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ABSTRACT

The mineral and organic phases of mineralized dentin contribute co-operatively to its strength and toughness. This study tested the null hypothesis that there is no difference in nano-dynamic mechanical behavior (complex modulus- E^* ; loss modulus- E'' ; storage modulus- E' ; in GPa) of dentin hybrid layers (base-line: E^* , 3.86 ± 0.24 ; E'' , 0.23 ± 0.05 ; E' , 3.85 ± 0.24) created by an etch-and-rinse adhesive in the presence or absence of biomimetic remineralization after *in vitro* aging. Using scanning probe microscopy and nano-dynamic mechanical analysis, we demonstrated that biomimetic remineralization restored the nano-dynamic mechanical behavior of heavily remineralized, resin-sparse regions of dentin hybrid layers (E^* , 19.73 ± 3.85 ; E'' , 8.75 ± 3.97 ; E' , 16.02 ± 2.58) to those of the mineralized dentin base (E^* , 19.20 ± 2.42 ; E'' , 6.57 ± 1.96 ; E' , 17.39 ± 2.0) [$p > 0.05$]. Conversely, those resin-sparse, water-rich regions degraded in the absence of biomimetic remineralization, with significant decline [$p < 0.05$] in their complex and storage moduli (E^* , 0.83 ± 0.35 ; E'' , 0.88 ± 0.24 ; E' , 0.62 ± 0.32). Intrafibrillar apatite deposition preserves the integrity of resin-sparse regions of hybrid layers by restoring their nanomechanical properties to those exhibited by mineralized dentin.

KEY WORDS: biomimetics, dentin bonding, dynamic mechanical behavior, hybrid layer, remineralization, scanning probe microscopy.

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Effect of Biomimetic Remineralization on the Dynamic Nanomechanical Properties of Dentin Hybrid Layers

INTRODUCTION

Controversy on the involvement of amorphous calcium phosphate (ACP) in mineralization of bone and teeth *in vivo* is tempered by the recent identification of transient amorphous precursors in the fin bones of zebrafish (Mahamid *et al.*, 2008) and tooth enamel (Beniash *et al.*, 2009). The purported role contributed by the non-classical crystallization pathway (Xu *et al.*, 2007; Cölfen, 2010) in collagen biomineralization is supported by the discovery of pre-nucleation clusters (Pouget *et al.*, 2009; Dey *et al.*, 2010) that aggregate in the presence of polyanionic molecules into amorphous precursors (Olszta *et al.*, 2007; Deshpande and Beniash, 2008; Nassif *et al.*, 2010), infiltrate collagen fibrils, and transform into intrafibrillar apatite crystallites (Weiner, 2008). When the electrostatic potential energy distribution is modeled along a collagen fibril, positively charged sites are recognized within the fibril that enable it to function as an active biomineralization template (Landis *et al.*, 2006) *via* attraction of negatively charged polyanion-stabilized ACPs (Nudelman *et al.*, 2010).

A biomimetic strategy has been developed that utilizes dual analogs to replicate the ACP sequestration motif and apatite nucleation motif of extracellular matrix proteins to promote hierarchical intrafibrillar apatite assembly within a collagen matrix (Tay and Pashley, 2008). The use of dual biomimetic analogs such as a combination of polycarboxylic acid and polyphosphate to induce hierarchical intrafibrillar mineralization has been further demonstrated in a single-layer reconstituted collagen model (Liu *et al.*, 2011a,b). This biomimetic mineralization strategy has been adopted to remineralize resin-sparse, water-rich zones within hybrid layers created by etch-and-rinse adhesives, to “back-fill” bonding defects *via* intrafibrillar remineralization of denuded collagen fibrils within the hybrid layer (Tay and Pashley, 2009; Kim J *et al.*, 2010) for increasing the durability of resin-dentin bonds (Kim YK *et al.*, 2010a; Liu *et al.*, 2011c).

Hierarchical association between collagen and the mineral phase is a strong determinant of the strength and toughness of mineralized tissues (Jäger and Fratzl, 2000; Gupta *et al.*, 2006). Hence, it is of interest to examine the nanomechanical behavior of dentin hybrid layers remineralized with the aforementioned dual-analog-based biomimetic strategy. Nano-dynamic mechanical analysis (nano-DMA) has been used to probe the nanomechanical properties of human tooth (Balooch *et al.*, 2004) and resin composites (Ilie *et al.*, 2009). The objectives of the present study were to employ nano-DMA to validate the use of nano-DMA

technology on resin-bonded dentin and to test the null hypothesis that there is no difference in dynamic mechanical behavior of dentin hybrid layers created by an etch-and-rinse adhesive in the presence or absence of biomimetic remineralization after *in vitro* aging.

MATERIALS & METHODS

Resin-bonded Dentin

Thirty extracted human third molars were obtained with the donors' informed consent in a protocol approved by the Georgia Health Sciences University. We prepared a 3-mm-thick dentin disk by making parallel cuts perpendicular to the longitudinal axis of each tooth using a low-speed Isomet saw (Buehler, Lake Bluff, IL, USA) under water cooling. Each disk was sectioned transversely to produce a 4-mm-wide slab. The coronal dentin surface was etched with 35% phosphoric acid for 15 sec to create a 5- to 6- μ m-thick layer of completely demineralized collagen on top of the mineralized base. The lack of both extrafibrillar and intrafibrillar mineral crystallites in this layer has previously been confirmed in unstained, non-demineralized transmission electron microscopy sections (Tay and Pashley, 2008).

Two layers of an unfilled etch-and-rinse adhesive (One-Step, Bisco Inc., Schaumburg, IL, USA) were applied to the moist etched dentin, air-dried to allow the adhesive solvent to evaporate, and light-polymerized for 20 sec at 600 mW/cm². A Ca²⁺- and OH⁻-releasing resin composite prepared with set Portland cement powder, fumed silica, and a hydrophilic resin blend of bisphenol A-diglycidylmethacrylate (BisGMA) and hydroxyethyl methacrylate (HEMA) (Kim *et al.*, 2010b) was placed on top of the cured adhesive and light-polymerized for 40 sec.

Each resin-bonded slab in the Experimental group (N = 10) was immersed in 10 mL of simulated body fluid at 37°C. The latter contained (in mM) 205.2 NaCl, 6.3 NaHCO₃, 4.5 KCl, 1.5 K₂HPO₄·3H₂O, 2.25 MgCl₂·6H₂O, 3.75 CaCl₂, and 0.75 Na₂SO₄, with 3.08 NaN₃ added to prevent bacterial growth. It also contained 500 μ g/mL polyacrylic acid (MW 1,800; Sigma-Aldrich, St. Louis, MO, USA) for stabilizing ACP as nanoprecursors and 2.5 wt% sodium tripolyphosphate (Mw 369, Sigma-Aldrich) as an analog of matrix phosphoproteins (pH adjusted to 7.4). Previous work demonstrated that complete remineralization of phosphoric-acid-etched resin-dentin interfaces required 3 mos (Kim *et al.*, 2010a; Liu *et al.*, 2011c). The remineralization medium was changed weekly during the initial 3-mo remineralization period and was substituted with SBF during the subsequent 6-mo aging period. The 6-mo period was based on the report by Hebling *et al.* (2005) that hybrid layers degraded significantly *in vivo* after 6 mos of bonding. For the Control group (N = 10), slabs were incubated at 37°C in SBF only (pH adjusted to 7.4), which was changed weekly during the same period. On retrieval, each slab was sectioned longitudinally into 2 halves, one for scanning probe microscopy (SPM) and nano-DMA and the other for TEM.

SPM and Nano-DMA

Each specimen half was cold-mounted in Epofix epoxy resin (Struers, Cleveland, OH, USA) and polished with diamond particle suspensions (Buehler) of sizes 9, 3, and 0.04 μ m to produce a highly polished resin-dentin interface with a surface roughness of less than 50 nm Root-Mean-Square. The surface quality of the

polished surface and the flatness of the mineralized specimens were verified from estimates of surface roughness by means of topography maps generated during SPM. The latter was performed with a Triboindenter (Model TI 900, Hysitron, Minneapolis, MN, USA) and a Berkovich diamond indenter with a 100-nm tip radius. Contact load and displacement signals were monitored and used to obtain surface topography and gradient images. These quantities were used to calculate the phase angle and to generate maps of the complex (E^*), loss (E''), and storage moduli (E'). Complex modulus (E^*) measures the total elastic and dampening capacity of a material. The storage modulus represents the elastic behavior of a material. The loss modulus represents the dampening capacity of a material. It is a measure of the loss of elastic energy. A 4- μ N set-point load, a 2- μ N dynamic loading amplitude, and a 100-Hz loading frequency were used, based on a pilot study on indenter calibration optimization of the scanning parameters. We calibrated those scanning parameters by comparing the dynamic material behavior of a light-cured neat resin blend (70% BisGMA, 28.75% HEMA, 0.25% camphorquinone, and 1% ethyl N,N-dimethyl-4-aminobenzoate; Ito *et al.*, 2005) generated by nano-DMA vs. conventional DMA (Appendix I). Specimens were scanned in the hydrated condition by the application of a layer of ethylene glycol over the specimen surface to prevent water evaporation during a typical 25- to 30-min scanning period for a 25 x 25 μ m area of the resin-dentin interface (Appendix II).

For the Baseline group, we used the remaining 10 teeth to prepare bonded slabs in the manner described previously, immersed in SBF for 1 wk to maximize water sorption (Ito *et al.*, 2005), embedded in Epofix, and examined with SPM/nano-DMA to obtain baseline data on the nanomechanical properties of the hybrid layer.

TEM

Half of the specimens were fixed in Karnovsky's fixative, post-fixed in 2% osmium tetroxide, dehydrated in ascending ethanol, and embedded in a TEM-grade epoxy resin. Non-demineralized 90-nm-thick sections were prepared and examined unstained with a JEM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV. The rest of the specimens were completely demineralized in formic acid/sodium formate buffer prior to TEM processing. Sections were stained with 2% uranyl acetate and Reynold's lead citrate prior to TEM examination.

Statistical Analysis

For nano-DMA, 4 scans were performed at different locations along the resin-dentin interface of each specimen. Each scan generated 256 x 256 datapoints (*i.e.*, 65,536 datapoints over a 25 x 25 μ m area) for each of the 3 parameters: complex modulus (E^*), loss modulus (E''), and storage modulus (E'). The datapoints were associated with different Regions Of Interests (ROI: mineralized dentin base, hybrid layer). For each property map, 5 sets of 225 datapoints were used to obtain the median property value for a particular ROI. That is, the 225 datapoints represent 1.47 x 1.47 = 2.15 μ m² of each 25 x 25 = 625 μ m² scan. The datapoints from 5 such non-overlapping squares were obtained for each ROI. Thus, for each nano-DMA parameter, 40 values (10 specimens x 4 scans) were generated for that ROI.

Figure 1. Dynamic material behavior of a representative hybrid layer (between arrowheads) in the Baseline group after the resin-bonded specimen was immersed in simulated body fluid for 7 days to maximize water sorption, without remineralization. The specimen was scanned in the hydrated condition. (A) Property map of the complex modulus as determined by nano-DMA. (B) Corresponding property map of the loss modulus. (C) Corresponding property map of the storage modulus. (D) Unstained TEM image of a specimen similar to the one examined by nano-DMA. R, adhesive resin; T, dental tubules; M, mineralized dentin base.

We analyzed each nano-DMA parameter separately by comparing different ROIs: (1) mineralized intertubular dentin, (2) hybrid layers from baseline specimens, (3) non-degraded regions of hybrid layers from SBF control, (4) degraded regions of hybrid layers from the SBF control, (5) sparsely remineralized regions of hybrid layers from biomimetically remineralized specimens, and (6) heavily remineralized regions of hybrid layers from biomimetically remineralized specimens. Since the normality (Shapiro-Wilk test) and homoscedasticity assumptions (modified Levene test) of the data for each parameter were violated, statistical analysis was performed by Kruskal-Wallis analysis of variance and Dunn's multiple-comparison tests at $\alpha = 0.05$.

RESULTS

The dynamic material behavior of a representative non-remineralized hybrid layer derived from a specimen from the Baseline group after 7 days of water sorption is illustrated in Figs. 1A-1C.

A corresponding TEM image of a similar specimen is shown in Fig. 1D. Property maps depicting the dynamic material behavior of a hybrid layer after biomimetic remineralization (Experimental group) are shown in Figs. 2A-2C. A corresponding TEM image of a specimen similar to that examined by SPM is shown in Fig. 2D. Remineralization occurred predominantly within the resin-sparse, water-rich regions of the hybrid layers. Collagen infiltration by a compliant polymerized resin invariably lowered the overall dynamic mechanical behavior of the sparsely remineralized, resin-rich regions of the hybrid layer. Nevertheless, the dynamic mechanical properties of the heavily mineralized, resin-sparse regions of the hybrid layer approximated those derived from the underlying mineralized intertubular dentin. Property maps and a corresponding TEM image of a specimen from the Control group that was aged in SBF only are shown in Figs. 3A-3D. In the absence of biomimetic analogs, not only did the hybrid layer not mineralize, but also the collagen fibrils in the basal regions were degraded. This resulted in decreases in the complex and storage moduli of those regions.

Fig. 4 represents nano-DMA data derived from different ROIs of the Baseline, Control, and Experimental remineralized specimens. Since there were no differences in the data obtained for mineralized intertubular dentin in the 3 groups, only the results of the

Baseline group were used for comparison [(ROI-1) E^* , 19.20 ± 2.42 ; E'' , 6.57 ± 1.96 ; E' , 17.39 ± 2.0 , in GPa]. For each parameter E^* , E'' , and E' , highly significant differences could be detected among the 6 ROIs [$p < 0.001$]. There was no difference between the dynamic mechanical behavior of baseline hybrid layers which had undergone water sorption [(ROI-2) E^* , 3.86 ± 0.24 ; E'' , 0.23 ± 0.05 ; E' , 3.85 ± 0.24] and that of the non-degraded regions of the hybrid layers in the Control specimens [(ROI-3) E^* , 4.09 ± 0.52 ; E'' , 0.18 ± 0.21 ; E' , 4.08 ± 0.50] [$p > 0.05$]. Conversely, the resin-sparse, degraded regions in the Control specimens exhibited significant decline in their complex modulus and storage modulus [(ROI-4) E^* , 0.83 ± 0.35 ; E'' , 0.88 ± 0.24 ; E' , 0.62 ± 0.32] when compared with the Baseline hybrid layers [ROI-2; $p < 0.05$]. The complex and storage moduli of the Baseline hybrid layers (ROI-2) and the non-degraded regions of the SBF Control hybrid layers (ROI-3) were significantly lower [$p < 0.05$] than those exhibited by the sparsely remineralized regions of the Experimental remineralized hybrid layers [(ROI-5) E^* , 6.13 ± 1.13 ; E'' , 0.98 ± 0.63 ; E' ,

5.95 ± 1.02]. The nano-dynamic mechanical behaviors of heavily remineralized regions of Experimental remineralized hybrid layers [(ROI-6) E^* , 19.73 ± 3.85 ; E'' , 8.75 ± 3.97 ; E' , 16.02 ± 2.58] were not significantly different from those of the mineralized intertubular dentin [ROI-1; $p > 0.05$].

DISCUSSION

Probing biomaterial properties at the nanoscale requires comparatively low indentation loads and the ability to examine materials in a hydrated state (Ebenstein and Pruitt, 2006). The advent of depth-sensing nanoindentation techniques coupled with simultaneous sinusoidal loading eliminates the need to visualize indents and offers the opportunity to measure, apart from *quasi*-static properties, the dynamic material behavior such as the storage and loss moduli (Balooch *et al.*, 2004; Donnelly *et al.*, 2006). At the nanometer level, bone exhibits considerable nanomechanical heterogeneity (Fantner *et al.*, 2005; Tai *et al.*, 2007). Since bone and dentin are ultrastructurally similar at their first 2 levels of hierarchy, nanomechanical heterogeneity is also likely to be present in dentin. Because of the use of adhesive resin to infiltrate a demineralized collagen matrix, heterogeneous remineralization within the hybrid layer was also anticipated. We did not use *quasi*-static nanoindentation in the present study, since only approximately 5 of those approximately placed indents could be placed across a 5- to 6- μ m-thick hybrid layer, which resulted in homogenization of both substrate and biomineralization variations.

The complex modulus of mineralized intertubular dentin is within the range of reduced modulus (16–27 GPa) reported by means of *quasi*-static nanoindentation techniques (Angker and Swain, 2006). The larger variation in individual dynamic properties of the heavily remineralized regions of the remineralized hybrid layers may be attributed to the lowering of those properties by a resin blend with considerably lower storage and loss moduli than those exhibited by mineralized tissues. Acquisition of 256×256 datapoints within a $25 \times 25 \mu\text{m}$ scanning window resulted in a linear resolution of 97.7 nm, which is within the dimensional range of collagen fibrils (*ca.* 50–200 nm in diameter). Appendix III represents high-magnification property maps (15×15 datapoints) taken from regions similar to those depicted in the $1.5 \times 1.5 \mu\text{m}^2$ TEM images of the remineralized hybrid layer and the mineralized intertubular dentin. Using a Lanczos

Figure 2. The effect of biomimetic remineralization on the dynamic material behavior of a representative remineralized hybrid layer (between arrowheads) from the Experimental group after *in vitro* aging in simulated body fluid. The specimen was scanned in the hydrated condition. (A) Property map of the complex modulus as determined by nano-DMA. (B) Corresponding property map of the loss modulus. (C) Corresponding property map of the storage modulus. (D) Unstained TEM image of a specimen similar to the one examined by nano-DMA. R, adhesive resin; T, dentinal tubules; M, mineralized dentin base; s, sparsely mineralized areas corresponding to resin-rich regions of the hybrid layer; h, heavily mineralized areas corresponding to resin-sparse regions of the hybrid layer.

pixel interpolation algorithm to resize the datapoints, we could identify individual mineralized collagen fibrils within the resin-supported collagen matrix. The dynamic mechanical properties of these individual or overlapping fibrils were similar to those present in the underlying mineralized intertubular dentin. We anticipate that adaptation of new nanoscopic scanning hardware will further enhance the resolution of these high-magnification property maps.

Dentin collagen matrices undergo gradual autolytic degradation by endogenous matrix metalloproteinases that are activated during acid demineralization (Mazzoni *et al.*, 2006). This feature enabled us to study the dynamic material behavior of a degraded hybrid layer in the absence of biomimetic remineralization. This is an important control, since successful remineralization of hybrid layers may be attributed to the presence of remnant non-collagenous proteins (NCPs) that function as

Figure 3. Dynamic material behavior and ultrastructure of a representative hybrid layer (between arrowheads) from the Control group that was subjected to *in vitro* aging in simulated body fluid in the absence of biomimetic remineralization. The specimen was scanned in the hydrated condition. Asterisk: Areas with degraded collagen within the hybrid layer. R, adhesive resin; T, dental tubules; M, mineralized tissue base. (A) Property map of the complex modulus as determined by nano-DMA. (B) Corresponding property map of the loss modulus. (C) Corresponding property map of the storage modulus. (D) Uranyl-acetate- and lead-citrate-stained TEM image of a specimen similar to the one examined by nano-DMA, showing basal regions within the hybrid layer that are devoid of collagen (asterisk). DM: laboratory-demineralized dentin.

natural apatite nucleation agents. Absence of remineralization in those basal regions provides evidence that remnant NCPs, if present, were incapable of inducing remineralization of denuded collagen fibrils in the absence of apatite seed crystallites as niduses for epitaxial growth.

Within the limits of the present study, we have to reject the null hypothesis that there is no difference in dynamic mechanical behavior of dentin hybrid layers created by an etch-and-rinse adhesive in the presence or absence of biomimetic remineralization after *in vitro* aging. Sodium polyphosphate has been used for chemical phosphorylation and cross-linking of proteins in food processing and is also a potent calcium-chelating agent. Since collagen fibrils exhibit size-exclusion characteristics (Toroian *et al.*, 2007), it is probably the small molecular mass of this molecule that enables it to infiltrate the intrafibrillar water compartments of the fibrils to function as an intrafibrillar site

orientation agent (Liu *et al.*, 2011a). Complementary use of nano-DMA, SPM, and TEM demonstrates that the present biomimetic remineralization strategy is capable of achieving the nanomechanical heterogeneity present in mineralized dentin. Biomimetic remineralization of the water-rich, resin-sparse regions of a hybrid layer also protects the denuded collagen fibrils from degradation and restores their dynamic material behavior to approximate the stiffness and damping modulus components of the mineralized tissue from which they are derived. Of all specimens examined in the Experimental group, approximately 50% of the spaces occupied by those hybrid layers were heavily remineralized and were located in the less-well-resin-infiltrated middle part of the hybrid layer. Conversely, the surface and base of those hybrid layers, as well as regions adjacent to the dentinal tubules, were only sparsely remineralized, since they were better resin-infiltrated because of anastomosis of the lateral branches of the dental tubules. Our results highlight the biomechanical significance of intrafibrillar collagen mineralization. Absence of intrafibrillar apatite in dentinogenesis imperfecta results in a severe compromise of the *quasi*-static mechanical properties of dentin (Kinney *et al.*, 2001). A recent simulation study with an osteogenesis-imperfecta-affected bone model indicated that alteration of mineral distribution within the mutated tropocollagen lowers the overall strength of the tropocollagen-apatite system (Dubey and Tomar, 2010). The use of

nano-DMA for probing the dynamic mechanical behavior of mineralized tissues at the nanoscale will have implications in understanding the interplay among healthy/alterd collagen, extracellular matrix proteins, and apatite assembly in mineralized tissues associated with these genetic diseases.

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Figure 4. Box plots of the dynamic mechanical properties obtained by nano-DMA from different regions of the baseline specimens, control specimens, and remineralized specimens. **(A)** Complex modulus (E^*). **(B)** Loss modulus (E''). **(C)** Storage modulus (E'). Each box plot represents the minimum, 25th percentile, median, 75th percentile, and maximum of 40 values (10 specimens $\times$ 4 scans). Boxes with the same letters are not statistically significant ($p > 0.05$). Group designations: (1) mineralized intertubular dentin (Baseline group), (2) hybrid layers from baseline water-sorbed specimens that had been stored in simulated body fluid for 7 days (Baseline group), (3) non-degraded regions of hybrid layers from the Control group, (4) degraded regions of hybrid layers from the Control group, (5) sparsely remineralized regions of hybrid layers in specimens from the Experimental group that were subjected to biomimetic remineralization, and (6) heavily remineralized regions of hybrid layers in specimens from the Experimental group that were subjected to biomimetic remineralization.

Weerasinghe for performing conventional dynamic mechanical analysis of the resin blend.

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