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***In-vitro* enzymatic biodegradation of adhesive resin in the hybrid layer**

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Abstract

Penetration of adhesives into the demineralized dentin surface and their subsequent conversion are critically important to longevity of the adhesive resin-dentin bond. The durability of the resin-dentin bond is investigated by monitoring the change of adhesive concentration within the hybrid layer of aged specimens using Raman spectroscopy. Absolute molar concentrations of Bis-GMA and HEMA were measured across the hybrid layer of resin-dentin specimens 24 h after photopolymerization and after 24-wk storage in one of three media: artificial saliva (SAL), SAL containing cholesterol esterase to attack resin (EST), and SAL containing bacterial collagenase to attack collagen (COL). No significant difference among these groups for both Bis-GMA and HEMA molar concentrations at 24-h storage was found; however, concentrations decreased from the adhesive resin to the middle of the hybrid layer. Concentrations remained unchanged at any resin-dentin position after aging in SAL. In the hybrid layer, concentrations significantly decreased with aging in COL and tended to decrease in EST. While showing potential enzymatic biodegradative effects of endogenous matrix metalloproteinases and salivary esterases, this methodology may also prove to be a valuable assessment of new chemistries and future approaches to improve resin-dentin bond performance.

Keywords

dentin; adhesive resin; Raman spectroscopy; hybrid layer; molar concentration

1. Introduction

Many accomplishments have been realized in dentin adhesion over the past three decades; however, improved durability of the resin-dentin bond is required for wider clinical application of adhesive bonding techniques.¹ Studies have indicated that the hybrid layer² is a weak link in the resin-dentin bond, and its failure could be time and adhesive-system dependent.^{3,4} Resin-dentin bond durability studies typically focus on the acquisition of either physical properties or morphological properties of aged bonds, with the most frequently used techniques including bond strength, fatigue resistance, leakage, and marginal analysis.^{3,5-8} For example, fracture toughness studies show that most failures occur, as observed clinically,⁹ in the interfacial region. Similar results have been also obtained based on fatigue-resistance¹⁰ and nanoleakage¹¹ experiments. However, these measurements are qualitative and indirect, and comparison among different groups is difficult, if not impossible. For example, micro-tensile and shear bond-strength-related techniques measure the decrease of bond strength after long-term storage with the assumption that the stronger the bonding, the better it will resist physical and chemical attack. However, the results depend not only on the properties of the bonding, but also several experimental factors, such as stress rate, sample shape, surface area, geometry, and

test method.^{12,13} Therefore, bond strength value is not a material property and lacks the ability to be used for comparison among data obtained from different groups.¹⁴ Although resin-dentin bond strength is a useful screening test for new products, absolute monomer concentrations within the hybrid layer may prove to be more predictive of clinical outcomes.

Successful penetration of adhesive resin into demineralized dentin has a positive effect on the long-term properties of the resin-dentin bond. However, in a previous study,¹⁵ adhesive infiltration was shown to be far from perfect, with absolute molar concentrations of both Bis-GMA and HEMA at the middle of the hybrid layer 10 and 19%, respectively, of expected concentrations in an ideally infiltrated hybrid layer. Since Bis-GMA is a di-functional monomer, necessary to form cross-linked polymer networks, it is unlikely that a strongly cross-linked polymer network is formed in the bottom half of the hybrid layer. Without the protection afforded by a strongly cross-linked network, acrylate-based polymers, which contain ester linkages, could be susceptible to hydrolysis and enzymatic biodegradation. Studies have shown that the water penetrating into the hybrid layer can cause elution of residual monomer, as well as biodegradation of polymer matrix.¹⁶⁻¹⁸ Biodegradation induced by hydrolysis includes the breaking of covalent bonds and the depletion of collagen fibrils and may reduce the mechanical strength of the bonding material.¹⁹ In addition, biodegradation of the hybrid layer could be expedited by enzymes existing in saliva.²⁰ For example, one study has shown that the cholesterol esterase in saliva could preferentially degrade monomers containing aromatic rings, such as Bis-GMA, in 16-day storage.²¹ Besides biodegradation of the polymer matrix, the collagen network of the hybrid layer may also be attacked after water storage.^{22,23} If the biodegradation of the collagen network in the hybrid layer was accelerated by enzyme activity, the exposed polymer matrix could be more susceptible to hydrolysis by incubation media. All of these effects would decrease the absolute concentrations of the comonomers (Bis-GMA and HEMA in this study) within the hybrid layer.

Therefore, the purpose of this study is to monitor the change of adhesive concentration within the hybrid layer of aged specimens. The objectives of this study are to: 1) measure the absolute molar concentration (c_i) of the comonomers Bis-GMA and HEMA within the hybrid layer after 24-wk storage for specimens stored in inorganic artificial saliva and in either cholesterol esterase or collagenase dissolved in the artificial saliva and 2) evaluate the effect of incubation media and storage time on adhesive resin within the hybrid layer by comparing c_i obtained after 24-wk storage to baseline at 24 h. The research hypotheses tested are: 1) the $c_{\text{Bis-GMA}}$ and c_{HEMA} in the adhesive resin are lower after 24-wk storage among all groups and 2) the $c_{\text{Bis-GMA}}$ and c_{HEMA} within the hybrid layer are lower after 24-wk storage, with the greatest change seen in the esterase and collagenase groups.

2. Materials and Methods

2.1 Materials

The resin-dentin bonded sample consisted of a photopolymerized commercial adhesive system (Scotchbond MultiPurpose, Lot # 9KU 3M ESPE, St. Paul, MN, USA) tested after it had been applied to the surface of an extracted human tooth, had composite placed and polymerized on top, and was subsequently stored in water. An experimental dentin primer, containing only HEMA and acetone, was used for experimental simplification and for comparative purposes to previous and ongoing studies.^{24,25} The resin-dentin bonded sample preparation has been described earlier.²⁶ Briefly, the bonded tooth was first sectioned into 2 mm × 2 mm sticks using a water-cooled, low-speed diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA). Then, a randomly selected side of each stick was wet polished with 600 and 800 grit SiC paper for 30 s, followed by a sequence of 6, 1, and 0.05- μm diamond pastes with brief distilled water rinsing between steps. To remove any remaining debris or

oil from abrasive papers and pastes, a soap solution was briefly applied to the stick with a cotton applicator before sonicating the specimen in distilled water for 30 min, followed by a 3–5 s application of aqueous 6% sodium hypochlorite (Clorox, The Clorox Company, Oakland, CA, USA). The polished specimens were then randomly placed in one of three storage media, sealed in vials, and stored in the dark for 24 wk at 37°C: (1) inorganic artificial saliva²⁷ with 1000 ppm sodium azide (Lot # 0631B36, Amresco, Solon, OH, USA) to prevent bacterial growth, pH 7.0 (SAL); (2) 1 unit cholesterol esterase (Lot # M8S2589, Worthington Biochemical Corp., Lakewood, NJ, USA) per mL of SAL (EST); and (3) 25 units bacterial (*Clostridium histolyticum*) collagenase (Lot # 40C3814, Worthington Biochemical Corp.) per mL of SAL (COL). Once a week, the incubation medium was exchanged, and the old medium was stored at –20°C for potential future analyses.

2.2 Methods

2.2.1 Raman Spectroscopy—Raman spectra were collected using an optical microscope (DM-LP, Leica Microsystems GmbH, Wetzlar, DEU) attached to a modular research Raman spectrograph (HoloLab5000R, Kaiser Optical Systems, Inc., Ann Arbor, MI, USA) using a 785 nm laser to induce the Raman scattering effect. The spectral coverage of this model ranges from 100 to 3450 cm^{-1} with an average spectral resolution of 5 cm^{-1} . The laser power was 8–10 mW with an exposure time of 60 s. Wavelength and intensity calibrations were performed using a calibration accessory (HCA-RXN, Kaiser Optical Systems, Inc.) according to manufacturer's specifications before each experiment. All measurements were carried out with auto-dark subtraction and cosmic ray correction. The Raman microscope was used to obtain three line scans per specimen across the hybrid layer (HL) in the intertubular region using a 100× objective. Scans were obtained at 24 h for each storage media and repeated on the same specimens after 24-wk storage at the same location in the specimen. To ensure the same location in the specimen was sampled each time, a notch was cut in one end of the polished specimen for orientation. The identical sampling location was found by stepping the computer-controlled stage a precise distance from the edge of the specimen and confirming the morphological characteristics of the hybrid layer at that position with specimen photos from previous experiments. Averaged integrated peak areas of 605 cm^{-1} and 1610 cm^{-1} were used in subsequent calculations to determine molar concentrations of Bis-GMA and HEMA. Because the hybrid layer thickness ranged from 5–8 microns, 6 standardized locations spanning the resin-dentin bond were used for data acquisition as described earlier,²⁶ namely: adhesive resin (AR), top of hybrid layer (THL), top quarter of hybrid layer (TQHL), middle of hybrid layer (MHL), bottom quarter of hybrid layer (BQHL), and bottom of hybrid layer (BHL).

2.2.2 Molar Concentration Determination—Bis-GMA and HEMA molar concentration is operationally defined as the number of monomers and/or polymer repeat units in the Raman sampling volume. Absolute molar concentration of Bis-GMA and HEMA from dentin-adhesive specimens were calculated from the Raman spectra collected across the hybrid layer. A detailed description of the method has been presented earlier.¹⁵ In brief, the hybrid layer was treated as a three-component system consisting of a binary-penetrated adhesive system and collagen. Separate calibration curves for comonomer relative molar concentration and Bis-GMA absolute molar concentration were constructed from adhesive standard specimens and Raman peak area ratios (1610 cm^{-1} for the Bis-GMA aromatic ring and 605 cm^{-1} for the methacrylate CCO). Since collagen and water were considered “Raman inactive” (i.e., the 785 nm laser used in these experiments does not induce a detectable Raman peak for either component in the fingerprint region), a dilution coefficient K_j for each sampling volume was defined to describe their impact on Raman peak area and to calculate HEMA absolute molar concentration.

2.2.3 Statistical Method—Molar concentrations for Bis-GMA and HEMA were separately tested comparing 24-h to 24-wk storage using a paired t-test for all storage media. Molar concentrations for Bis-GMA and HEMA were also compared across storage media using repeated-measures ANOVA with Bonferroni *post hoc*. Statistical significance was set at $\alpha = 0.05$ for all tests using a statistical software package (Prism v. 4.00, GraphPad Software, Inc., San Diego, CA, USA).

3. Results

Since the adhesive peak intensity significantly decreased at depths greater than MHL, reliable absolute molar concentrations of adhesive components could be reported only for the first four locations (AR – MHL). Because the THL is the interface between the AR and TQHL and contains contributions from both sides during Raman acquisition, THL data were excluded from the statistical analyses.

Absolute $c_{\text{Bis-GMA}}$ and c_{HEMA} in the AR and across the HL at 24 h and 24 wk were determined from specimens stored in three different storage media and are reported in Table 1. Repeated-measures ANOVA confirmed that no significant difference ($p > 0.05$) existed for $c_{\text{Bis-GMA}}$ and c_{HEMA} at each location (AR, TQHL, and MHL) across the groups (SAL, EST, and COL) after 24 h. Therefore, there is no significant difference among these three groups for both Bis-GMA and HEMA molar concentrations at baseline storage. However, after 24 wk, absolute $c_{\text{Bis-GMA}}$ and c_{HEMA} differences were observed within storage groups as tested by ANOVA with Bonferroni *post hoc* across each media, as did paired t-tests between 24-h and 24-wk sets (Table 1).

Analysis of absolute $c_{\text{Bis-GMA}}$ and c_{HEMA} in the AR was performed by comparing results obtained at 24-h and 24-wk storage. Paired t-tests showed that absolute $c_{\text{Bis-GMA}}$ was significantly lower in EST ($p = 0.001$) and tended to be lower in SAL ($p = 0.0571$). However, a significantly higher $c_{\text{Bis-GMA}}$ after 24-wk storage was observed for the COL group ($p = 0.026$). Repeated ANOVA analysis showed that after 24-wk storage, $c_{\text{Bis-GMA}}$ in the SAL and EST groups were equal and were significantly lower than in the COL group ($p = 0.0049$). For absolute c_{HEMA} in the AR after 24-wk storage, t-test analysis showed: c_{HEMA} tended to be lower in the COL group ($p = 0.0555$), significantly lower in the EST group ($p = 0.001$), and tended to be higher in the SAL group ($p = 0.0568$). Repeated ANOVA analysis showed that absolute c_{HEMA} was significantly higher in the SAL and EST groups than in the COL group ($p = 0.005$).

Analyses of absolute $c_{\text{Bis-GMA}}$ and c_{HEMA} at TQHL and MHL were performed by comparing results obtained at 24-h and 24-wk storage. For Bis-GMA, t-test analysis showed that absolute $c_{\text{Bis-GMA}}$ tended to be lower after 24-wk storage; however, a statistically significant decrease was only observed for the COL group at both TQHL ($p = 0.043$) and MHL ($p = 0.013$). For the EST group, a similar trend was observed at TQHL and MHL. Repeated ANOVA analysis showed that lower absolute $c_{\text{Bis-GMA}}$ after 24-wk storage in EST and COL was significant at TQHL ($p < 0.0001$), but not significant at MHL ($p = 0.767$). For HEMA, t-test analysis showed that the absolute c_{HEMA} tended to be lower after 24-wk storage for the EST group at TQHL ($p = 0.058$) and significantly decreased in COL at MHL ($p = 0.028$). The c_{HEMA} in SAL was not significantly different after 24-wk storage. Repeated ANOVA analysis showed that lower absolute c_{HEMA} after 24-wk storage in EST and COL was significant at TQHL ($p < 0.0001$), but not significant at MHL ($p = 0.0647$).

4. Discussion

No significant differences in molar concentrations were found for specimens at 24 h at each acquisition position across all groups; therefore, any variations observed after 24-wk storage should be due to time or incubation media. In the AR, after 24-wk storage, the absolute $c_{\text{Bis-GMA}}$ tended to be lower in the EST and SAL groups, while the COL group witnessed an increase. The absolute c_{HEMA} increased in the EST and SAL groups, but decreased in the COL group. Therefore, the first hypothesis (i.e., $c_{\text{Bis-GMA}}$ and c_{HEMA} in AR are lower after 24-wk storage among all groups) was rejected. Within the HL, after 24-wk storage, the absolute $c_{\text{Bis-GMA}}$ demonstrated a decrease in the COL group at both TQHL and MHL; a similar trend was observed for the EST group at TQHL, but not MHL. The absolute c_{HEMA} tended to be lower in the EST and COL groups, but was significant only at MHL in the COL group. Therefore, the second hypothesis (i.e., $c_{\text{Bis-GMA}}$ and c_{HEMA} within the HL are lower after 24-wk storage, with the greatest change seen in the esterase and collagenase groups) was accepted.

In this present study, durability of the HL was investigated, where EST media contained cholesterol esterase to attack resin and COL media contained bacterial (*Clostridium histolyticum*) collagenase to attack collagen. Within the HL (TQHL and MHL), absolute $c_{\text{Bis-GMA}}$ and c_{HEMA} was unchanged after 24-wk storage in SAL; however, molar concentrations tended to be lower in EST and were significantly lower in COL. This indicates that both esterase and collagenase enzymes can expedite the biodegradation of the HL. Although no large difference in absolute $c_{\text{Bis-GMA}}$ was observed in AR after 24-wk storage, for data acquired in the center of the HL (TQHL and MHL), the absolute $c_{\text{Bis-GMA}}$ exhibited a decrease during 24-wk storage in both EST and COL. This decrease in $c_{\text{Bis-GMA}}$ may indicate that the hydrolysis or biodegradation of Bis-GMA-related compounds could be accelerated by the enzyme in the incubation media.²⁴ Consistent with the results from a previous study,¹⁵ molar concentrations measured in the present study were ~10-50% of expected values at the center of the HL. Without the protection of the polymer matrix, exposed collagen network would be susceptible to attack by water, and collagen biodegradation would be expedited by the collagenase. Continued biodegradation of the collagen network would, in turn, expose more polymer matrix to water, leading to hydrolysis and elution. This biodegradation of the polymer matrix and elution of the degradation products would further expose the collagen network. Such biodegradation cycles may explain why only significant decreases of $c_{\text{Bis-GMA}}$ and c_{HEMA} were observed in the EST and COL groups as compared to the SAL group.

The novel Raman-based method fully described earlier¹⁵ was applied to determine the change of absolute $c_{\text{Bis-GMA}}$ and c_{HEMA} in different incubation media at AR, TQHL, and MHL after 24-wk storage. Compared to relative values, such as monomer weight fraction or molar fraction of adhesive system in the HL, absolute c_i provides direct measurement of adhesive components at specific positions in the HL. This method also has the capability to evaluate the accelerating effect of enzymes on the biodegradation of adhesive stored in media for long time periods. This methodology may prove to be a valuable assessment of new chemistries and approaches introduced to improve resin-dentin bond performance, such as: enhancement of resin infiltration via ethanol-wet bonding,²⁸ inhibition of matrix metalloproteinases with chlorhexidine,²⁹ or higher degrees of conversion with new photoinitiation systems.³⁰

Data acquisition for this long-term aging study began before the establishment of the absolute molar concentration methodology previously reported,¹⁵ where the number of line scans per specimen were increased from 3 to 9 and laser output was fixed at 8 mW. Focusing on the HL for data acquisition is relatively easy and straightforward for freshly

prepared specimens; however, for aged specimens, debris accumulated on the surface, which may have affected Raman spectra or sampling. These surface differences may explain the unexpected increases after 24-wk storage observed for $c_{\text{Bis-GMA}}$ stored in COL and c_{HEMA} stored in EST in the AR; whereas, only decreases in molar concentration were measured in the hybrid layer. Future studies of this kind should fix the laser output, optimize sampling protocols, and evaluate less complex systems to include neat monomers not bonded to a tooth substrate. Additionally, measurement of a non-polished specimen may more accurately yield true concentrations,³¹ which could be obtained by using confocal capabilities of this Raman instrument. This study only begins to elucidate resin-dentin bond formation and its complex interaction with pulp-dentin complex and the oral environment.

5. Conclusion

The absolute molar concentrations of the comonomers in this adhesive system changed over time across the hybrid layer when stored in enzymatic media designed to attack either the adhesive polymer (EST) or collagen (COL) matrix. Within the limitations imposed by the experimental design, the following conclusions can be drawn: 1) $c_{\text{Bis-GMA}}$ and c_{HEMA} decreased from the AR to the MHL, and after 24-wk aging; 2) $c_{\text{Bis-GMA}}$ and c_{HEMA} was unchanged at any resin-dentin position in SAL; 3) in the AR, $c_{\text{Bis-GMA}}$ significantly decreased in EST and increased in COL, while c_{HEMA} significantly increased EST; and 4) in the HL, $c_{\text{Bis-GMA}}$ and c_{HEMA} significantly decreased in COL and tended to decrease in EST.

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7. References

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Table 1Absolute Bis-GMA and HEMA molar concentrations at 24-h and 24-wk storage.¹

Location	Storage Media	24-h c _i (SD)	24-wk c _i (SD)	t-test (p)
c^{Bis-GMA} ²				
AR	SAL	1.602 (0.031) ^a	1.461 (0.059) ^a	0.057
	EST	1.678 (0.029) ^a	1.445 (0.015) ^a	0.001*
	COL	1.595 (0.077) ^a	1.686 (0.086) ^b	0.026*
TQHL	SAL	0.696 (0.266) ^a	0.808 (0.095) ^a	0.396
	EST	0.812 (0.312) ^a	0.171 (0.026) ^b	0.071
	COL	0.747 (0.124) ^a	0.287 (0.050) ^b	0.043*
MHL	SAL	0.214 (0.128) ^a	0.198 (0.143) ^a	0.914
	EST	0.264 (0.192) ^a	0.013 (0.020) ^a	0.173
	COL	0.323 (0.050) ^a	0.032 (0.047) ^a	0.013*
c^{HEMA} ³				
AR	SAL	2.915 (0.133) ^a	3.505 (0.248) ^a	0.057
	EST	2.598 (0.123) ^a	3.575 (0.064) ^a	0.001*
	COL	2.947 (0.325) ^a	2.560 (0.364) ^b	0.056
TQHL	SAL	1.896 (0.531) ^a	1.853 (0.244) ^a	0.922
	EST	1.890 (0.562) ^a	0.500 (0.047) ^c	0.058
	COL	1.824 (0.568) ^a	1.083 (0.065) ^b	0.179
MHL	SAL	0.597 (0.267) ^a	1.012 (0.757) ^a	0.505
	EST	0.879 (0.530) ^a	0.050 (0.073) ^a	0.137
	COL	1.079 (0.244) ^a	0.092 (0.131) ^a	0.028*

AR = adhesive resin; TQHL = top quarter of hybrid layer MHL = middle of hybrid layer

¹ Within columns **sets**, groups with a different letter are significantly different ($p < 0.05$). No difference was found at baseline (24 h) for any group.² Bis-GMA 24-wk storage: AR $p = 0.0049$; TQHL $p < 0.0001$; MHL $p = 0.767$.³ HEMA 24-wk storage: AR $p = 0.0050$; TQHL $p < 0.0001$; MHL $p = 0.0647$.