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Changes in Stiffness of Resin-infiltrated Demineralized Dentin after Remineralization with a Bottom-up Biomimetic Approach

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Abstract

This study examined changes in elastic modulus, mineral density and ultrastructure of resin-infiltrated dentin after biomimetic remineralization. Sixty demineralized dentin beams were infiltrated with Clearfil Tri-S Bond, One-Step or Prime&Bond NT. They were immersed in simulated body fluid (SBF) for one week to maximize water sorption before determining the baseline elastic moduli. For each adhesive (N=20), half of the beams remained immersed in SBF (control). The rest were immersed in a biomimetic remineralization medium. The elastic moduli were measured weekly for 15 additional weeks. Representative remineralized specimens were evaluated with X-ray microtomography and transmission electron microscopy (TEM). The elastic moduli of control resin-infiltrated dentin remained consistently low, while those immersed in the biomimetic remineralization medium increased by 55–118% after 4-months. X-ray microtomography of the remineralized specimens revealed decreases in mineral density from the beam surface to the beam core that was indicative of external mineral aggregation and internal mineral deposition. Interfibrillar and intrafibrillar remineralization of resin-sparse intertubular dentin were seen under TEM together with remineralized peritubular dentin. Biomimetic remineralization occurs by diffusion of nanoprecursors and biomimetic analogs in completely demineralized resin-infiltrated dentin and proceeds without the contribution of materials released from a mineralized dentin base.

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Keywords

Bio-remineralization; Macro-hybrid layer; Intrafibrillar remineralization; Elastic modulus; Peritubular dentin

1. Introduction

Dental caries continues to be the most prevalent oral disease in spite of remarkable progress achieved in the last half of a century to reduce its prevalence. To date, treatment of dental caries is focused mainly on a surgical model of removing the carious tooth structure followed by replacement with an inert restorative material. It is estimated that half of all resin composite restorations fail within 10 years [1] and replacing them consumes 60% of the dentist's practice time. Composite-dentin bonds are continuously challenged by the harsh mechanical and chemical environments of the oral cavity [2], with the risk of secondary caries being 3.5 times higher in resin composite than in amalgam restorations [3]. Clearly, there is a compelling need to pursue alternative methods to preserve resin-dentin bond integrity and extend the longevity of resin-based restorations.

The consensus that hydrophilic resin monomers are mandatory for dentin bonding results in manufacturers incorporating increasing concentrations of these resin monomers into dentin adhesives. Thus, resin-dentin bonds created by contemporary adhesives are susceptible to degradation via water sorption, hydrolysis of resin ester linkages and activation of endogenous matrix metalloproteinases [4]. Although collagen degradation within imperfect hybrid layers may be postponed by application of chlorhexidine as a matrix metalloproteinase inhibitor [5], a zone of resin-sparse demineralized dentin inadvertently remains that is potentially susceptible to cyclic fatigue during function [6].

Mineralized collagen fibrils stabilized by intrafibrillar and interfibrillar apatites do not degrade over time [7,8]. Thus, remineralization of incompletely resin-infiltrated demineralized collagen matrices appears to be an alternative approach for extending the longevity of resin-dentin bonds. A bottom-up, particle-mediated biomineralization approach [9] based on sequestration of liquid-like amorphous mineral nanoprecursors [10,11] and subsequent fusion of mesocrystalline phases [12,13] has been described. Using two separate biomimetic analogs of noncollagenous proteins (NCPs) [14], Tay and Pashley adopted this approach to achieve remineralization of acid-etched dentin [15] and resin-dentin interfaces [16,17], with evidence of intrafibrillar and interfibrillar apatite deposition. These findings provided valuable information on the ultrastructure of interfacial components such as hybrid layers, but not on the mechanical properties of resin-infiltrated dentin. Mineral formation alone is insufficient for determining dentin functionality after remineralization [18]; a more appropriate endpoint to evaluate the effectiveness of dentin remineralization is the re-establishment of mechanical properties that are consistent with those derived from hydrated mineralized dentin [19].

Hybrid layers created by contemporary dentin adhesives are usually less than 10 μm thick. This makes them difficult to be evaluated for changes in mechanical properties without resorting to the use of nanoindentation methods. A macro resin-infiltrated dentin model created from a 200–300 μm thick layer of demineralized dentin has been developed for quantifying collagen matrix shrinkage during dentin bonding [20,21]. It is anticipated that elastic modulus testing of idealized macro-hybrid layers should provide preliminary data on how biomimetic remineralization affects the mechanical properties of hydrated resin-infiltrated collagen matrices. Thus, the objectives of this study were to evaluate the distribution in flexural modulus and mineral density, and to examine the ultrastructure of resin-infiltrated dentin after biomimetic remineralization. The null hypothesis tested was that there are no differences in

the flexural moduli of water-sorbed macro-models of resin-infiltrated dentin after 4 months of immersion in either a simulated body fluid (SBF) only or in a biomimetic remineralization medium.

2. Materials and methods

2.1 Preparation of macro-hybrid layers

Sixty pieces of a macro model of resin-infiltrated dentin, approximately 7 (length) $\times$ 3 (width) $\times$ 0.3 (depth) mm in dimensions, were prepared from demineralized dentin beams that were infiltrated with Clearfil Tri-S Bond (Kuraray, Medical Inc, Tokyo, Japan), One-Step (Bisco Inc., Schaumburg, IL, USA) or Prime&Bond NT (Dentsply De Trey, Konstanz, Germany) (N = 20). The actual dimensions of these beams were measured to the nearest 0.01 mm using a pair of digital calipers. The dentin beams were derived from sixty noncarious human third molars collected after receiving the patients' informed consent under a protocol approved by the MCG Human Assurance Committee. A 0.32 ± 0.03 mm thick disk of mid-coronal dentin was prepared perpendicular to the longitudinal axis of each tooth with a slow-speed Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. One 7 mm long and 3 mm wide dentin beam was prepared from the center of the dentin disk to ensure that dentinal tubules were oriented parallel to the plane of maximum stress during three-point flexure [22]. The beams were completely demineralized in 0.2 M formic acid/sodium formate (pH = 2.96) containing protease inhibitors to prevent collagen degradation, with the end point of demineralization monitored using digital radiography.

As the macro models of resin-infiltrated dentin (300 μ m) were much thicker than the hybrid layers (0.2–8 μ m thick) created during clinical bonding, a prolonged ethanol replacement protocol was employed to ensure optimal adhesive infiltration into those thick demineralized collagen matrices [21]. Infiltration of the respective adhesive was conducted under vacuum for 60 min under amber laboratory lighting to prevent premature curing of the light-curable dentin adhesives. After polymerization, each beam was polished to expose the resin-infiltrated collagen matrix. The polished beams had a thickness of 0.30 ± 0.03 mm in order to establish a 16:1 span-to-depth ratio to minimize shear and local deformation effects during three-point flexure [23].

It is known that the flexural moduli of hydrophilic dentin adhesives dropped up to 70% after water sorption for 72 h [24]. Thus, the resin-infiltrated dentin beams were immersed in a simulated body fluid (SBF) for one week to maximize water sorption [24] before determining the baseline flexural moduli. The SBF was prepared by dissolving 136.8 mM NaCl, 4.2 mM NaHCO₃, 3.0 mM KCl, 1.0 mM K₂HPO₄·3H₂O, 1.5 mM MgCl₂·6H₂O, 2.5 mM CaCl₂ and 0.5 mM Na₂SO₄ in deionized water [25] and adding 3.08 mM sodium azide to prevent bacterial growth.

2.2 Remineralization medium

White Portland cement (Lehigh Cement Company, Allentown, Pennsylvania, USA) was mixed with deionized water in a water-to-powder ratio of 0.35:1, placed in flexible silicone molds and incubated at 100% relative humidity for one week before use. For the biomimetic remineralization medium, 500 μ g/mL of polyacrylic acid (Mw = 1,800; Sigma-Aldrich, St. Louis, Illinois, USA) and 200 μ g/mL of polyvinylphosphonic acid (Mw = 24,000; Sigma-Aldrich), were added to the SBF as dual biomimetic analogs [15]. The control medium consisted only of SBF.

2.3 Biomimetic Remineralization

For each adhesive, the resin-infiltrated beams were randomly divided into a control subgroup and an experimental subgroup ($N = 10$). Each beam was placed on top of a set Portland cement block (*ca.* 1 g) inside a glass scintillation vial. The latter was filled with 15 mL of SBF for the control or 15 mL of the biomimetic remineralization medium for the experimental specimens. Each capped vial was incubated at 37°C. The media were changed every month, with their pH monitored weekly so that they were above 9.25. This ensured that apatite was formed instead of octacalcium phosphate [26].

2.4 Three-point flexure

After one week of immersion in SBF, the baseline elastic moduli (i.e. flexural moduli) of the resin-infiltrated beams were measured using a miniature three-point bending device [21]. Each beam was centrally loaded under water at 1% strain using a universal testing machine (Vitrodyne V100, Liveco Inc., Burlington, VT, USA) at a crosshead of 1 mm/min. Flexural modulus (FM) was calculated using the following formula: $FM = L^3 F_1 / 4dbh^3$, where L is the supporting span length (mm), F_1 is the load at a convenient point in the straight line portion of the load-displacement curve (N), d is the deflection at load F_1 (mm), b is the width of the test specimen (mm) and h is the thickness of the test specimen (mm).

After immersion in the respective media, the control and experimental specimens were retrieved weekly for further three-point flexure evaluation for 15 additional weeks. The data obtained for the control and the experimental specimens at the end of the four-month period were statistically analyzed using Mann-Whitney rank sum test at $\alpha = 0.05$.

2.5 X-ray microtomography

At the end of the four month period, five experimental specimens from each adhesive were examined with X-ray microtomography for the extent of remineralization within the macro models of resin infiltrated dentin. Mineralized dentin beams and resin-infiltrated dentin beams that had not been subjected to remineralization served as the respective positive and negative controls. Each specimen was fixed perpendicularly in the specimen holder turntable of a micro-CT scanner (SkyScan 1174, SkyScan N.V., Aartselaar, Belgium). Scanning was performed with a spatial resolution of 6 μm at 50 kV and 800 μA , a 0.6° rotation step and 360° rotation. After reconstruction, two-dimensional virtual slices were prepared using the CT-analyser software (Skyscan) and saved in a 256 gray scale format. As the mineral densities of bone and dentin were similar [27,28], the gray scale was converted to bone mineral density (BMD) by calibrating with phantoms of known BMDs. The extent of remineralization was expressed as BMD and compared with the positive and negative controls.

2.6 Fourier transform-infrared spectroscopy (FT-IR)

A Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a diamond attenuated total reflection (ATR) setup was used to collect FT-IR spectra from the demineralized dentin, the polymerized adhesives, the resin-infiltrated dentin beams after one week of immersion in SBF, and after 4 months of biomimetic remineralization. A reference spectrum was also obtained for hydroxyapatite powder (Sigma-Aldrich) using the ATR-IR technique. Spectra were obtained between 400–4000 cm^{-1} at 4 cm^{-1} resolution using 32 scans.

2.7 Transmission electron microscopy (TEM)

After X-ray microtomography and FT-IR spectroscopy, representative specimens were processed for TEM to examine the characteristics of remineralization within the resin-infiltrated dentin. Five control specimens were also processed for TEM according to the protocol reported by Tay and Pashley [15]. Briefly, the specimens were dehydrated in ethanol

(50–100%), immersed in propylene oxide and embedded in epoxy resin. Non-demineralized, 90 nm thick sections were prepared and examined unstained using a JEM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV.

3. Results

3.1 Three-point flexure

Progression increase in the flexural modulus as a result of biomineralization is summarized in Fig. 1. The medians as well as 25–75% quartiles of the flexural moduli (GPa) of resin-infiltrated dentin beams before and after 4 months of biomimetic remineralization are presented in Table I. Before remineralization, the baseline flexural modulus of the resin-infiltrated dentin beams was highest for Clearfil Tri-S Bond (0.63 GPa), followed by Prime&Bond NT (0.62 GPa) and One-Step (0.49 GPa). For all adhesives, the flexural modulus of the control specimens remained low throughout the 4-month period. When specimens prepared from Clearfil Tri-S Bond were immersed in the biomimetic analog-containing remineralization medium, the flexural modulus increased linearly over the first six weeks, reaching a plateau after six weeks (Fig. 1A). There was an overall 92% increase in the flexural modulus (0.63 GPa to 1.21 GPa) after 4 months of remineralization. Similar changes were observed for the One-Step (Fig. 1B) and Prime&Bond NT (Fig. 1C) specimens. Their flexural moduli increased by 118% (0.49 GPa to 1.07 GPa) and 55% (0.62 GPa to 0.96 GPa), respectively. For each adhesive, a highly significant difference was observed between the flexural modulus data in the control and the experimental subgroups after 6 weeks of remineralization ($p < 0.01$).

3.2 X-ray microtomography

Figure 2A is a schematic depicting how the profiles were derived from the voxelized data of the reconstructed images. To examine the extent of remineralization, the BMDs of the experimental beams were compared with the mean BMD data derived from mineralized dentin beams (positive control; BMD = 2.23 g/cm²) and resin-infiltrated demineralized dentin beams (negative control; BMD = 1.06 g/cm²). The mean BMD profiles of the positive and negative controls were used to represent 100% and 0% mineralization, respectively. Since the X-ray microtomography results were similar for the adhesives utilized in this study, the results presented are generic and representative of all the adhesives. The BMD profile of a Clearfil Tri-S Bond specimen is illustrated in Fig. 2B. The two dotted vertical lines “A” represent the two external surfaces of the 0.324 mm thick (i.e. 492–168 = 324 μm) specimen. As there was a crust of heavy mineral deposits on the beam surface (see Fig. 4A), the edge of the beam was arbitrarily taken as a point that was 6 μm internal to the peak density recorded on either side of the beam. There was an overall decrease in mineral density from the beam surface to the center. Nevertheless, the partially remineralized dentin was not as mineral-dense as the natural mineralized dentin. Based on the established 0–100% scale, the surface of the resin-infiltrated dentin beam (mean of both sides) was remineralized to 87% of the density of mineralized dentin while the center was only 32%. Likewise, the respective heaviest and sparsest extent of remineralization for the Tri-S Bond experimental specimens were $82.0 \pm 7.1\%$ and $27.2 \pm 6.2\%$, while those for One-Step were $76.0 \pm 12.2\%$ and $26.2 \pm 7.2\%$, and for Prime&Bond NT were $72.2 \pm 5.4\%$ and $17.6 \pm 3.4\%$.

3.3 FT-IR spectroscopy

The three adhesives displayed different IR profiles after polymerization. Nevertheless, they exhibited a similar trend after the resin-infiltrated dentin beams were subjected to 4 months of biomimetic remineralization. Figure 3 represents a series of IR spectra generated from a macro-hybrid layer created by One-Step adhesive. Prior to adhesive infiltration, the completely demineralized dentin beam [spectrum DD] revealed characteristic collagen related bands (amide I, II and III at 1200–1700 cm⁻¹; amide A and B at 2900–3400 cm⁻¹) [29]. The FT-IR

profiles of the polymerized One-Step adhesive and the corresponding resin-infiltrated dentin beam after one week of SBF immersion were depicted in spectra AD and MH, respectively. The amide I-III bands of the demineralized dentin [spectrum DD] were obscured in the MH spectrum by the fingerprint region of the adhesive ($\sim 800\text{--}1700\text{ cm}^{-1}$). The adhesive related bands were reduced in intensity (solid arrows) when compared with those exhibited by the pure adhesive. Likewise, the amide A region of the collagen matrix were also reduced in intensity (open arrow) when compared with that of the adhesive-free demineralized dentin. The broader appearance of this region could have been caused by the convolution of a broad water sorption band ($2500\text{--}3700\text{ cm}^{-1}$) representing the $\nu_1\nu_3\text{OH}$ stretching mode of adsorbed water molecules [30]. The characteristic bands of the One-Step adhesive were absent from the FT-IR spectrum of the remineralized resin-infiltrated dentin beam [spectrum MH-BR]. This was caused by a thin layer of surface mineral deposition that was devoid of the adhesive. The faint amide I and II bands of the remineralized collagen matrix corresponded to those in the demineralized dentin (vertical dotted lines). Distinct amide A and amide B peaks could no longer be discerned. The apatite related PO_4 bands corresponded well with those exhibited by the reference hydroxyapatite reference [spectrum HAP]. The broad phosphate band at $900\text{--}1200\text{ cm}^{-1}$ assigned to the ν_1 and ν_3 stretching modes, and the two peaks at 566 cm^{-1} and 605 cm^{-1} assigned to the ν_4 banding mode of the phosphate group in hydroxyapatite could be clearly discerned.

3.4 TEM

Four month old control specimens of all adhesives exhibited no remineralization except for a surface layer of mineral deposition (not shown). Figure 4 represents a generic overview of the extent of remineralization in superficial parts of a resin-infiltrated dentin beam after four months of biomimetic remineralization. Apart from the superficial mineral deposits, partial remineralization of the resin-infiltrated intertubular dentin could be seen (Fig. 4A). The collagen matrix immediately beneath the beam surface exhibited evidence of both extrafibrillar and intrafibrillar apatite deposition (Fig. 4B). The intrafibrillar apatites demonstrated a highly ordered pattern of mineral deposition that recapitulated the banded apatite arrangement in natural mineralized dentin [31]. Selected area electron diffraction of these crystallites demonstrated ring-like patterns that are characteristic of apatite (inset, Fig. 4B). The intensity of mineral deposition decreased from the beam surface to the beam core (Fig. 4B–4D). Nevertheless, the dimensions of the needles and platelets remained unaltered and evidence of intrafibrillar remineralization could be observed from regions close to the beam core (Fig. 4D).

Control specimens incubated in SBF for 4 months never exhibited any trace of peritubular dentin that was completely dissolved during specimen preparation. Conversely, for the experimental specimens, remineralization of the peritubular dentin could be observed around the periphery of dentinal tubules (Fig. 4A, Fig. 5A and 5C). The layer of remineralized peritubular dentin was not always complete (Fig. 5B). A higher magnification view of junction between the remineralized intertubular and peritubular dentin revealed that the latter consisted of much smaller nanocrystals (*ca.* 5–10 nm) that were continuous with the larger mineral platelets (*ca.* 50 nm) in the intertubular dentin (Fig. 5D).

4. Discussion

In the present study, a macro model consisting of a $300\text{ }\mu\text{m}$ thick layer of resin-infiltrated demineralized dentin was employed to evaluate the extent of biomimetic remineralization that occurs in resin-infiltrated hybrid layers. This model was different from our previous acid-etched dentin model that consisted of a $5\text{--}8\text{ }\mu\text{m}$ thick hybrid layer on top of a mineralized dentin base [16,17]. One reason for adopting a completely demineralized dentin model was to eliminate the ambiguity in differentiating the remineralized apatite crystallites from remnant

apatite seed crystallites present in partially-demineralized dentin. The latter could have contributed to a top-down remineralization mechanism via epitaxial growth of calcium phosphate salts over existing seed crystallites [32,33]. Such a remineralization mechanism would be considerably easier than the bottom-up biomimetic approach employed in the current study. However, in the absence of seed crystallites, the ability of the top-down approach to generate new apatite crystallites with dimensions that approximate those in natural mineralized dentin had never been demonstrated at the ultrastructural level. Another reason for using the completely demineralized dentin model was to ascertain that remineralization of collagen matrices could occur without contribution from noncollagenous proteins (NCPs) and proteoglycans released from mineralized dentin. One might argue that a completely demineralized collagenous matrix could still contain bound NCPs that induced apatite nucleation or growth [34]. However, no remineralization of the collagen matrix could be discerned in the present study when control resin-infiltrated dentin beams were immersed in SBF only. Our results were also supported by a recent study showing that reconstituted collagen fibrils without NCPs exhibited intrafibrillar remineralization when polyaspartic acid was present in the remineralization medium [35].

An alternative measurement technique to the flexural test is the quasistatic and dynamic nanoindentation techniques [36,37]. Nanodynamic assessment of mechanical properties can produce mappings showing the variation in complex, storage and loss moduli across a selected area of interest on the specimen surface, using sinusoidal loads at variable frequencies. It has advantages over the conventional methods for its high resolution of force and accurate indent positioning. However, as the nanoindentation method specifically analyzes the near-surface layer of the specimen, it might be difficult to detect overall changes in the whole dentin specimen. By contrast, three-point flexure testing using macro models of resin-infiltrated dentin can measure overall changes in bulk specimens. Based on results acquired from the present work, by slicing the reconstructed voxelized data obtained from X-ray microradiography, we had been able to examine 6 μm thick virtual slices of the remineralized specimens. One example of the variation in mineral density across the thickness of a specimen is illustrated in Fig. 2. This variation is usually undetectable using conventional microradiography. Apart from the variation in mineral density along the specimen width-thickness plane, we also observed such variation produced in the length-width sectioning plane (not shown). Thus, assigning values to represent the mean quasistatic or dynamic mechanical properties of a bulk specimen may not truly reflect how the variation in mineral distribution influences the nanomechanical properties of the biomimetically-remineralized dentin.

A similar disadvantage in evaluating the chemical composition of bulk specimens could be seen from the FT-IR results. Although we had gently removed the visible mineral crust deposited on the specimen surface with a razor blade prior to FT-IR examination, a thin layer of mineral deposit invariably remained (Fig. 4A). As the penetration depth of the IR radiation is in the order of its wavelength ($< 10 \mu\text{m}$), the diamond-coupled ATR-IR technique only provided spectroscopic information for the surface 10 μm of the specimen. This probably accounted for the detection of relatively weak collagen signals when the remineralized specimen was scanned non-destructively along its surface. Although the IR data did not provide definitive evidence of intrafibrillar remineralization within the collagen matrix, it clearly indicated that the minerals formed were apatites, thereby complementing the selected electron diffraction data obtained from the TEM specimens. In the future, it would be helpful if FT-IR microspectroscopic imaging [38] is available for chemical analysis of the calcium phosphate composition within the entire remineralized specimen.

Of great interest was the observation that apatitic crystallites grew in “resin-bonded collagen fibrils” in a repeated order that is similar to normal mineralized dentin. Although the contribution of intrafibrillar remineralization to the mechanical properties of dentin has been

emphasized [39], it is unknown whether intrafibrillar remineralization alone can recover the elastic modulus of the hydrated collagen fibrils from 0.1 MPa [40] to the 16–27 GPa exhibited by mineralized dentin (see [41] for review). A recent simulation study demonstrated that the overall mechanical properties of a collagen matrix are enhanced [42] when highly organized interfibrillar crystallites are present on the collagen fibrillar surfaces [43]. It has been suggested that mineralization increases the stiffness of collagen fibrils by two mechanisms. Intrafibrillar apatites strengthen the collagen fibrils in tension/compression along the fibril axis and in shear along the plane of the mineral platelets, while interfibrillar minerals further strengthen the fibrils in all remaining deformation modes [42]. Thus, intrafibrillar remineralization is not the only contributor to the biomechanical properties of dentin [18]. Moreover, the presence of a remineralization gradient from the specimen surface to the specimen core suggests that infiltration of the polymer-stabilized fluidic nanoprecursors [10] through the resin-infiltrated collagen matrix is a time-dependent, diffusion-controlled process [44]. Although individual collagen fibrils have been shown to remineralize within 16–24 hr [35], it is likely that the rate of remineralization will decrease as diffusion channels become blocked with minerals. It is noteworthy that the ~150 μm wide diffusion gradient (i.e. from either side of the beam surface) in the present study far exceeds the distance in which macromolecules have to traverse in natural mineralization. These macromolecules reach the mineralization front via the odontoblast-predentin interface (i.e. < 30 μm) or directly via the odontoblast process [45]. Under these circumstances, a diffusion gradient would have been minimal or non-existent. This challenging issue should be considered in future designs of biomimetic delivery systems for remineralizing caries-affected dentin which may be more than 100 μm thick [46].

The elastic modulus of hydrated single non-mineralized collagen fibrils has been reported to increase three orders of magnitude from 1.2 MPa to 1.9 GPa after dehydration [47]. Bertassoni *et al.* [18] cautioned that properties obtained from dehydrated dentin matrices may not reflect the true mechanical behaviour of the remineralized tissues under hydrated conditions. This is the rationale for maximizing the water sorption of resin-infiltrated dentin beams prior to flexural modulus measurement, and for flexing those specimens under water. As there were significant differences in the flexural moduli between the control and experimental subgroups in all adhesives, we have to reject the null hypothesis tested. However, it is worth noting that the hydrated, remineralized resin-infiltrated dentin exhibited much lower flexural moduli (1–1.2 GPa) than natural mineralized dentin (16–27 GPa). While the aforementioned remineralization gradient certainly would have contributed to this low increase in beam stiffness, it is prudent to point out that the specimens we examined were infiltrated with hydrophilic resins. Adhesive resins used in dentin bonding have flexural moduli far inferior to that of sound dentin, typically in the range of 2–5 GPa. Rule of mixtures [48] predicts the effective modulus of elasticity (E_{eff}) from the fractional volume (Vol) and the moduli of elasticity (E) of resin and collagen [i.e. $E_{\text{eff}} = (\text{Vol}_{\text{resin}})(E_{\text{resin}}) + (\text{Vol}_{\text{coll}})(E_{\text{coll}}) + (\text{Vol}_{\text{min}})(E_{\text{min}})$]. Similar to the much thinner authentic hybrid layers [49], the elastic moduli of the macro models of these hybrid layers are largely determined by the resins that have much lower stiffness than mineralized dentin [20]. It has been estimated that in mineralized dentin, 50% of the volume of the dentin matrix is occupied by apatitic crystallites, 30 vol% by collagen and 20 vol% is occupied by water [50]. After complete demineralization of the dentin matrix by 37% phosphoric acid during the acid-etch phase of resin bonding, all of the mineral phase (50 vol%) is extracted and replaced by rinse water which, when combined with the 20 vol% of intrinsic water yields 70 vol% water and 30 vol% collagen. During the comonomer infiltration phase of resin bonding, 67–68 vol% of that 70 vol% water is replaced by polymerized resin leaving only 2–3 vol% of residual water within collagen fibrils that did not become enveloped by resin. This 2–3 vol% water is the theoretical volume fraction that is available for remineralization. Because 67–68 vol% became filled with resin, it is no longer available for remineralization. This is why the stiffness of “remineralized” resin-infiltrated dentin is so much closer to resin alone (*ca.* 2 GPa) than to fully mineralized dentin (*ca.* 16 GPa) (Table II). Our

intent was not to restore the original stiffness of resin-infiltrated dentin to that of fully mineralized dentin, but to “backfill” water-filled uninfiltrated demineralized dentin matrices with apatitic mineral to create a near-perfect seal of dentin. Using the rule of mixtures allowed us to estimate the volume fraction of the demineralized dentin matrix that did not become infiltrated with resin during bonding. Note from Table II that this value would be about 2 vol%. Water is known to exert a plasticizing effect on hydrophilic resins. Up to 70% of the original elastic moduli of these resins were lost after three days of water sorption [20]. Thus, even if the collagen matrix is completely remineralized with apatite, it is unrealistic to expect that remineralized hybrid layers can have elastic moduli that fully approximate that of natural mineralized dentin.

Our previous studies on biomimetic remineralization of acid-etched, completely demineralized dentin showed that dentinal tubules within the remineralized intertubular dentin were completely devoid of peritubular dentin. Interestingly, remineralization of peritubular dentin could be identified in the present study. The nanocrystals deposited in peritubular space were much smaller in dimensions, very closely packed and continuous with the larger apatite crystallites in the adjacent intertubular dentin. The organic matrix in peritubular dentin is devoid of a collagen scaffold [51,52] but contains a matrix that is rich in glutamic acid-containing protein and a high-molecular mass calcium-proteolipid-phospholipid-phosphate complex [53]. Presumably, this delicate peritubular dentin organic matrix was disrupted during the creation of thin hybrid layers by agitation of etchants directly on top of the dentin surface. Conversely, this organic matrix could have been preserved during demineralization of the much thicker macro models of resin-infiltrated dentin. As hard tissue mineralization can not occur without an accompanying scaffold, this organic matrix probably serves as a scaffold for the deposition of the smaller crystalline platelets in a brick-and-mortar-like manner within the peritubular space. It is noteworthy that the intratubular contents within the lumen of the dentinal tubules were remineralized with highly oriented crystallites that had the same dimensions as those deposited within the peritubular space (Fig. 5C). They resembled the intratubular apatites found in the transparent zone of carious dentin [54]. This suggests that the formation of peritubular dentin and tubular occlusion in caries-affected dentin may be regulated by a similar biomineralization process. This phenomenon requires further clarification in future remineralisation studies of partially-demineralized caries-affected dentin using the bottom-up biomimetic approach.

5. Conclusions

Within the limits of this study, it may be concluded that mineral-free, resin-infiltrated collagen matrices can be remineralized when the biomimetic analogs polyacrylic acid and polyvinylphosphonic acid are present in the remineralization medium. Both intrafibrillar and interfibrillar remineralization can be observed within the resin-infiltrated, water-permeable collagen matrices. The 0.55–1.18 times increase in the flexural modulus of the water-plasticized resin-infiltrated dentin is modest when compared to that of fully mineralized dentin. This may be partly explained by the presence of water-plasticized adhesive resins that have very low stiffness within the collagen matrix of the experimental specimens, and partly by the presence of a mineral density gradient from the specimen surface to the specimen core. The observation of this remineralization gradient suggests that diffusion of amorphous calcium phosphate nanoprecursors through the polymerized adhesive resin matrix is diffusion controlled. This mineral density gradient is a challenging issue that needs to be considered in the translation of the current liquid-based, proof-of-concept approach into a biomimetic remineralization delivery system for remineralizing thick layers of caries-affected dentin.

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Fig.1. Time-dependent progression changes in the flexural moduli (in GPa) in macro models of resin-infiltrated dentin created by **A.** Clearfil Tri-S Bond; **B.** One-Step and **C.** Prime&Bond NT during 4 months storage in either the biomimetic remineralization medium (solid lines) or the control simulated body fluid medium (dotted lines).

Fig.2.

A. A schematic illustrating how the variation in mineral density across the thickness of a macro-hybrid layer was retrieved from the reconstructed X-ray microtomography data. i) A series of 2-D length-thickness virtual slices were made from the reconstructed voxelized data. Slices containing intact images of the entire beam were stacked in the Z-direction to produce ii) a composite image that represented the mean mineral density of the individual specimens. iii) A rectangular area of the composite image including the entire length of the beam except for its two ends was used to obtain the final mineral density variation through the thickness of the specimen. The information obtained thus represented the variation in mineral density within a volume rather than 2-D information. **B.** Mineral density profile of a representative experimental

specimen from Clearfil Tri-S bond after 4 months of biomimetic remineralization. Decrease in mineral densities from points “A” (beam surface) to “B” (beam subsurface) to “C” (halfway between surface and least demineralized part of the beam) to “D” (least demineralized part of the beam core) revealed a diffusion-controlled remineralization process.

Fig.3.

A representative series of FT-IR spectra of a demineralized dentin beam bonded with One-Step adhesive, followed by 4 months of biomimetic remineralization. DD: demineralized dentin; AD: polymerized adhesive without oxygen inhibition layer; MH: resin-infiltrated demineralized dentin after one week of immersion in simulated body fluid. MH-BR: resin-infiltrated demineralized dentin after four months of biomimetic remineralization; HAP: hydroxyapatite reference.

Fig.4.

Representative TEM images taken from 4-month specimens immersed in the biomimetic remineralization medium. **A.** A low magnification view of a specimen created by Clearfil Tri-S Bond, showing heavy external mineral aggregation (asterisk) along the surface of the resin-infiltrated demineralized dentin. This layer corresponded with the location labeled by “A”s in Fig. 2. Partial remineralization of the underlying intertubular dentin (D) could be seen. T: dentinal tubules. **B.** A high magnification view of Fig. 4A showing the extent of remineralization from a region closed to the surface of the resin-infiltrated demineralized dentin (depicted by the label “B” in Fig. 2). Apatite platelets were deposited in an ordered, overlapping manner (open arrowhead) within the collagen fibrils (i.e. intrafibrillar remineralization). Longer needle-shaped apatites (arrows) were also deposited around the collagen fibrils (i.e. extrafibrillar remineralisation). Inset: selected area electron diffraction of the minerals. **C.** A high magnification view of a less heavily remineralized region located at the midpoint between the surface and the core of the resin-infiltrated demineralized dentin (depicted by label “C” in Fig. 2). Although remineralization was less intense, intrafibrillar remineralization was still evident (open arrowheads). **D.** A high magnification view of the region located in the area

depicted by the label “D” in Fig. 2. Mineral distribution was sparse. Nevertheless, the dimension of the mineral platelets (*ca.* 50 nm) remained unchanged and intrafibrillar remineralization was still evident (open arrowheads).

Fig.5.

Ultrastructural characterization of the remineralized peritubular dentin derived from 4-month specimens immersed in the biomimetic remineralization medium. **A.** A low magnification cross sectional view of a dentinal tubule (T) from a Prime&Bond NT-bonded macro-hybrid layer. P: peritubular dentin; I: intertubular dentin. **B.** A moderately high magnification of Fig. 5A showing the incompletely remineralized layer of peritubular dentin (between open arrows). The surface that approximated the dentinal tubule (T) was more heavily remineralized than the surface that was continuous with the intertubular dentin (I). **C.** A low magnification view of a longitudinal section of a dentinal tubule (T) from a One-Step-bonded specimen. The peritubular dentin (P) was more heavily remineralized than the previous specimen. Some of the tubular contents were also remineralized (arrow). I: intertubular dentin. **D.** A high magnification view of Fig. 5C showing the junction between the remineralized intertubular dentin (I) and peritubular dentin (P). Apatite nanocrystals formed within the peritubular dentin were much smaller (10 nm in the area depicted by the pointer and 5 nm in the region depicted by the arrow) than the mineral platelets (ca. 50 nm; open arrowhead) present in the intertubular dentin.

Table I

Medians and 25–75% quartiles of the flexural moduli (GPa) of the resin-infiltrated dentin beams before and after 4 months of biomimetic remineralization

Adhesive	Before remineralization		After 4 months of remineralization	
	Median	25–75% quartiles	Median	25–75% quartiles
Clearfil Tri-S Bond	0.63	0.59~0.66	1.21	1.15~1.23
One-Step	0.49	0.46~0.52	1.07	1.03~1.10
Prime&Bond NT	0.62	0.61~0.63	0.96	0.95~0.98

Table II
Computed effective moduli of elasticity (E_{eff}) of macro-hybrid layer beams based on the rule of mixtures

	Resin		Collagen		Mineral		E_{eff}^*
	Vol_{resin}	E_{resin}	Vol_{coll}	E_{coll}	Vol_{min}^{***}	E_{min}	
Assuming no mineralization	0.7	2 GPa	0.3	0.001 GPa	0	16 GPa	1.4003 GPa
Assuming 2% mineralization	0.68	2 GPa	0.3	0.001 GPa	0.02	16 GPa	1.6803 GPa
Assuming 4% mineralization	0.66	2 GPa	0.3	0.001 GPa	0.04	16 GPa	1.9603 GPa
Assuming 6% mineralization	0.64	2 GPa	0.3	0.001 GPa	0.06	16 GPa	2.2403 GPa

* $E_{eff} = (Vol_{resin})(E_{resin}) + (Vol_{coll})(E_{coll}) + (Vol_{min})(E_{min})$

** The mineral density is not uniform and varied from a low of 17.6% to a high of 82% of the mineral density of normal mineralized dentin. But mineral density is not volume.