Chitosan-Based Extrafibrillar Demineralization for Dentin Bonding

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APPENDIX

Bonding procedures for bond testing

Application methods of the two experimental adhesives examined in the present study are summarized in the Appendix Table. For water-wet bonding of the hydrophilic adhesive, dentin surfaces were conditioned with 15 wt% phosphoric acid for 15 sec, or 1 wt% chitosan for 30 sec or 60 sec, rinsed with water and blot-dried with lint-free paper. Two consecutive coats of the experimental hydrophilic adhesive were applied. The first coat was applied with an applicator tip for 15 sec to thoroughly wet the conditioned dentin surface. The adhesive was gently air-dried with oil- and moisture-free air for 10 sec to evaporate the ethanol solvent. Drying/evaporation was performed approximately 10-15 cm away from the adhesive-coated surface and gradually bringing the air-source to within 10 mm of the surface over the 10-sec period, producing a surface with a uniform glossy appearance. The second layer of adhesive was subsequently applied, air-dried and polymerized with a visible light-emitting diode curing unit with an energy intensity of 1,000 mW/cm^2 (Valo Plus, Ultradent Products, Inc., South Jordan, UT) for 20 sec. For the experimental hydrophobic adhesive, the wet bonding mode was the ethanol-wet bonding mode. After water rinsing and blot drying, the conditioned dentin was saturated with 100% ethanol. The latter was left on the conditioned dentin surface for 30 sec prior to blot drying with lint-free paper. The adhesive was applied and light-cured as described previously.

For dry bonding of the hydrophilic or the hydrophobic adhesive, the dentin surfaces were conditioned with 15 wt% phosphoric acid for 15 sec, or 1 wt% chitosan for 30 sec or 60 sec. The conditioned dentin was air-dried with oil- and moisture-free air for 5 sec. Each adhesive was applied and light-cured in the manner described for wet bonding.

After light-curing of each adhesive, two 2-mm thick layers of a light-curable resin composite (Clearfil AP-X, Kuraray Noritake Dental Inc., Tokyo, Japan) were placed over the bonded dentin. Each layer was light-cured for 40 sec. Bonded specimens were stored in deionized water at 37 °C

for 24 hours prior to sectioning vertically into 0.9 mm thick resin-dentin slabs. The two slabs adjacent to the central slab of each tooth were further sectioned into 0.9 × 0.9 mm sticks. The four longest sticks from those two slabs were selected, yielding 4 sticks per tooth (i.e. 80 sticks/group) for bond strength evaluation. Each stick was attached to a testing jig with cyanoacrylate adhesive and stressed to failure under tension with a universal testing machine using a crosshead speed of 1 mm/min. The maximum force recorded at failure was divided by the cross-sectioned area of each stick to yield the tensile bond strength in megaPascals (MPa). Bonding and bond strength tesing were performed by one trained operator. The dentin side of each fractured beam was examined with a stereoscopical microscope at 40x magnification to identify the failure mode. Failure modes were classified as adhesive failure, mixed failure (failure extending into dentin or resin composite), cohesive failure in resin composite or cohesive failure in dentin.

Appendix Table. Composition and application mode of the two experimental resins

Resin	pH of solvated experimental resin (resin 70 wt%, ethanol 30 wt%)	Wet-bonding mode	Dry-bonding mode
Hydrophobic resin blend	6.53	1. Apply 15 wt% phosphoric acid for 15 sec or 1 wt% chitosan for 30 sec or 60 sec, then rinse and blot-dry with lint-free paper. 2. Apply 100% ethanol to the conditioned dentin surface, leave in place for 30 sec, and blot dry with lint-free paper. 3. Apply the first coat of solvated resin to thoroughly wet all the tooth surfaces with applicator tip for 15 sec, using light brushing motion. 4. Gently air-dry the adhesive for approximately 3 sec for the solvent to evaporate. 5. Apply the second layer of solvated resin with applicator tip for 30 sec, using light brushing motion, and gently air-dry for 3 sec. 6. Light cure for 20 sec.	1. Apply 15 wt% phosphoric acid for 15 sec or 1 wt% chitosan for 30 sec or 60 sec, then rinse and apply oil-and moisture-free air for 5 sec. 2. Apply the first coat of solvated resin to thoroughly wet all the tooth surfaces with applicator tip for 15 sec, using light brushing motion. 3. Gently air-dry the adhesive for approximately 3 sec for the solvent to evaporate. 4. Apply the second layer of solvated resin with applicator tip for 30 sec, using light brushing motion, and gently air-dry for 3 sec. 5. Light cure for 20 sec.
Hydrophilic resin blend	6.84	1. Apply 15 wt% phosphoric acid for 15 sec or 1 wt% chitosan for 30 sec or 60 sec, then rinse and blot-dry with lint-free paper. 2. Apply the first coat of solvated resin to thoroughly wet all the tooth surfaces with applicator tip for 15 sec, using light brushing motion. 3. Gently air-dry the adhesive for approximately 3 sec for the solvent to evaporate. 4. Apply the second layer of solvated resin with applicator tip for 30 sec, using light brushing motion, and gently air-dry for 3 sec. 5. Light cure for 20 sec.	1. Apply 15 wt% phosphoric acid for 15 sec or 1 wt% chitosan for 30 sec or 60 sec, then rinse and apply oil-and moisture-free air for 5 sec. 2. Apply the first coat of solvated resin to thoroughly wet all the tooth surfaces with applicator tip for 15 sec, using light brushing motion. 3. Gently air-dry the adhesive for approximately 3 sec for the solvent to evaporate. 4. Apply the second layer of solvated resin with applicator tip for 30 sec, using light brushing motion, and gently air-dry for 3 sec. 5. Light cure for 20 sec.

Abbreviations: Bis-GMA, bisphenol A diglycidyl ether dimethacrylate; CQ, camphorquinone; EDMAB, ethyl *N*,*N*-dimethyl-4-aminobenzoate; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, triethylene-glycol dimethacrylate

Procedures for *in-situ* zymography

Due to the highly cross-linked nature of dentin collagen, degradation of hybrid layers by activated endogenous matrix metalloproteinases requires specimens to be aged for 9-12 months before visible evidence of collagen breakdown can be identified. *In-situ* zymography is an expedited, quantifiable laboratory technique for comparing the relative degradation potential of resin-dentin interfaces, without relying on actual degradation of the resin-sparse, water-rich collagen fibrils. Rapid localization of matrix metalloproteinase activities in histological sections is achieved by supplementing the sections with a quenched fluorescein-conjugated substrate. The supplemental gelatin substrate can be easily degraded within 48 hours by the activated forms of dentin matrix-bound matrix metalloproteinases.

Each bonded dentin slab was attached with cyanoacrylate cement to a glass slide and polished sequentially with 600- and 1200-grit wet silicon carbide papers under running water to obtain an approximately 50 μm thick section. The coarsely-polished section was further refined by polishing with 4000-grit wet silicon carbide paper for 5 min to obtain a highly-glossy surface. Fifty microliters of quenched fluorescein-conjugated gelatin (E-12055, Molecular Probes, Eugene, OR) was then placed on top of each slab and protected with a cover slip. Light-protected gelatin-loaded slide assemblies were incubated in 100% relative humidity at 37 °C for 24 hours. Endogenous gelatinolytic activity within the resin-dentin interface could be visualized after hydrolysis of the quenched fluorescein-conjugated gelatin. This evaluated using a two-photon confocal laser scanning microscope (LSM 780, Carl Zeiss, Oberkochen, Germany). Microscopy was performed by an independent observer who was unaware of the treatment applied to the crown segments. Three areas, each with a surface area of 84.9 μm x 84.9 μm, were used for evaluation of gelatinolytic activity for each specimen derived from the 16 subgroups (see Main Text). For consistency, one area was taken from the center between the two dentinoenamel junctions. The two other areas were taken at 2 mm away from each respective dentinoenamel junction. For each area, twenty 350 nm thick optical sections were acquired from different focal planes. Stacked images were processed with the Zeiss ZEN 2010 software. Fluorescence emitted by the hydrolyzed fluorescein-conjugated gelatin was quantified using Image J software (National Institutes of Health, Bethesda, MD). Gelatinolytic activity was expressed as a percentage of the green fluorescence within the hybrid layer (3 areas × 10 specimens; N = 30).

Procedures for interfacial water permeability evaluation

Three drops of each adhesive were mixed with 1 μL of a yellow fluorescent dye (Alexa Fluor™ 532, $\lambda_{ex}/\lambda_{em}$ 532/553 nm; ThermoFisher Scientific, Waltham, MA) and light-protected until use. Bonded crown segments were attached to perforated plexiglass platforms that were connected with polyethylene 18-gauge polyethylene tubings to water-containing syringes to simulate 20 cm physiologic pulpal pressure. The water used for permeability testing incorporated a blue fluorescent dye (Alexa Fluor™ 405, ThermoFisher Scientific) for identification with CLSM. Adhesive procedures and restoration build-up was performed under water pressure. The two experimental adhesives were applied using the corresponding wet bonding technique (i.e. water-wet bonding for the hydrophilic adhesive and ethanol-wet bonding for the hydrophilic adhesive) on dentin conditioned with 15 wt% phosphoric acid for 15 sec (control) or 1 wt% chitosan for 60 sec. After resin composite build-up with Clearfil AP-X hybrid composite, each bonded crown segment was removed from the pulpal pressure simulation setup. A 1-mm thick section containing the resin-dentin interface was sectioned from each crown segment, highly-polished under running water and stabilized on a glass slide with cyanoacrylate glue for CLSM imaging. Three 84.9 μm x 84.9 μm optical section series were acquired per slab from different focal planes (N = 15 images). One image was taken from the center of each slab, and the other two images were taken from each side where the remaining dentin thickness was the thinnest from the dentin surface. Blue fluorescence within and above the hybrid layer in the stacked images was taken to represent the permeability of the interface to water movement during bonding. The most extensive blue fluorescence among the three locations was used to represent the worst water permeability scenario for a particular tooth.

***In-situ* zymography of resin-dentin interfaces bonded with experimental hydrophilic adhesive: 3-factor ANOVA and Holm-Sidak pairwise comparisons**

Normality Test (Shapiro-Wilk) Passed (P = 0.779)

Equal Variance Test: Passed (P = 0.178)

Source of variation	**F value**	**P value**
Conditioner	2099.949	<0.001*
Bonding mode	6.697	0.014*
Thermomechanical cycling	20.141	<0.001*
Conditioner x Bonding mode	18.016	<0.001*
Conditioner x Thermomechanical cycling	7.194	0.011*
Bonding mode x Thermomechanical cycling	0.321	0.575
Conditioner x Bonding mode x Thermomechanical cycling	0.146	0.705

*Factors and interactions marked with asterisks are significantly different ($p < 0.05$)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method): Overall significance level = 0.05

Comparisons for factor: **Conditioner**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	3.924	45.825	<0.001	Yes

Comparisons for factor: **Bonding mode**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	0.222	2.588	0.014	Yes

Comparisons for factor: **Thermomechanical cycling (TMC)**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Before TMC *vs* After TMC	0.384	4.488	<0.001	Yes

Comparisons for factor: **Bonding mode** within **Chitosan**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	4.287	35.405	<0.001	Yes

Comparisons for factor: **Bonding mode** within **Phosphoric acid**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	3.560	29.402	<0.001	Yes

Comparisons for factor: **Conditioner** within **Wet bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.585	4.831	<0.001	Yes

Comparisons for factor: **Conditioner** within **Dry bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.142	1.171	0.250	No

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Chitosan**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	4.153	34.300	<0.001	Yes

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Phosphoric acid**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	3.694	30.507	<0.001	Yes

Comparisons for factor: **Conditioner** within **Before TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.614	5.070	<0.001	Yes

Comparisons for factor: **Conditioner** within **After TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.155	1.277	0.211	No

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Wet bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Before TMC *vs* After TMC	0.270	2.231	0.033	Yes

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Dry bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Before TMC *vs* After TMC	0.173	1.429	0.163	No

Comparisons for factor: **Bonding mode** within **Before TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	0.433	3.574	0.001	Yes

Comparisons for factor: **Bonding mode** within **After TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* After bonding	0.336	2.773	0.009	Yes

***In-situ* zymography of resin-dentin interfaces bonded with experimental hydrophobic adhesive: 3-factor ANOVA and Holm-Sidak pairwise comparisons**

Normality Test (Shapiro-Wilk) Passed (P = 0.136)

Equal Variance Test: Passed (P = 0.088)

Source of variation	**F value**	**P value**
Conditioner	1086.638	<0.001*
Bonding mode	55.009	<0.001*
Thermomechanical cycling	2.298	0.139
Conditioner x Bonding mode	82.427	<0.001*
Conditioner x Thermomechanical cycling	1.933	0.174
Bonding mode x Thermomechanical cycling	0.226	0.638
Conditioner x Bonding mode x Thermomechanical cycling	0.00395	0.950

*Factors and interactions marked with asterisks are significantly different (p < 0.05)

All Pairwise Multiple Comparison Procedures (Holm-Sidak method): Overall significance level = 0.05

Comparisons for factor: **Conditioner**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	3.699	32.964	<0.001	Yes

Comparisons for factor: **Bonding mode**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	0.832	7.417	<0.001	Yes

Comparisons for factor: **Thermomechanical cycling (TMC)**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Before TMC *vs* After TMC	0.170	1.516	0.139	Yes

Comparisons for factor: **Bonding mode** within **Chitosan**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	4.717	29.729	<0.001	Yes

Comparisons for factor: **Bonding mode** within **Phosphoric acid**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	2.680	16.889	<0.001	Yes

Comparisons for factor: **Conditioner** within **Wet bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	1.581	11.664	<0.001	Yes

Comparisons for factor: **Conditioner** within **Dry bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.186	1.175	0.249	No

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Chitosan**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	3.885	24.292	<0.001	Yes

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Phosphoric acid**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	3.543	22.326	<0.001	Yes

Comparisons for factor: **Conditioner** within **Before TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.326	2.055	0.048	Yes

Comparisons for factor: **Conditioner** within **After TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Chitosan *vs* Phosphoric acid	0.014	0.089	0.930	No

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Wet bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Before TMC *vs* After TMC	0.885	5.581	<0.001	Yes

Comparisons for factor: **Thermomechanical cycling (TMC)** within **Dry bonding**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Before TMC *vs* After TMC	0.779	4.908	<0.001	Yes

Comparisons for factor: **Bonding mode** within **Before TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* Dry bonding	0.223	1.408	0.169	No

Comparisons for factor: **Bonding mode** within **After TMC**

Comparison	**Difference of means**	**t**	**P value**	**P<0.05**
Wet bonding *vs* After bonding	0.117	0.736	0.467	No