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Comparison of depth of dentin etching and resin infiltration with single-step adhesive systems

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Summary *Objectives*. Adhesion of resin composites to dentin is currently believed to result from impregnation of adhesive resin into superficially demineralized dentin. The purpose of this study was to use micro-Raman spectroscopy and scanning electron microscopy (SEM) to investigate the extent of resin penetration into etched dentin with single-step adhesive systems.

Methods. Adhesive systems used were One-Up Bond F (Tokuyama Dental) and Reactmer Bond (Shofu, Inc.). A self-etching primer system Mac Bond II (Tokuyama Dental) was employed as a control. Resin composites were bonded to bovine dentin with the adhesive systems, and specimens were sectioned parallel to dentinal tubules. Raman spectra were successively recorded along a line perpendicular to the dentin-adhesive interface in steps of 0.2 μm and the spectra were obtained. SEM observations of the resin–dentin interface were also conducted.

Results. The dentin–resin interface of single-step adhesive systems showed a gradual transition in the relative amount of adhesive from the resin side to dentin side. The widths of resin penetration into demineralized dentin detected by Raman microscopy were greater than those obtained by the morphological analysis using SEM.

Conclusions. From the results of this study, a gradual variation in the composition of the dentin–resin interface was detected, and the degree of resin impregnation observed with SEM observation was less than that detected with the Raman microscopy.

Introduction

Since the introduction of acid etch technique for bonding to enamel surface,[1] many attempts have been made to develop restorative materials that bond to both enamel and dentin. The development of promising adhesive system is dependent on understanding the bonding mechanism and characterizing the bonding interface between resin composites and tooth substrates. Compared to the clinical success of enamel bonding, predictable and clinically durable dentin bonding has been a more challenging goal to achieve. More factors

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contribute to the difficulty in dentin bonding than enamel, including higher organic component in dentin, higher water content, fluid pressure from the dentinal tubules, and the presence of smear layer.[2-5]

Adhesive systems can be categorized by how they treat the smear layer to obtain micromechanical retention through resin monomer infiltration into the demineralized dentin to create the so-called hybrid layer.[6-8] One-bottle or total-etch adhesive systems remove the smear layer and smear plugs with phosphoric acid, and demineralize the subsurface intact dentin. Self-etching primer systems use acidic functional monomers mixed with hydrophilic monomers and are applied to the smear layer covered dentin for a certain period of time. These primers are designed to dissolve the mineral component of the smear layer and alter the underlying superficial dentin.[9-11] The etching potential of the self-etching primers results in the creation of a thin resin–dentin impregnated layer. Use of the self-etching system is attractive for clinicians because the primers are used on dry dentin followed by air drying without water rinsing. This system is generally less technique sensitive compared with systems that utilize separate acid etching and water rinsing steps.[12]

Recently single step-adhesive systems, which combine the function of the self-etching primer and bonding agent, have been developed. Theoretically, the acidic adhesive dissolves the smear layer incorporating it into the mixture, and demineralizes the superficial dentin, then hardens after light irradiation. Although the bond strengths are not as high as two-step bonding systems, the single-step adhesives have advantage of a simple bonding procedure with a final goal of decreasing the technique sensitivity of the bonding procedure. As acidic components in the single-step adhesives dissolve the smear layer, the acidity of the adhesive may be neutralized by the mineral components of the smear layer. It has been suggested that poor infiltration of adhesive resin into the demineralized dentin leaves nano-spaces in the hybrid layer, and unprotected collagen fibrils in such a region might be susceptible to degradation from oral fluids and bacterial enzymes.[13-15] It is a major concern that the degree of resin monomer infiltration into demineralized dentin correspond to the depth of etching.[16]

Numerous morphological and chemical studies have been done in an effort to understand and obtain a structural image of the dentin–resin interface created by several adhesive systems.[17-21] Because of the very small thickness of the resin impregnated dentin layer, methods to analyze this layer must have a very high resolution. One of the most widely used tools for this purpose has been scanning electron microscopy (SEM). An argon-ion-beam etching technique has been used for selective removal of substrate atoms to visualize the morphology of the resin impregnated dentin layer. In a study to evaluate the resin impregnated dentin layer with this technique, it was suggested that the resin penetration into the exposed collagen fibril network might not be uniform.[22] Though a clear picture of dentin–resin interface has been obtained with the argon-ion-beam technique, no conclusion can be drawn about the resin saturation of the exposed collagen fibrils and superficial demineralized dentin.

Raman micro-spectroscopy is a useful analytical technique for studying the composition and structure of adhesive systems.[23] Since the acquired spectra are attributed to molecules rather than to single elements, and the laser beam can be focused to a very small spot size, a high spatial resolution at the sample surface can be achieved. Dehydration of the sample is not required and the measurements can be done under room conditions. Several studies using Raman Microscopy to analyze the resin–dentin interface have been published.[24-30] Differences in the extent of resin monomer penetration among the adhesive systems have been fully characterized with the use of Raman microscopy.

The purpose of this study was to analyze the degree of demineralization of bovine dentin and the extent of resin monomer penetration into demineralized dentin with the use of Raman microscopy and SEM to better understand the bonding mechanism of the single-step adhesives. The null hypotheses to be tested were: (1) there were no differences in the degree of demineralization and the extent of resin monomer infiltration of the systems used in this study; and (2) there was no difference in the thickness of resin impregnated dentin layer detected by two analytical methods.

Materials and methods

Adhesive systems and restoratives

Adhesive systems with the resin composites employed in this study were two single-step adhesive systems, One-Up Bond F with Palfique Estelite (Tokuyama Dental, Tokyo, Japan) and Reactmer Bond with Reactmer (Shofu, Inc., Kyoto, Japan) as listed in Table 1. A two-step adhesive system including a self-etching primer system Mac

Table 1 Bonding systems used in this study.

Adhesive (Lot No.)	Resin composite (Lot No.)	Manufacturer
One-Up Bond F (A: 0712, B: 0709) Water, MAC-10, HEMA, MMA, multifunctional methacrylic monomer fluoroaluminosilicate glass, photoinitiator (aryl borate catalyst)	Palfique Estelite (210)	Tokuyama Dental (Tokyo, Japan)
Reactmer Bond (A: 100005, B: 100005) Water, acetone, 4-AET, 4-AETA, UDMA, HEMA, PRG filler, fluoroaluminosilicate glass, trimethyl barbituric acid, photoinitiator *p*-toluenesulfinic acid sodium salt	Reactmer (110007)	Shofu, Inc. (Kyoto, Japan)
Mac-Bond II Primer (A:0251, B:013) A: MAC-10, HEMA, acetone, isopropyl alcohol, phosphate monomer B: Ethanol, water Adhesive resin (0181) MAC-10, HEMA, bis-GMA, TEGDMA, CQ	Palfique Estelite (210)	Tokuyama Dental (Tokyo, Japan)

Abbreviations: Mac-10, 10-methacryloxydecyl di-hydrogen phosphate; HEMA, 2-hydroxyethyl methacrylate; MMA, methyl methacrylate; 4-AET, 4-acryloyloxyethyl trimellitic acid; 4-AETA, 4-acryloyloxyethyl trimellitate anhydride; UDMA, urethane dimethacrylate; bis-GMA: 2, 2bis[4-(2-hydroxy-3-methacryloyloxypropoxy)]phenyl; TEGDMA, triethylene glycol di-methacrylate; CQ, *dl*-camphorquinone.

Bond II with Palfique Estelite (Tokuyama Dental, Tokyo, Japan) was used as a control.

Light-curing was performed using an Optilux 501 curing unit (Demetron/Kerr, Danbury, CT, USA), with the light intensity adjusted to 600 mW/cm^2 as measured with a dental radiometer (Model 100, Demetron/Kerr).

Sample preparation

Mandibular incisors extracted from 2–3 year old cattle and stored frozen for up to 2 weeks were used as a substitute for human teeth. After removing the roots with a low-speed saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA), the pulps were removed, and final finish was accomplished by grinding on wet 600-grit silicon carbide paper until a sufficient area of dentin was exposed. After ultrasonic cleaning with distilled water for 1 min to remove the excess debris, these surfaces were washed and dried with a three-way syringe.

For single-step systems, equal amounts of the adhesive A and B were dispensed into a mixing well. They were mixed for 5 s until homogenous liquid mixtures were obtained. The mixed adhesives were applied to dentin surface for 20 s, and then light irradiated for 20 s.

For the two-step self-etching system, equal amounts of Mac Bond II Primer A and B were dispensed into the well and mixed for 5 s. Then the mixed primer was applied to the dentin surface for 20 s followed by gentle air blowing to evaporate the volatile ingredients. A coat of Mac Bond II bonding resin was applied to the primed dentin, and light irradiated for 10 s.

A Teflon mold, 2.0 mm high and 4.0 mm diameter was used to form and hold the restorative materials to the tooth surface. Resin composite was condensed into the mold and cured for 40 s according to each manufacturer's instructions. After 24 h storage in 37 °C water, these specimens were embedded in self-curing epoxy resin (Epon 812, Nisshin EM, Tokyo, Japan) and stored at 37 °C for 12 h. After setting, the epoxy casts were sectioned parallel to the dentinal tubules and the sectioned surfaces of the cut halves were polished to high gloss using silicon carbide papers of 600, 1200 and 4000-grit size, successively. Then the surface was mirror polished on a special soft cloth with diamond paste to a grit size of 1 μm (Buehler Ltd). Care was taken to avoid aggressive polishing that might make a gap between dentin and adhesive or change the specimen height, and no destructive effect was found at the dentin–resin interface where the laser beam was focused. At least three specimens from different bovine teeth were prepared for each adhesive system

Laser Raman microscopy

Raman spectra were obtained with a computer controlled laser Raman microscope equipped with

a monochrometer and a back thinned multi-phase pinned (MPP) type charge-coupled device (CCD) camera (System 2000, Renishaw, UK). A He-Ne laser (GLG-5900, NEC Co., Tokyo, Japan) tuned to a wavelength of 632.8 nm with an output level of 75 mW was used as an excitation source. The focus of the laser beam in conjunction with the CCD (70% quantum yield) provided a spatial resolution of 0.6–0.8 μm. Instrument calibration was determined before data acquisition by comparison of spectra from pure Si (520 cm^{-1}).

The spectral resolution of the laser Raman microscopy was determined by use of a silicon solid plate ion-coated with thin Au. The plate was put on a precision X–Y stage and moved from the Au-coated to Au-uncoated portion of the plate surface in steps of 0.2 μm. The spectra of Si (520 cm^{-1}) were obtained at positions corresponding to each measuring point and the relative intensities of the spectra were calculated. From the acquired data, the spatial resolution determined as 0.6–0.8 μm. Since this device was configured as a confocal microscope, the sampling depth of the Raman scattering from the 1×1 μm area was calculated as 2 μm.

The sample was placed on a precision X–Y stage with a spatial resolution of 0.1 μm. An optical microscope (BH2-UMA, Olympus, Tokyo, Japan) allowed for visual identification of the position from which the signal was obtained. The laser beam was focused on the sample surface with a 100× microscope objective taking care to avoid resin tags, which would disturb the measurement. The sample was moved perpendicular to the interface in steps of 0.2 μm, and the spectra were obtained at each position across the dentin-adhesive interface with an integration time of 120 s for each measurement. Raman spectra of unaltered dentin and cured adhesive were also recorded as references for each obtained spectra. The acquired spectra in the region of interest were analyzed using a curve fitting program with the Raman microscope software (Renishaw), and the relative amounts of hydroxyapatite, adhesive resin, and organic substance in the dentin–resin interface were calculated. The calculated Raman intensities were extracted to provide plots of intensity versus perpendicular position to the bonded interface. These plots were fitted to a diffusion model as previously described.[27] The interface width was defined as the distance between the position of 10 and 90% intensity. The measurements were done three times with different specimens for each bonding system, and the widths of resin-impregnated dentin layer were determined.

Statistical analysis

The results were analyzed by calculating the mean and standard deviation for each adhesive system. The data were subjected to a one-way ANOVA followed by Tukey's multiple comparison tests at a *p*-value of 0.05. The statistical analysis was carried out with Sigma Stat® software system (SPSS, Inc., Chicago, IL, USA).

SEM observation

The treated dentin surface and the restorative/dentin interface were observed by scanning electron microscopy (SEM). For the treated dentin surface observation, the dentin surfaces were treated with adhesive according to each manufacturer's instructions as described above and then rinsed with acetone and water. For the ultrastructure observation of resin–dentin interface, bonded specimens stored in 37 °C distilled water for 24 h were embedded in epoxy resin and then longitudinally sectioned with a diamond saw. The sectioned surfaces were polished to a high gloss with abrasive discs followed by diamond pastes down to 0.1 μm particle size. They were fixed in a 2.5% glutaraldehyde in cacodylate buffer solution, dehydrated in ascending grades of *tert*-butyl alcohol (50% for 20 min, 75% for 20 min, 95% for 20 min, and 100% for 2 h), and then transferred from the final 100% bath to a critical-point dryer (Model ID-3, Elionix, Tokyo, Japan) for 30 min. The polished surfaces were then subjected to argon-ion beam etching (EIS-200ER, Elionix, Tokyo, Japan) for 15 s with the ion beam (accelerating voltage 1.0 kV, ion current density 0.4 mA/cm^2) directed perpendicular to the polished surface. The surfaces were coated in a vacuum evaporator with a thin film of Au. Observation was done under a scanning electron microscope (SEM, JSM-5400, JEOL, Tokyo, Japan) at an operating voltage of 15 kV. Three different measurements of resin impregnated dentin layer thickness were obtained for each adhesive system.

Results

Representative Raman spectra of bovine dentin and resin impregnated dentin with One-Up Bond F recorded in the region of 500–1800 cm^{-1} are shown in Fig. 1. The intense peak at 960 cm^{-1} is associated with the P–O stretching vibration in the mineral apatite component of dentin.[31] Weak bands at 1242, 1450 and 1669 cm^{-1} are attributed to the organic components of dentin such as collagen

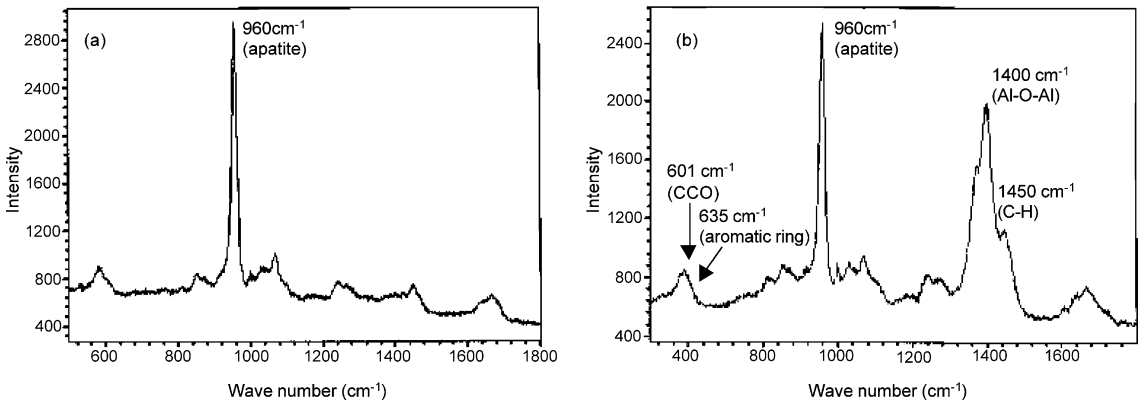

Figure 1 The representative Raman spectra of (a) bovine dentin, and (b) resin impregnated dentin (One-Up Bond F) recorded in the region of 500–1800 cm^{-1}.

matrix.[32,33] Several intense peaks are observed for the Raman spectrum of resin impregnated dentin; from these 635 cm^{-1}, which is assigned to the aromatic ring group of Bis-GMA,[34] and 1450 cm^{-1}, which is assigned to the C–H alkyl group,[25,33] were selected as measures of the resin adhesive. Since the Raman peak associated with aromatic ring (635 cm^{-1}) was compound with the Raman peak of CCO (601 cm^{-1}) associated with an ester functional group, the individual band was obtained from the peak areas by the curve fitting software, which was designed to calculate a best-fit curve.

Because the question arises whether Bis-GMA, whose molecular weight (MW = 512) is significantly larger than HEMA (MW = 130), is representative of all the monomers in the hybrid layer, the C–H alkyl group was selected as a measure of all of the organic components in the hybrid layer. Since collagen has a peak at1450 cm^{-1} this choice does not allow one to separate the organic components of the adhesive system from those of the dentin.

The intensities of the selected Raman bands scanned across the resin-dentin interface of the One-Up Bond F adhesive system are shown in Fig. 2. Scans were started from the dentin side to the resin side. A gradual decrease in intensity of the P–O peak from the dentin to resin side was observed. On the other hand, the peak of the bis-GMA and the peak of the organic substrate increased across the dentin-resin interface. Since the C–H alkyl groups also appear in the collagen spectrum, the changes in relative intensities of this peak were related to both dentin and resin. From these curves, mineral removal seemed to start at 1.4 μm and end at 4.0 μm, and the resin penetration corresponded to this same 2.6 μm wide region. We speculate that this width corresponded to the resin impregnated dentin layer, where mineral components of the dentin were gradually replaced by resin monomers.[30]

Representative SEM photographs of the resin-dentin interface etched with an argon-ion-beam are shown in Fig. 3. A thin layer from which more surface material was removed by argon-ion bombardment than from underlying dentin indicated resin impregnated layer detected by this technique. The thickness of this layer was thinner for the single-step systems than for the two-step self-etch system.

Cross-sectional and longitudinal observations of the treated dentin are shown in Figs. 4 and 5. Differences in extent of etching by the adhesive/primer were observed on the longitudinally sectioned dentin. For the dentin treated with One-Up Bond F, remnants of smear plugs were observed with mild etching of the superficial peritubular dentin. Reactmer Bond and Mac Bond II primer

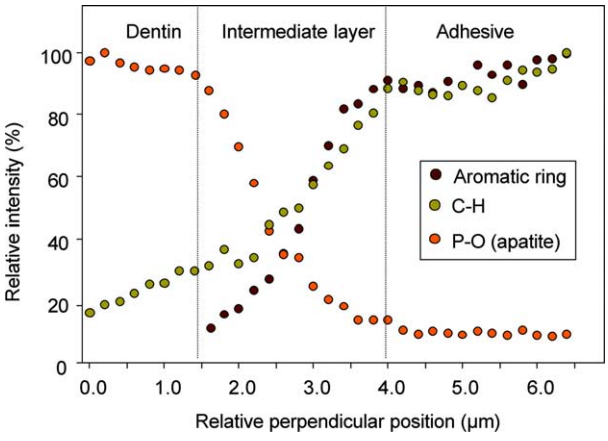

Figure 2 Representative line scans across the resin-dentin interface of One-Up Bond F adhesive system. In the Raman spectrum of resin impregnated dentin, a peak at 635 cm^{-1}, due to the aromatic ring group of Bis-GMA, and a peak at 1450 cm^{-1}, due to scissoring vibration of the $>CH_2$ bond, and a peak at 1400 cm^{-1}, due to the Si–O–Si of glass fillers, were observed.

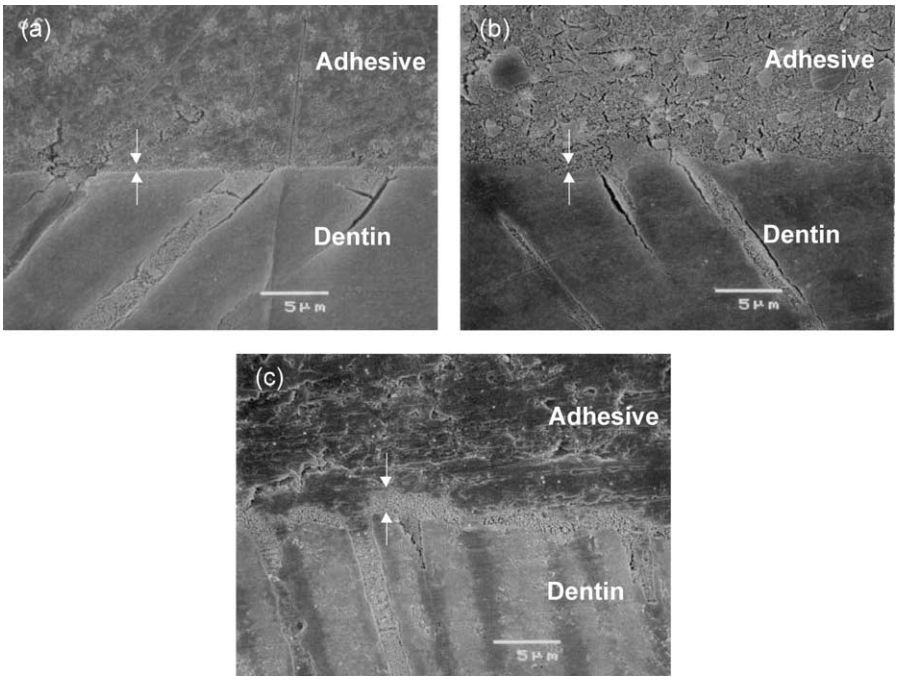

Figure 3 Representative SEM pictures of the resin–dentin interface of the three adhesive systems after argon-ion-etching, (a) One-Up Bond F, (b) Reactmer Bond, and (c) Mac Bond II (original magnification; ×3500).

application resulted in a prominent stripping of the smear layer and left dentinal tubules entirely free of smear plugs.

Comparisons of the widths of resin impregnated dentin layer created by adhesive systems are summarized in Table 2. All pairwise multiple comparison procedure (Tukey test) indicated that a significant greater width of resin impregnated dentin layer was determined from the Raman microscopy ($p<0.01$) than from the SEM observations.

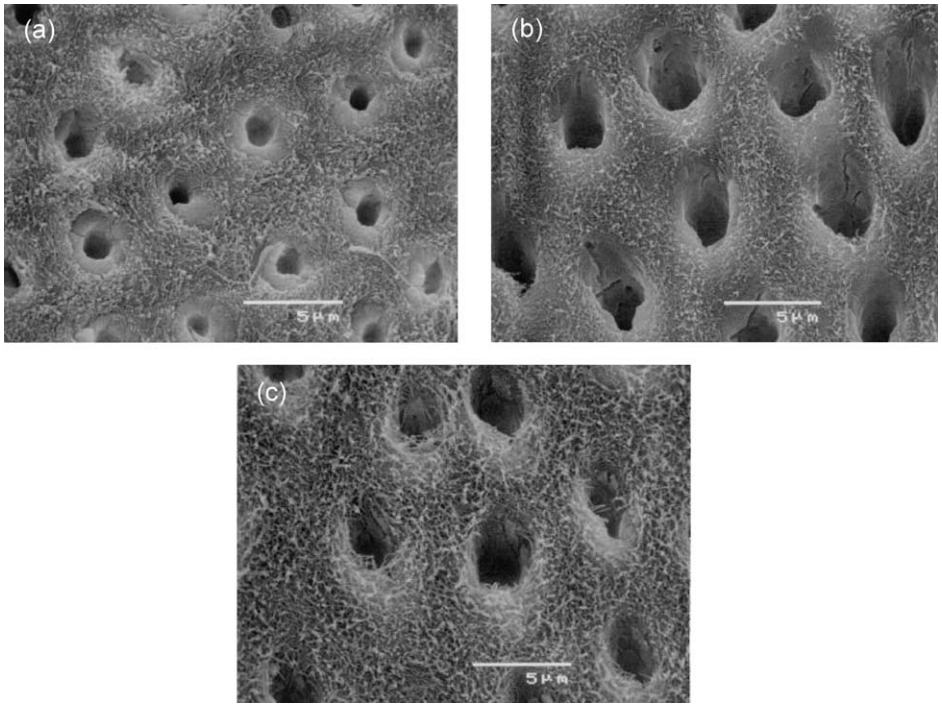

Figure 4 Representative SEM pictures of treated dentin surface with adhesive/primer followed by rinsing with acetone and water, (a) One-Up Bond F, (b) Reactmer Bond, and (c) Mac Bond II (original magnification; ×5000).

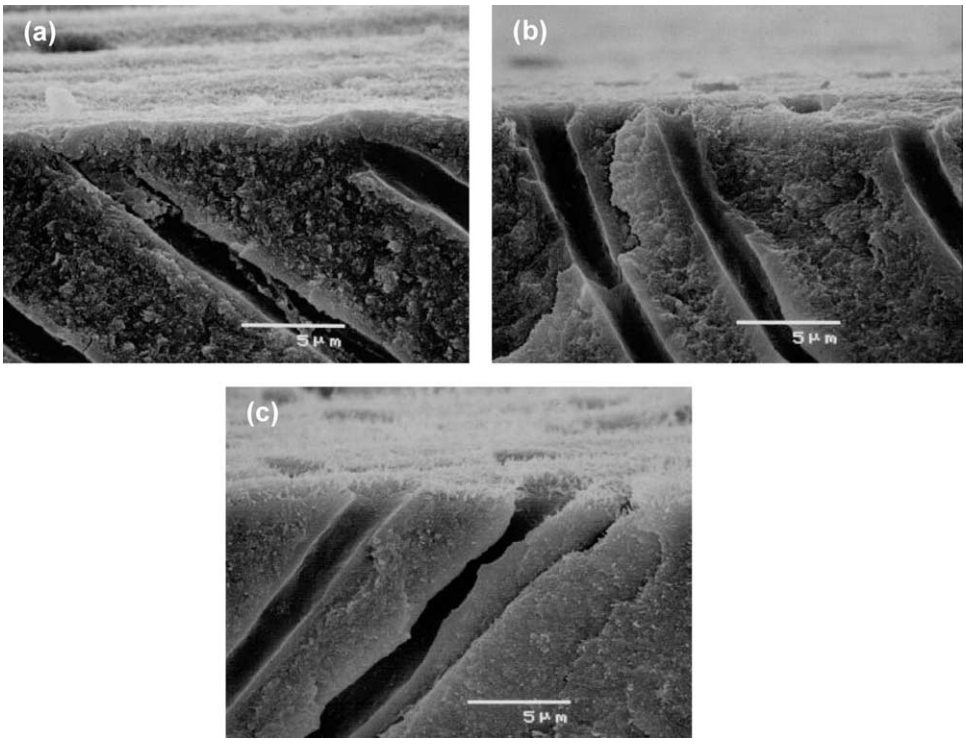

Figure 5 Representative SEM pictures of cross-sectional view of treated dentin, (a) One-Up Bond F, (b) Reactmer Bond, and (c) Mac Bond II (original magnification; ×5000). Differences in extent of etching were observed on the longitudinally sectioned dentin with the appearance of exposed collagen fibers.

Discussion

Laser Raman spectroscopy is a useful analytical technique for study of the bonding structure of samples and determining their composition. Using this technique, the problems associated with morphological analysis of the dentin–resin interface with infrared spectroscopy (IR) can be avoided.[23] Specimens can be observed under normal atmospheric conditions without ultra-thin sectioning or other preparatory procedures that might damage the interface. In contrast to conventional IR microscopy, water is a weak Raman scatterer so that Raman spectra can be acquired from moist dentin specimens. Despite these advantages, the background from fluorescence dominates the weaker Raman signal. The fluorescence is due to electronic excitation of the organic component when irradiated by laser radiation. To help overcome the problem of high background noise, a He-Ne laser was used as an excitation source in this study. In addition the light generated from a He–Ne laser (632.8 nm) does not contribute to initiating the polymerization reaction of visible-light cured resins that use camphorequinone as a photoinitiator.[35]

The adhesive of the single-step adhesive systems is a hydrophilic solution that is extremely effective in wetting the tooth surface.[36–38] The etching effect of these systems is related to the acidic monomers that interact with the mineral component of the tooth substrate, and enhance monomer penetration.[39] These single-step adhesives may form a continuum between the tooth surface and

Table 2 Widths of resin impregnated dentin layer created by adhesive systems.

Method	Adhesive	Range	Mean (std)	Tukeyh's group*
Raman microscopy	One-up bond F	2.2–2.6	2.40 (0.14)	a
	Reactmer bond	2.4–2.8	2.55 (0.18)	a
	Mac bond II	3.0–3.8	3.40 (0.28)	b
SEM	One-up bond F	0.4–0.6	0.50 (0.12)	c
	Reactmer bond	0.8–1.2	1.00 (0.23)	d
	Mac bond II	1.6–2.0	1.75 (0.46)	a

Unit: μm, *: $\alpha = 0.05$.

the adhesive by the simultaneous demineralization and resin penetration followed by polymerization to create a stable bond. To generate the hydrogen ions required for effective dissolution and demineralization, the single-step system has to contain water as well as water-soluble hydrophilic monomers such as 2-hydroxyethyl methacrylate (HEMA). Water is an essential component in the adhesives, so that the acidic monomer can dissociate. Protons in solution derived from the acidic monomer then interact with the mineral component of the tooth substrate.

The representative Raman bands scanned across the resin–dentin interface showed transitional changes in the hydroxyapatite and resin component (Fig. 2). Gradual changes in intensity of Raman peaks might indicate that the minerals removed by the acid were gradually replaced by resin. The line scans of the dentin–resin interface of the single-step adhesive systems suggested that the depth of dentin demineralization was 2.2–2.8 μm and resin penetration was detected to the total depth of demineralization. Within the limitation of this study, two single-step and a two-step adhesive systems exhibited different degrees of demineralization and resin monomer impregnation, with One-Up Bond F being the least and Mac Bond II being the most aggressive. In the case of these bonging systems, penetration of the adhesive into the dentin substrate occurs simultaneously with the demineralization of the mineral component in the dentin. There is little possibility that a region of demineralized dentin that was not infiltrated by the resin monomers exists with these systems.

The thickness of the hybrid layer depends on the bonding system used, and variations of the thickness were observed among specimens using the same bonding system and different dentin substrates.[40–42] Though different hybrid layer thickness was observed, no correlation with the dentin bond strength was found. The hybrid layer and the presence of resin tags may not be the only mechanisms influencing dentin bond strengths. Smaller width of the resin-impregnated resin was observed for the single-step systems. The thickness of resin impregnated dentin layer detected by SEM were significantly smaller than those obtained with the Raman microscopy. During the specimen preparation for SEM, drying is necessary to obtain a high vacuum condition. It has been reported that all forms of SEM specimen preparation cause significant shrinkage artifacts, leading to final volumes of approximately 55–70% of the original volumes.[43] This might relate to the differences in the measured width by the two different techniques.

The poor resistance to argon-ion bombardment of the resin–dentin interface raised questions about the real degree of demineralization impregnated with resin monomers. From micromorphological study of the polished resin–dentin interface, heterogeneous features of the resin impregnated dentin layer have been detected.[22] Since the collagen phase is removed at a much faster rate than hydroxyapatite phase during ion bombardment,[44] the partially mineralized dentin at the bottom part of the resin impregnated dentin layer is more likely to resist the effect of argon-ion etching. Therefore, the widths detected by SEM observations may not particularly reflect the true width of this layer.

The micromechanical entrapment of resin in the demineralized dentin substrate is of importance to create a hybrid layer. The dentin–resin interface has been investigated by use of the Raman microscopy and SEM, and the importance of resin penetration through the entire depth of decalcified dentin has been emphasized. After infiltration of the resin monomers into the decalcified dentin, subsequent polymerization of monomers is required to create a stable bond. If the polymerization of these monomers is not complete, hydrophilic monomers or small oligomers might be extracted or hydrolyzed from areas containing nanoleakage.[13,14] The quality of the hybrid layer and uniformity of resin impregnation are important factors to understand the contributions of the hybrid layer to dentin bonding.

The Raman spectroscopic technique provides chemical and morphologic information on the dentin–resin interface. Though deconvolution techniques could improve the special resolution, the instrument function must be known before these technique can be applied accurately.[28] Due to its limited special resolution, the obtained data should be considered as relative base from adhesive side to dentin side for the single-step adhesive systems used in this study.

Conclusions

Laser Raman microscopy used in this study was a useful tool for study of the variation in chemical composition across the dentin–resin interface. The hybrid layer represents a gradual decrease in the relative amount of adhesive from the resin side to dentin side. The diffusion of resin monomers into demineralized dentin may produce a gradient resin penetration with the highest concentration at the surface of the adhesive, lower concentration in the middle of the hybrid layer, and little resin in the deepest portion of the demineralized zone.

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