Published in final edited form as:

J Dent. 2010 November ; 38(11): 908–915. doi:10.1016/j.jdent.2010.08.004.

Grape seed proanthocyanidins increase collagen biodegradation resistance in the dentin/adhesive interface when included in an adhesive

Bradley Green, Xiaomei Yao, Arindam Ganguly, Changqi Xu, Vladimir Dusevich, Mary P Walker, and Yong Wang

University of Missouri-Kansas City School of Dentistry, Kansas City, MO 64108, USA

Abstract

Objectives—Contemporary methods of dentin bonding could create hybrid layers (HLs) containing voids and exposed, demineralized collagen fibers. Proanthocyanidins (PA) have been shown to crosslink and strengthen demineralized dentin collagen, but their effects on collagen degradation within the HL have not been widely studied. The purpose of this study was to compare the morphological differences of HLs created by BisGMA/HEMA model adhesives with and without the addition of grape seed extract PA under conditions of enzymatic collagen degradation.

Methods—Model adhesives formulated with and without 5% PA were bonded to the acid etched dentin. Five- μ m-thick sections cut from the bonded specimens were stained with Goldner's trichrome. The specimens were then exposed to 0.1% collagenase solution for zero, one, or six days. Following collagenase treatment, the specimens were analyzed with SEM/TEM.

Results—Staining did not reveal a difference in the HLs created with the two adhesives. SEM showed the presence of intact collagen fibrils in all collagenase treatment conditions for specimens bonded with adhesive containing PA. These integral collagen fibrils were not observed in the specimens bonded with adhesive without PA after the same collagenase treatment. TEM confirmed that the specimens containing PA still showed normal collagen fibril organization and dimensions after treatment with collagenase solution. In contrast, disorganized collagen fibrils in the interfacial zone lacked the typical cross-banding of normal collagen after collagenase treatment for specimens without PA.

Conclusions—The presence of grape seed extract PA in dental adhesives may inhibit the biodegradation of unprotected collagen fibrils within the HL.

Keywords

collagen crosslinking; adhesive/dentin interface; proanthocyanidins; bonding durability; collagenase

Corresponding Author: Dr. Yong Wang, University of Missouri-Kansas City, School of Dentistry, 650 E. 25th St., Kansas City, MO 64108, Fax: 816-235-5524, Phone: 816-235-2043, Wangyo@umkc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Contemporary composite restorations rely on adhesive systems to bond the restorative material to the underlying tooth structure. These restorations most often fail due to marginal failures at the gingival floors, suggesting that the tooth/adhesive interface is the weakest area of the restoration¹. When bonding to dentin, it is generally accepted that a high quality interface is achieved when adhesive monomers thoroughly infiltrate and encapsulate exposed collagen fibrils, creating the so-called “hybrid layer”². Although modern adhesive systems generally achieve high quality marginal seals and high bond strengths immediately after placement, these properties begin to deteriorate in a matter of months both *in vitro* and *in vivo*^{1,3}.

The deterioration of the hybrid layer is due to a variety of physical and chemical factors, including hydrolysis and enzymatic degradation of exposed collagen as well as adhesive resin^{3–5}. It has been suggested that as the components of the hybrid layer begin to deteriorate, water-filled canals form within the layer. These canals allow oral and dentinal fluids to access the hybrid layer, increasing the likelihood of further degradation⁶. Although bacterial enzymes may be involved in the degradation of the hybrid layer, recent studies suggest that host-derived matrix metallo-proteinases (MMPs) play a pivotal role⁷. It has also been shown that the application of acid etching or self-etching procedures can lead to activation and increased expression of MMPs^{8,9}, increasing the risk of enzymatic breakdown of the newly formed hybrid layer.

A partial solution to the problem of hybrid layer deterioration may be the incorporation of appropriate degradation inhibitors into adhesive bonding systems^{1, 10}. One possible component of such a system is a class of plant compounds known as proanthocyanidins. Proanthocyanidins (PA), which form a complex subgroup of the flavonoid compounds, have been found in a wide variety of fruits, vegetables, flowers, nuts, seeds, and bark¹¹. They have been shown to lack toxicity, and have been reported to demonstrate a large variety of health-promoting actions¹². PA from grape seed extract has been shown to safely and effectively crosslink collagen in both *in vitro* and *in vivo* models¹³. Grape seed extract PA has also been shown to promote bone formation in the mandibular condyles of rats¹⁴, increase the stiffness of demineralized dentin¹⁵, and inhibit the progression of artificial root caries^{16,17}. Similar PA from elm tree and cranberry extracts has been shown to inhibit MMP production and activity^{18,19}.

Although interactions between PA and dentin collagen have been investigated, only one study has specifically focused on the dentin/adhesive bond²⁰. To date, no studies have been conducted on PA at the dentin/adhesive interface using clinically appropriate techniques. Incorporating PA into dentin adhesives may provide a new delivery method that allows the substance to remain in the hybrid layer for an extended period of time, enhancing the degree of collagen cross-linking. The purpose of this study was to qualitatively compare the morphological differences of hybrid layers created by BisGMA/HEMA model adhesives with and without the addition of grape seed extract PA under conditions of enzymatic collagen degradation. The research hypothesis was that the presence of PA in the adhesive system would enhance resistance to collagen biodegradation *in situ* and lead to morphological differences of the hybrid layer after enzymatic degradation compared to the pure model adhesive.

2. Materials and methods

2.1. Model Adhesives

Two model adhesives with compositions similar to Single Bond Plus (3M ESPE Dental Products, St. Paul, MN, USA) were used in this study²¹. For both adhesives, the monomer mixture consisted of 2-hydroxyethyl methacrylate (HEMA, Acros Organics, Morris Plains, NJ)

and 2,2-bis[4(2-hydroxy-3-methacryloyloxy-propyloxy)-phenyl] propane (Bis-GMA, Polysciences, Washington, PA, USA) with a mass ratio of 45/55. Three component visible light photo initiators (Aldrich, Milwaukee, WI) were also used: camphorquinone (CQ, 0.5 wt %), ethyl (4-dimethyl amino) benzoate (EDMAB, 0.5 wt%), and diphenyliodonium hexafluorophosphate (DPIHP 1.0 wt%). Concentrations of photo initiators were calculated with respect to the total amount of monomer. Both adhesives used ethanol (Fisher, Fair Lawn, NJ, USA) as a solvent. The pure adhesive had a solvent to monomer mass ratio of 40/60. The adhesive with PA was prepared by adding a powdered grape seed extract (MegaNatural Gold Grape Seed Extract containing over 90% PA, Polyphenolics, Madera, CA, USA) to the solvent at 5.0 wt% with respect to the monomer. This yielded an adhesive with a solvent/monomer/grape seed extract mass ratio of 37/60/3.

2.2 Specimen Preparation

Eight extracted non-carious human molars stored in 0.96% (w/v) phosphate buffered saline (PBS) containing 0.002% sodium azide at 4° C were used. The teeth were collected after the patient's informed consent was obtained under a protocol (02-99e) approved by the University of Missouri-Kansas City Adult Health Sciences Institutional Review Board. The roots of the teeth were removed 2–3 mm below the cemento-enamel junction using a water-cooled low-speed diamond saw (Buehler, Lake Bluff, IL, USA). This created a flat base, and allowed the teeth to be attached to an aluminum block with cyanoacrylate adhesive (Zapit, Dental Ventures of America, Corona, CA, USA). The occlusal one-third of the crowns was then removed using the same saw. A uniform smear layer was created by abrading the dentin surface with 600 grit silicon carbide grinding paper (Buehler, Lake Bluff, IL, USA) under water until light microscopy revealed a smooth surface, free of residual enamel.

For all specimens, the dentin surface was acid etched for 15 s with 35% phosphoric acid (Scotchbond Etchant, 3M ESPE, St. Paul, MN, USA) and then rinsed for 10 s with distilled water. The dentin surface was kept moist, but not excessively wet by removing excess water with absorbing paper (Kimwipes, Kimberly-Clark Professional, Roswell, GA, USA). Two layers of adhesive were applied using a brush applicator, with gentle air thinning for 10 s following the application of each layer. The adhesive was then light-polymerized for 20 s (550 mW/cm², Spectrum 800, Dentsply, Milford, DE, USA). Four teeth were bonded using the model adhesive without PA, and four teeth were bonded using the model adhesive with PA.

Following adhesive application, all specimens were stored in distilled water at room temperature for 24 hours before being cut into slabs. The same water-cooled low-speed saw was used to make perpendicular cuts into the bonded dentin surface at 1.7 mm increments. A single cut was then made parallel to and approximately 2 mm beneath the bonded surface, which freed the slabs from the remaining tooth structure. Because some excess material was removed due to the thickness of the saw blade, the final dimensions of the slabs were ~ 10 × 1.4 × 2 mm. All slabs were stored in PBS at 4°C.

Three slabs from each specimen were mounted on polymethylmethacrylate supports using the previously mentioned cyanoacrylate adhesive, and several 5 µm thick sections were cut from each slab using a tungsten carbide knife mounted on a Polycut "S" sledge microtome (Leica, Deerfield, IL, USA). The slabs were cut with the microtome in order to prepare a smooth and uniform surface for collagenase or control (non-collagenase) treatment and subsequent analysis. The sections cut from slabs were collected on glass slides and air-dried to prepare for Goldner's trichrome staining.

2.3 Collagenase Treatment

The collagenase solution was prepared by dissolving 1 mg of collagenase with a molecular weight of ~110 kDa (Collagenase Type I, Clostridiopeptidase A from *Clostridium histolyticum*, 125 U/mg, Sigma-Aldrich, St. Louis, MO, USA) per mL of TESCA buffer (5.75 g N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 25 mg sodium azide, 26.5 mg CaCl₂, dissolved in distilled water to make 500 mL of buffer, adjusted to pH 7.4). One slab from each specimen was treated with the collagenase solution for 1 day, while a second slab from each specimen was treated for 6 days. The third slab from each tooth received no treatment, and these slabs served as a control (non-collagenase) group. All treated slabs were individually incubated in wells of a cell culture plate (Costar 24, Corning, Lowell, MA, USA) holding 2.5 ml collagenase solution per well, 37°C, with shaking. The collagenase solution was changed every 24 hours for those specimens in the 6 day treatment condition.

2.4 Differential Staining Technique

The 5µm thick sections were mounted on glass slides using Haupt's adhesive (1 g gelatin dissolved in 10 ml distilled water at 50°C, add 15 ml glycerol and 2 g phenol crystals), and allowed to dry for a minimum of 48 hours. The sections were then stained with Goldner's trichrome²² using the technique described by Wang et al²³. Stained slides were photographed through a 100X oil immersion lens (Pan Fluor 1.30, Nikon Corporation, Tokyo, Japan) using a light microscope (E800, Nikon Corporation, Melville, NY, USA) equipped with a CCD camera (Optronics, Goleta, CA, USA).

2.5 Scanning Electron Microscopy (SEM)

Following treatment in the collagenase or non-collagenase control conditions, the slabs were dehydrated in increasing concentrations of ethanol, starting with 33%, 50%, 70%, and 85% for 15 min each. This was followed by 95%, 100%, and 100% ethanol for 30 min each. The slabs were then dried overnight, mounted to aluminum stubs with conductive tape, and sputter coated with ~20 nm of gold-palladium alloy. The slabs were examined at a variety of magnifications in a FEI/Philips XL30 Field-Emission Environmental SEM (Philips, Eindhoven, Netherlands) at 5kV.

2.6 Transmission Electron Microscopy (TEM)

Specimens for TEM were prepared from the same slabs observed with SEM. The slabs, which were not embedded, were cut with 35° diamond knife (Diatome, Biel, Switzerland) on EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Cutting was performed so that hybrid layer and underlying layer of dentin were preserved. The 90–100 nm-thick thin sections were stained with 3% uranyl acetate and 1% phosphotungstic acid stains. Micrographs were taken at 80 kV accelerating voltage with CM12 TEM (FEI, Hillsboro, OR, USA) equipped with SC1000 ORIUS® digital camera (Gatan, Pleasanton, CA, USA).

3. Results

Representative images of the Goldner's trichrome stained sections are presented in Figure 1. It has been previously reported that this technique will cause mineralized collagen to stain green, adhesive to stain beige, and exposed protein to stain red, while protein that has been encapsulated by adhesive will stain orange^{24,25}. Our results showed that the exposed protein of the hybrid layer stained closer to burgundy, rather than the red or orange stains reported in previous studies. However, there were no obvious differences in color between the model adhesive without PA and the model adhesive with PA. In both groups, the burgundy representation of the hybrid layer and the green representation of mineralized dentin were separated by a thin violet layer.

The scanning electron micrographs revealed morphological differences between the specimens bonded with and without PA, across all collagenase treatment conditions. In the non-collagenase (control) condition, the model adhesive without PA (Fig. 2A) produced a hybrid layer with a smoother and more glass-like surface topography compared to the model adhesive with PA (Fig. 3A), which exhibited more crumpled and more easily identifiable collagen fibrils. The arrows in Fig. 2A mark the less easily identified collagen fibrils found in the hybrid layer of model adhesive without PA. After one day of treatment with the collagenase solution, collagen fibrils are no longer identifiable with the adhesive without PA (Fig. 2B). The interface became less smooth and porous. Arrows mark areas of adhesive that may have partially encapsulated collagen before enzymatic degradation. In contrast, the specimens bonded with model adhesive containing PA (Fig. 3B) show a large number of visible collagen fibers, although they exhibit a more linear and less web-like appearance compared to the group that received no collagenase treatment (Fig. 3A). For both adhesive groups, the results after 6 days of treatment with collagenase are similar to those after 1 day of collagenase treatment. It should be noted, however, that the quality of the interface seen in the groups bonded with model adhesive containing PA and exposed to collagenase treatment (Fig. 3B and C) was not consistent. Some areas showed many collagen fibrils with relatively little adhesive (similar to Fig. 3B), while other areas showed a greater amount of adhesive within the interface (similar to Fig. 3C).

The results from transmission electron micrographs further confirmed the above micro-morphologic observation by SEM. In the adhesive/dentin interface from the groups that received no collagenase treatment, intertubular collagen fibrils revealed intact cross-banding patterns (Figs. 4 and 5). However, after six days of treatment with the collagenase solution, for the adhesive without PA, the collagen fibrils within the interface were sparsely observed in the intertubular zone (Fig. 6A). Disorganized collagen fibrils (Fig. 6B) in the interfacial zone lacked the typical 67 nm cross-banding of normal collagen. In contrast, the specimens bonded with model adhesive containing PA still showed normal collagen fibril organization and dimensions after 6 days of treatment with collagenase solution (Fig. 7).

4. Discussion

The results of this study indicated that the presence of grape seed extract PA in dental adhesives inhibited the biodegradation of unprotected collagen fibrils within the hybrid layer when challenged by the collagenase solution. Based on these results, the research hypothesis was supported. The SEM images showed that demineralized, exposed collagen fibrils would remain in the hybrid layer after six days of digestion with a 0.1% collagenase solution when they were exposed to PA from grape seed extract during adhesive bonding. In areas where there was less adhesive encapsulation, exposed collagen fibrils were easily identified and numerous, with no visually apparent differences in quality or quantity between the one and six day collagenase treatment groups. In contrast, the specimens prepared using model adhesive without PA clearly lacked any intact, exposed collagen fibrils after treatment with the collagenase solution. The increase in collagen degradation resistance could be attributed to the release of PA from the experimental adhesive.

The quality of the adhesive component of the hybrid layer did seem to be slightly lower in the specimens prepared with adhesive containing PA, as indicated by the increased hybrid layer porosity of the non-collagenase group (Fig. 3A), and variability in the amount of hybrid layer adhesive visible in the collagenase treated groups (Fig. 3, B and C). This could be a result of less collagen encapsulation by resin due to lower degree of conversion. Although the two adhesive formulations used in this study differed only in the concentration of grape seed extract (none vs. 5% of adhesive weight), they exhibited different polymerization properties. Previous unpublished work from our laboratory showed that the degree of monomer conversion of the

model adhesive varied inversely with the concentration of grape seed extract. With 20 s of exposure to the same curing lamp used in this study, the degree of conversion of the model adhesive without PA was found to be ~86%. The degree of conversion of the model adhesive with 5% PA was found to be ~68%, although these numbers are within the range of commercially available adhesives^{21,26,27}. The addition of PA decreased the degree of conversion to some extent. It seems likely that the somewhat lower degree of conversion may have allowed some monomers/oligomers to leach out of the hybrid layer, thereby exposing some collagen fibrils³.

The less than optimum quality of the hybrid layer formed between dentin and the model adhesive containing PA actually facilitated the observation of the effects of PA on collagen fibrils degradation by SEM. In other words, we were able to see the interfacial collagen fibrils clearly due to less collagen encapsulation. Even though the interface quality is relatively poor, after the collagenase treatment, the collagen fibrils were evidently visible, indicating that collagen fibrils degradation resistance increased by PA crosslinking reactions. These results were further confirmed by TEM observation. The dentin specimens bonded with model adhesive containing PA showed intact cross-banding patterns after 6 days of treatment with collagenase solution (Fig. 7), which is similar to the normal collagen fibril organization and dimensions. In contrast, in the adhesive/dentin interface from the group without PA that exposed to similar collagenase treatment, the collagen fibrils were sparingly observed in the intertubular zone (Fig. 6). Disorganized collagen fibrils (Fig. 6B) in the interfacial zone lacked the typical cross-banding of normal collagen.

Previous studies have indicated that nearly all demineralized dentin collagen fibrils should be digested after 1 day of treatment with the collagenase solution^{28–30}. However, in the adhesive/dentin interface even from the group without PA, most of collagen is still retained after 6 days of treatment. The negligible effect of bacterial collagenase at the dentin bonded interface has also been indicated in previous studies, especially with short term storage^{31–33}. The presence of the infiltrated adhesive is believed to restrict enzyme activities. Solubilizing collagen fibrils depends on the cleavage of cross-links along their lengths into many peptide chains/fragments. It is possible that collagen fragments would only solubilize from the surface of these specimens. The infiltrated adhesive may make collagenase more difficult to reach the inside of the specimens or cause soluble collagen fragments deep in the specimen much harder to leach out. In addition, the large size of the collagenase molecules (with a high molecular weight of ~110 kDa) may also account for this. Our SEM/TEM results seem to support these statements, as observed morphological changes were limited to the surface of the specimens after the collagenase treatments. However, it is likely that collagenase molecules could also penetrate through tubules and digest the bottom of the hybrid layer. Even though fibrous collagen fragments are retained in the interface, these fragments would dramatically reduce mechanical strength or bonding of the interface. Further micro-mechanical tests are needed to evaluate the effect of inclusion of PA in the adhesive on the bond strength and its stability under conditions such as the collagenase solution treatment.

In previous studies, morphological evidence of collagen degradation has also been found using SEM and TEM in the resin-dentin interface in specimens stored in water for over 1 year^{34, 35}. Collagen degradation significantly affects the long-term bond durability of the adhesive/dentin interface. Not much information is available on the mechanism of collagen degradation both *in vitro* and *in vivo*. Although gelatinolytic and collagenolytic activities induced by oral bacteria may play a role in the collagen degradation, recently, collagenase (matrix metalloproteinases, MMPs)^{36,37} activated by acids has been demonstrated to be involved in the degradation process of dentin collagen. Pashley *et. al.*³⁶ have reported that the degradation of collagen in the demineralized dentin is due to host-derived MMPs that are induced by acid etching and released slowly over time. Recent studies have shown that PA could inhibit MMP

production and activity^{18,19}. To further explore this collagen self-destruction mechanism, instead of using the collagenase solution, long-term (>1 year) water storage experiments are underway to investigate the effects of host-derived MMPs on PA crosslinked collagen degradation.

In summary, the incorporation of the grape seed extract within dental adhesives appears to be a viable technique to protect exposed collagen fibrils of the hybrid layer from degradation by collagenase solution. Morphological differences between the hybrid layers created with model adhesives prepared with and without PA were apparent from the SEM and TEM micrographs. The collagen degradation resistance was increased by the presence of PA in the model adhesive. Although a recent study found that treatment of demineralized dentin with grape seed extract prior to adhesive application significantly increased microtensile bond strength²⁰, the cross-linking effect on collagen degradation within the hybrid layer has not been studied. In addition, the previous study used a technique in which the prepared teeth were submersed in a grape seed extract solution for an hour, which is not possible in clinical situations.

Although our method is clinically feasible and has shown the inhibition of collagen degradation in the adhesive/dentin interface, the issue on the decreased degree of conversion of the resultant adhesive and hybrid layer needs to be addressed. Based on our preliminary studies, this issue could be resolved by changing the concentration and type of photo-initiators or the PA concentration. These studies are currently underway in our lab. Recently, the studies on the degree of conversion and modulus of elasticity of experimental adhesives formulated with different chlorhexidine concentrations were reported³⁸. Similar studies should be performed on adhesives prepared with different concentrations of grape seed extract and the impact of PA on the physical properties of the adhesive/dentin interface. Finally, it may be advantageous to use chromatography or other methods to isolate various components of the grape seed extract, so that unnecessary compounds which do not exert a protective effect on collagen are not incorporated into adhesive mixtures.

Acknowledgments

This investigation was supported in part by USPHS Research Grants DE 015281 and DE 021023 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892.

References

1. De Munck J, Van Landuyt K, Peumans M, Poitevin A, Lambrechts P, Braem M, et al. A critical review of the durability of adhesion to tooth tissue: methods and results. *Journal of Dental Research* 2005;84:118–32. [PubMed: 15668328]
2. Nakabayashi N, Kojima K, Masuhara E. The promotion of adhesion by the infiltration of monomers into tooth substrates. *Journal of Biomedical Materials Research* 1982;16:265–73. [PubMed: 7085687]
3. Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano Dorigo E. Dental adhesion review: aging and stability of the bonded interface. *Dental Materials* 2008;24:90–101. [PubMed: 17442386]
4. Kostoryz EL, Dharmala K, Ye Q, Wang Y, Huber J, Park JG, et al. Enzymatic biodegradation of HEMA/bisGMA adhesives formulated with different water content. *Journal of Biomedical Materials Research B Applied Biomaterials* 2009;88:394–401.
5. Tay FR, Pashley DH. Have dentin adhesives become too hydrophilic? *Journal of Canadian Dental Association* 2003;69:726–31.
6. Tay FR, Hashimoto M, Pashley DH, Peters MC, Lai SC, Yiu CK, et al. Aging affects two modes of nanoleakage expression in bonded dentin. *Journal of Dental Research* 2003;82:537–41. [PubMed: 12821715]

7. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, et al. Collagen degradation by host-derived enzymes during aging. *Journal of Dental Research* 2004;83:216–21. [PubMed: 14981122]
8. Sano H. Microtensile testing, nanoleakage, and biodegradation of resin-dentin bonds. *Journal of Dental Research* 2006;85:11–4. [PubMed: 16373674]
9. Lehmann N, Debret R, Romeas A, Magloire H, Degrange M, Bleicher F, et al. Self-etching increases matrix metalloproteinase expression in the dentin-pulp complex. *Journal of Dental Research* 2009;88:77–82. [PubMed: 19131322]
10. Xu C, Wang Y. Collagen cross-linking by glutaraldehyde increases its biodegradation resistance in wet dentin bonding. *Dental Materials*. 2010 submitted.
11. Ferreira D, Slade D. Oligomeric proanthocyanidins: naturally occurring O-heterocycles. *Natural Product Reports* 2002;19:517–41. [PubMed: 12430722]
12. Fine AM. Oligomeric proanthocyanidin complexes: history, structure, and phytopharmaceutical applications. *Alternative Medicine Review* 2000;5:144–51. [PubMed: 10767669]
13. Han B, Jaurequi J, Tang BW, Nimni ME. Proanthocyanidin: a natural crosslinking reagent for stabilizing collagen matrices. *Journal of Biomedical Materials Research A* 2003;65:118–24.
14. Ishikawa M, Maki K, Tofani I, Kimura K, Kimura M. Grape seed proanthocyanidins extract promotes bone formation in rat's mandibular condyle. *European Journal Oral Sciences* 2005;113:47–52.
15. Bedran-Russo AK, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. *Journal of Biomedical Materials Research B Applied Biomaterials* 2008;86B:330–4.
16. Xie Q, Bedran-Russo AK, Wu CD. In vitro remineralization effects of grape seed extract on artificial root caries. *Journal of Dentistry* 2008;36:900–6. [PubMed: 18819742]
17. Walter R, Miguez PA, Arnold RR, Pereira PN, Duarte WR, Yamauchi M. Effects of natural cross-linkers on the stability of dentin collagen and the inhibition of root caries in vitro. *Caries Research* 2008;42:263–8. [PubMed: 18523385]
18. La VD, Howell AB, Grenier D. Cranberry proanthocyanidins inhibit MMP production and activity. *Journal of Dental Research* 2009;88:627–32. [PubMed: 19641150]
19. Song SE, Choi BK, Kim SN, Yoo YJ, Kim MM, Park SK, et al. Inhibitory effect of procyanidin oligomer from elm cortex on the matrix metalloproteinases and proteases of periodontopathogens. *Journal of Periodontal Research* 2003;38:282–9. [PubMed: 12753366]
20. Al-Ammar A, Drummond JL, Bedran-Russo AK. The use of collagen cross-linking agents to enhance dentin bond strength. *Journal of Biomedical Materials Research B Applied Biomaterials* 2009;91:419–24.
21. Ye Q, Spencer P, Wang Y, Misra A. Relationship of solvent to the photopolymerization process, properties, and structure in model dentin adhesives. *Journal of Biomedical Materials Research A* 2007;80:342–50.
22. Goldner J. A modification of the masson trichrome technique for routine laboratory purposes. *American Journal of Pathology* 1938;14:237–43. [PubMed: 19970387]
23. Wang Y, Spencer P, Hager C, Bohaty B. Comparison of interfacial characteristics of adhesive bonding to superficial versus deep dentine using SEM and staining techniques. *Journal of Dentistry* 2006;34:26–34. [PubMed: 15907359]
24. Spencer P, Swafford JR. Unprotected protein at the dentin-adhesive interface. *Quintessence International* 1999;30:501–7. [PubMed: 10635264]
25. Spencer P, Wang Y, Walker MP, Wieliczka DM, Swafford JR. Interfacial chemistry of the dentin/adhesive bond. *Journal of Dental Research* 2000;79:1458–63. [PubMed: 11005728]
26. Cadenaro M, Antonioli F, Sauro S, Tay FR, Di Lenarda R, Prati C, et al. Degree of conversion and permeability of dental adhesives. *European Journal of Oral Sciences* 2005;113:525–30. [PubMed: 16324144]
27. Breschi L, Cadenaro M, Antonioli F, Sauro S, Biasotto M, Prati C, et al. Polymerization kinetics of dental adhesives cured with LED: correlation between extent of conversion and permeability. *Dental Materials* 2007;23:1066–72. [PubMed: 17109951]

28. Coli P, Alaeddin S, Wennerberg A, Karlsson S. In vitro dentin pretreatment: surface roughness and adhesive shear bond strength. *European Journal of Oral Sciences* 1999;107:400–13. [PubMed: 10515206]
29. Ahmed AA, Garcia-Godoy F, Kunzelmann KH. Self-limiting caries therapy with proteolytic agents. *American Journal of Dentistry* 2008;21:303–12. [PubMed: 19024256]
30. Ganss C, Schlueter N, Hardt M, von Hinckeldey J, Klimek J. Effects of toothbrushing on eroded dentine. *European Journal of Oral Sciences* 2007;115:390–6. [PubMed: 17850428]
31. Armstrong SR, Jessop JL, Vargas MA, Zou Y, Qian F, Campbell JA, et al. Effects of exogenous collagenase and cholesterol esterase on the durability of the resin-dentin bond. *Journal of Adhesive Dentistry* 2006;8:151–60. [PubMed: 16830661]
32. Jung YJ, Hyun HK, Kim YJ, Jang KT. Effect of collagenase and esterase on resin-dentin interface: a comparative study between a total-etch adhesive and a self-etch adhesive. *American Journal of Dentistry* 2009;22:295–8. [PubMed: 20225473]
33. Toledano M, Osorio R, Osorio E, Aguilera FS, Yamauti M, Pashley DH, et al. Effect of bacterial collagenase on resin-dentin bonds degradation. *Journal of Materials Science Materials in Medicine* 2007;18:2355–61. [PubMed: 17569004]
34. Hashimoto M, Ohno H, Kaga M, Endo K, Sano H, Oguchi H. In vivo degradation of resin-dentin bonds in humans over 1 to 3 years. *Journal Dental Research* 2000;79:1385–91.
35. Hashimoto M, Ohno H, Sano H, Tay FR, Kaga M, Kudou Y, et al. Micromorphological changes in resin-dentin bonds after 1 year of water storage. *Journal Biomedical Materials Research* 2002;63:306–11.
36. Hannas AR, Pereira JC, Granjeiro JM, Tjälðerhane L. The role of matrix metalloproteinases in the oral environment. *Acta Odontologica Scandinavica* 2007;65:1–13. [PubMed: 17354089]
37. Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjälðerhane L. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Archives of Oral Biology* 2007;52:121–7. [PubMed: 17045563]
38. Cadenaro M, Pashley DH, Marchesi G, Carrilho M, Antonioli F, Mazzoni A, et al. Influence of chlorhexidine on the degree of conversion and E-modulus of experimental adhesive blends. *Dental Materials* 2009;25:1269–74. [PubMed: 19570568]

Figure 1. Representative light micrographs of the adhesive/dentin interface of the specimens that received no collagenase treatment stained with Goldner's trichrome. **A.** Model adhesive without PA. **B.** Model adhesive with PA.

Figure 2.

Representative scanning electron micrographs of the model adhesive without PA, 16,000X. A. The group that received no collagenase treatment. Arrows mark collagen fibrils. B. 1 day of treatment in the collagenase solution. Arrows mark voids in adhesive that may have partially encapsulated collagen prior to degradation. C. 6 days of treatment in the collagenase solution. Arrows mark voids in adhesive that may have partially encapsulated collagen prior to degradation.

Figure 3.

Representative scanning electron micrographs of the model adhesive with PA, 16,000X. A. The group that received no collagenase treatment. B. 1 day of treatment in the collagenase solution C. 6 days of treatment in the collagenase solution.

Figure 4. Representative transmission electron micrographs of the model adhesive without PA. A. Undemineralized micrograph of the group that received no collagenase solution treatment. B. Higher magnification of the hybrid layer region of Fig. 4A. The intertubular collagen fibrils revealed intact cross-banding patterns.

Figure 5. Representative transmission electron micrographs of the model adhesive with PA. A. Undemineralized micrograph of the group that received no collagenase solution treatment. B. Higher magnification of the hybrid layer region of Fig. 5A. The intertubular collagen fibrils revealed intact cross-banding patterns.

Figure 6.

Representative transmission electron micrographs of the model adhesive without PA. A.

Undemineralized micrograph of the specimens that received 6 days of treatment in the collagenase solution. B. Higher magnification of the hybrid layer region of Fig. 6A.

Disorganized collagen fibrils in the interfacial zone lacked the typical 67 nm cross-banding of normal collagen.

Figure 7. Representative transmission electron micrographs of the model adhesive with PA. A. Undemineralized micrograph of the specimens that received 6 days of treatment in the collagenase solution. B. Higher magnification of the hybrid layer region of Fig. 7A. It showed normal collagen fibril organization and dimensions in the interfacial zone, which has the typical 67 nm cross-banding.