DENTAL MATERIALS XXX (2015) XXX–XXX

Available online at www.sciencedirect.com

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journal homepage: www.intl.elsevierhealth.com/journals/dema

On the stiffness of demineralized dentin matrices

Heonjune Ryou[a], *Gianluca Turco*[b], *Lorenzo Breschi*[b], *Franklin R. Tay*[c,d], *David H. Pashley*[c], *Dwayne Arola*[e,f,*]

[a] *Department of Mechanical Engineering, University of Maryland Baltimore County, Baltimore, MD, USA*
[b] *Department of Biomedicine, Unit of Dental Sciences and Biomaterials, University of Trieste, Trieste, Italy*
[c] *Department of Oral Biology, College of Dental Medicine, Georgia Health Sciences University, Augusta, GA, USA*
[d] *Department of Endodontics, College of Dental Medicine, Georgia Health Sciences University, Augusta, GA, USA*
[e] *Department of Materials Science and Engineering, University of Washington, Seattle, WA, USA*
[f] *Department of Restorative Dentistry and Department of Oral Health Sciences, School of Dentistry, University of Washington, Seattle, WA, USA*

ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form
17 November 2015
Accepted 30 November 2015
Available online xxx

Keywords:
Collagen
Crosslinking
Dentin bonding agents
Durability
EDC
Endogenous proteinases
Stiffness

ABSTRACT

Resin bonding to dentin requires the use of self-etching primers or acid etching to decalcify the surface and expose a layer of collagen fibrils of the dentin matrix. Acid-etching reduces the stiffness of demineralized dentin from approximately 19 GPa–1 MPa, requiring that it floats in water to prevent it from collapsing during bonding procedures. Several publications show that crosslinking agents like gluteraladehyde, carbodiimide or grape seed extract can stiffen collagen and improve resin–dentin bond strength.

Objective. The objective was to assess a new approach for evaluating the changes in stiffness of decalcified dentin by polar solvents and a collagen cross-linker.

Methods. Fully demineralized dentin beams and sections of etched coronal dentin were subjected to indentation loading using a cylindrical flat indenter in water, and after treatment with ethanol or ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The stiffness was measured as a function of strain and as a function of loading rate from 1 to 50 μm/s.

Results. At a strain of 0.25% the elastic modulus of the fully demineralized dentin was approximately 0.20 MPa. It increased to over 0.90 MPa at strains of 1%. Exposure to ethanol caused an increase in elastic modulus of up to four times. Increasing the loading rate from 1 to 50 μm/s caused an increase in the apparent modulus of up to three times in both water and ethanol. EDC treatment caused increases in the stiffness in fully demineralized samples and in acid-etched demineralized dentin surfaces *in situ*.

Significance. Changes in the mechanical behavior of demineralized collagen matrices can be measured effectively under hydration via indentation with cylindrical flat indenters. This approach can be used for quantifying the effects of bonding treatments on the properties of decalcified dentin after acid etching, as well as to follow the loss of stiffness over time due to enzymatic degradation.

* *Corresponding author at*: Department of Materials Science and Engineering, University of Washington, Roberts Hall, 333, Box 352120, Seattle, WA 98195-2120, USA. Tel.: +1 206 685 8158; fax: +1 206 543 3100.
E-mail address: darola@uw.edu (D. Arola).

http://dx.doi.org/10.1016/j.dental.2015.11.029
0109-5641/

1. Introduction

Mineralized dentin contains about 50% mineral by volume. If it is acid-etched with phosphoric acid in preparation for resin–dentin bonding, approximately the top 10 μm of mineralized dentin is completely demineralized [1]. After rinsing off the excess acid, the rinse water extracts the solubilized mineral and leaves the insoluble collagen fibrils floating in approximately 70 vol% water. The goal in adhesive dentistry is to replace that volume of water with adhesive resin monomers. However, the monomer concentration is low (3–4 mol/L) when compared to the water concentration (56 mol/L). Consequently, it is impossible for dentists to remove all of the water using solvated comonomer mixtures in the relatively short period (60–90 s) that most dentists generally allot for monomer infiltration.

The presence of excess residual water [2] in demineralized dentin is problematic. Application of bonding blends that contain dimethacrylates like BisGMA, TEGDMA or UDMA that are insoluble in water, undergo phase changes when they encounter that residual water [3,4]. Consequently, some collagen fibrils remain infiltrated by water instead of monomers [5,6]. These collagen fibrils also contain bound endogenous proteases (MMPs and cathepsins) that become uncovered and activated by acid-etching [5–10]. They are hydrolases that use the residual water to slowly destroy the collagen fibrils, which are critical to anchor the adhesive and resin composite to the underlying mineralized dentin.

If the water is removed from demineralized dentin, there is a rapid, spontaneous development of new interpeptide hydrogen bonds between adjacent collagen peptides. Formation of these bonds increases collagen stiffness [11,12] and causes shrinkage [13]. Both changes are rapidly reversible if the collagen is rehydrated because the Hoy's solubility parameter describing the hydrogen bonding cohesive forces (δ_h) is 40 $(J/cm^2)^{1/2}$, which is much greater than that of collagen peptides 14.8 $(J/cm^2)^{1/2}$ [14]. Water preferentially H-bonds to collagen peptides and prevents interpeptide H-bonds from forming, which is how water plasticizes collagen and synthetic polymers [14].

Solvents like acetone and ethanol produce reversible interpeptide hydrogen bonds, which also cause reversible increases in matrix stiffness [11,15,16]. However, proanthocyanidin agents like Grape Seed Extract (GSE) and tannic acid produce covalent cross-links and irreversible increases in matrix stiffness [17–19] and strength [20]. Such cross-linking may also make the dentin too stiff to undergo complete shrinkage if the matrix is inadvertently air-dried. The increase in stiffness of the demineralized dentin reflects the degree of interfibrillar cross-linking achieved within the collagen matrix [21]. Consequently, measurements of the changes in matrix stiffness provide a quantitative means for assessing the degree of cross-linking and the treatment's effectiveness [1,22]. Recently, Liu et al. [23] showed that addition of 2 wt% GSE to 20 vol% phosphoric acid produced an acid-etched dentin matrix that was apparently stabilized against the action of microbial collagenase within 15–20 s. Nevertheless, they did not measure the increase in stiffness of the demineralized matrix caused by the GSE/phosphoric acid mixture. That would require an approach capable of measuring the stiffness of the etched layer of dentin *in situ* with adequate sensitivity.

The changes in stiffness of demineralized dentin after application of cross-linkers or other treatments reflects the degree of intermolecular bonding achieved. Thus, quantifying the changes in matrix stiffness is important. The stiffness of demineralized dentin collagen matrices has been measured using atomic force microscopy [24–26] and flexure methods [11,17–20,27]. While AFM is a potent method for evaluating the stiffness of collagen, it is generally applied to evaluate single fibrils and not the matrix as a continuum [28]. Quantifying the degree of collagen cross-linking requires measures at the meso-scale, not at the fibril level. Existing flexure and tensile testing methods are macro-scale and not capable of measuring the small changes in stiffness of a 1–10 μm layer of demineralized dentin matrix *in situ* that results from application of polar solvents. Therefore, the purpose of this investigation was to examine a new approach for evaluating the stiffness of demineralized dentin collagen matrices. Here we attempt to perform macroscopic "indentations" with a cylindrical flat indenter to measure reversible and irreversible changes in stiffness of demineralized dentin collagen matrices resulting from application of various clinically-relevant treatments. The null hypothesis of the investigation is that macroscopic indentations are not capable of quantifying the stiffness of decalcified layers of dentin resulting from acid etching *in situ*.

2. Materials and methods

2.1. Dentin specimens

Human third molars were obtained from the Oral Surgery Clinics of the Georgia Regents University with signed informed consent using a document approved by the GRU Human Assurance Committee. The teeth came from young patients (18–24 yrs of age) and were examined at receipt under light microscopy for signs of caries or cracks. Those teeth without signs of carious lesions or mechanical degradation were stored under refrigeration (4 °C) until ready for use.

The occlusal enamel and superficial dentin were removed using an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) at right angles to the long axis of the tooth. A second, parallel section was made to create a disk of mid-coronal dentin of either 1.0 or 2.0 mm thick thickness (±0.1 mm). The 2 mm thick disks were further sectioned to prepare beams approximately 3 mm × 2 mm × 5 mm. Three beams and three disks were prepared, all from different teeth. After preparation the samples were stored in 0.9% NaCl containing 0.02% sodium azide to prevent microbial growth.

2.2. Dentin demineralization

To create completely demineralized dentin, single beams or dentin disks were suspended in 10 ml of 10 wt% phosphoric acid (PA) in screw top tubes. Multiple tubes were tumbled at room temperature for 16 h to completely demineralize the specimens after [29]. At the end of demineralization the enamel periphery had been completely removed from the

Please cite this article in press as: Ryou H, et al. On the stiffness of demineralized dentin matrices. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.11.029