff. The mechanisms responsible for the “dissipative” characteristics of the mineralized cuff are elusive and require further examination.

The absence of significant differences in the storage modulus with parametric conditions (Tables 2 and 3) showed that the elastic component of the intertubular dentin was not affected by the change in static load. Based on the degree of difference in elastic modulus of apatite and collagen, the elastic response is undoubtedly controlled by the mineral content. In contrast to this behavior, the dampening component or viscoelastic component of the intertubular dentin decreased with rise of static load. That implies that the relative viscous behaviors was a function of the indentation magnitude and could be a combination of elastic or plastic (i.e. permanent) deformation. One aspect of the mechanical behavior that was not explored (and that is not directly available from the DMA) was the relative contribution of elastic, plastic and viscous deformation to the total indentation response. The indentation response of mineralized tissues is a function of both elastic and plastic deformation, as well as a time-dependent viscous component [Oyen, 2006]. There are means of extracting these individual components of deformation from the load-indentation depth responses [Oyen and Cook, 2009]. An understanding of these behavioral differences between the intertubular and peritubular dentin could be important to tooth function. Future studies will be required to identify the exact mechanisms contributing to deformation under indentation of hydrated intertubular and peritubular dentin.

CONCLUSIONS

An experimental evaluation of the dynamic mechanical behavior of intertubular and peritubular dentin was performed using a nanoscopic Dynamic Mechanical Analysis (nanoDMA). On the basis of the results obtained, the following conclusions may be drawn:

1. The average storage modulus of intertubular dentin ($18.1 \text{ GPa} < E' < 21.6 \text{ GPa}$) obtained from discrete indentations was significantly less ($p < 0.001$) than that obtained for the peritubular cuff ($28.6 \text{ GPa} < E' < 34.2 \text{ GPa}$) over the range of parameter space examined. There was no significant influence of loading frequency or quasi-static indentation load on the storage modulus of either the intertubular or peritubular dentin.
2. The average loss modulus of the intertubular dentin ($0.30 \text{ GPa} < E'' < 1.44 \text{ GPa}$) was not significantly different ($p > 0.05$) from that obtained for the peritubular cuffs ($0.40 \text{ GPa} < E'' < 1.83 \text{ GPa}$) over the parameter space evaluated. There was a significant increase in the loss modulus with reduction of the indentation load and with increasing loading frequency for both constituents.
3. Results for the scanning mode nanoDMA provided results for the storage modulus that was in agreement with the range of responses obtained from the discrete indentation mode of evaluation. However, the estimated loss modulus obtained from scanning mode was nearly three times greater than that obtained from the indentation approach. The difference in behavior obtained from the two methods appears to be attributed to the dependence of the viscous behavior to the magnitude of quasi-static load.
4. Hydration is an important contributor to the dynamic mechanical behavior of dentin, particularly the loss modulus. Also, a comparison of mechanical behavior between sections of tissue using nanoDMA must be conducted with consistent parameters of evaluation (including the quasi-static and dynamic components), as the interpreted behavior is a large function of measurement protocol.

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APPENDIX

Nanoindentation and DMA Basics

In conventional nanoindentation a quasi-static indent is introduced according to a prescribed load or displacement history. From knowledge of the load, indentation area and a characteristic unloading stiffness the hardness and elastic modulus of the substrate are determined. There are various methods available for quantifying these properties, with the most common approach utilizing the “Oliver and Pharr” or multi-point method of evaluation [Oliver and Pharr, 1992]. One drawback of using a quasi-static approach for characterizing hard tissues is that the response can be a function of the indentation protocol. Specifically, time-dependent deformation occurring during the indentation contributes to the interpreted hardness and the elastic modulus [Oyen and Cook, 2009].

In nanoDMA the properties are measured by superposing a sinusoidal load on the static contact load during an indentation, which is akin to the loading methodology used in conventional DMA [Menard, 2008]. Though the load and displacement signals have the same frequency, dampening imposed by the material under evaluation causes a time delay (or phase difference) between the two signals. The dynamic mechanical response associated with the interaction of the machine, the substrate and its mounting foundation can be modeled analytically using an appropriate combination of spring and dashpot elements. There are various models used in simulating the dynamic aspect of the indenter responses in nanoindentation [Fischer-Cripps, 2004]. For the instrument used in the present study a specific dynamic mechanical model is employed that includes the compliance of the load frame and the tip/specimen interaction [Odegaard et al., 2005]. Using a force balance to describe the dynamic response of the loading frame, indenter and substrate during loading, the contact stiffness (S) is defined by

$$S = \left| \frac{F_0}{z_0} \right| \cos\phi + m\omega^2 - K_s \quad (\text{A1})$$

and the dampening (D_s) behavior is described by

$$\omega D_s = \left| \frac{F_0}{z_0} \right| \sin\phi - \omega D_i \quad (\text{A2})$$

These two quantities define the apparent spring stiffness and dampening coefficients of the contact response, respectively. The quantities F_o and z_o are the force and displacement amplitudes of the sinusoidal load history, ϕ is the phase angle (i.e. offset) between the two signals and ω is the angular frequency. Note that the quantities m , K_s and D_i represent the indenter mass, frame stiffness and the indenter dampening constants, respectively, which can be determined precisely using a calibration procedure [Odegaard et al., 2005].

The stiffness and dampening coefficients identified from the dynamic indentation response (as defined by Eqn's A1 and A2) are used in quantifying characteristic moduli for the material, which represent these two components of mechanical behavior. Specifically, the storage modulus (E') describing the linear elastic response is determined from

$$E' = \frac{S}{2\beta} \sqrt{\frac{\pi}{A}} \quad (A3)$$

and the loss modulus (E'') representing the dampening behavior of the material is estimated by

$$E'' = \frac{\omega D_s}{2\beta} \sqrt{\frac{\pi}{A}} \quad (A4)$$

In these two equations, A is the contact area under the indentation load and β is a shape function for the indenter, which is a known constant for most indenter shapes [Fisher-Cripps, 2004]. Again, the dynamic stiffness (S) and dampening (ωD_s) coefficients are known precisely from the indentation, provided that the measured load and displacement signals are documented and used to estimate the phase angle. For a linear viscoelastic material the storage and loss moduli are combined in terms of a complex elastic modulus (E^*) according to

$$E^* = E' + iE'' \quad (A5)$$

Also, the ratio of the loss and storage moduli can be used to obtain another measure of the dynamic response according to

$$\tan \delta = \frac{E''}{E'} \quad (6)$$

Note from the description of E' and E'' in Eqn's A3 and A4, that $\tan \delta$ is not dependent explicitly on the tip geometry. It provides a relative measure of the viscous response of the tissue.

Figure 1. Details concerning specimen preparation. (a) approximate location of the dentin slice, (b) finished section mounted in resin

a) intertubular dentin. The reduced elastic modulus (E_R) and hardness for the two indentations with 2 second and 20 second hold times are 25.63 GPa and 1.31 GPa and 25.85 GPa and 1.00 GPa, respectively.

b) peritubular dentin. The reduced elastic modulus (E_R) and hardness for the two indentations with 2 second and 20 second hold times are 37.23 GPa and 2.38 GPa, and 31.82 GPa and 2.55 GPa, respectively.

Figure 2.

Load displacement responses for single indents performed using quasistatic indentation. Indentation responses are shown for both intertubular and peritubular dentin and result from indentations performed with 2 sec and 20 sec hold periods.

Figure 3. Single nanoscopic indents within intertubular (I) and peritubular (P) dentin. This image is of the surface topography of dentin obtained using scanning probe microscopy (SPM).

a)

b)

c)

d)

Figure 4.

Influence of static load on the (a) complex modulus, (b) loss modulus, (c) storage modulus, and (d) $\tan \delta$. Significant differences in the moduli with the evaluation parameters are identified in Tables 2 and 3.

a)

b)

c)

d)

Figure 5.

Influence of dynamic loading frequency on the (a) complex modulus, (b) loss modulus, (c) storage modulus, and (d) $\tan\delta$. The overall trend of all moduli shows an increase with higher loading frequency. Significant differences in the moduli with the evaluation parameters are identified in Tables 2 and 3.

a)

b)

c)

d)

e)

f)

g)

h)

Figure 6.

Influence of the evaluation parameters on the (a) complex modulus of peritubular cuff, (b) complex modulus of intertubular dentin, (c) loss modulus of peritubular cuff, (d) loss modulus of intertubular dentin, (e) storage modulus of peritubular cuff, (f) storage modulus of intertubular dentin, (g) $\tan\delta$ of peritubular cuff, (h) $\tan\delta$ of intertubular dentin.

Figure 7.

Property maps of the dynamic elastic moduli obtained from nanoDMA in scanning mode over a 30 μm square window of evaluation. Results from two representative sections of coronal dentin are presented in terms of the Complex (E^*), Loss (E'') and Storage Moduli (E'). a) sample 1 (E^* ; ID = 26.68 ± 8.87 GPa, PD = 41.63 ± 23.55 GPa; E'' ; ID = 9.61 ± 6.65 GPa, PD = 19.70 ± 18.52 GPa; E' ; ID = 20.76 ± 8.05 GPa, PD = 32.21 ± 21.23 GPa). b) sample 2 (E^* ; ID = 23.34 ± 7.84 GPa, PD = 48.12 ± 31.11 GPa; E'' ; ID = 6.83 ± 4.88 GPa, PD = 16.99 ± 17.04 GPa; E' ; ID = 19.48 ± 6.98 GPa, PD = 29.25 ± 15.62 GPa).

Table 1

Results of nanoDMA of calibration samples with and without hydration using ethylene glycol. The measure are presented in terms of the average and (standard deviation). Those measures tethered by brackets are significantly different ($p < 0.05$).

Material/Treatment	Complex (GPa)	Loss (GPa)	Storage (GPa)
Discrete Indent (N=10)			
<u>Polycarbonate</u>			
Dry	2.91 (0.79)	0.18 (0.05)	2.89 (0.78)
Ethy. Glycol	2.55 (0.32)	0.13 (0.03)	2.53 (0.32)
Scanning Mode (N=3)			
<u>Fused Silica</u>			
Dry	70.57 (21.63)	31.72 (16.39)	50.09 (18.89)
Ethy. Glycol	62.19 (19.37)	25.98 (13.90)	45.95 (16.48)
<u>Polycarbonate</u>			
Dry	3.13 (0.52)	0.68 (0.27)	2.81 (0.50)
Ethy. Glycol	1.54 (0.24)	0.18 (0.11)	1.47 (0.21)

Table 2

Statistical analysis performed on average responses for intertubular dentin.

Freq.	Complex Modulus			Loss Modulus			Storage Modulus			Tan delta		
	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd	F	p
2	18.6±3.0 ^a	1.89	.07	.30±0.4 ^a	6.24	.0005	18.1±3.0	1.90	.07	.015±0.02 ^a	5.22	.0005
4	17.9±2.4 ^a			.42±0.4 ^{ab}			17.5±2.4			.024±0.02 ^{ab}		
6	18.3±2.2 ^a			.52±0.4 ^{ab}			17.8±2.2			.030±0.02 ^{abc}		
8	18.8±2.3 ^a			.74±0.6 ^{bc}			18.4±2.2			.038±0.03 ^{abcd}		
10	19.8±3.5 ^a			.86±0.5 ^{cd}			19.1±2.5			.042±0.03 ^{abcd}		
20	19.6±2.6 ^a			.93±0.6 ^{cd}			19.3±3.3			.047±0.03 ^{abcd}		
40	20.1±2.7 ^a			1.06±0.7 ^{bd}			19.6±2.5			.054±0.03 ^{bcd}		
60	21.0±3.3 ^a			1.29±1.3 ^{cd}			20.5±3.1			.061±0.06 ^{cd}		
80	21.0±2.8 ^a			1.34±0.9 ^{cd}			20.4±2.7			.066±0.04 ^d		
100	22.1±3.0 ^a			1.44±0.9 ^d			21.6±2.9			.067±0.04 ^d		
Load	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd	F	p
1000	19.4±2.2 ^{ab}	3.83	.03	.45±.24 ^a	67.7	.0005	19.0±2.2 ^a	2.75	.07	.023±.01 ^a	58.5	.0005
700	18.7±3.6 ^a			.45±.27 ^a			18.4±3.5 ^a			.025±.01 ^a		
400	20.7±2.8 ^b			1.55±.88 ^b			20.0±2.6 ^a			.075±.04 ^b		

* Groups with the same letter are not significantly different

Table 3

Statistical analysis performed on average responses for peritubular dentin.

Freq.	Complex Modulus			Loss Modulus			Storage Modulus			Tan delta		
	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd [*]	F	p
2	29.3±3.6 ^a	2.94	.005	.40±.44 ^{ab}	12.28	.0005	28.6±3.5	3.08	.004	0.12±.02 ^a	12.24	.0005
4	29.2±4.4 ^a			.30±.26 ^a			28.5±4.2			.014±.01 ^a		
6	29.0±3.2 ^a			.58±.50 ^{ab}			28.4±3.1			.018±.02 ^a		
8	29.8±3.5 ^{ab}			.69±.56 ^{abc}			29.1±3.3			.026±.02 ^{ab}		
10	30.2±3.4 ^{ab}			.72±.53 ^{abc}			29.5±3.2			.023±.02 ^{ab}		
20	30.8±3.6 ^{ab}			.99±.80 ^{cd}			30.0±3.5			.031±.03 ^{abc}		
40	32.0±3.9 ^{ab}			1.29±1.10 ^{de}			31.2±3.6			.040±.03 ^{bcd}		
60	31.8±3.6 ^{ab}			1.54±1.27 ^{de}			31.0±4.2			.055±.04 ^d		
80	33.1±4.2 ^{ab}			1.42±1.42 ^{de}			32.4±4.2			.049±.03 ^{cd}		
100	35.0±7.2 ^b			1.83±.80 ^e			34.2±5.5			.053±.03 ^d		
Load		F	p	Mean±Sd	F	p	Mean±Sd	F	p	Mean±Sd	F	p
1000	29.6±5.6 ^a	5.21	.008	.48±.36 ^a	88.24	.005	29.1±5.5	5.92	.004	.016±.01 ^a	105.2	.0005
700	32.8±3.5 ^b			.53±.40 ^a			32.2±3.5			.056±.03 ^a		
400	30.7±2.8 ^{ab}			1.69±.95 ^b			29.8±2.6			.016±.01 ^b		

* Groups with the same letter are not significantly different