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ABSTRACT

It is widely held that the hardness and modulus of dentin increase in proportion to the mineral concentration. To test this belief, we measured hardness and modulus of normal dentin and an altered form of dentin without gap-zone mineralization in wet and dry conditions by AFM nanoindentation to determine if the modulus and hardness scale linearly with mineral concentration. Mineral concentrations in the mid-coronal location of the normal and altered dentins were 44.4 vol% and 30.9 vol%, respectively. Surrounding the pulp of the altered dentin was a region of higher mineralization, 40.5 vol%. The indentation modulus of normal dentin was 23.9 (SD = 1.1) GPa dry and 20.0 (SD = 1.0) GPa wet. In mid-coronal regions of the altered dentin, the indentation modulus was 13.8 (SD = 2.0) GPa dry and 5.7 (SD = 1.4) GPa wet. In the more mineralized regions of the altered dentin, the modulus was 20.4 (SD = 1.8) GPa dry and 5.3 (SD = 0.8) GPa wet; the properties of the altered wet dentin did not correlate with mineral concentration. The results of this study raise doubt as to whether mineral concentration alone is a sufficient endpoint for assessing the success or failure of remineralization approaches in restorative dentistry.

KEY WORDS: dentin, Young's modulus, hardness, mineralization, collagen.

The Importance of Intrafibrillar Mineralization of Collagen on the Mechanical Properties of Dentin

INTRODUCTION

Most naturally mineralizing load-bearing tissues are composed of type I collagen fibrils and a reinforcing nanocrystalline apatite mineral phase. The mineral phase is partitioned between two sites: intrafibrillar mineral, which is confined within or immediately adjacent to the gap zones of the collagen fibrils; and extrafibrillar mineral, which lies within the interstitial spaces separating the fibrils (Landis *et al.*, 1996). The fraction of mineral that is extrafibrillar is not well-established, although small-angle neutron scattering in bone (Bonar *et al.*, 1985) suggests that as much as 70-75% of the mineral may be extrafibrillar. The mechanical consequences of this partitioning are also unknown, although the two phases (extra- and intrafibrillar) are usually modeled as linear elastic (Pidaparti *et al.*, 1996). Accordingly, if ϕ_c , ϕ_{im} , and ϕ_{em} are the volume fractions of collagen, intrafibrillar, and extrafibrillar mineral, respectively, then the Young's modulus of the tissue is approximated by (Pidaparti *et al.*, 1996):

$$E_{11} \approx \{\phi_c a_o + \phi_{im} C_m\} + C_m \phi_{em} \tag{1}$$

In 1, a_o is the elastic stiffness of the unmineralized collagen, and C_m is the elastic stiffness (C_{11}) of the pure hydroxyapatite phase. This model predicts that the elastic stiffness will increase with increasing mineral concentration, implying that the elastic properties of a demineralized collagen scaffold can be restored through remineralization.

The major difficulty with the above analysis is that the model, particularly its treatment of mineral partitioning, has never been rigorously tested. In particular, while it might be relatively straightforward to remineralize the extrafibrillar compartments, restriction of access might make it difficult to remineralize the gap zones within the interior of the collagen fibrils. In the absence of intrafibrillar mineral, Eq. 1 reduces to:

$$E \approx \phi_c a_o + \phi_{em} C_m \tag{2}$$

Thus, the elastic properties of the tissue could be restored by increasing the amount of extrafibrillar mineral, even without the contribution of the intrafibrillar mineral. Unfortunately, it is difficult to conceive of an experiment that could elucidate the separate contributions of the intra- and extrafibrillar mineral.

In an earlier study, we provided evidence that intrafibrillar mineralization may be absent in dentinogenesis imperfecta (DI-II) dentin (Kinney *et al.*, 2001a), and proposed that this altered form of dentin might provide a unique resource for studying the mechanical importance of extrafibrillar mineral in isolation. Regardless of whether these specimens

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reflected the true nature of DI-II, since the population of teeth was so small, no evidence of intrafibrillar mineral was found, yet at the same time the extrafibrillar mineral appeared normal in crystallite size, consistent with earlier findings in DI-II dentin (Kerebel *et al.*, 1981). Therefore, in this study we measured the hardness and indentation Young's modulus of this altered dentin to test the hypothesis that the elastic modulus is linear in the mineral concentration (Eqs. 1 and 2), and to measure the separate contribution of extrafibrillar mineral to the elastic properties of dentin. A positive outcome would justify using the mineral concentration as an endpoint for assessing the success or failure of remineralization approaches in restorative dentistry.

MATERIALS & METHODS

The experiments were conducted in three stages. First, the specimens were imaged intact in their original storage solution. Second, the specimens were cut into thin wafers, dried, and imaged with small-angle x-ray scattering (SAXS) (Kinney *et al.*, 2001a,b). Third, the specimens were polished, rehydrated, and indented with the atomic force microscope (AFM, Nanoscope III Digital Instruments, Santa Barbara, CA, USA). These stages are described below in greater detail.

Specimens

Four unerupted and intact human third molars were obtained from the UCSF Dental Hard Tissue Specimen Bank, and we certify that the use of the teeth conformed to an informed consent protocol approved by the Institutional Review Boards of the University of California-San Francisco, Lawrence Livermore National Laboratory, and Stanford University. The donors were female and ranged in age from 18 to 21 yrs old. The altered dentin specimens came from a single female donor, age 20 yrs, with a clinical diagnosis of dentinogenesis imperfecta type II (DI-II) based on physical evidence (see Table) as well as a family history of this trait and no clinical or familial evidence of osteogenesis imperfecta (OI). The altered specimens were unerupted third molars, and the enamel was largely intact. After extraction, the teeth were sterilized with gamma irradiation. All teeth, both altered and normal controls, were stored under identical conditions in de-ionized water at 4°C.

Synchrotron Radiation Computed Tomography (SRCT)

Prior to performing the indentations, we imaged the teeth whole

with SRCT on beamline 10-2 at the Stanford Synchrotron Radiation Laboratory. SRCT provided a non-invasive quantitative mapping of the mineral concentration in the vicinity of the indentations (Kinney *et al.*, 1994). The x-ray energy was 25.0 keV; image resolution was 17 μm .

The image data were reconstructed into three-dimensional mappings of the x-ray opacity with Fourier-filtered back-projection. The x-ray opacities were converted to mineral concentration by methods described in a previous study (Kinney *et al.*, 2000).

AFM Indentations

After images were made, 1-mm-thick specimens were cut from the teeth about 1 mm superior to the cervical margin, leaving the peripheral enamel intact. After performing small-angle x-ray scattering (Kinney *et al.*, 2001a), we glued the specimens onto a metal disk and polished them with a succession of SiO₂ papers and diamond paste; final polishing was with 0.25- μm paste.

Specimens were studied with the use of an atomic force microscope (Nanoscope III Digital Instruments, Santa Barbara, CA, USA) with the standard head replaced with a Triboscope indenter system (Hysitron Inc., Minneapolis, MN, USA), as described elsewhere (Balooch *et al.*, 1998; Marshall *et al.*, 2001). A liquid cell Berkovich diamond indenter was used for indentation and imaging. Fused silica was used to calibrate the indentation Young's modulus and to define the tip area function for indentation depths between 30 and 240 nm. Loads were adjusted to maintain the indentation depth between 150 and 200 nm, which required a load of 400 μN for normal hydrated and dehydrated dentin, as well as for the dehydrated altered dentin. For the altered dentin in its hydrated state, the load was lowered to 200 μN .

Specimens were first tested in the hydrated state by immersion in de-ionized water in a liquid cell. Subsequently, specimens were dehydrated by air-blowing and dried at room temperature for at least 1 hr prior to being re-tested. From the load-displacement data, the hardness, H, and the indentation Young's modulus, E, were determined (Oliver and Pharr, 1992). A minimum of 25 indentations was performed on the intertubular dentin of each specimen. These 25+ measurements at each location were averaged, and paired two-tailed *t* tests were applied to these averages to test for significant differences between specimens.

RESULTS

The mineral concentration in normal dentin was relatively constant from the DEJ inward about 1 mm (44.4V% SD = 1.6), at which point it began to decrease gradually toward the pulp. In an approximately 0.3-mm layer surrounding the pulp, there was a sudden drop in mineral concentration to 35.0V% (SD = 1.8). The mineral concentration in the middle dentin of the altered teeth was also uniform (30.9V% SD = 1.5); however, in a similar boundary layer surrounding the abbreviated pulp chambers, the mineral density was actually higher (40.5V% SD = 1.9). We labeled this region DI+ to distinguish the region of higher mineralization surrounding the pulp from the middle dentin (DI). It was not established whether the layer surrounding the pulp of the altered dentin was a tertiary dentin (reparative response) or resulted from a change in the formation rate (secondary dentin).

Table. Physical Characteristics Usually Present in the Dentin of Patients with Dentinogenesis Imperfecta Type II (left column after Waltimo *et al.*, 1995) and the Characteristics of the Altered Dentin Used in This Study

Known Characteristics of DI-II Dentin	Presence (+) or Absence (-) in Altered Dentin ^a
Permanent teeth discolored	+
Rapid attrition of permanent teeth	+
Bulbous crowns	+
Obliteration of pulp chambers	+
Dentinal tubules scarce	+
Aberrant orientation of collagen fibrils	Not examined in this study

^a The similarity between the physical characteristics of the altered dentin used in this study with those of known DI-II, combined with the clinical diagnosis from the attending dentist, lead us to believe that the altered dentin used in this study was in fact DI-II.

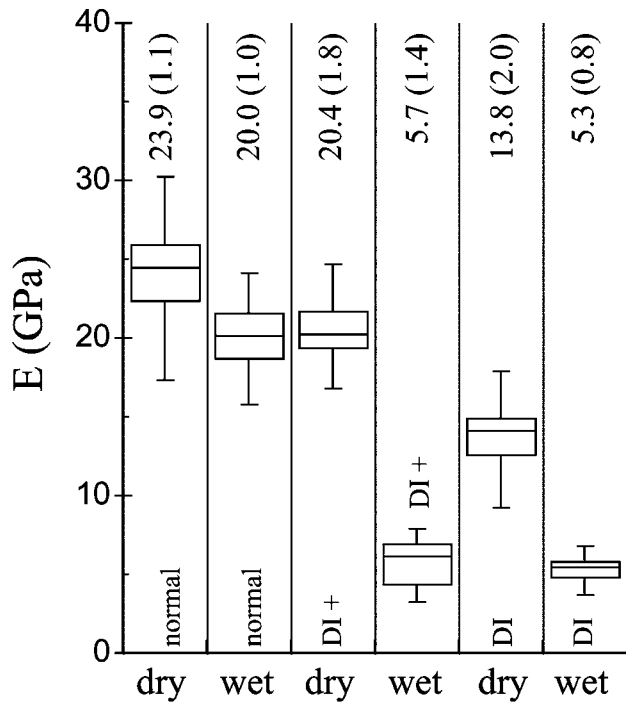


Figure 1. A box plot of the magnitudes of the indentation Young's modulus (pooled from all specimens) in dry and wet normal dentin, the more highly mineralized near-pulp regions of the altered dentin (DI+), and the less-mineralized mid-coronal altered dentin (DI). The box encloses the 50% of the data lying between the upper and lower quartiles, and the solid horizontal bar is the median. All of the data from multiple measurements of the N = 3 teeth are contained within the error bars. The means and standard deviations of the specimen averages in each group are written above the graph (N = 3). The differences between wet and dry were significant ($p < 0.001$). The difference between the wet DI+ and wet DI was not significant.

The indentation Young's modulus of dry normal dentin was 23.9 (SD = 1.1) GPa; the modulus decreased by 15%, or 3.9 GPa, with wetting. This decrease was significant ($p < 0.001$). The indentation Young's modulus of dry DI+ dentin was 20.4 (1.8) GPa; the modulus decreased by nearly a factor of four with wetting. The modulus of the less mineralized altered dentin (DI) was 13.8 (2.0) GPa; the modulus decreased nearly three-fold with wetting. A box plot displaying the results of all indentations is shown in Fig. 1.

The hardness of normal dentin did not change with wetting (0.83-0.85 GPa), whereas the hardness of the altered dentin displayed the same, anomalous, wet/dry behavior as the Young's modulus. A box plot of the hardness data is shown in Fig. 2.

The indentation Young's modulus of the dry dentin was correlated with mineral density ($R^2 = 0.99$ in a linear regression model). This correlation vanished when the same specimens were wet (Fig. 3). A similar behavior was observed in the hardness data ($R^2 = 0.97$ dry).

DISCUSSION

Micromechanics models of mineralized tissues usually have assumed that both the intrafibrillar and extrafibrillar mineral phases are linear elastic at small strains (Pidaparti *et al.*, 1996).

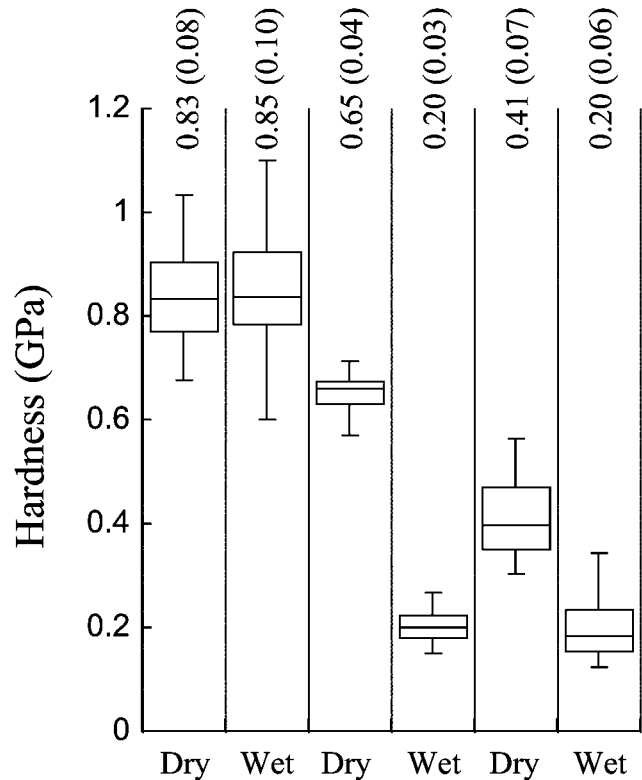


Figure 2. A box plot of the magnitudes of the hardness of the dry and wet dentins. See caption in Fig. 1 for additional explanation.

According to these models, the more abundant extrafibrillar mineral should contribute significantly to the elastic behavior of the tissue. In the absence of intrafibrillar mineralization, the elastic modulus should be linear with the mineral concentration (Eq. 1.2). Indeed, a linear correlation between the Young's modulus and mineral concentration was observed in the dry tissues. However, when the specimens were measured wet, this correlation vanished (Fig. 3), contrary to the prediction of the model. Furthermore, the indentation Young's modulus of the hydrated altered dentin was only a quarter of the magnitude of normal dentin, even though the mineral concentration was between 70% and 90% of normal. The mechanical importance of intrafibrillar mineralization appears to have been greatly underestimated in micromechanics models.

The hardness of normal dentin did not appear to depend on water content. The hardness of the altered dentin, on the other hand, exhibited the same, anomalous, hydration behavior as the Young's modulus. In normal dentin, it appears that the effects of the decreased yield strength must be coupled with the decreased elastic modulus in such a way that the hardness remains constant. This coupling must be broken in the absence of intrafibrillar mineralization.

Since dentin is hydrated in the oral environment, the lack of correlation of elasticity and hardness with mineral density is troubling. Current research is increasingly oriented toward efforts to arrest and re-mineralize caries lesions in dentin with either solution chemistry or more fundamental tissue engineering approaches (Mellberg and Sanchez, 1986; Clarkson and Rafter, 2001; Lynch and Baysan, 2001; ten Cate,

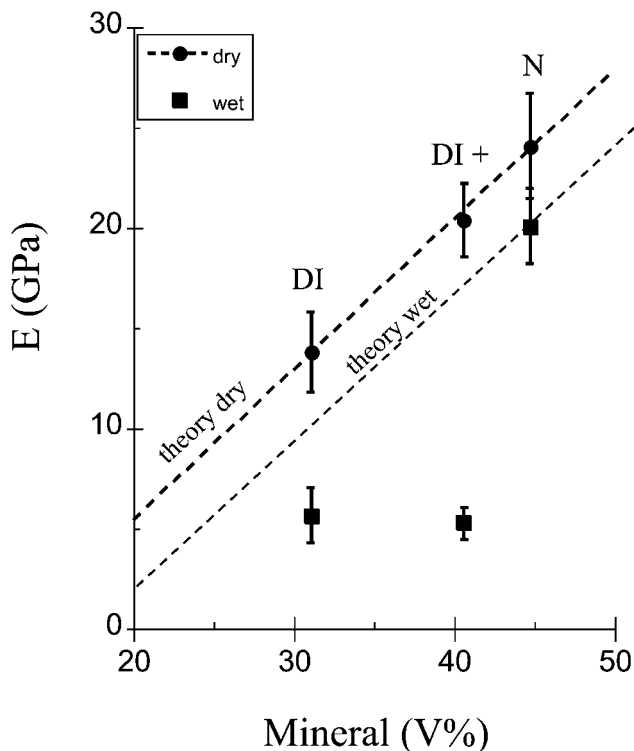


Figure 3. Dry (solid circles) and wet (solid squares) magnitudes of the indentation Young's modulus (E) of normal (N), pulpal region of the altered dentin (DI+), and mid-coronal region of the altered (DI) dentin. The error bars represent the standard deviations in the measured magnitudes ($N = 3$). There was a linear relationship ($R^2 = 0.99$) between the mineral concentration and the Young's modulus in the dry dentins, in agreement with Eq. 1.2. The correlation with mineral concentration vanished when the dentin was hydrated. Similar behavior was observed for hardness.

2001). Though remineralization of enamel shows great potential (Shen *et al.*, 2001; Lagerweij and ten Cate, 2002), the results of this study raise doubt as to whether mineral concentration alone is an appropriate endpoint for assessing the success or failure of these approaches in dentin. It would appear, instead, that a more appropriate endpoint in remineralization studies should be the establishment of mechanical properties consistent with the normal tissue. While in certain circumstances it might be possible to achieve high elastic moduli and abrasion resistance with low concentrations of mineral (Lichtenegger *et al.*, 2002), it now appears that there are circumstances where dentin can display low elastic moduli and abrasion resistance in spite of near-normal (90%) levels of mineral concentration.

To explain these experimental observations, we propose that the traditional approach of treating the extrafibrillar mineral as linear elastic should be reconsidered. We hypothesize instead that the extrafibrillar mineral is granular, and that the contacts between the crystalline grains are highly compliant due to moisture or attached proteins. This unconsolidated granular phase is held together and reinforced by interpenetrating collagen fibrils. The intrafibrillar mineral, which stiffens the collagen fibrils, dominates the elastic behavior under normal loading conditions. With drying,

however, small contractions in the collagen fibrils impose compressive stresses that consolidate the granular matrix. Compression of the granular matrix, which acquires rigidity solely as a result of these shrinkage stresses (Norris and Johnson, 1997), may be responsible for the observed increases in the Young's modulus with drying. In the absence of intrafibrillar mineralization, the elastic constants are determined by the compliant contacts of the extrafibrillar mineral, and are therefore quite low when hydrated. With drying, however, the decrease in the contact compliance, combined with possible compressive stresses induced by the shrinking of the unmineralized collagen fibrils, could account for the observed dependence of the elastic modulus with mineral concentration. In principle, this hypothesis is testable; there should be a measurable pressure dependence of the elastic constants. This test, however, will require more specimens than we can access at this time.

Because all of the altered dentin specimens were from a single patient, we caution against inferring that our results are representative of dentin in all patients with DI-II, even though both clinical and histological findings were consistent with DI-II (Malmgren *et al.*, 1988; Waltimo *et al.*, 1995; Modesto *et al.*, 1996). However, in the altered dentin examined in this study, we were unable to detect intrafibrillar mineral (Kinney *et al.*, 2001a). Therefore, we expect that the absence of intrafibrillar mineralization would lead to similar, anomalous, behavior regardless of the dentin pathology. In addition, since dentin is a naturally hydrated biological composite of collagen and apatite similar to other calcified tissues such as cementum and bone, it will be important to determine if the same findings can be generalized to these other tissues. Further study is warranted.

In summary, we have shown that when intrafibrillar mineralization is absent, the expected linear relationship between mineral concentration and Young's modulus or hardness vanishes. Further research into the coupling between the collagen and mineral phases is warranted.

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