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Original Article

Riboflavin-ultraviolet-A collagen crosslinking treatments in improving dentin bonding and resistance to enzymatic digestion

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KEYWORDS
Riboflavin;
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Cross-linking;
Collagen;
Dentin bonding;
Enzymatic digestion

Abstract *Background/purpose:* The efficacy of riboflavin-ultraviolet-A (RF-UVA) treatment in crosslinking collagen and improving dentin bonding has been proven. However, biodegradation of the hybrid layer may compromise the bonding. The purpose of this study was to evaluate different RF-UVA treatments regarding their ability to preserve dentin bonding from enzymatic digestion.

Materials and methods: Collagen subjected to different RF (0.1 %, 1 %)-UVA (1, 2, 5 min) treatments and 5 % glutaraldehyde (GA), without or with enzymatic digestion, were examined by gel electrophoresis (SDS-PAGE). Twenty-five teeth with exposed dentin were primed with one of three RF-UVA treatments (0.1 %RF/1-minUVA, 0.1 %RF/2-minUVA, and 1 %RF/1-minUVA), GA, or distilled water after acid-etching, then restored with an adhesive and a resin composite. After 24-h storage, these teeth were sectioned into microbeams. Half of them received an early microtensile bond strength (μTBS) test, while the other half was stored in enzyme solution for 7 days before testing. Nanoleakage and hybrid layer degradation were examined by TEM.

Results: According to SDS-PAGE results, all groups showed the dissipation of intense γ bands of collagen after digestion. For the early bonded specimens and after enzymatic digestions, 0.1 % RF/2-minUVA treated group presented the highest μTBS and none of premature failure. Its TEM images showed less nanoleakage after digestion, which is contributed to the well suspended collagen fibrils and resin infiltration in the hybrid layer.

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Conclusion: RF-UVA treatment attained collagen crosslinking effects to improve resin-dentin bonding. 0.1 %RF/2-minUVA effectively enhanced dentin bond strength and resistance to enzymatic digestion by optimally expanding dentinal collagen matrix to facilitate hybrid layer formation.

Introduction

The success in dentin bonding is the fundamental of dental restorations. Contemporary dentin bonding process involves etching the dentin surface with an acidic solution or monomer, which removes the smear layer and the minerals on dentin.[1] The resin adhesive is then applied and infiltrates into the collagen network of the demineralized dentin, creating a hybrid layer (HL).[2] To establish effective and durable resin-dentin bonding, the HL should be structurally stable. However, it is well known that primer and adhesive resin may not completely encapsulate the denuded collagen in the deepest part of HL.[3] Water permeation into the defective HLs causes hydrolytic breakdown of either the adhesive resin or collagen, thereby compromising the bonding.[4] On the other hand, the denuded collagen fibrils are vulnerable to collagenolysis by endogenous dentinal enzymes, cathepsin-K enzymes, and other enzymes from bacteria or saliva.[5–7] These enzymatic degradations adversely affect dentin bond durability.

Dentin is a complex mineralized connective tissue, while fibrillar type I collagen is responsible to its stiffness and toughness.[8] With the inter- and intramolecular covalent and hydrogen bonds, dentinal collagen matrix can withstand the acid etching and adhesive treatments and maintain its structural integrity.[9] However, the etching procedure substantially increases the intrinsic dentin gelatinolytic and collagenolytic activity those might induce the degradation of HL.[5] Since 2000s, the use of extrinsic collagen crosslinking agents has been advocated to improve resin-dentin bonds. Bedran-Russo et al. have demonstrated successful biomodification of demineralized dentin with proanthocyanidin and genipin.[10,11] These natural agents induce additional inter- and intra-molecular crosslinks and keep the network in a relatively expanded state to facilitate the interdiffusion of resin monomers.[12] Although these crosslinking agents exhibit high potency and low cytotoxicity, their treatments take a long duration (at least 4 h), and the polymeric polyphenols caused marginal staining of restorations.[13] Glutaraldehyde (GA) is the most widely used protein fixer due to its high reactivity with collagen. Previous studies has demonstrated that GA treatment significantly improved resin-dentin bond strengths after only 1-min application,[14] and reduced its degradation after collagenase challenge and long-term water storage.[15] On the other hand, GA exhibits noticeable cytotoxicity on human cells at a low concentration,[16] and reveals even more adverse effects in combining with HEMA and other resin monomers.[17]

Riboflavin (RF, vitamin B_2) is a natural and effective photosensitizer which may be activated under 270, 365, and 445 nm wavelength ultraviolet-A (UVA) light to create covalent bonds between and within the collagen fibers.[18] For its use in dentistry, Cova et al. reported that a pretreatment of 0.1 % RF and 2-min UVA irradiation before dentin adhesives increased the bond strength and reduced interfacial nanoleakage of composite restorations.[19] In our previous study, the same RF-UVA treatment effectively enhanced the stiffness of collagen matrix and maintained its expanded states in the HL, thereby improved dentin adhesion.[20] The effect of RF in inactivating endogenous matrix metalloproteinases has also been confirmed.[19] In a meta-analysis, only glutaraldehyde and riboflavin are evidence to improve the resin-dentin bond strength.[21]

The preservation of HL is crucial to sustain oral functioning. However, the degradation of collagen may happen due to the endogenous or exogenous enzymatic digestions. The collagen crosslinking could be an effective strategy in reducing HL degradation by both strengthening the collagen network and inhibiting collagenolytic enzyme. RF-UVA treatment has shown promising effect in stabilization and strengthening dentin collagen fibrils, while it is not perfectly clear whether it can also resist the enzymatic degradation. The purpose of this study was thus to evaluate the effects of RF-UVA photooxidative crosslinking treatments on resisting collagenase digestion and maintaining resin-dentin bonding, and to find out the proper protocol.

Materials and methods

In this study, type I collagen after cross-linking treatment and enzymatic digestion was first characterized by the electrophoretic pattern. Different RF-UVA protocols were examined in regard to enhance resin-dentin bond strength, resistance to enzymatic degradation, and prevent the occurrence of nanoleakage in the HL. The experimental materials, their manufactures, and ingredients are listed in Table 1.

Gel electrophoresis

In this study, RF-UVA treatments consisted of 0.1 % or 1 % aqueous solutions of riboflavin 5′-phosphate (Sigma-Aldrich, St. Louis, MO, USA) (denoted as RF0.1 or RF1) and 1, 2, or 5 min UVA irradiation (denoted as UV1, UV2, UV5). An 5 % aqueous glutaraldehyde (GA)-based desensitizer (Gluma Desensitizer, Heraeus Kulzer, South Bend, IN, USA) application for 1 min was used for comparison. Meanwhile, the distilled water (DW) was used as a negative control group.

The crosslinking effects of these treatments were examined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 5 μL of bovine collagen solution (Sigma-Aldrich) was diluted to 10 μL, then mixed with 5 μL of

Table 1 Experimental materials, their manufacturers, and ingredients.

Material	Classification	Manufacturers	Ingredient
Collagen Solution, Ultrapure, bovine	Collagen solution	Sigma-Aldrich, St. Louis, MO, USA	3 mg/mL; ~97 % Type I collagen, ~3 % Type III collagen
Riboflavin 5′-phosphate	Cross-linker	Sigma-Aldrich	Riboflavin 5′-phosphate sodium salt hydrate
Gluma Desensitizer	Cross-linker	Heraeus Kulzer, South Bend, IN, USA	36.1 wt-% HEMA and 5.1 wt-% glutaraldehyde in water
Collagenase Type I	Enzyme	Invitrogen, Carlsbad, CA, USA	Type I collagenase (from clostridium histolyticum)
Denfil Etchant-37	Etchant	Vericom Co., Chuncheon, Korea	37 % phosphoric acid
Adper Scotchbond Multi-Purpose Plus	3-step etch-and-rinse adhesive system	3M ESPE, St. Paul, MN, USA	Primer: water, 55 % HEMA, 5 % polyalkenoic acid copolymer Adhesive: Bis-GMA, HEMA, initiators, stabilizers
Filtek Z250	Resin composite	3M ESPE	Zirconia/silica filler, Bis-GMA, UDMA, Bis-EMA, and camphorquinone

Bis-EMA: bisphenol A-ethoxylated-glycidyl dimethacrylate; Bis-GMA: bisphenol A-glycidyl dimethacrylate; HEMA: hydroxyethyl methacrylate; UDMA: urethane-dimethacrylate.

either one of the crosslinkers (RF0.1, RF1, GA) or DW in Eppendorf tubes. For RF0.1 and RF1 groups, the solutions were irradiated in a UVA light box (Bio-LX E—365, Vilber Lourmat, Marnela-Vallée, France) with a peak intensity at 365 nm and 65 W output for either 1, 2, or 5 min. Subsequently, the solution in each group was mixed with 5 μL of 4 × dye (bromophenol blue). After heat denaturation in 100 °C boiling water for 15 min, they were loaded onto an SDS-polyacrylamide (SDS-PA) 4.5 % stacking gel overlayed on an SDS-PA- 8 % separating gel. Electrophoresis was carried out at constant voltage of 100 V. Afterward, the gels were stained with 0.1 % Coomassie blue and photographed after removing the dye excess. Molecular mass markers of 70, 100, 130, and 170 kDa (PageRuler™ Prestained Protein Ladder, Fermentas, Hanover, MD, USA) were run in a parallel lane.

Five collagen-based samples (DW, RF0.1UV1, RF0.1UV2, RF1UV1, and GA) were further examined for their resistance to enzymatic digestion. For this test, 5 μL of bovine collagen solution was mixed with DW or the crosslinkers, then RF groups were subjected to the assigned UVA irradiation. 1 mg/mL type I collagenase (lyophilized from Clostridium Histolyticum, Invitrogen, Carlsbad, CA, USA) was dissolved in a collagenase buffer (10 mM Tris(hydroxymethyl)aminomethane and 10 mM $CaCl_2$ in 0.15 M NaCl, pH 7.4) at 37 °C for 30 min. 5 μL of collagenase solution was added in the 10 μL of treated collagen solutions and treated for 5 min. SDS-PAGE was then performed to characterize their molecular compositions. The densities of γ, β, and α bands of individual groups in the electrophoretogram were further quantify using the Gel Analysis function of ImageJ software (NIH, Bethesda, MD, USA), and calibrated with the original DW specimen.

Microtensile bond strength tests

Twenty-four sound extracted human molars were used with approval by the Institutional Review Board, National Cheng Kung University Hospital (B-ER-101-028). Teeth with caries, cracks, endodontic treatment, or restorations were excluded from this study. The obtained teeth were stored in 4 °C normal saline containing 0.02 % sodium azide until use. To prepare the specimens, the teeth were embedded in epoxy resin and ground to expose dentin. The dentin surfaces were serially polished with 180-, 320-, 600- grit silicon-carbide abrasive papers (Nihon Kenshi Co., Osaka, Japan) under running water at 70 rpm on a grinding machine (Ecomet 3; Buehler Ltd., Lake Bluff, IL, USA).

The teeth were evenly distributed into 5 groups to receive five treatments: DW, RF0.1UV1, RF0.1UV2, RF1UV1, and GA. The flat dentine surfaces were acid-etched for 15 s with 37 % phosphoric acid (Vericom Co., Chuncheon, Korea), then thoroughly rinsed off and dried using a gentle air stream for 2 s to leave slightly moist. In RF-UVA groups, specimens were irradiated in the UVA light box. After these treatments, a single coat of Scotchbond Multi-purpose primer (3M ESPE, St. Paul, MN, USA) was applied, followed by gentle air drying for 5 s. The Scotchbond multi-purpose adhesive was then placed, and light cured for 20 s. After completion of the bonding procedure, a hybrid composite (Z250, A2 shade; 3M ESPE) was used to build up a 4-mm high cylinder on dentin in two increments, each was light-cured for 40 s. After storage for 24 h in DW at 37 °C, these restored teeth were sectioned perpendicular to the bonded interface into serial slabs, then further sectioned into 0.9 × 0.9 mm^2 resin-dentin beams with a low-speed diamond saw (Isomet 2000; Buehler Ltd.) under water cooling. For each group, at least 24 beams were collected. Half of the resin-dentin beams were examined by the μTBS test immediately after cutting, while the other half was subjected to enzymatic degradation (enz) before test. The enz subgroups were treated with the type I collagenase for 7 days at 37 °C. The collagenase solution was changed every 24 h.

In the microtensile bond strength (μTBS) test, each beam was fixed with cyanoacrylate glue to a jig on a universal testing machine (AG-1; Shimadzu, Kyoto, Japan). Individual resin-dentin beams were stressed under tension at a crosshead speed of 1 mm/min until failure. The cross-section areas at the site of fracture were measured using a

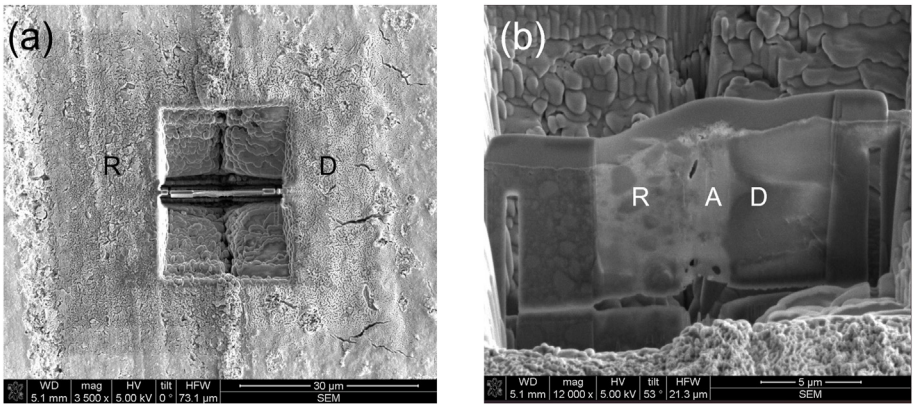

Figure 1 The FIB-TEM specimen preparation process. SEM image of (a) the top view of the TEM specimen prepared using DB-FIB and of (b) the thin electron-transparent microplate after the completion of ion beam trimming. A: adhesive layer; R: resin composite; D: dentin.

digital caliper. The μTBS was then calculated by dividing the load at failure by the area. Statistical analysis was performed by two-way ANOVA and Tukey multiple comparison test using SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA). The differences were considered significant at $P < 0.05$.

After the microtensile bond strength test, all fractured beams were dried in a desiccator, mounted on stubs with conductive tape, sputter-coated with gold for 3 min and examined by scanning electron microscopy (SEM) (JSM-6390 LV; JEOL, Tokyo, Japan) connected with an energy dispersive spectrometer (EDS) (INCA 350; Oxford Instruments, Abingdon, UK) at 20 kV for failure patterns observation. The failure patterns were classified as: P, premature failure before the μTBS test; A, 100 % adhesive failure; Cd, 100 % cohesive failure in dentin; Cr, 100 % cohesive failure in composite resin, and M, mixed failure with partially adhesive and partially cohesive failures. Data were analyzed by a chi-square test and Fisher's exact test at a significant level $P < 0.05$.

Transmission electron microscopy observation

A tracer solution was prepared as the aqueous solution of 50 wt. % ammoniacal silver nitrate. Two resin-dentin beams from each early and enz subgroups were immersed in the tracer solution for 24 h. They were then placed in a photo developing solution for 8 h and irradiated under a fluorescent light to reduce silver nitrate to metallic silver. The beams were fixed with 2.0 % paraformaldehyde and 1.25 % glutaraldehyde in 0.1 M phosphate-buffer saline (PBS) for 2 h. The sections were double stained for protein with 1 % uranyl acetate, followed by 1 % lead oxalate.

Cross-sectional transmission electron microscopy (TEM) specimens were produced with dual-beam focused ion beam (DB-FIB) (Nova-200 NanoLab, Thermo Fischer, Waltham, MA, USA). The specimens were platinum-sputtered to enhance electrical conductivity. A layer of carbon thin film was deposited via gas injection systems on the region of interest to avoid damage caused by the ion and electron bombardment. A gallium ion beam was operated at 5 kV to cut two parallel 15 μm long slots across the resin-dentin interfaces to form a 1-μm microplate in between. The microplate was gradually trimmed to 100 nm thickness by serially reducing the beam currents from 20,000−100 pA (Fig. 1). The finished specimen was placed onto a carbon-coated copper grid for TEM (HR-TEM 2100F, JEOL) examination operated at 200 kV. Nanoleakage was observed as the degree of infiltrated tracer silver grains. X-ray energy dispersion detector (INCA Energy 250 X-max, Oxford Instruments) allowed chemical composition characterization. The structure of collagen fibers near the HL was also examined on the TEM images.

Results

Gel electrophoresis analysis

The DW group showed the typical pattern of type I collagen consisting of 1 γ trimer band (300 kDa), a β dimer band (260 kDa), and two α1 and α2 monomer bands (130 and 100 kDa) (Fig. 2a). In all RF-UVA groups, the γ band was dense and accompanied with multiple bands between γ and β bands. These groups also showed deposits on the base of the stacking gels. The γ bands in RF0.1 groups (UV1-UV5) seemed to be denser than their corresponding RF1 groups. Contrarily, heavier aggregation deposits were found in RF1 groups. GA-treated collagen aggregated heavily in the Eppendorf tube (Fig. 2b). Its large-molecular-weight cannot migrate in the separating gel and resulted in empty GA lane (Fig. 2a).

Considering that 5-min UV irradiation does not fit the clinical demands and neither cause relevant changes, only the RF0.1UV1, RF0.1UV2, RF1UV1, and GA crosslinking treatments were subjected to the enzymatic digestion. The densities of γ and β bands in all groups decreased after digestion, and bands between γ and β bands seemed to be vanished. RF0.1UV1, RF0.1UV2, and RF1UV1 groups still showed higher intensity compared to the original DW group (Fig. 2a). Those insoluble aggregates in the GA group reduced in size (Fig. 2b) but failed to show characteristic bands on the electrophoretogram (Fig. 2a).

The density of γ, β, and α bands were calibrated with DW group and plotted (Fig. 3). For undigested groups,

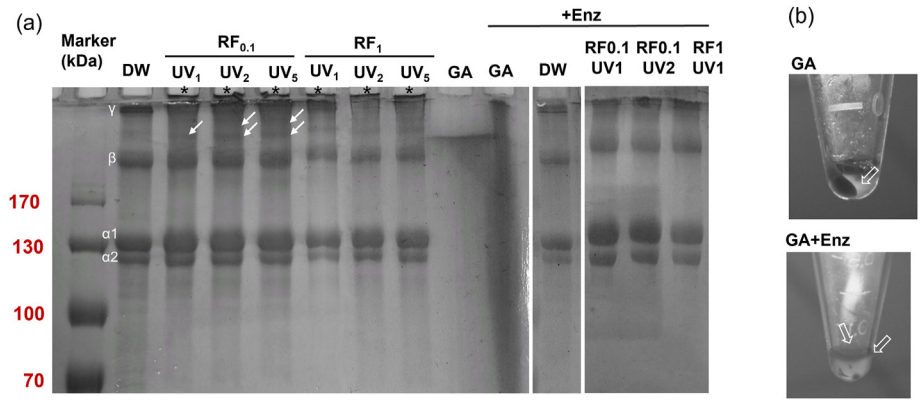

Figure 2 (a) Electrophoretogram (SDS-PAGE) of type I collagen receiving different treatments. The asterisks indicate insoluble high molecular weight polymer piled on the stacking gel; the white arrows indicate the intermediary dimer/trimer-like fractions. (b) GA-treated and enzyme-digested specimen showed the over-crosslinked collagen (indicated by hollow arrows) as aggregates in the tubes. DW: distilled water; RF: riboflavin; UV: ultraviolet-A; GA: glutaraldehyde; Enz: enzymatic degradation.

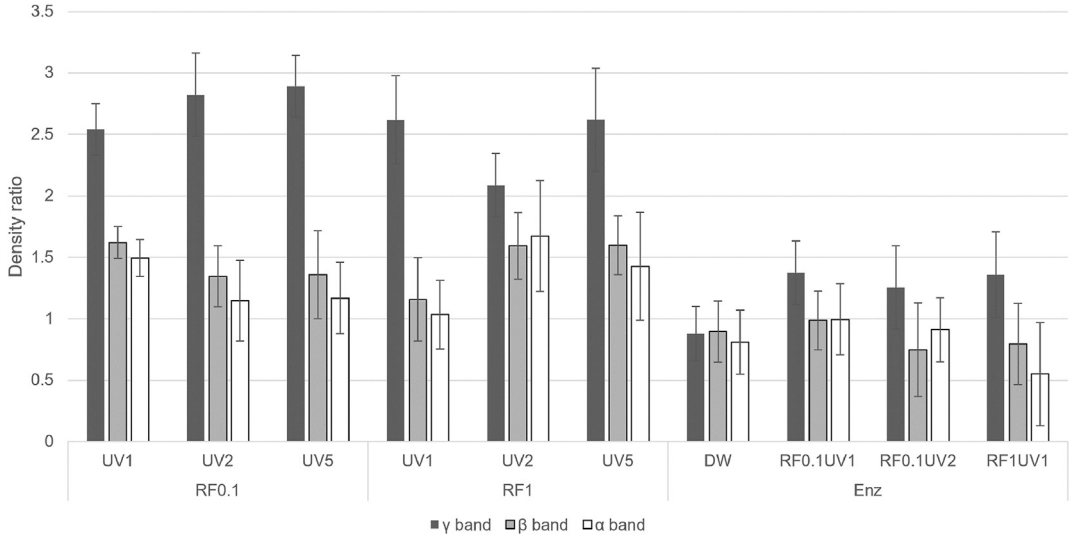

Figure 3 Density ratio plot of the SDS-PAGE migrated bands. All the γ, β, and α bands are calibrated with the original DW group. DW: distilled water; RF: riboflavin; UV: ultraviolet-A; GA: glutaraldehyde; Enz: enzymatic degradation.

RF0.1UV2 and RF0.1UV5 exhibited the highest γ trimer and crosslinked dimer-trimer bands, while RF0.1UV1, RF1UV2 and RF1UV5 showed increased densities of β and α bands. After enzymatic digestion, each group showed decreased γ, β, and α bands compared to corresponding bands of the undigested groups.

Microtensile bond strengths and fracture patterns

For the early μTBS test, significant differences existed among groups ($P < 0.05$) (Table 2). DW group showed the lowest μTBS among the five groups collected in the table below. All the crosslinking treatments enhanced the bond strengths. RF0.1UV2 showed the highest μTBS (54.41 ± 3.68 MPa), followed by RF1UV1, GA, and RF0.1UV1. These four groups were not significantly different. For the fracture patterns, there was no significant difference among groups. All of them contained adhesive and mixed fracture as their major failure patterns. DW samples showed 25 % premature failures (3 of 12) before the microtensile test.

After 7-day enzymatic digestion, all groups showed reduced μTBSs. R0.1U2 group still retained the highest bond strength, then in order were RF1UV1, GA, RF0.1UV1, and DW. Except GA groups, μTBS values of four groups were not significantly different from its early μTBS value after enzymatic digestion. DW showed premature failure in half of specimens (6 of 12), which was significantly different from RF0.1UV2, RF1UV1, and GA.

Transmission electron microscopy observation

In the early stage, DW and GA groups showed condensed silver grain deposits in the vicinity of hybrid layer (Fig. 4). The silver grain even infiltrated into the dentin in these two groups. RF0.1UV1 presented sparsely small silver grains in the adhesive layer, while RF0.1UV2 and RF1UV1 exhibited even less stain.

Table 2 The mean (standard error) values of μTBS[a] (MPa) and failure patterns in experimental groups.

Group (*n* = 12)	Early		Enz[a]	
	μTBS[b,c]	Failure pattern *P/A/M/Cd/Cr*[a]	μTBS[b,c]	Failure pattern *P/A/M/Cd/Cr*[a]
DW	29.18 (5.94) ^{Ba}	3/5/4/0/0 ^{A}	26.30 (8.01) ^{Ba}	6/1/0/2/3 ^{A}
RF0.1UV1	40.40 (5.89) ^{ABa}	2/4/5/1/0 ^{A}	27.88 (5.09) ^{Ba}	2/4/4/1/1 ^{AB}
RF0.1UV2	54.41 (3.68) ^{Aa}	0/6/4/1/1 ^{A}	51.70 (4.21) ^{Aa}	0/4/2/0/6 ^{B}
RF1UV1	49.51 (4.31) ^{ABa}	0/5/6/1/0 ^{A}	42.70 (5.13) ^{ABa}	0/5/2/2/3 ^{B}
GA	47.84 (5.29) ^{ABa}	1/4/3/1/3 ^{A}	31.48 (4.65) ^{ABb}	1/4/5/1/1 ^{B}

[a] μTBS: microtensile bond strength; Enz: after enzymatic digestion; P: pre-failure before the μTBS test; A: adhesive failure; M: mixed failure; Cd: completely cohesive fracture in dentin; Cr: completely cohesive fracture in resin composite.
[b] Identical uppercase letters represent no significant differences among treatments in the same column.
[c] Identical lowercase letters represent no significant differences between early and enzymatic degradation tests in the same row.

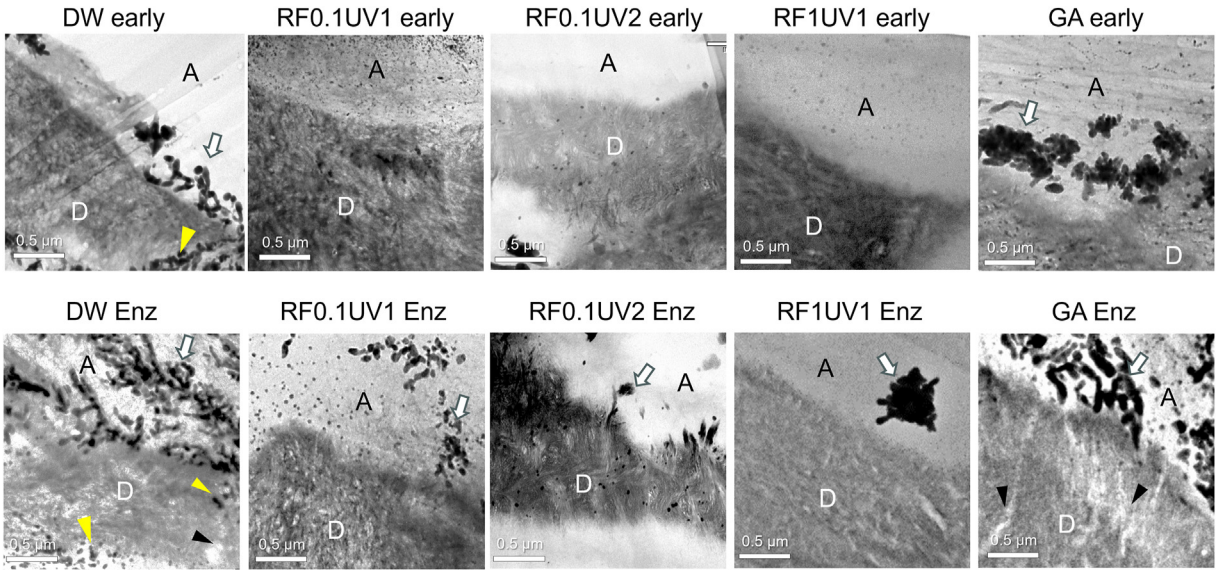

Figure 4 The TEM images in the early and enzyme-treated specimens (6000 × magnification). A: adhesive layer; D: dentin. Hollow arrows: silver grain deposit; yellow arrowheads: infiltration of silver grain into dentin collagen matrix; black arrowheads: crack lines in the collagen matrix. DW: distilled water; RF: riboflavin; UV: ultraviolet-A; GA: glutaraldehyde; Enz: enzymatic degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

After the enzymatic digestion, the silver grain deposits increased and aligned in a water-tree pattern in DW and GA groups. In these two groups, loosened collagen matrix and more infiltration of silver grains was also noticed. In RF0.1UV1 group, the amounts of silver grains increased. RF0.1UV2 and RF1UV1 showed the least nanoleakage. In RF0.1UV2, some grains are deposited on the collagen matrix. In RF1UV1, only isolated silver deposits were found in the adhesive layer.

With a higher magnification (× 100,000), the structure of collagen matrix in the enzyme treated specimens were revealed (Fig. 5). In the DW group, collagen fibrils in the interfacial zone were disorganized and somewhat loosened. In all RFUV groups, a layer of densely stained fibrils at the top of collagen matrix were found even after enzymatic digestion. In RF0.1UV2 and RF1UV1, the collagen fibrils were significantly denser and well-aligned compared to those in DW group. In the GA group, the densely stained fibril band was also shown. The typical characteristic of 67 nm cross-banding of type I collagen fibrils was found, indicating a highly crosslinked fibril structure (Fig. 5e). However, GA also presented dense deposition of silver grain near the HL, crack line of collagen matrix, and infiltration of silver stain into the matrix.

Discussion

Although the biomodification of dentinal collagen by using natural crosslinkers and GA has been investigated extensively, the potential of photo-activated RF-UVA strategy in this specific purpose remained to be elucidated. In this study, three practicable protocols RF0.1UV1, RF0.1UV2, and RF1UV1 were chosen based on our previous results.[20] Their abilities to crosslink collagen, enhance dentin bonding, and prevent collagen degradation in dentin specimens were examined.

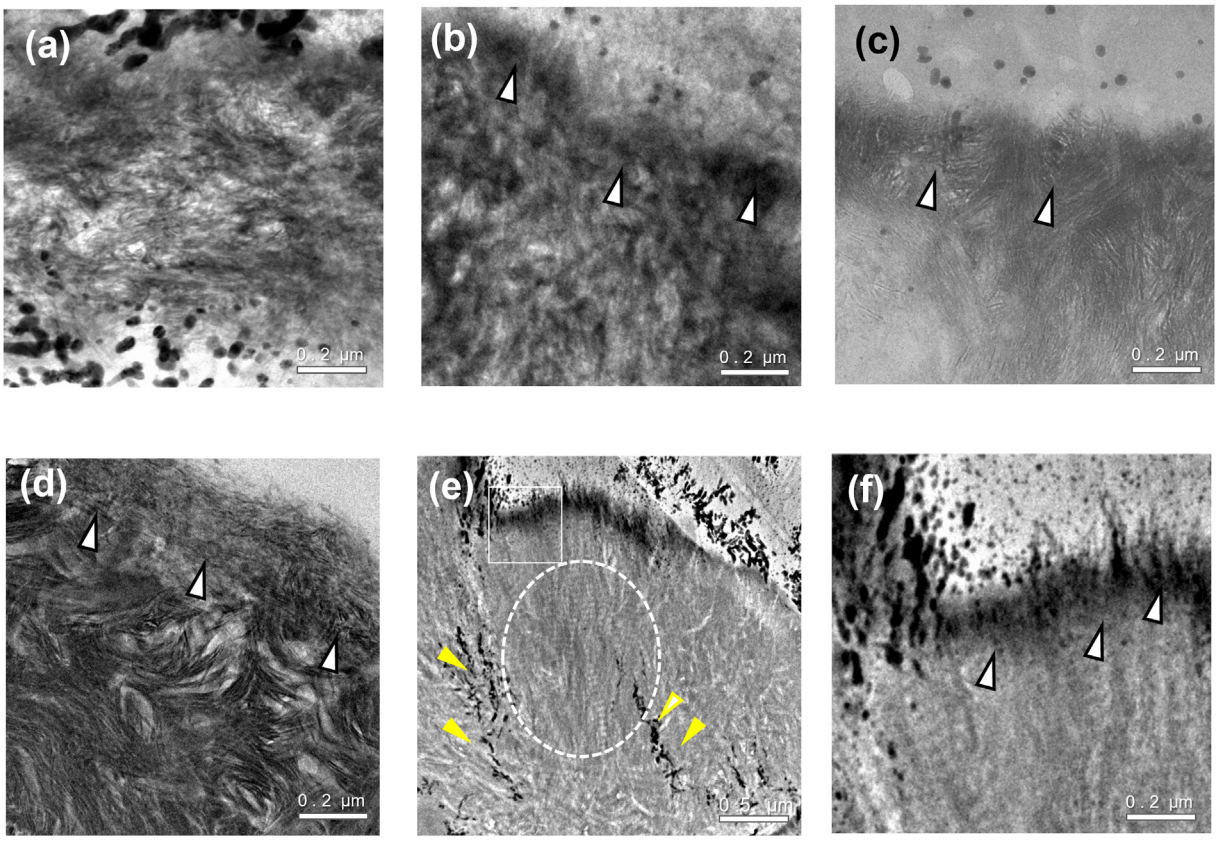

Figure 5 The collagen matrix in the enzyme-treated specimens. (a) DW. (b) RF0.1UV1. (c) RF0.1UV2. (d) RF1UV1. (e) GA. (f) The enlarge view of the islet in (e). Hollow arrowheads: collagen fibrils; yellow arrowheads: infiltration of silver grain into dentin collagen matrix; dashed circle: the area presenting 67 nm cross-banding of type I collagen. a-d, and f: 100,000 × magnification; e: 20,000 × magnification.

In this study, the SDS-PAGE test showed that all the RF-UVA groups enhanced collagen cross-linking, as evidenced by intense polypeptide deposits on the stacking gel, increased intensity of the γ trimer, and high molecular weight bands between the β and γ bands. The existence of the insoluble aggregates indicated the formation of cross-linked collagen, which is in accordance with previous studies.[20,22] Increasing the RF concentration from 0.1 % to 1 % did not increase the density of γ bands. Extending the UVA irradiation slightly enhanced the crosslinking in 0.1 % RF groups but not in 1 % RF groups. It is speculated that the 0.1 % RF may cross-link α-polypeptide chains of the collagen molecules into dimers and trimers in thermally denatured entities, while 1 % RF caused intensive aggregations which are immobilized in the stacking gels rather than separating in the running gel.

SDS-PAGE of GA group specimens also exhibited the ultra-high-molecular-weight aggregates formation, with no migrated polypeptide bands into the separating SDS-PA gel. After collagenase treatment, the aggregated collagen partially decomposed which led to a continuous band in the separating gel. As one of the most effective protein cross-linking reagents, GA can be applied in various aqueous forms and conditions. The crosslinking process induced by GA involves a large variety of pathways like aldol condensation or Michael-type addition reaction.[23] It is generally assumed that GA crosslinking of collagen occurs through reaction of the aldehyde groups of GA with the ε-amino groups of lysine or hydroxylysine residues of the collagen molecules. Subsequently, the intermediate products (a Schiff base) might be involved in either the formation of GA polymers due to aldol condensation reactions, or the formation of a secondary amine-type compound as a precursor to create quaternary pyridinium-type crosslinkage between collagen entities. The degree and products of GA cross-linking may vary with treatment time, temperature, concentration, and pH values.[24] There is no clear agreement about the main reactive species that participates in the crosslinking process because monomeric and polymeric forms of GA are in a dynamic equilibrium. Therefore, the enzymatic digested GA did not reveal the regular pattern of natural collagen but instead showed protein fragments of different molecular weights.

Compared to GA, the RF-UVA treatment works by forming covalent bonds to bridge amino groups of individual collagen fibrils.[25] This photochemical reaction involves UVA irradiation to cause subtle damage of the collagen, but also excite RF.[26] An energy transfer between the excited RF and molecular oxygen produces singlet oxygen that then interacts with the collagen fibrils in an oxidation reaction. The natural lysyl oxidase pathway is activated to connect the e-amino group of lysine or hydroxylysine to carboxy telopeptides and form new cross-links.[27] These physical cross-links did not alter the structure of collagen fibrils.

Therefore, RF-UVA presented typical type I collagen pattern after enzymatic digestion. Their γ band density decreased but still remained high compared to the negative control.

The present study demonstrated that all RF-UVA and GA cross-linkers increased early bond strength. Although RF0.1UV1 increased the early bond strength, its μTBS value reduced dramatically after digestion. RF0.1UV2 achieved the highest bond strengths in both stages, followed by RF1UV1. Both also showed favorable fracture patterns with most cohesive failure and no premature failure. The effectiveness of RF0.1UV2 has been demonstrated in previous studies for enhancing resin-dentin bond strength and decreasing interfacial nanoleakage,[19] and it was found to be superior to other agents such as carbodiimide and GSE.[28] RF0.1UV2 actually presented a similar degree of collagen gel crosslinking and resistance to enzymatic digestion compared to RF1UV1. Its high bond strength might be attributed to optimal stiffening of dentinal collagen matrix. Our previous study assessed the orientation of collagen fibrils in TEM images after the crosslinking treatments, and found a projecting alignment of collagen fibrils after RF0.1UV2 treatment and an enveloped matrix form in GA group.[20] In this study, RF0.1UV2 also owned the stiffened and expanded collagen matrix to facilitate the infiltration of resin monomer in the HL, which is contributed to the enhanced resin-dentin adhesion. Accordingly, RF0.1UV2 showed significantly improved bond strengths and reduced nanoleakage at both stages.

In this study, both R0.1UV2 and RF1UV1 displayed a well-organized collagen matrix after exposing to enzymatic degradation, indicating their ability to resist collagenase digestion. Previous studies have indicated that nearly all demineralized dentin collagen fibrils should have been digested after 1 day of collagenase treatment.[29,30] However, RF0.1UV2 preserved bond strength after 7-days of digestion. The capability of RF0.1UV2 in preserving bond durability after 18 months has been reported and is related to the inactivation of host-derived metalloproteinases.[31] Some investigators attribute this to the masking of the recognition site by the crosslinks, or to the retention of the cleaved peptide fragments by the newly formed cross-links within the collagen network.[32] The enhanced infiltration of resin monomers into the HL also contributes to increased resistance to enzymatic degradation, by either restricting enzyme release or reducing collagen matrix exposure.[33]

Although GA also improved the early μTBS, it did not maintain the bond strength after enzyme digestion. The TEM image of GA treated dentin revealed multifaceted changes, corresponding to the over-crosslinked reactions observed in the gel electrophoresis. GA group showed the presence of a densely stained fibril band, and coarse collagen fibers at the rim and within the dentin. Additionally, many silver grains were deposited near the HL, indicating the presence of nanoleakage. Considering the clinical availability, the material used in the GA group was the Gluma Desensitizer, which is a water-based adhesive containing HEMA, glutaraldehyde, and water. The water and HEMA contents of Gluma might cause the overwetting of the dentin surface,[34] and the strong crosslinking reaction rendered the collagen fibrils a barrier, restraining the infiltration of resin monomers. According to Munksgaard, GA in Gluma may crosslink albumin to form protein precipitates, which further react with poly-HEMA to occlude open dentinal tubules.[35] In the enzyme-treated GA specimen, the collagen fibers in the superficial layer were stained but became loosened, while those in the deep layer remained intact and resisted the enzyme digestion. Another specific finding in GA-treated dentin was the presence of crack lines and infiltration of silver grains deeply into the dentin. GA crosslinking may cause strong tension in the denuded collagen matrix, thus inducing the cracks. Similar findings have been reported as Gluma and other GA-containing adhesives improved initial bond strengths and decreased collagen solubilization, but did not counteract the bond strength reduction (7.2—28.9 %) after 6-month storage.[36] With these findings, GA treatment might render over-crosslinking of collagen fibrils and thus impaired the long-term resin-dentin bonding.

For the use of RF-UVA in conjunction with contemporary dentin bonding system, there is still limitations. In this study, the adhesive was a 3-step etch-and-rinse system. RF was applied after acid etching to strengthen the denuded collagen. When RF-UVA was used as an intermediate treatment in a 2-step etch-and-rinse system, its effectiveness in enhancing the durability and strength of the resin—dentin bond was also confirmed.[37] However, the crosslinking effects may not occur in self-etch systems since the collagen is not sufficiently exposed from the dentin surface. When attempting to pre-treat dentin surfaces with RF before applying a 2-step self-etch adhesive, a declined μTBS below the untreated samples was observed.[38] Recently, some investigators tried incorporating RF into universal adhesive to treat dentin.[39] Modified adhesives with 0.1 % RF under blue light activation improved the mechanical properties of demineralized dentin as well as stable resin-dentin bonding. This new application might simplify the use of RF crosslinking in this field and expand its clinical use.

With the limitations of this study, it can be concluded that RF-UVA treatment attained collagen crosslinking effect to improve resin-dentin bonding. The regimen combining 0.1 % RF and 2-min UVA is optimal to create a stiffen and expanded collagen matrix to allow resin infiltration and resists the enzymatic digestion. GA treatment, on the other hand, over-crosslinked the collagen matrix, leading to negative impacts such as tension on dentin. RF-UVA as a potential dentin bonding enhancer, its action mechanism and long-term effects of require further investigation.

Declaration of competing interest

The authors declare no conflict of interest relevant to this study.

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References

1. Nakabayashi N, Kojima K, Masuhara E. The promotion of adhesion by the infiltration of monomers into tooth substrates. *J Biomed Mater Res* 1982;16:265—73.
2. Nakabayashi N, Nakamura M, Yasuda N. Hybrid layer as a dentin-bonding mechanism. *J Esthetic Restor Dent* 1991;3:133—8.
3. Sano H, Takatsu T, Ciucchi B, Russell CM, Pashley DH. Tensile properties of resin-infiltrated demineralized human dentin. *J Dent Res* 1995;74:1093—102.
4. Breschi L, Prati C, Gobbi P, et al. Immunohistochemical analysis of collagen fibrils within the hybrid layer: a FEISEM study. *Operat Dent* 2004;29:538—46.
5. Pashley DH, Tay FR, Yiu C, et al. Collagen degradation by host-derived enzymes during aging. *J Dent Res* 2004;83:216—21.
6. Tjäderhane L, Nascimento FD, Breschi L, et al. Optimizing dentin bond durability: control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. *Dent Mater* 2013;29:116—35.
7. Mazzoni A, Tjäderhane L, Checchi V, et al. Role of dentin MMPs in caries progression and bond stability. *J Dent Res* 2015;94:241—51.
8. Linde A. Dentin matrix proteins: composition and possible functions in calcification. *Anat Rec* 1989;224:154—66.
9. Breschi L, Maravic T, Cunha SR, et al. Dentin bonding systems: from dentin collagen structure to bond preservation and clinical applications. *Dent Mater* 2018;34:78—96.
10. Bedran-Russo AK, Pereira PN, Duarte WR, Drummond JL, Yamauchi M. Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. *J Biomed Mater Res B Appl Biomater* 2007;80:268—72.
11. Bedran-Russo AK, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. *J Biomed Mater Res B Appl Biomater* 2008;86:330—4.
12. Carvalho RM, Mendonca JS, Santiago SL, et al. Effects of HEMA/solvent combinations on bond strength to dentin. *J Dent Res* 2003;82:597—601.
13. Silva JC, Cetira Filho EL, de Barros Silva PG, Costa FWG, Saboia VPA. Is dentin biomodification with collagen cross-linking agents effective for improving dentin adhesion? A systematic review and meta-analysis. *Restor Dent Endod* 2022;47:e23.
14. Cilli R, Prakki A, de Araujo PA, Pereira JC. Influence of glutaraldehyde priming on bond strength of an experimental adhesive system applied to wet and dry dentine. *J Dent* 2009;37:212—8.
15. Bedran-Russo AKB, Castellan CS, Shinohara MS, Hassan L, Antunes A. Characterization of biomodified dentin matrices for potential preventive and reparative therapies. *Acta Biomater* 2011;7:1735—41.
16. Eyüboğlu G, Yeşilyurt C, Ertürk M. Evaluation of cytotoxicity of dentin desensitizing products. *Operat Dent* 2015;40:503—14.
17. Scheffel DLS, Soares DG, Basso FG, de Souza Costa CA, Pashley D, Hebling J. Transdentinal cytotoxicity of glutaraldehyde on odontoblast-like cells. *J Dent* 2015;43:997—1006.
18. Wollensak G. Crosslinking treatment of progressive keratoconus: new hope. *Curr Opin Ophthalmol* 2006;17:356—60.
19. Cova A, Breschi L, Nato F, et al. Effect of UVA-activated riboflavin on dentin bonding. *J Dent Res* 2011;90:1439—45.
20. Chiang YS, Chen YL, Chuang SF, et al. Riboflavin-ultraviolet-A-induced collagen cross-linking treatments in improving dentin bonding. *Dent Mater* 2013;29:682—92.
21. Hardan L, Daood U, Bourgi R, et al. Effect of collagen cross-linkers on dentin bond strength of adhesive systems: a systematic review and meta-analysis. *Cells* 2022;11:2417.
22. Wollensak G, Redl B. Gel electrophoretic analysis of corneal collagen after photodynamic cross-linking treatment. *Cornea* 2008;27:353—6.
23. Migneault I, Dartiguenave C, Bertrand MJ, Waldron KC. Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques* 2004;37:790—802.
24. Ruijgrok J, De Wijn J, Boon M. Glutaraldehyde crosslinking of collagen: effects of time, temperature, concentration and presoaking as measured by shrinkage temperature. *Clin Mater* 1994;17:23—7.
25. Seseogullari-Dirihan R, Tjäderhane L, Pashley DH, Tezvergil-Mutluay A. Effect of ultraviolet A-induced crosslinking on dentin collagen matrix. *Dent Mater* 2015;31:1225—31.
26. Menter JM, Patta AM, Sayre RM, Dowdy J, Willis I. Effect of UV irradiation on type I collagen fibril formation in neutral collagen solutions. *Photodermatol Photoimmunol Photomed* 2001;17:114—20.
27. Ashwin PT, McDonnell PJ. Collagen cross-linkage: a comprehensive review and directions for future research. *Br J Ophthalmol* 2010;94:965—70.
28. Venigalla BS, Jyothi P, Kamishetty S, Reddy S, Cherukupalli RC, Reddy DA. Resin bond strength to water versus ethanol-saturated human dentin pretreated with three different cross-linking agents. *J Conserv Dent Endod* 2016;19:555—9.
29. Bedran-Russo AKB, Vidal CMP, Dos Santos PH, Castellan CS. Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds. *J Biomed Mater Res B Appl Biomater* 2010;94:250—5.
30. Macedo GV, Yamauchi M, Bedran-Russo AK. Effects of chemical cross-linkers on caries-affected dentin bonding. *J Dent Res* 2009;88:1096—100.
31. Hass V, Luque-Martinez IV, Gutierrez MF, et al. Collagen cross-linkers on dentin bonding: stability of the adhesive interfaces, degree of conversion of the adhesive, cytotoxicity and in situ MMP inhibition. *Dent Mater* 2016;32:732—41.
32. Charulatha V, Rajaram A. Influence of different crosslinking treatments on the physical properties of collagen membranes. *Biomaterials* 2003;24:759—67.
33. Green B, Yao X, Ganguly A, et al. Grape seed proanthocyanidins increase collagen biodegradation resistance in the dentin/adhesive interface when included in an adhesive. *J Dent* 2010;38:908—15.
34. Tay FR, Gwinnett JA, Wei SH. Micromorphological spectrum of acid-conditioned dentin following the application of a water-based adhesive. *Dent Mater* 1998;14:329—38.
35. Munksgaard E. Amine-induced polymerization of aqueous HEMA/aldehyde during action as a dentin bonding agent. *J Dent Res* 1990;69:1236—9.
36. Lee J, Sabatini C. Glutaraldehyde collagen cross-linking stabilizes resin—dentin interfaces and reduces bond degradation. *Eur J Oral Sci* 2017;125:63—71.
37. Abunawareg M, Abuelenain D, Elkassas D, et al. Role of dentin cross-linking agents in optimizing dentin bond durability. *Int J Adhesion Adhes* 2017;78:83—8.
38. Kasraei S, Mojtahedi M, Goodarzi MT, Azarsina M, Khamverdi Z. The effect of dentin pre-treatment with activated riboflavin on the bond strength of a two-step self-etch adhesive system. *Dent Med Probl* 2019;56:143—8.
39. Fu C, Deng S, Koneski I, et al. Multiscale in-vitro analysis of photo-activated riboflavin incorporated in an experimental universal adhesive. *J Mech Behav Biomed Mater* 2020;112:104082.