10-2020

Removal of Water Binding Proteins from Dentin Increases the Adhesion Strength of Low-Hydrophilicity Dental Resins

Ana Paula Farina
University of Illinois at Chicago

Doglas Cecchin
University of Illinois at Chicago

Cristina M. P. Vidal
University of Illinois at Chicago

Ariene A. Leme-Kraus
University of Illinois at Chicago

Ana K. Bedran-Russo
Marquette University, ana.bedran-russo@marquette.edu

Dental Materials, Vol. 36, No. 10 (October 2020): e302-e308. DOI.

Removal of Water Binding Proteins from Dentin Increases the Adhesion Strength of Low-Hydrophilicity Dental Resins

Ana Paula Farina
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL
Department of Restorative Dentistry, Passo Fundo Dental School, University of Passo Fundo, UPF, Passo Fundo, RS, Brazil
Doglas Cecchin
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL
Department of Restorative Dentistry, Passo Fundo Dental School, University of Passo Fundo, UPF, Passo Fundo, RS, Brazil
Cristina M.P. Vidal
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL,
Ariene Arcas Leme-Kraus
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL
Ana K. Bedran-Russo
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL

Abstract

Objectives

To investigate the role of proteoglycans (PGs) on the physical properties of the dentin matrix and the bond strength of methacrylate resins with varying hydrophilicities.

Methods

Dentin were obtained from crowns of human molars. Enzymatic removal of PGs followed a standard protocol using 1 mg/mL trypsin (Try) for 24 h. Controls were incubated in ammonium bicarbonate buffer. Removal of PGs was assessed by visualization of glycosaminoglycan chains (GAGs) in dentin under transmission electron microscopy (TEM). The dentin matrix swelling ratio was estimated using fully demineralized dentin. Dentin wettability was assessed on wet, dry and re-wetted dentin surfaces through water contact angle measurements. Microtensile bond strength test (TBS) was performed with experimental adhesives containing 6% HEMA (H_6) and 18% HEMA (H_{18}) and a commercial dental adhesive. Data were statistically analyzed using ANOVA and post-hoc tests ($\alpha = 0.05$).

Results

The enzymatic removal of PGs was confirmed by the absence and fragmentation of GAGs. There was statistically significant difference between the swelling ratio of Try-treated and control dentin ($p < 0.001$). Significantly lower contact angle was found for Try-treated on wet and dry dentin ($p < 0.002$). The contact angle on re-wet dentin was not recovered in Try-treated group ($p = 0.9$). Removal of PGs significantly improved the TBS of H_6 (109% higher, $p < 0.001$) and H_{18} (29% higher, $p = 0.002$) when compared to control. The TBS of commercial adhesive was not affected by trypsin treatment ($p = 0.9$).

Significance

Changing the surface energy of dentin by PGs removal improved resin adhesion, likely due to more efficient water displacement, aiding to improved resin infiltration and polymerization.

Keywords

Dentin, Proteoglycans, Wettability, Swelling, Adhesion

1. Introduction

The dentin constitutes the bulk tissue of teeth providing biological shielding to the vascularized pulp and mechanical support to enamel [1]. The extracellular matrix (ECM) comprises approximately 30% of the dentin and includes collagen and non-collagenous components [1], serving as a scaffold for the controlled deposition of hydroxyapatite crystallites (50%) [2]. Small leucine rich proteoglycans (SLRPs), the second most predominant non-collagenous component in dentin, are not only essential for dentin formation, but also the dentin's ultra-structural and biomechanical features. These biomacromolecules are major constituents of mineralized and non-mineralized connective tissues [3]. Proteoglycans (PGs) contain GAGs chains polysaccharides covalently attached to a core protein [4]. Chondroitin-4-sulfate (C4S)/chondroitin-6-sulfate (C6S) are the most predominant glycosaminoglycans (GAGs) in dentin [5]. The high water affinity of GAGs and their tight relationship with the collagen fibrils network [6,7] assures to the PGs the fundamental structural and biomechanical regulatory functions in the dentin matrix.

The dentin ECM is often exposed for the effective usage of man-made reparative/restorative biomaterials [8], such as resin composites. Dental resin-based restorations rely on the infiltration of a hydrophilic/hydrophobic blend of resin monomers into partially demineralized dentin creating a micromechanical interlocking with the exposed collagen network [[9], [10], [11], [12]]. Hydrophilicity and residual water at the adhesive interface interfere with both the resin adhesion and interfacial stability over time [11,12]. Pitfalls leading to proteolytic and hydrolytic degradation of the dentin-resin interface could be minimized if successful infiltration and polymerization of the adhesive resin is attained [13]. Hydrophilic monomers, such as HEMA, improve wettability and infiltration of dental adhesives into moist dentin [14,15]. However, hydrophilic monomers create a less stable hybrid layer [16]. From the substrate perspective, the collagen inter-fibrillar spaces are essential for resin infiltration [17]. The negative charges of GAGs contribute to incomplete water displacement from the demineralized dentin matrix [18], which can impair collagen encapsulation by adhesive monomers [4,19], leading to faster breakdown of the dentin-resin interface.

Despite large evidence of the ultrastructural and mechanical roles of PGs in dentin, there is limited knowledge of the roles these non-collagenous proteins have in the infiltration and interactions of dental resins with the dentin ECM. The aim of this study was to evaluate the effect of PGs on the physical properties of the dentin matrix and the bond strength of experimental adhesive systems with varying hydrophilicity. The investigated null hypotheses were: (1) enzymatic removal of PGs does not affect the swelling and wettability of the dentin matrix, and (2) the dentin-resin bond strength of low-hydrophilicity experimental dental adhesive to dentin will not be affected by the enzymatic removal of PGs.

2. Material and methods

2.1. Substrate selection and protocols for enzymatic removal of proteoglycans

Dentin was obtained from the crown portion of sound extracted human molars, kept frozen for no longer than 2 months after extraction (Institutional Review Board protocol #2011-0312). An enzymatic digestion protocol was selected to remove PGs [20,21], using 1 mg/mL trypsin – Try (TPCK, Sigma-Aldrich, St. Louis, MO) in 0.2 M ammonium bicarbonate buffer (pH 7.5) for 24 h at 37 °C.

2.2. Visualization of PGs-GAGs – transmission electron microscopy (TEM)

Removal of PGs was determined by visualization of GAGs following staining with cupromeronic blue (CB) as published previously [20,21]. Five (5) teeth were pre-fixed in 10% buffered neutral formalin at 4 °C for 24 h. Then, mid-coronal dentin sections (1.5 × 1.5 × 0.5 mm) were obtained using a low-speed diamond saw (series 15LC, Buehler, Lake Bluff, IL, USA) under water irrigation and further fixed in 10% buffered neutral formalin for 3 days at 4 °C. Specimens were demineralized with 10% phosphoric acid (Ricca Chemical Company, Arlington, TX, USA) for 5 h under agitation and thoroughly washed with distilled water. The enzymatic removal of PGs was performed as described above (n = 5). Untreated dentin (control, n = 5) were incubated in 0.2 M ammonium bicarbonate. Specimens were immersed in 0.05% CB diluted in 25 mM NaAc containing 0.1 M $MgCl_2$ and 2.5% glutaraldehyde (pH 5.8) for 10 min (repeated 3 times), followed by immersion in 34 mM sodium tugnstate (pH 9.4) for 30 min. After, specimens were dehydrated in ascending concentration of ethanol (25%, 50% and 75% ethanol for 15 min each; 90% for 30 min; 95% for 30 min twice and 100% for 1 h twice). Specimens were embedded in

LR white resin, sectioned into 70 nm thick sections, and sections were mounted on copper grids. No staining of collagen was performed to facilitate visualization of GAGs staining. Sections were analyzed and imaged in TEM (JEM-1220, JEOL, Peabody, MA) at 80 kV.

2.3. Water content of the dentin matrix – swelling ratio

The mid-coronal dentin of twelve (12) teeth were serially sectioned into 1.7 width × 6.0 length × 0.5 mm thickness specimens with a slow speed diamond saw (series 15LC, Buehler). The specimens were demineralized as described in section 2.2. The enzymatic removal of PGs was performed as described in 2.1. The dry mass of demineralized dentin specimens of each group (n = 12) was measured after 24 h in a desiccator containing anhydrous calcium sulfate. Specimens were then immersed in distilled water for 1 h, kept overnight in 10 M PBS (pH 7.4), excess moisture was blotted dry and weighed immediately (wet mass) [22]. The specimens were then placed in distilled water for 10 min to remove buffer salts, dried in a desiccator and weighed until a constant mass was obtained (no variance above 0.02 mg, approximately 1 h drying). The swelling ratio (Q) was calculated as the ratio between the weight of the swollen (wet mass) specimen to that of the dry specimen [22]. Data were statistically analyzed by one-way Anova and Tukey's post-hoc tests ($\alpha = 0.05$).

2.4. Wettability of the dentin matrix – contact angle method

The wettability of the dentin surface was assessed by measuring the water contact angle. Twenty (20) teeth were divided into two groups (n = 10). The occlusal enamel was removed with #180 silicon carbide paper, followed by #320 and #600 grits under water to simulate a smear-layer covered dentin surface. The wettability was assessed on the dentin surfaces after acid etching with 35% phosphoric acid gel (3M Oral Care, St. Paul, MN, USA) (control) or acid etching + PG removal (Try-treated) under different conditions: wet (dentin kept visually moist as for the wet-bond adhesive technique) and dry (air-dried for 3 min) and re-wet (air-dried for 3 min and re-wetted for 1 min). One drop of water (2 μL) was placed on the dentin surface and the contact angle (θ) was assessed using a standard contact angle goniometer model 200-F4 (Ramé-hart instrument co., Succasunna, NJ, USA). The distance between the tip and the dentin surface was kept constant and all tests were performed under controlled temperature (23 ± 1 °C) and humidity (greater than 30%) [23]. Images were acquired within 10 s and the water contact angle was calculated using the software DropImage (Ramé-hart instrument co.). Data were statistically analyzed by two-way ANOVA and Games-Howell post-hoc tests ($\alpha = 0.05$).

2.5. Resin adhesion to dentin – microtensile bond strength

The dentin-resin adhesion was evaluated using a standard microtensile bond strength test (TBS). Thirty (30) teeth were divided into three groups (n = 10), had their occlusal enamel removed with #180 silicon carbide paper, and flat mid-coronal dentin exposed using #320 and #600 grits under running water. Dentin surfaces were etched with 35% phosphoric acid gel (3M Oral Care) for 30 s, rinsed for 60 s and either treated with trypsin (Try-treated) or not (control), as described in section 2.1. Surfaces were restored with either a low hydrophilicity experimental adhesive (H_6) containing 6% HEMA or a high hydrophilicity experimental adhesive (H_{18}) containing 18% HEMA (composition in Table 1). A commercial adhesive system was used as control for adhesion experiments [Adper Single Bond Plus – SB (3M Oral Care, St. Paul, MN, USA)]. All adhesive systems were applied as recommended for SB. After bonding procedures, a resin composite (Filtek Supreme, 3M Oral Care) was placed in three increments of 2 mm as build-up for testing. Each increment was light polymerized for 40 s. A halogen light-curing

unit operated at 600 mW/cm^2 (Optilux, Demetron; Danbury CT, USA) was used for adhesive and resin composite polymerization. Teeth were stored in distilled water at 37 °C for 24 h and then sectioned longitudinally across the bonded interface with a low-speed diamond saw (Buehler) under water irrigation to obtain specimens of approximately 1.0 mm^2 (± 0.02 mm^2), measured with a digital caliper (Digimatic Caliper, Mitutoyo; Tokyo, Japan). Specimens were mounted onto a Ciucchi's jig and tested at crosshead speed of 0.5 mm/min using EZ-Graph instrument (Shimadzu Co., Tokyo, Japan). TBS was determined and data statistically analyzed by two-way ANOVA and Scheffe's post hoc tests ($\alpha = 0.05$). The fracture pattern was observed under a stereomicroscope at 40× magnification, and classified as adhesive, cohesive or mixed failures [24].

Table 1. Composition and application protocol of the investigated dental adhesive systems.

Adhesive System	**Composition**	**Application protocol**
Adper Single Bond Plus – SB (3M Oral Care)	Bis-GMA, DMA, UDMA, HEMA, CQ, nanofillers, water, ethanol, methacrylate functional copolymer of polyacrylic and polytaconic acids	Leave dentin surface moist Apply three consecutive coats of adhesive Lightly dry to ensure removal of the solvent Light cure for 40 s
H_6 (Experimental adhesive)	41.25% Bis-GMA, 12% TEGDMA, 6% HEMA, 0.6% EDMAB, 0.15% CQ, ethanol	
H_{18} (Experimental adhesive)	41.25% Bis-GMA, 18% HEMA, 0.6% EDMAB, 0.15% CQ, ethanol	

Bis-GMA: 2,2-bis[4-(2-hydroxy-3-methacryloylpropoxy)]-phenyl propane; TEGDMA: triethyleneglycol dimethacrylate; HEMA: 2-hydroxyethyl methacrylate; CQ: camphorquinone; EDMAB: 2-ethyl dimethyl-4-aminobenzoate; GDMA: glycerol dimethacrylate; UDMA: urethane dimethacrylate.

3. Results

Representative TEM images are shown in Fig. 1. A higher density of GAGs was depicted around the intratubular dentin region surrounding the tubules and distributed throughout the intertubular dentin of the control group. No particular orientation of the GAGs was apparent; however their aggregation was evident. GAGs were mostly absent, but also fragmented GAGs could be observed in Try-treated dentin matrix (Fig. 1).

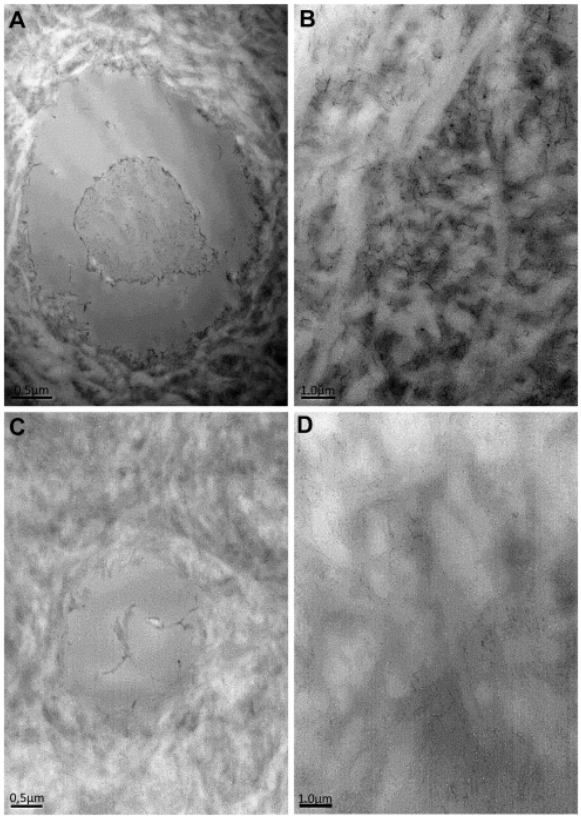

Fig. 1. Representative transmission electron microscopy images of demineralized dentin stained with cupromeronic blue dye only. **A** and **B** depict a low and high magnification of untreated dentin. Glycosaminoglycans chains – GAGs (filaments) are evident throughout the dentin, and more predominantly found surrounding and within the dentin tubules. **C** and **D** depict a low and high magnification images of trypsin-treated dentin. The low image contrast indicates the drastic reduction on GAGs staining as well as the presence of fragmented GAGs. Collagen was not stained, therefore not evident. Low magnification: 45000x; high magnification: 80000x.

The physical properties of the dentin matrix (Fig. 2) were significantly modified by the enzymatic removal of PGs. There was statistically significant increase in the swelling ratio (Fig. 2A) of Try-treated when compared to untreated dentin ($p < 0.001$). The surface wettability results showed statistically significant interactions between groups ($p < 0.001$). Overall dry dentin exhibited lower wettability than wet dentin ($p < 0.001$). Significantly lower water contact angle on wet dentin was observed on Try-treated dentin when compared to control ($p < 0.001$). Likewise, the water contact angle (Fig. 2B) was statistically lower for Try-treated on dry dentin when compared to untreated dentin ($p < 0.001$). There was no statistically significant difference between dentin on re-wetted surfaces. The water contact angle on re-wetted dentin recovered in control, but not in Try-treated group ($p < 0.001$).

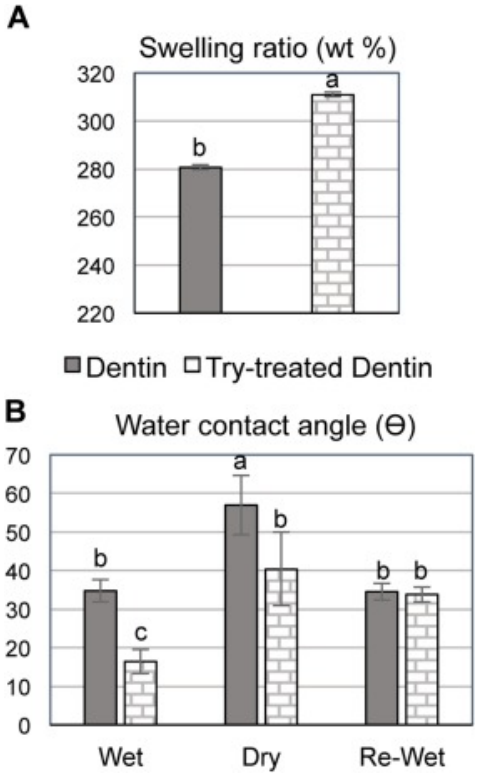

Fig. 2. Results of the (**A**) swelling ratio (weight %) and (**B**) water contact angle. Different lower case letters indicate statistically significant difference ($p < 0.05$) within each method.

There were significant differences ($p < 0.001$) in the dentin-resin TBS among the three dental adhesives and between dentin treatment (try-treated and untreated) (Fig. 3A); as well as a significant interaction between both factors (dentin treatment vs. dental adhesives, $p < 0.001$). The experimental dental adhesives (H_6 and H_{18}) exhibited statistically lower TBS when compared to SB on untreated dentin ($p < 0.05$). The bond strength of SB was not affected by the dentin treatment ($p = 0.98$), however Try treatment significantly affected the TBS of the experimental adhesives ($p < 0.001$). Trypsin treatment of dentin matrix resulted in a significant increase in the TBS of the low hydrophilicity (H_6, $p < 0.001$) and high hydrophilicity experimental adhesives (H_{18}, $p = 0.002$), of 109% and 29%, respectively. The TBS mean values of Try-treated dentin were statistically similar among the three adhesives ($p > 0.05$). Only adhesive and mixed failures were observed for the fractured specimens, with the highest number of adhesive failures found in the H_6 adhesive system in the control group (Fig. 3B).

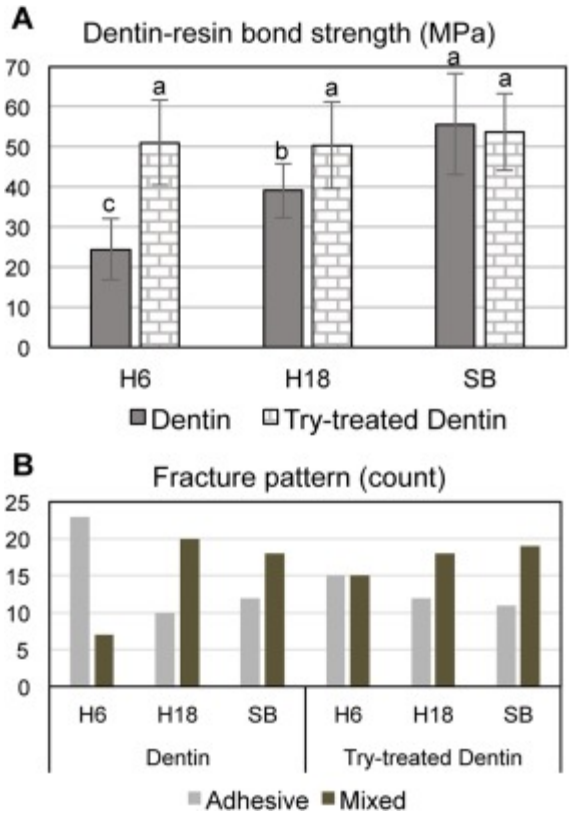

Fig. 3. (**A**) Results of dentin-resin microtensile bond strength (MPa) of untreated dentin (control) and Try-treated dentin. Different lower case letters indicate statistically significant difference ($p < 0.05$) within each method. (B) Results of fracture pattern analysis of debonded specimens for the different groups. Only mixed and adhesive failures were found and the occurrence varied among groups.

4. Discussion

PGs regulate many functional and structural aspects of dentin [1,25], thus unraveling the functional roles of PGs to the dentin matrix could lead to the improvement of restorative therapies. Herein, the enzymatic digestion of PGs by a 24 h Try digestion protocol [20] removed GAGs (Fig. 1), thus enabling the assessment of PG-depleted dentin matrices. The physical properties of the dentin matrix as well as the resin-dentin bond strength of the experimental dental adhesives were significantly affected by Try-treatment, thus both research hypothesis were rejected.

Interactions between PGs/GAGs-collagen are a result of binding of the core protein to collagen fibrils by multiple hydrogen bonds, forming interfibrillar aggregates; while the GAGs interact with one another, bridging fibrils and expanding the collagen network [1]. The electrostatic repulsive forces between GAGs regulate the nano-scale water interactions between PGs and collagen and within collagen fibrils. This system keeps the hydrostatic pressure that results in increased compressive strength, expansibility and sliding of the collagen network [26]. Higher weight variation of Try-treated dentin matrices was observed, suggesting that the PG-depleted matrix is more prone to water imbalance (swelling ratio, Fig. 2A). Increased size of porosities following enzymatic removal of PGs [20,26] may have contributed to the significantly higher mean values of swelling ratio of Try-treated specimens. This finding complements prior observation that PGs regulate the amount of bound and unbound water in the ECM [27], thus their removal results in increased swelling. Hence, this complex

interaction and water dynamics are necessary for the maintenance of structure and biomechanics of hydrated collagenous networks [25,28].

The increased wettability (Fig. 2B), and possibly permeability, of the wet Try-treated group could have occurred due to ionic imbalance between the dentin ECM and water [26], causing altered amounts of bound and unbound water within the collagen matrix in the absence of PGs. Since GAGs are highly hydrophilic molecules and attach to collagen through the PG's core proteins, it is conceivable that these molecules may contribute to the maintenance of collagen fibril architecture by filling the interfibrillar space in the fully hydrated state. The negative charges of GAGs also regulate the fluid flow within the dentin matrix, functioning as an interfibrillar semi-permeable barrier within the tissue [[29], [30], [31]]. Moreover, increased porosity of the matrix has been reported following digestion of non-collagenous molecules using enzymatic treatments [30,31], contributing to the higher wettability observed on the PG-depleted dentin surface. Interestingly, re-wetted Try-treated dentin did not recover the water contact angle values to the wet condition. The findings indicate a structural disorganization of the dentin matrix impairing rapid hydration recovery, as the PGs/GAGs no longer regulate water intake of the collagen network, and consequently increasing the surface energy of the Try-treated dentin matrix.

The experimental adhesives of different hydrophilicities, H_6 and H_{18} (containing 6% and 18% wt. HEMA, respectively), exhibited lower TBS than the commercial brand (Fig. 3A). As expected, resin hydrophilicity played an important role in the adhesion of untreated dentin, where the more hydrophilic adhesive H_{18} achieved higher bond strength than the less hydrophilic H_6. This is due to improved diffusion of the more hydrophilic resin (H_{18}) within the wet, non-treated dentin matrix. HEMA serves as a solvent for other monomers to improve miscibility of hydrophobic and hydrophilic components in adhesive blends [[11], [12], [13], [14], [15]]. However, the increased hydrophilicity promoted by HEMA may result in the entrapment of water molecules within the polymeric chains impairing the stability of the dentin-resin interface [32,33]. Both experimental adhesives (H_6 and H_{18}) experienced significant increase in TBS following Try treatment compared with untreated dentin (109% and 29%, respectively), most remarkably that of the low-hydrophilicity resin (H_6). The findings suggest that the trypsin digestion changed the displacement and evaporation pattern of the bound and unbound water within the dentin matrix, likely improving the diffusion of the less hydrophilic resin; thus contributing to the higher bond strength observed in this group. In fact, Try-treatment affected the wettability and water flow of the dentin matrix in such a way that the hydrophilicity of the adhesive system had no influence on the dentin-resin bond strength. Thus, the increased surface energy of the Try-treated dentin matrix likely improved wettability of both experimental adhesives, regardless of the amount of HEMA. Fracture pattern analysis of dentin-resin interfaces (Fig. 3B) showed higher number of adhesive fractures in control group with H_6, however when applied on a Try-treated dentin surface, the number of mixed and adhesive failures were similar, regardless of the adhesive system.

A commercial adhesive system was used as reference for standard bond strength values, in whichthe resin-dentin bond strength remained similar between control and Try-treated dentin, under the experimental protocols. However, earlier studies have reported that Try-digestion decreased the bond strength to wet dentin surfaces [20,34], including one study [20] using an earlier generation of Adper

Single Bond. The differences in trypsin protocol (48 h vs. 24 h), substrate (human vs. bovine) and formulation of adhesive system could explain the differences in the outcomes among studies.

Besides cleavage of the core protein, resulting in the removal of PGs (core protein + GAGs), Try-digestion removes other non-collagenous components of the ECM [20]. Thus, it is possible that the findings observed here can be also a result of the removal of other ECM components. Given the structural roles of PGs, elucidated by various enzymatic and chemical removal protocols of PGs/GAGs [20,21,[34], [35], [36], [37], [38], [39]], the removal of PGs by Try-digestion is a major cause of changes to the physical properties of the dentin ECM and the outcomes of increased bond strength of the low-hydrophilicity dental adhesive investigated herein. The use of low- rather than high-hydrophilicity resins, will likely improve the stability of the dentin-resin interface. Finally, this investigation does not present Try-digestion as a potential strategy to improve resin-dentin adhesion, rather it provides additional insights for future strategies aiming to improve and facilitate resin adhesion to dentin by inactivation of PGs at the dentin-resin interface.

This study reinforces the role of non-collagenous dentin components in the adhesion of methacrylate resin to the dentin matrix. It showed that a low-hydrophilicity experimental adhesive could bond to Try-treated dentin, resulting in statistically similar bond strength values to a commercially available dental adhesive.

5. Conclusion

Trypsin digestion altered the water balance, surface wettability of the dentin matrix and improved the bond strength of a low hydrophilicity dental adhesive to dentin. The removal of water binding dentin ECM components at the dentin-resin interface may facilitate the diffusion of resin monomers under wet-bond condition and evaporation of bound and unbound water.

Acknowledgments

The study was supported by CAPES Foundation-Brazil [grant BEX 17764/12-2].

References

[1] L.E. Bertassoni, J.P. Orgel, O. Antipova, M.V. Swain. **The dentin organic matrix - limitations of restorative dentistry hidden on the nanometer scale.** *Acta Biomater*, 8 (7) (2012), pp. 2419-2433

[2] P. Dechichi, J.C. Biffi, C.C. Moura, A.W. Ameida. **A model of the early mineralization process of mantle dentin.** *Micron*, 38 (2007), pp. 486-491

[3] M. Goldberg, M. Ono, D. Septier, M. Bonnefoix, T.M. Kilts, Y. Bi, *et al.* **Fibromodulin-deficient mice reveal dual functions for fibromodulin in regulating dental tissue and alveolar bone formation.** *Cells Tissues Organs* (Print), 189 (2009), pp. 198-202

[4] R.V. Iozzo. **Matrix proteoglycans: from molecular design to cellular function.** *Annu Rev Biochem,* 67 (1998), pp. 609-652

[5] R.J. Waddington, R.C. Hall, G. Embery, D.M. Lloyd. **Changing profiles of proteoglycans in the transition of predentine to dentine.** *Matrix Biol,* 22 (2003), pp. 153-161

[6] E. Hedbom, D. Heinegård. **Binding of fibromodulin and decorin to separate sites on fibrillar collagens.** *J Biol Chem,* 268 (1993), pp. 27307-27312

[7] E. Schonherr, H. Hausser, L. Beavan, H. Kresse. **Interaction of biglycan with type I collagen.** *J Biol Chem,* 270 (1995), pp. 2776-2783
[8] J. Perdigão, M. Lopes. **Dentin bonding-questions for the new millennium.** *J Adhes Dent,* 1 (1999), pp. 191-209
[9] H. Sano, T. Takatsu, B. Ciucchi, J.A. Horner, W.G. Matthews, D.H. Pashley. **Nanoleakage: leakage within the hybrid layer.** *Oper Dent,* 20 (1995), pp. 18-25
[10] F.R. Tay, J.A. Gwinnett, K.M. Pang, S.H.Y.J. Wei. **Resin permeation into acid-conditioned, moist, and dry dentin: a paradigm using water-free adhesive primers.** *Dent Res,* 75 (1996), pp. 1034-1044
[11] L. Breschi, A. Mazzoni, A. Ruggeri, M. Cadenaro, R. Di Lenarda, E.S. Dorigo. **Dental adhesion review: aging and stability of the bonded interface.** *Dent Mater,* 24 (2008), pp. 90-101
[12] M. Cadenaro, F. Antoniolli, B. Codan, K. Agee, F.R. Tay, E.S. Dorigo, *et al.* **Influence of different initiators on the degree of conversion of experimental adhesive blends in relation to their hydrophilicity and solvent content.** *Dent Mater,* 26 (2010), pp. 288-294
[13] L. Tjäderhane, F.D. Nascimento, L. Breschi, A. Mazzoni, I.L. Tersariol, S. Geraldeli, *et al.* **Strategies to prevent hydrolytic degradation of the hybrid layer a review.** *Dent Mater,* 29 (2013), pp. 999-1110
[14] F.R. Tay, D. Pashley. **Have dentin adhesives become too hydrophilic?** *J Can Dent Assoc,* 69 (2003), pp. 726-731
[15] L. Hitmi, D. Bouter, M. Degrange. **Influence of drying and HEMA treatment on dentin wettability.** *Dent Mater,* 18 (2002), pp. 503-511
[16] J. De Munck, B. Van Meerbeek, Y. Yoshida, S. Inoue, M. Vargas, K. Suzuki, *et al.* **Four-year water degradation of total-etch adhesives bonded to dentin.** *J Dent Res,* 82 (2003), pp. 136-140
[17] D.H. Pashley, F.R. Tay, R.M. Carvalho, F.A. Rueggeberg, K.A. Agee, M. Carrilho, *et al.* **From dry bonding to water-wet bonding to ethanol-wet bonding. A review of the interactions between dentin matrix and solvated resins using a macro model of the hybrid layer.** *Am J Dent,* 20 (2007), pp. 7-20
[18] K.A. Agee, A. Prakki, T. Abu-Haimed, G.H. Naguib, M.A. Nawareg, A. Tezvergil-Mutluay, *et al.* **Water distribution in dentin matrices: bound vs. Unbound water.** *Dent Mater,* 31 (2015), pp. 205-216
[19] D. Septier, R.C. Hall, D. Lloyd, G. Embery, M. Goldberg. **Quantitative immunohistochemical evidence of a functional gradient of chondroitin 4-sulphate/dermatan sulphate, developmentally regulated in the predentine of rat incisor.** *Histochem J,* 30 (1998), pp. 275-284
[20] A.K. Bedran-Russo, P.N.R. Pereira, W.R. Duarte, K. Okuyama, M.J. Yamauchi. **Removal of dentin matrix proteoglycans by trypsin digestion and its effect on dentin bonding.** *Biomed Mater Res Part B: Appl Biomater,* 85 (2008), pp. 261-266
[21] C.M.P. Vidal, A.A. Leme-Kraus, M. Rahman, A.P. Farina, A.K. Bedran-Russo. **Role of proteoglycans on the biochemical and biomechanical properties of dentin organic matrix.** *Arch Oral Biol,* 82 (2017), pp. 203-208
[22] C.S. Castellan, P.N. Pereira, R.H. Grande, A.K. Bedran-Russo. **Mechanical characterization of proanthocyanidin–dentin matrix interaction.** *Dent Mater,* 26 (2010), pp. 968-973
[23] A.A. Leme, C.M. Vidal, L.S. Hassan, A.K. Bedran-Russo. **Potential role of surface wettability on the long-term stability of dentin bonds after surface biomodification.** *J Biomech,* 48 (2015), pp. 2067-2071

[24] D. Cecchin, A.P. Farina, C.M.P. Vidal, A.K. Bedran-Russo. **A Novel Enamel and Dentin Etching Protocol Using a-hydroxy Glycolic Acid: Surface Property, Etching Pattern, and Bond Strength Studies.** *Oper Dent,* 43 (2018), pp. 101-110

[25] J.E. Scott. **Proteoglycan fibrillar collagen interactions.** *Biochem J,* 52 (1998), pp. 313-323

[26] S.P. Ho, R.M. Sulyanto, S.J. Marshall, G.W. Marshall. **The cementum-dentin junction also contains glycosaminoglycans and collagen fibrils.** *J Struct Biol,* 151 (2005), pp. 69-78

[27] J.E. Scott, A.M. Thomlinson. **The structure of interfibrillar proteoglycan bridges ('shape modules') in extracellular matrix of fibrous connective tissues and their stability in various chemical environments.** *J Anat,* 192 (1998), pp. 391-405

[28] L.E. Bertassoni, M.V. Swain. **The contribution of proteoglycans to the mechanical behavior of mineralized tissues.** *J Mech Behav Biomed Mater,* 38 (2014), pp. 91-104

[29] S. Habelitz, M. Balooch, S.J. Marshall, G. Balooch, S.Jr G.W. Marshall. **In situ atomic force microscopy of partially demineralized human dentin collagen fibrils.** *J Struct Biol,* 138 (2002), pp. 227-236

[30] J.E. Scott. **Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues.** *FASEB J,* 6 (1992), pp. 2639-2645

[31] L. Cardoso, S.C. Cowin. **Role of structural anisotropy of biological tissues in poroelastic wave propagation.** *Mech Mater,* 44 (2012), pp. 174-188

[32] S. Ito, M. Hashimoto, B. Wadgaonkar, N. Svizero, R.M. Carvalho, C. Yiu, *et al..* **Effects of resin hydrophilicity on water sorption and changes in modulus of elasticity.** *Biomaterials*, 26 (2005), pp. 6449-6459

[33] Y. Nishitani, M. Yoshiyama, K. Hosaka, J. Tagami, A. Donnelly, M. Carrilho, *et al.* **Use of Hoy's solubility parameters to predict water sorption/solubility of experimental primers and adhesives.** *Eur J Oral Sci,* 115 (2007), pp. 81-86

[34] P.N. Pereira, A.K. Bedran-de-Castro, W.R. Duarte, M. Yamauchi. **Removal of noncollagenous components affects dentin bonding.** *J Biomed Mater Res B Appl Biomater*, 80 (2007), pp. 86-91

[35] L.E. Bertassoni, M.V. Swain. **Removal of Dentin Non-collagenous Structures Results in the Unraveling of Microfibril Bundles in Collagen Type I.** *Connect Tissue Res,* 58 (2017), pp. 414-423

[36] A. Mazzoni, D.H. Pashley, A. Ruggeri Jr., F. Vita, M. Falconi, R. Di Lenarda, *et al.* **Adhesion to chondroitinase ABC treated dentin.** *J Biomed Mater Res B Appl Biomater*, 86 (2008), pp. 228-236

[37] A.P. Farina, C.M.P. Vidal, D. Cecchin, T.R. Aguiar, A.K. Bedran-Russo. **Structural and biomechanical changes to dentin extracellular matrix following chemical removal of proteoglycans.** *Odontology*, 107 (2019), pp. 316-323

[38] T. Boukpessi, S. Menashi, L. Camoin, J.M. Tencate, M. Goldberg, C. Chaussain-Miller. **The effect of stromelysin-1 (MMP-3) on non-collagenous extracellular matrix proteins of demineralized dentin and the adhesive properties of restorative resins.** *Biomaterials*, 29 (2008), pp. c4367-73

[39] Y. Alania, J. Creighton, L.T. Trevelin, C.A. Zamperini, A.K. Bedran-Russo. **Regional contribution of proteoglycans to the fracture toughness of the dentin extracellular matrix.** *J Biomech*, 101 (2020), Article 109633