



**New Concept of Resin-Dentin
Interfacial Adhesion Mechanisms:
*The Protected Layer***

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H.Nurrohman, The Protected layer



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The Protected Layer



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Preface

This thesis is based on the original research works by the author, to which the following articles refer.

- Chapter 1. Nurrohman H, Nikaido T, Sadr A, Takagaki T, kitayama S, Ikeda M, Waidyasekera K, Tagami J. Long-term regional bond strength of three MMA-based adhesive resins in simulated vertical root fracture. *Dental Materials Journal* 2011;30(5): 655-663.
- Chapter 2. Nurrohman H, Nikaido T, Takagaki T, Sadr A, kitayama S, Ikeda M, Waidyasekera K, Tagami J. Dentin bonding performance and ability of four MMA-based adhesive resins to prevent demineralization along the hybrid layer. *The Journal of Adhesive Dentistry* 2012.
- Chapter 3. Nurrohman H, Nikaido T, Takagaki T, Sadr A, Ichinose S, Tagami J. Apatite crystal protection against acid-attack beneath resin-dentin interface with four adhesives: TEM and crystallography evidence. *Dental Materials* 2012;28(7):e89-98.

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Chapter 1

Long-term regional bond strength of three MMA-based adhesive resins in simulated vertical root fracture

Introduction

Complete vertical root fractures (CVRF) of endodontically treated teeth are frequently encountered in the dental practice (Tamse, 2006). Removal of tooth structure during endodontic and restorative treatments increases the risk of tooth fracture, with fatigue mechanisms mediating the fracture of root tissues over time (Kishen, 2006). However, there are no reliable methods for treating the fractured teeth completely (Pitts and Natkin, 1983; Trope and Rosenberg, 1992; Selden, 1996; Dederich, 1999). Sugaya *et al* (2001) developed a new and promising method for CVRF by atraumatic intentional extraction, and rotational replantation of the tooth restored with an adhesive resin. Intentional extraction of the tooth fragments offers the advantage of enabling removal of the granulation tissue. In addition, rotational replantation brings the fracture line in contact with the healthy periodontal tissue (Kawai and Masaka, 2002).

The complex structure of root dentin varies by its location; several studies have reported that dentin bond-strength of different adhesives was influenced by the region of the dentin substrate (Yoshiyama *et al.*, 1996; Pereira *et al.*, 1999), and that the regional bond-strength durability was different between adhesives (De Munck *et al.*, 2011). To date, the effect of regional structure of root dentin on the long-term bond strength of the repaired root has not been investigated.

4-methacryloxyethyltrimellitate anhydride/methyl methacrylate-tri-n-butyl borane resin (4-META/MMA-TBB resin; Sun Medical, Moriyama, Japan) has been used to bond the CVRF segments because of its tolerance against blood contamination of the dentin surface

(Miles *et al.*, 1994) and excellent bonding performance (Miles *et al.*, 1994; Nakabayashi *et al.*, 1982). Formation of a hybrid layer that consists of a molecular-level mixture of adhesive polymers and dental hard tissues is recognized as one of the contributing factors to the high bond strength achieved by this material (Nakabayashi *et al.*, 1982, 1991). However, the time required for application and polymerization of this material (more than 20 min) appeared to be too long for bonding the fragments of the fractured root in the clinical situation.

In order to simplify the application procedures, MMA-based adhesive resins with self-etching primers either in two bottles or in a single bottle have been developed (M-Bond and M-Bond II; Tokuyama Dental, Tokyo, Japan). In the self-etching primer system, etching and priming steps are combined into a single application step, and the self-etching primer agent is not rinsed off after application. A self-curing resin layer is then applied directly on the treated surface. In addition to the simplified application procedure, these MMA adhesive resins have a shorter setting time and a longer handling time compared to the 4-META/MMA-TBB resin. However, the literature has reported conflicting results on bond strengths of some simplified systems to dentin (Van Meerbeek *et al.*, 1999; Bouillaguet *et al.*, 2001; Swift, 2003), raising concerns over the durability of the interface achieved with these systems. Moreover, little information is available about the performance of the self-etching MMA-based adhesive resins used to reattach root fragments in CVRF.

The aim of the present study was to investigate bond durability of three MMA-based adhesive resins to different regions of root dentin in a simulated CVRF over a period of 1 year, by evaluating microtensile bond strength and failure analysis. The null hypothesis of this study was that the microtensile bond strengths and failure modes of the tested MMA-based adhesive resin systems were not affected by long-term water storage and dentin location.

Materials and methods

Adhesive materials and teeth

Three self-cured MMA-based adhesive resins, Super-Bond C&B (SB; Sun Medical), M-Bond and M-Bond II (MB and MB II, Tokuyama Dental), were used in this study (Table 1). SB is a two-step acid-etching 4-META/MMA-TBB adhesive resin, while MB and MB II are self-etching systems.

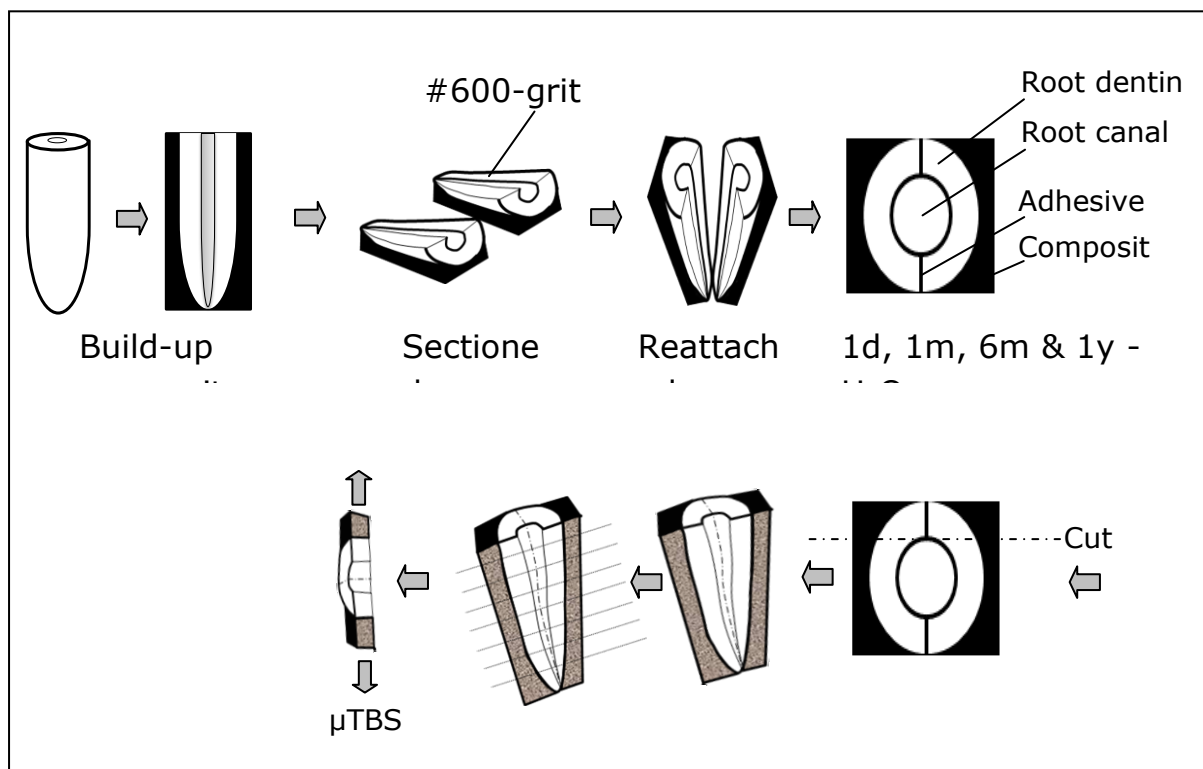


Figure 1. Sample preparation of the μ TBS test setup.

Sixty-six human lower premolars with single root were used as bonding substrates. They were collected after the individuals' informed consent, and used according to a protocol approved by the Institutional Review Board of Tokyo Medical and Dental University. The teeth were stored in water at 4°C and used within one month after extraction. The teeth were randomly distributed into three groups according to the adhesive resins. Each tooth was sectioned approximately 1 mm below the cemento-enamel junction (CEJ) using a low-speed diamond saw (Isomet; Buehler, Lake Bluff, IL, USA) under water

stream. The pulpal tissue was removed from root canal with a barbed broach. The root surfaces were then cleaned from of all remnants of periodontal ligament by scouring using a scalpel blade.

Bonding procedures and bond strength test

The outline of the microtensile bond strength (μ TBS) test is schematically presented in Fig. 1. In order to provide sufficient bulk for handling, resin buildups were made on the buccal and lingual surfaces of the roots using Clearfil SE Bond (Kuraray Medical, Tokyo, Japan) as adhesive and 2-mm-thick increments of resin composite (Clearfil APX; Kuraray Medical).

Cervical and apical halves of each root were identified and painted with different colors on the composite surface. In order to simulate a CVRF, the roots were vertically sectioned at the middle in the mesio-distal direction to obtain 2 fragments. The dentin surface on each fragment was then lightly ground with 600-grit SiC paper under a water spray to create a standard smear layer, similar to the clinical treatment method, in which the smear layer was created by rotary instruments on the fracture lines (Arikan *et al.*, 2008; Ogata *et al.*, 2002). Each pair of fragments obtained from the same tooth in each of the groups were then reattached using one of the three MMA-based adhesive resins; SB, MB and MB II according to the manufacturers' instructions. Immediately prior to setting (approximately 20 min for SB and 10 min for MB and MB II), excess of the adhesive resin was carefully removed with a hand instrument (Arikan *et al.*, 2008). The reattached specimens were then placed in water maintained at 37°C for up to 1 y. The solution was changed daily and no preservative or antimicrobial agents were used (Kitasako *et al.*, 2000). To prevent thermocycling effects, the water was heated to 37°C prior to changing it. After 1 d, 1 m, 6 m or 1 y, five premolars were used for μ TBS test. At each period, the specimens were longitudinally sectioned in a bucco-lingual direction from its central part, yielding two halves, each one containing the resin-bonded dentin interfaces.

Table 1 - Adhesive materials used in this study.

Material/Lot/Manufacturer		Composition	pH	Application
Super-Bond C&B;SK1 Sun Medical; Moriyama, Japan	Green activator (10-3) Catalyst Powder Liquid	10% citric acid, 3% FeCl ₃ TBB PMMA MMA, 4-META	0.74	Etch dentin with 10-3 solution for 10 s, rinse and air-dry. Powder : Liquid : Catalyst = 1 : 4 : 1 Mixed for 5 s, applied to dentin surface.
M-Bond; X760532 Tokuyama Dental; Tokyo, Japan	Primer A Primer B Powder Liquid	Phosphoric acid monomers, acetone Water, acetone, borate catalyst PMMA, BPO MMA, MAC-10, amine, multifunctional methacrylate	1.6	Mixed equal amounts of Primer A and B, apply for 30 s and gently air-dry for 10 s. Powder : Liquid = 1 : 3 Mixed for 10 s, applied to dentin surface.
M-Bond II; 0090Y8 Tokuyama Dental; Tokyo, Japan	Primer Powder Liquid	Phosphoric acid monomer, water, acetone, UDMA, co-activator PMMA, co-activator MMA, UDMA, HEMA, MTU-6, borate catalyst	1.7	Apply primer for 20 s and gently air-dry for 10 s. Powder : Liquid = 1 : 3 Mixed for 5 s, applied to dentin surface.
TBB, tri-n-butyl borane; MMA, methyl methacrylate; 4-META, 4-methacryloxyethyl trimellitate anhydride; BPO, benzoyl peroxide; MAC-10, 10- methacryloyloxydecamethylene malonic acid; HEMA, 2-hdroxyethyl methacrylate; UDMA, urethane dimethacrylate; MTU-6, 6-methacryloxyhexyl 2-thiouracil-5-carboxylate.				

Table 2 - μ TBS to human root dentin after different water storage periods.

Groups	Dentin region	1 d	1 m	6 m	1 y
SB	Cervical	23.2 (7.5)	27.4 (8.2)	25.3 (3.7)	21.0 (6.6)
	Apical	24.6 (7.9)	27.7 (4.3)	26.0 (2.7)	22.1 (5.2)
	Total	23.9 (7.5) ^{ab A}	27.5 (6.4) ^{a B}	25.6 (3.2) ^{a D}	21.5(5.9) ^{b G}
MB	Cervical*	24.6 (5.6)	26.4 (3.7)	24.2 (4.3)	18.6 (6.9)
	Apical*	22.5 (3.8)	25.2 (5.2)	22.1 (3.4)	12.8 (5.1)
	Total	23.6 (5.0) ^{c A}	25.8 (4.5) ^{c B}	23.2 (4.0) ^{c E}	15.7 (6.7) ^{d H}
MB II	Cervical**	22.8 (4.2)	20.8 (4.8)	18.7 (2.8)	14.8 (4.0)
	Apical**	20.9 (3.8)	18.3 (2.1)	17.1 (2.8)	12.1 (5.9)
	Total	21.9 (4.1) ^{c A}	19.6 (3.9) ^{cf C}	18.0 (2.9) ^{f F}	13.5 (5.2) ^{g H}

Means (SD) in MPa.(n =15).
^{a-g} Within each material, total values with the same lowercase letters showed no statistically significant difference ($p > 0.05$).
^{A-H} At each time period, total values with the same capital letters showed no statistically significant difference ($p > 0.05$).
^{*,**} The region (cervical or apical) was a significant factor for within the adhesive material ($p < 0.05$).

The resulting specimens were serially sectioned into beams with cross-sectional areas of 1 mm², cut at right angles to the long axis of the root. Three beams were collected from each dentin region of each specimen, resulting in a total of 15 beams per period and dentin region (n=15).

These specimens were then fixed to the testing jig of a handy-type universal testing machine (EZ-Test; Shimadzu, Kyoto, Japan) with a cyanoacrylate adhesive (Zapit; DVA, Anaheim, CA, USA) and subjected to microtensile bond strength testing at a crosshead speed of 1 mm/min. To determine the mode of failure, all specimens were immediately examined after fracture under a stereomicroscope (Nikon SMZ1000; Nikon, Tokyo, Japan) at a magnification of 40x. Failure modes were categories as: (A) adhesive failure at the resin-dentin interface; (B) cohesive failure completely within the adhesive resin; (C) partially adhesive failure, where remnants of resin remained on the dentin surface; (D) cohesive failure in the demineralized or intact dentin. Representative specimens for each failure mode were

additionally examined by a scanning electron microscope (SEM) (JSM-5310LV; JOEL, Tokyo, Japan) to confirm the stereomicroscope observations. Prior to the SEM observations, the specimens were air-dried and sputter-coated with gold.

SEM examination of the conditioned dentin surfaces

The six remaining premolars were sectioned longitudinally in the bucco-lingual direction and a standard smear layer was created on the fractured dentin surfaces by wet grinding in the same way as for the specimens in the μ TBS test. Dentin specimens in SB group were conditioned using SB green activator (10% citric acid with 3% ferric chloride: 10-3 solution) for 10 s, rinsed and air-dried. In MB and MB II groups, the specimens were treated with the self-etching primer of MB (Primer A and B) for 30 s or that of MB II (Primer) for 20 s, respectively and gently air-dried. Immediately after conditioning, the surface was subjected to an ascending series of ethanol (30, 50, 70 and 95%) for 1 min each. In case of MB and MB II, the specimens were further ultrasonically cleaned in absolute acetone for 1 min in order to completely dissolve the primer and dehydrate the specimens for SEM observation. After air-drying, the samples were coated with gold and examined using the SEM to observe surface texture and etching pattern.

Statistics

The μ TBS data of the three MMA-based adhesives to cervical or apical dentin after different storage time periods were statistically analyzed using a three-way ANOVA (adhesive material vs. dentin region vs. storage period). Separate two-way ANOVAs were also conducted within each material or within each storage time followed by Dunnett's T3 post-hoc tests. The statistical significance level was always set at $\alpha=0.05$.

Results

Microtensile bond strength testing

Mean μ TBS are presented in Table 2. Three-way ANOVA revealed that each of the factors (adhesive material, dentin region, and storage period) had a significant effect on the μ TBS ($p < 0.05$); however, there were no significant interactions between the three factors ($p = 0.857$). Significant interactions were detected between adhesive material and dentin region ($p < 0.05$), and also between adhesive material and storage period ($p < 0.05$).

Separate two-way ANOVA within each material showed that unlike SB, for both MB and MB II, the dentin region was a significant factor. Further analysis with multiple comparisons by Dunnett's T3 showed that in MB the μ TBS at 1 y decreased significantly compared to 1 d ($p < 0.05$). In MB II, the μ TBS at 6 m and 1 y was significantly lower than that at 1 d ($p < 0.05$). For SB, there was no significant difference in μ TBS between 1 d and any other storage periods ($p > 0.05$).

Multiple comparisons between materials at each storage period indicated that there was no significant difference among materials at 1 d ($p > 0.05$); however, at 1 y, SB showed significantly higher μ TBS compared to MB and MB II ($p < 0.05$), while MB and MB II were not significantly different ($p > 0.05$).

Failure modes of all the test groups are summarized in Fig. 2 and representative SEM micrographs of each mode are presented in Fig. 3. For SB, cohesive failure in adhesive resin (mode B) was predominant in 1 d and 1 m, for both cervical and apical dentin (Fig. 3a). Cohesive failure in demineralized dentin under the hybrid layer (mode D) appeared in low percentages at 6 m and 1 y (Fig. 3b). Moreover, there was an increase in partial adhesive failure with a thin layer of adhesive resin remaining on the dentin surface (mode C) at 6 m and 1 y. For MB, cohesive failure in adhesive resin cement (mode B) was the dominant failure mode during the experimental periods for cervical dentin, while

adhesive failure mostly occurred for apical dentin. In this adhesive resin, failure mode D (cohesive failure in dentin) was not detected in any of the specimens. For MB II, most of the failures to regional dentin were found to be adhesive along the dentin surface (mode A) up to 1 y (Fig. 3c and 3d). Similar to MB, no cohesive failure in dentin (mode D) was observed. Several defects (voids or blisters) were observed within the remnants of the cement for MB and MB II on the dentin surface after failure (Figs. 3c and 3e).

SEM examination of the conditioned dentin surfaces

The result of SEM examination of the dentin surface treated by SB, MB and MB II are shown in Figs. 4a to c. After the dentin was etched with 10-3 solution (SB), the smear layer and smear plugs appeared to be removed and the tubule orifices were completely exposed (Fig. 4a). SEM observation showed that the self-etching primers of MB or MB II had a similar effect on the dentin surface. The smear layer appeared to be demineralized, exposing collagen fibrils on the intertubular dentin surface. Some smear plugs were only partially removed, occasionally leaving smear debris in the tubules. Some tubules remained occluded in MB II specimens (Figs. 4b and c).

Discussion

There is a growing demand for a reliable treatment method to deal vertical root fracture (Pitts and Natkin, 1983; Trope and Rosenberg, 1992; Selden, 1996; Dederich, 1999; Sugaya et al., 2001; Kawai and Masaka, 2002). In this study, a promising method (Sugaya et al., Kawai and Masaka, 2002; Arikan et al., 2008); to reattach the root fragments in CVRF was investigated. Although this method is debatable and root resorption caused by ankylosis cannot be avoided (Panzarini et al., 2008), an immediate replantation and long-term maintenance of the natural tooth would be of great value to patients, avoiding advanced surgical procedures and financial costs resulting from advanced prosthetic replacement.

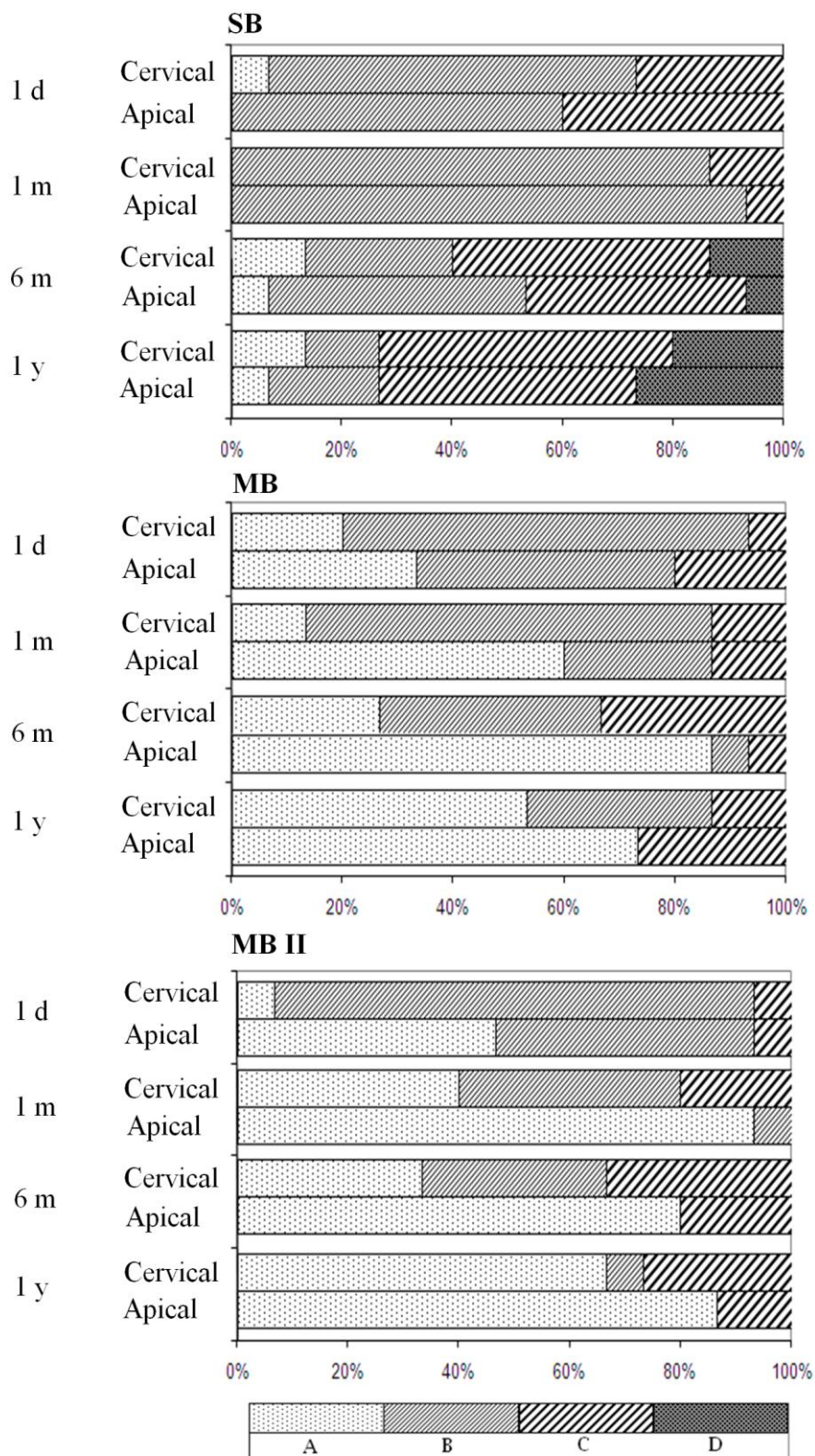


Figure 2. Fracture mode distribution after tensile testing. (A) Adhesive failure at the resin-dentin interface; (B) Cohesive failure completely within the adhesive resin; (C) Partial adhesive failure, where remnants of resin remained on the dentin surface; (D) Cohesive failure in the demineralized dentin and intact dentin.

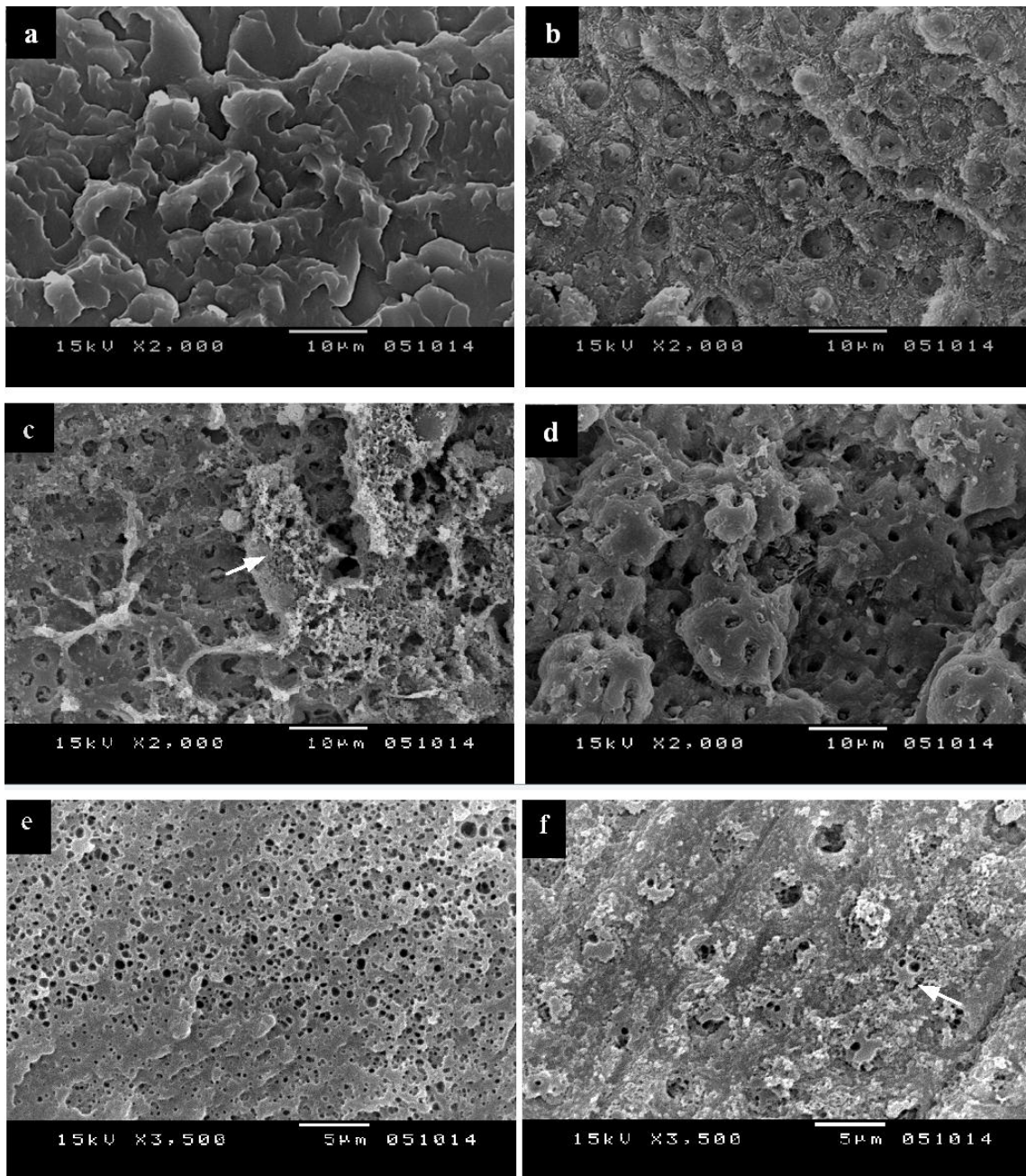


Figure 3. SEM micrographs of the fractured surface at 2000X (a-d) and 3500X (e,f) magnifications. (a) SB at 1 m; the observed mode was cohesive failure within the adhesive resin. (b) SB at 6 m; failure has occurred at the bottom of the hybrid layer. (c) MB II at 6 m; adhesive failure at resin-dentin interface with remnants of a porous adhesive resin. (d) MB II at 1 y; adhesive failure can be observed at the interface of the adhesive resin and a superficially demineralized dentin. (e) MB at 1 y; cohesive failure within adhesive resin, numerous voids are observed within the material. (f) MB at 1 y; adhesive failure at the resin-dentin interface with small remnants of the resin.

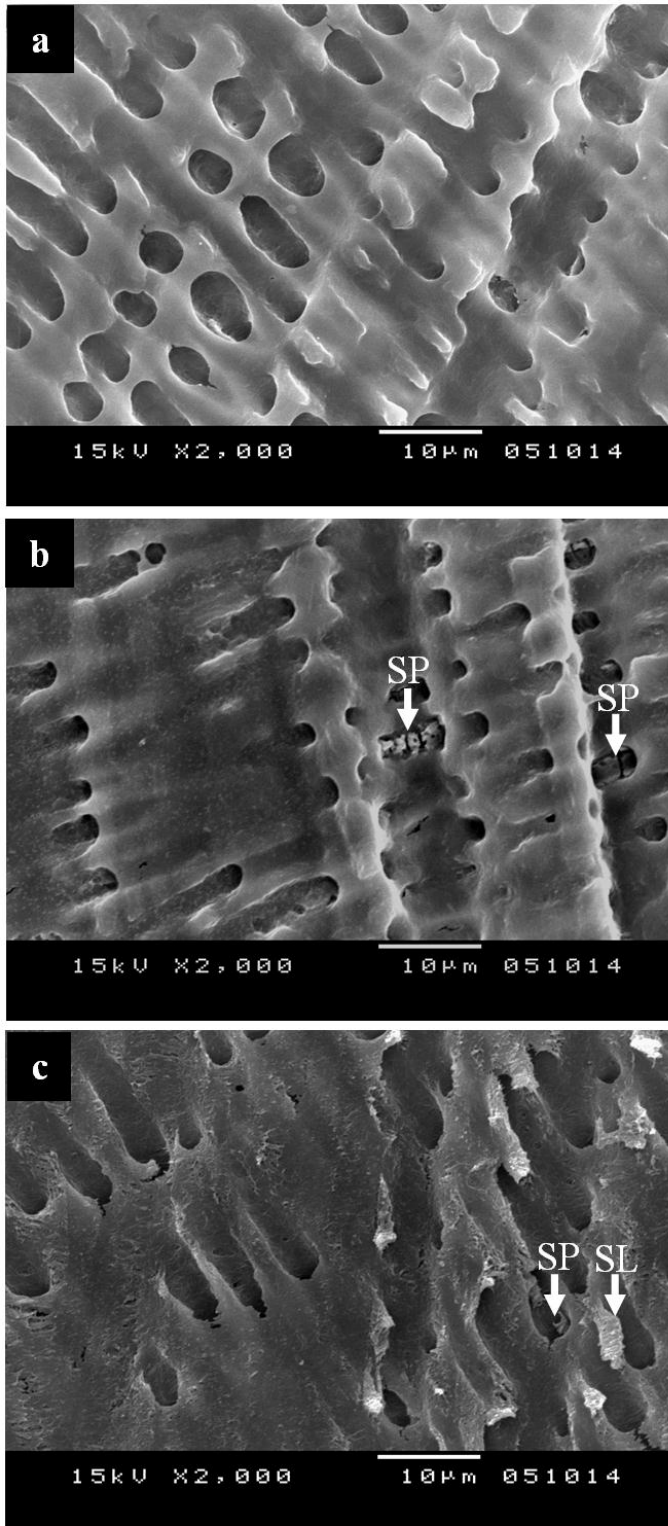


Figure 4. SEM micrographs of conditioned dentin surfaces, as the bonding substrates in the three groups. (a) Dentin surface etched with 10% citric acid with 3% ferric chloride (group SB); the smear layer and smear plugs appeared to be completely removed and the tubule orifices were opened wide. (b) Dentin surface treated with self-etching primer of MB; the smear layer was removed with small traces of the smear plugs (SP) remaining. (c) Dentin surface treated with self-etching primer of MB II; residual smear layer (SL) and smear plug (SP) partially obliterating the dentinal tubules orifices are observed.

In this study, the fracture line was lightly polished to refresh the adhesion sites on dentin as suggested by Arikan *et al* (2008); however, it should be noted that in the clinical situation, as much healthy root dentin tissue as possible should be preserved while cleaning and the cement layer should be made as thin as possible by pushing the fragments together. Regeneration of cementum and periodontium can be expected when the space of the cement is small (Kawai and Masaka, 2002; Arikan *et*

al., 2008).

The tensile strength of root dentin is considered important in understanding not only the characteristics of root dentin but also the high occurrence of CVRF. Moreover, the

fracture resistance of the repaired root in the long-term will impact the success of this treatment. A combination of the microtensile method developed by Sano *et al* (1994) and the CVRF reattachment technique enabled measurement and comparison of the regional bond strengths in the same tooth. In addition, the repaired specimens were stored in water for up to 1 y to evaluate long-term performance.

In the present study, three MMA-based adhesives, including one acid-etching system (SB) and two self-etching systems (MB and MB II), were evaluated. Interestingly there was no statistically significant difference in μ TBS of SB between cervical *versus* apical dentin. It is known that in the cervical region, intrinsic dentin water content increases with increased tubule diameter and density (number/mm²) (Tagami *et al.*, 1990). It was reported that the polymerization initiator in SB utilized oxygen and water to initiate radical polymerization of the resin monomers (Okamoto *et al.*, 1998). Therefore, considering the stability of bond strength in SB within different regions and over time, it maybe stated that SB had a certain tolerance to the surface moisture or water content of dentin (Tagami *et al.*, 1990; Tao *et al.*, 1991). Moreover, tubule diameter and tubule density decrease from cervical to apical dentin (Carrigan *et al.*, 1984; Mjör and Nordahl, 1996). If resin tag formation was the only factor affecting the adhesion, the μ TBS would have decreased in the apical region. However, in both regions, etching with the 10-3 solution resulted in complete removal of the smear layer and exposure of the intertubular and circumferentially oriented collagen fibrils, allowing hybridization of dentin and resin tag formation (Pashley *et al.*, 1995). These findings are in agreement with previous report which concluded that SB performed well in bonding to both superficial and deep dentin (Tao *et al.*, 1991). It is noteworthy that the MMA-based adhesive resin is hydrophobic; therefore, a very low water uptake is seen into the body of adhesive over time (Unemori *et al.*, 2003). In this context, a long-term degradation of this material should involve the interface or the underlying dentin rather than the adhesive layer itself.

While the μ TBS of SB to dentin was stable over time, the failure modes showed a remarkable change. The location of the failure shifted from cohesive failure within the adhesive resin towards failure at the bottom of hybrid layer and underlying dentin. It was suggested that SB monomers did not fully diffuse through the collagen network after acid etching, and the unprotected collagen acted as pathway for hydrolytic degradation of the hybrid layer and change in failure mode (Kitasako *et al.*, 2000, 2002). Changing the storage solution might also induce the loss of calcium from the etched dentin, resulting in further exposure of the dentin collagen, which could have an important effect on the hydrolysis of unprotected collagen fibrils (Kitasako *et al.*, 2000).

While the null hypothesis could be accepted for SB, it was rejected for MB and MB II, as the μ TBS values were decreased by storage and the μ TBS was significantly influenced by the dentin region; bond strength to cervical dentin was higher than that to apical dentin for both materials. It was speculated that the higher μ TBS in cervical dentin was due to increased permeability of dentin to resin penetration and an increased cross-sectional area in the cervical region (El Zohairy *et al.*, 2005). In addition, apical dentin is more sclerotic or transparent than the other region depending on the age (Kinney *et al.*, 2005). Formation of sclerotic dentin begins at the apical end of the root and often extending into the coronal dentin (Micheletti Cremasco, 1998). In this region, not only the tubules are filled with acid-resistant whitlockite minerals⁹, but also the intertubular dentin seems to be more acid resistant (Van Meerbeek *et al.*, 1994). The self-etching primers of MB (pH 1.6) and MB II (pH 1.7), with a mild etching capacity demineralize dentin only partially and parts of smear layers and smear plugs remain on the dentin (Figs. 4b and c), hampering the diffusion of monomers into the apical dentin (Yoshiyama *et al.*, 2000) and forming a thin interfacial zone in this region. Unlike SB specimens which tended to fail within demineralized dentin overtime, in MB and MB II frequent adhesive failures were observed, which generally became more prevalent

especially in apical dentin, confirming that the resin-dentin interface was the weak-link in these groups.

A significant decrease was found in μ TBS of both self-etching systems among the different experimental periods. Numerous voids were seen along the fractured interfaces of both materials (Figs. 3c and 3e). Several reports exist on the incompatibility between chemically activated resin composites combined with acidic monomers (Sanares *et al.*, 2001; Swift *et al.*, 2001). Despite the improved initiator system of MB and MB II (Tay *et al.*, 2001A), the voids may form due to the low conversion degree of primer components and/or excessive water in the formulation (Cadenaro *et al.*, 2005). A high concentration of hydrophilic components such as phosphoric acid monomers in both MB and MB II primers could lead to an increased water sorption, and consequently, decreased hydrolytic stability of the interface (Yiu *et al.*, 2004). In addition, apart from the primer, the body of these resin cements is composed of an MMA-based resin. It was reported that upon effective polymerization, a hydrophobic MMA-based resin showed lower water sorption when compared to most methacrylate-based resin adhesives that contained high amount of hydrophilic solvents (Unemori *et al.*, 2003; Hashimoto *et al.*, 2006). However, the stability and water sorption of the new mixtures of MMA and other monomers as in the formulation of MB and MB II are not known.

While micromechanical interlocking of resin into etched and primed dentin was historically considered to be the most important mechanism of bonding to dentin (Nakabayashi *et al.*, 1982, 1991), with mild self-etching primers, a stable chemical interaction between acidic monomers and the hydroxyapatite minerals remaining within the substrate may provide additional benefits to achieve a strong durable bond (Yoshida *et al.*, 2004). However, one should be aware that in order to take full advantage of the mild self-etching primer systems, adhesive systems with balanced formulations are necessary. Further

research is warranted on the development and evaluation of self-etching primer based self-cure adhesive resin cements.

Conclusions

Based on our results it can be concluded that the long-term bond strengths of three MMA-based adhesive resins to different regions of root dentin in a simulated CVRF after aging in water was material dependent. The μ TBS of SB was stable over time and not affected by dentin region, whereas MB and MB II showed a lower bond strength to apical dentin and a significant decrease over 1 y period.

Chapter 2

Dentin bonding performance and ability of four MMA-based adhesive resins to prevent demineralization along the hybrid layer

Introduction

Achieving good adhesion to tooth substrate is still challenging, as the preparation of the hydrophilic dentin surface for application of a hydrophobic adhesive resin is a technique-sensitive and time-consuming procedure (Sadek *et al.*, 2008).

Adhesive resin cements were introduced to dentistry to improve the longevity of indirect restorations (Hikasa *et al.*, 2010). Resin cements with various chemical formulations have been launched; methyl-methacrylate (MMA)-based systems that have a linear polymer structure were among the first generations of resin cements that could effectively adhere to dentin (Nakabayashi *et al.*, 1982). The MMA-based materials differ in physical and mechanical properties from dimethacrylate-based systems that are more common in dental adhesives and have a heavily-cross linked polymer structure (Imai *et al.*, 1997).

MMA-based adhesive resins such as 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl borane resin (4-META/MMA-TBB resin) (Super-Bond C&B, Sun Medical, Moriyama, Japan) and M-Bond (Tokuyama Dental, Tokyo, Japan) have been available in the market for several years now. The 4-META/MMA adhesive resin, initiated with TBB, is a two-step etch-and-rinse system. Through the application of 10% citric acid and 3% ferric chloride (10-3) solution the dentin is etched and upon application of the resin a hybrid layer is formed that consists of a molecular-level mixture of adhesive polymers and a collagen scaffold (Nakabayashi *et al.*, 1982, 1991; 1998). The formation of a hybrid layer was recognized as one of the contributing factors to the high bond strength (Nakabayashi *et al.*, 1982, 1991; Yang *et al.*, 2005, 2006, 2010) and good clinical retention

rates of this material (Hikasa *et al.*, 2010; Yang *et al.*, 2010). On the other hand, M-Bond is a two-bottle self-etching system that needs mixing before application. M-Bond contains a tertiary amine in the MMA liquid and benzoyl peroxide (BPO) in the PMMA powder as a polymerization accelerator. Two-functional monomers, 10-methacryloyloxy decamethylene malonic acid (MAC-10) and a multifunctional monomer are also present in the liquid. However, these materials have become less popular due to their complicated multi-step clinical application procedure and long curing periods for bonding the restoration to the tooth surface (Shimada *et al.*, 2005).

Recently, new MMA-based adhesive resin systems have been developed; a single-bottle self-etching primer has been introduced (M-Bond II, Tokuyama Dental) to eliminate the need for mixing of the primer components before application as in M-Bond. M-Bond II contains a borate catalyst in the MMA liquid as a polymerization accelerator and co-activator in the PMMA powder. The self-etching primer in both M-Bond and M-Bond II contains a phosphoric acid monomer and a borate derivate, which acts as a surface activator. An experimental self-etching primer has also been developed (SBP-40TX, Sun Medical) which replaces the 10-3 solution in the 4-META/MMA-TBB system. The pretreatment solution for Super-Bond C&B, contains 4-META and a reductant dissolved in solvents. These MMA-based adhesives have a shorter setting time and a longer working time compared to their predecessors M-Bond and 4-META/MMA-TBB.

Hybridization or *in situ* polymerization of monomers that infiltrated into a demineralized microporous collagen scaffold, which is essential to obtain good bonding to dentin (Nakabayashi *et al.*, 1991). Recent studies have documented formation of an acid-resistant zone adjacent to the hybrid layer with self-etching adhesive systems (Inoue *et al.*, 2006; 2009; Nikaido *et al.*, 2009; Tsuchiya *et al.*, 2004; Waidyasekera *et al.*, 2009). This zone is characterized by resistance to an acid and base challenge, and is different from the

conventional hybrid layer and caries-inhibition zone formed with fluoride-releasing materials. It was supposed that the so-called “acid-base resistant zone (ABRZ)” may effectively retard initial demineralization of the resin-dentin interface, and contribute to the sealing of restoration margins and the promotion of restoration durability (Inoue *et al.*, 2006; Tsuchiya *et al.*, 2004; Waidyasekera *et al.*, 2009). The existence of the ABRZ was thought to be related to monomer penetration into the partially demineralized dentin, when a mild self-etching primer adhesive system was used (Waidyasekera *et al.*, 2009). Takagaki *et al.* (2009) evaluated the hybridization effects on bond strength and ultrastructural change of the resin-dentin interface after acid-base challenge using 4-META/MMA-TBB resin with etch-and-rinse adhesive systems. In that study, the MMA monomer penetration was sufficient to create a hybrid layer, however, the ABRZ was not observed probably because of acid etching of dentin during the bonding procedures (Waidyasekera *et al.*, 2009).

Microtensile bond strength (μ TBS) test with thermal cycling have been frequently used as a laboratory method to evaluate dentin bonding performance (De Munck *et al.*, 2005; Yang *et al.*, 2005). The combination of μ TBS test and observation of resin-dentin interface after acid-base challenge can be a beneficial method to evaluate the durability of adhesion and the potential of the adhesive systems to inhibit early demineralization along the hybrid layer.

The aim of this study was to evaluate the tensile bond durability and ability of four MMA-based adhesive resins to prevent demineralization along the hybrid layer when exposed to a demineralising solution. The null hypotheses of this study were that the microtensile bond strengths of the tested MMA-based adhesive resin systems were not affected by thermal cycling, and that the formation of an ABRZ could be verified in all adhesives tested.

Materials and methods

Adhesive materials used

Four self-cured MMA-based adhesive resins were used in this study (Table 1); Super-Bond C&B (SB) is a 4-META/MMA-TBB resin, SBP-40TX (SBP) is an experimental self-etching primer, M-Bond (MB) and M-Bond II (MB II) are self-etching systems.

Microtensile Bond Strength (μ TBS) Testing

Sixty-four human third molars were used in this study. The non-carious molars were collected after the individuals' informed consent and used according to a protocol approved by the Institutional Review Board of Tokyo Medical and Dental University. After disinfection in 0.1% thymol solution for 72 h, the teeth were stored in water at 4°C and used within 1 month after extraction. The teeth were randomly allocated into four groups. For each group, ten teeth were used for μ TBS evaluation and failure mode analysis, and the remainder (n = 6) were used for SEM observation of the interface.

The outline of the μ TBS test is schematically presented in Fig. 1. The coronal portion of each tooth was removed to expose flat, mid-coronal dentin surface using a low-speed diamond saw (Isomet; Buehler, Lake Bluff, IL, USA) under water cooling. The dentin surface was ground with 600-grit SiC paper to produce a standardized smear layer. The area of adhesion was limited using a piece of 300- μ m-thick aluminium foil masking tape. Following this, the dentin surfaces were treated with one of the four MMA-based adhesive resins; SB, SBP, MB and MB II according to the manufacturers' instructions (shown in Table 1). Using the adhesive resins, a 10 mm-diameter polymethyl methacrylate (PMMA) rod (Mitsubishi Rayon, Tokyo, Japan) was placed perpendicularly on the dentin surface of the specimens to serve as a handle. Excess adhesive resin was removed gently with hand instruments.

Table1 Composition of the adhesive systems used in the study

Adhesive Material	Component	Composition/pH	Batch number	Application
Super-Bond C&B;SB Sun Medical; Moriyama, Japan	Green activator (10-3) Catalyst Powder Liquid	10% citric acid, 3% FeCl ₃ /0.74 TBB PMMA MMA, 4-META	TF1	Etch dentin with 10-3 solution for 10 s, rinse and air-dry. Powder : Liquid : Catalyst = 1 : 4 : 1 Mix for 5 s, apply to dentin surface.
Experimental primer SBP-40TX;SBP Sun Medical; Moriyama, Japan	Primer Catalyst Powder Liquid	4-META, reductant, water, acetone/3.1 TBB PMMA MMA, 4-META	090413 TF1	Apply primer for 20 s and gently air-dry for 10 s. Powder : Liquid : Catalyst = 1 : 4 : 1 Mix for 5 s, apply to dentin surface.
M-Bond; MB Tokuyama Dental; Tokyo, Japan	Primer A Primer B Powder Liquid	Phosphoric acid monomers, acetone Water, acetone, borate catalyst /1.6 PMMA, BPO MMA, MAC-10, amine, multifunctional methacrylate	015088	Mix equal amounts of Primer A and B, apply for 30 s and gently air-dry for 10 s. Powder : Liquid = 1 : 3 Mix for 10 s, apply to dentin surface.
M-Bond II; MB II Tokuyama Dental; Tokyo, Japan	Primer Powder Liquid	Phosphoric acid monomer, water, acetone, UDMA, co-activator/1.7 PMMA, co-activator MMA, UDMA, HEMA, MTU-6, borate catalyst	0090Y8	Apply primer for 20 s and gently air-dry for 10 s. Powder : Liquid = 1 : 3 Mixed for 5 s, applied to dentin surface.
TBB, tri-n-butyl borane; MMA, methyl methacrylate; 4-META, 4-methacryloxyethyl trimellitate anhydride; BPO, benzoyl peroxide; MAC-10, 10-methacryloyloxy-decamethylene malonic acid; HEMA, 2-hydroxyethyl methacrylate; UDMA, urethane dimethacrylate; MTU-6, 6-methacryloxyhexyl 2-thiouracil-5-carboxylate.				

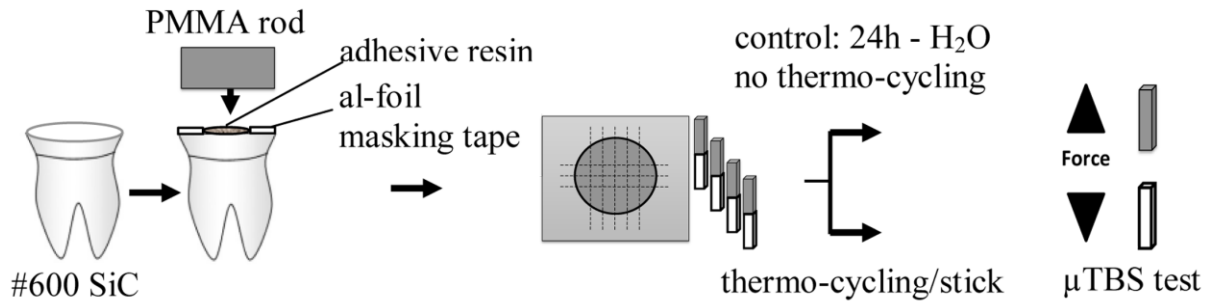


Figure 1. Sample preparation of the μ TBS test setup.

Table 2 Microtensile bond strengths and failure modes of the tested adhesives to dentin

Time	Group	Bond strength MPa \pm SD	Failure mode			ptf/n
			A	C	AC	
Prior to thermal cycling	SB	28.2 \pm 4.1 ⁺	10	22	8 ^{ab}	0/40
	SBP	27.8 \pm 3.6 ⁺	2	32	6	0/40
	MB	26.8 \pm 3.9 ⁺	11	21	8 ^c	0/40
	MB II	25.4 \pm 3.8 ⁺	13	22	5	0/40
After thermal cycling	SB	26.1 \pm 3.7 ⁺	15	3	22 ^d	0/40
	SBP	24.1 \pm 9.9 ⁺	3	11	26 ^{bc}	0/40
	MB	18.6 \pm 8.6 [*]	25	0	15 ^e	0/40
	MB II	16.5 \pm 6.5 [*]	24	8	5 ^{ade}	3/40

Values identified with different superscript symbols in this column are significantly different ($p < 0.05$). A: adhesive failure; C: cohesive in resin failure; AC: adhesive and cohesive failure. Values identified with the same superscript letters in this column are significantly different in the distribution of failure modes ($p < 0.05$). ptf = pre-testing failures, n = number of specimens.

The bonded specimens were then left at room temperature for 30 min to ensure the initial polymerization. The specimens were then immersed in 37°C distilled water for 24 h. The specimens were then perpendicularly sectioned at the resin-dentin interface into serial slabs. Each slab was further sectioned into 0.9 mm x 0.9 mm PMMA-dentin beams. Eight beams were obtained from each of ten teeth from each material yielding 80 beams for bond strength evaluation. The exact dimension of each beam was measured using a pair of digital callipers

(Mitutoyo, Tokyo, Japan). Half of the specimens were tested after 24 h (n = 40), and the remaining beams were thermo-cycled for 10,000 times between 5°C and 55°C with a dwell time of 30 s, and then tested.

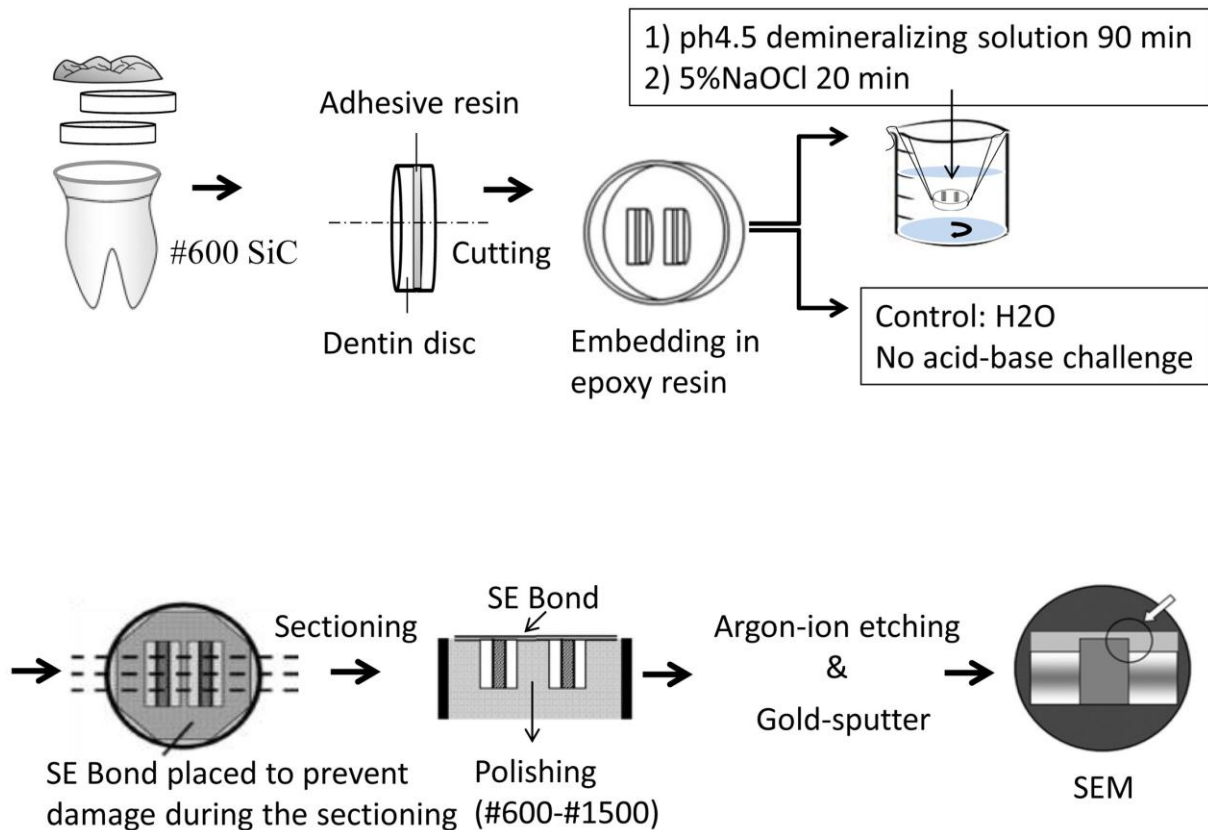


Figure 2. Sample preparation for SEM observation of ABRZ.

At the time of the test, each beam was attached to the testing apparatus with a cyanoacrylate adhesive (Zapit; DVA, Anaheim, CA, USA) and tested in a universal testing machine (EZ-Test; Shimadzu, Kyoto, Japan) at a crosshead speed of 1 mm/min. Beams that exhibited pre-testing failure during specimen preparation were recorded as null bond strength, and those values were excluded from the statistical analysis in order to maintain the normal distribution of data for parametric statistical analysis (pre-testing failures per each group are reported in Table 2).

The fractured surfaces were examined with a stereomicroscope (Nikon SMZ1000; Nikon, Tokyo, Japan) at 40x magnification to determine the mode of failure. Failures were classified into the following categories; adhesive failure along the resin-dentin interface, cohesive failure within adhesive resin, and mixed failure including adhesive failure along the resin-dentin interface and cohesive failure within adhesive resin, according to a previous study (Yang *et al.*, 2006). Representative fractured beams from each group were desiccated, gold-coated and observed with SEM (JSM-5310LV; JOEL, Tokyo, Japan) at an accelerating voltage of 20 kV to examine the morphology of the bonded interface.

SEM analysis of the adhesive interface after acid-base challenge

The sample preparation for assessment of demineralization inhibition potential, as illustrated in Fig. 2, was conducted according to the previous methodology.¹² Two dentin discs with a thickness of approximately 1.5-mm were prepared from the mid-coronal portion of each of the 24 remaining molars using a diamond saw under running water. The dentin surfaces were ground with 600-grit SiC paper to standardize the smear layers under a water spray. The ground dentin surfaces were then treated with one of the four MMA-based adhesive resins; SB, SBP, MB or MB II, which were applied between two random disks to produce a dentin disk sandwich. Six specimens were bonded in this manner in each group, with three specimens serving as control and the other three served for observation of ABRZ. The bonded specimens were stored in distilled water at 37°C for 24 h. Subsequently, each of the specimens was vertically sectioned at the resin-dentin interface into two halves and embedded in an epoxy resin (Epoxyure Resin; Buehler). The resin-dentin interface was ground with SiC papers from 400-grit to 1200-grit. The specimens allocated for ABRZ observation were subjected to an acid-base challenge, while the control specimens were stored in distilled water. For the acid challenge, each specimen was stored in 100 ml buffered demineralizing solution, containing 2.2 mmol/L calcium chloride, 2.2 mmol/L of sodium

dihydrogen phosphate, and 50 mmol/L of acetic acid adjusted at pH 4.5 for 90 min to assess the inhibition of demineralization (Wefel *et al.*, 1995). For the base challenge, the specimens were immersed in 5% sodium hypochlorite solution for 20 min to remove any demineralized dentin collagen fibrils, and then rinsed with running water for 60 s.

After the treatment, a light-cured adhesive resin, Clearfil SE Bond (Kuraray Medical, Tokyo, Japan), was applied to the acid-base treated surface without the prior use of any self-etching primer, in order to protect the structure and prevent wear of the adhesive during polishing, because otherwise the edge of the adhesive would be torn away during specimen polishing (Inoue *et al.*, 2006). The specimens were then vertically sectioned at the resin-dentin interface and polished in turn with diamond pastes with particle sizes of 6, 3, 1 and 0.25 μm (DP-Paste; Struers, Ballerup, Denmark). The polished surfaces were etched with an argon-ion beam (EIS-IE; Elionix, Tokyo, Japan) for 7 min to bring the hybrid layer into sharp relief. The operating conditions for the argon ion beam etching were an accelerating voltage of 1 kV and an ion current density of 0.2 mA/cm^2 , with the ion beam directed perpendicular to the polished surface (Inokoshi *et al.*, 1993). The specimens were then gold-sputter coated. Finally, the interface in control specimens and morphological changes of the resin-dentin interface in acid-base challenged specimens were observed using an SEM (JSM-5310LV).

Statistical analysis

The distribution of μTBS data was first checked for normality with the Kolmogorov-Smirnov test, and then statistically analyzed with two-way ANOVA to test the influence of two factors, “adhesive material” and “thermal cycling”. Bonferroni’s t-test was used to identify statistical differences between pairs of means at the significance level of $\alpha = 0.05$. The failure mode results were compared for each adhesive material using the chi-square test at a significance level of $\alpha = 0.05$.

Results

μTBS

The μ TBS values and failure modes are shown in Table 2. Two-way ANOVA revealed that the μ TBSs were affected by the “adhesive material” ($F = 16.458$; $p < 0.0001$) and “thermal cycling” ($F = 73.716$; $p < 0.0001$). The interactions were statistically significant between two factors ($p < 0.0001$).

In the control groups without thermal cycling, there were no significant differences in μ TBS among the four adhesives ($p > 0.05$). However, the μ TBSs of MB and MB II decreased significantly after thermal cycling ($p < 0.001$), while the μ TBSs of SB and SBP decreased slightly but not statistically significantly after thermal cycling. In the thermal cycling groups, the μ TBSs of SB and SBP were significantly higher than those of MB and MB II ($p < 0.001$).

Pre-testing failures of the specimens during the specimen preparation were not observed, except for MB II thermal cycling group ($n = 3$). The chi-square test revealed that there was a significant difference in distribution of failure mode among the groups ($p < 0.05$). Without thermal cycling, the predominant mode of failure was cohesive failure within adhesive resin in all the groups. After thermal cycling, for SB and SBP, more than half of the failures were mixed failure including cohesive and adhesive failures (Figs. 3a and b). In contrast, for MB and MB II, two thirds of the failures were adhesive failure at the resin-dentin interface. Blister-like structures were revealed along the fractured interfaces of MB and MB II thermal cycling groups (Figs. 3c and d).

SEM observations of ultrastructural features after acid-base challenge

SEM photomicrographs of the resin-dentin interface in control specimens and after acid-base challenge are shown in Figs. 4. An outer lesion (OL), created by mineral loss due to

the acid-base challenge, was observed in all groups after the challenge. The depth of the OL ranged from 15 to 20 μm .

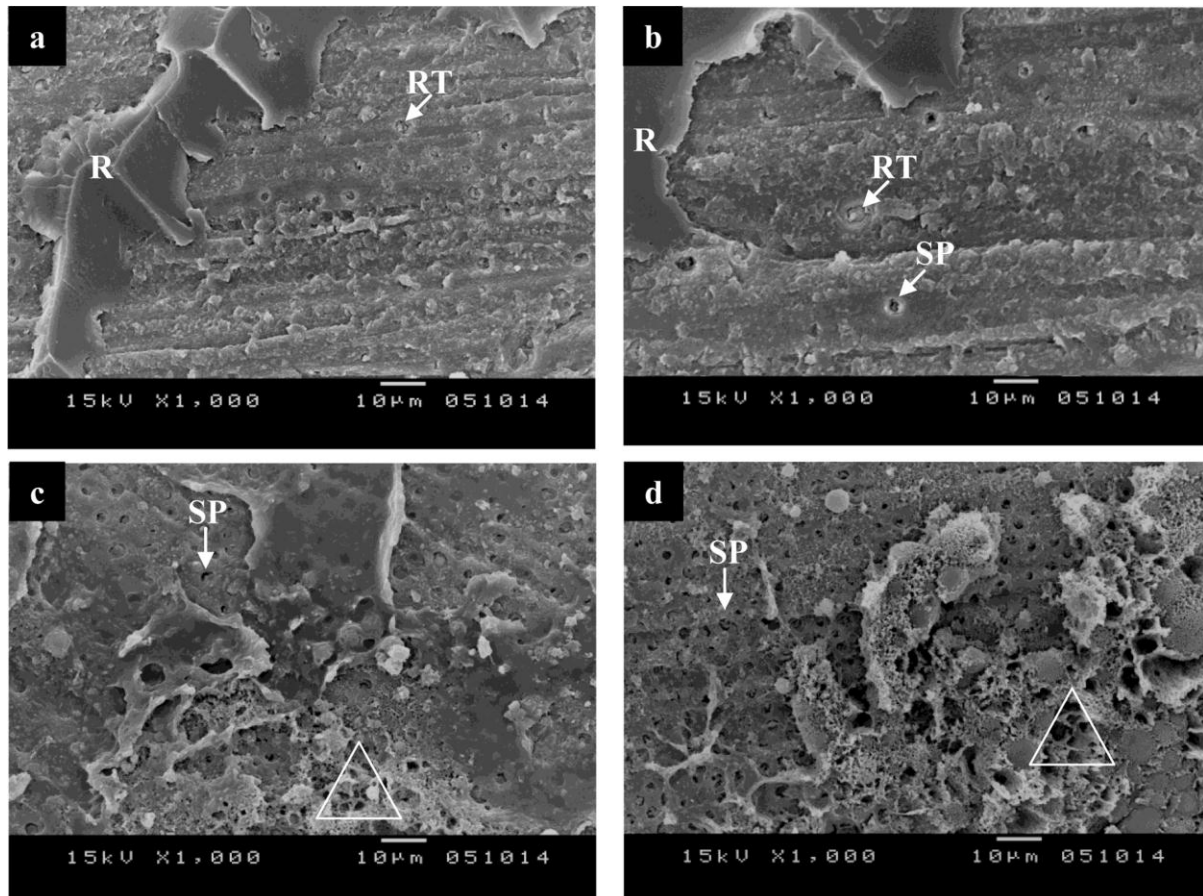


Figure 3. SEM micrographs of a debonded specimen. (a) Dentin side of SB-TC, showing cohesive failure within adhesive resin and at the top of the hybrid layer where most of the resin tags remained firmly embedded in the tubules. (b) Dentin side of SBP-TC, showing cohesive failure within adhesive resin and at the top of the hybrid layer where few tubules remained sealed. Some dentinal tubules were obstructed with smear plugs (SP). (c) Dentin side of MB-TC, showing formation of blisters or droplets (triangle) at the resin-dentin interface. (d) Dentin side of MB II-TC, showing a porous structure of the adhesive resin caused by formation of voids. R, adhesive resin; RT, resin tags; SP, smear plugs; DT, dentinal tubules.

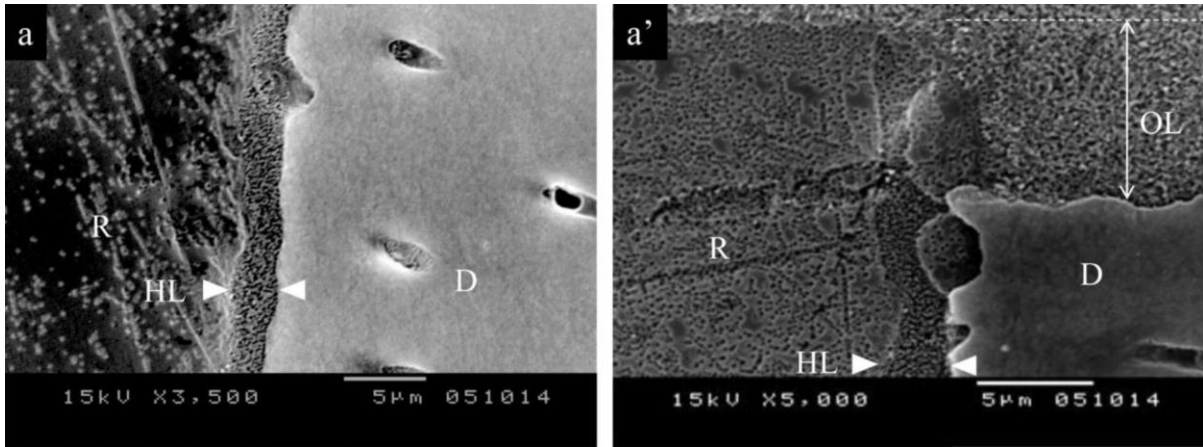


Figure 4. (a) SEM micrographs of resin-dentin interface of a control group under magnification 3500X. SB: Thick hybrid layer (HL) of approximately 3 μm in width could be observed. (a') After the acid-base challenge under magnification 5000X, an ABRZ (acid-base resistant zone) is not present for SB. R, adhesive resin; HL, hybrid layer; ABRZ, acid-base resistant zone; D, dentin; OL, outer lesion.

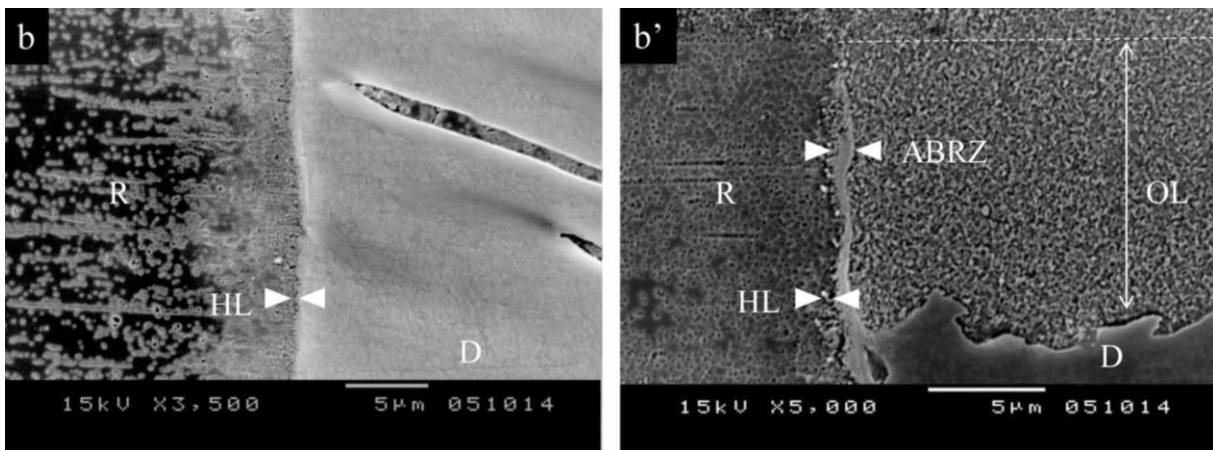


Figure 4. (b) SEM micrographs of resin-dentin interface of a control group under magnification 3500X. SBP: It was difficult to observe a hybrid layer less than 0.5 μm in thickness. (b'): After the acid-base challenge under magnification 5000X, an ABRZ of approximately 1.0 μm in width (white triangles) could be clearly observed beneath the HL of SBP. R, adhesive resin; HL, hybrid layer; ABRZ, acid-base resistant zone; D, dentin; OL, outer lesion.

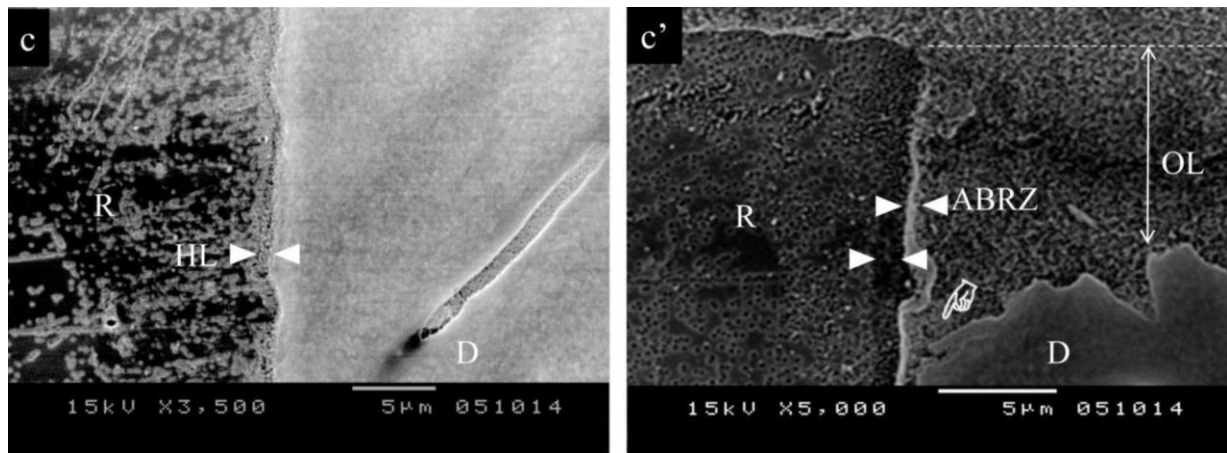


Figure 4. (c) SEM micrographs of resin-dentin interface of a control group under magnification 3500X. MB: a hybrid layer 1 μm in thickness is observed (white triangles). (c') After the acid-base challenge under magnification 5000X, a thin acid-base resistant zone less than 1.0 μm in width (white triangles) was observed for MB. A funnel-shaped area of demineralization (pointer) is seen at the region where ABRZ and dentin integrate beyond the outer lesion (OL). R, adhesive resin; HL, hybrid layer; ABRZ, acid-base resistant zone; D, dentin; OL, outer lesion.

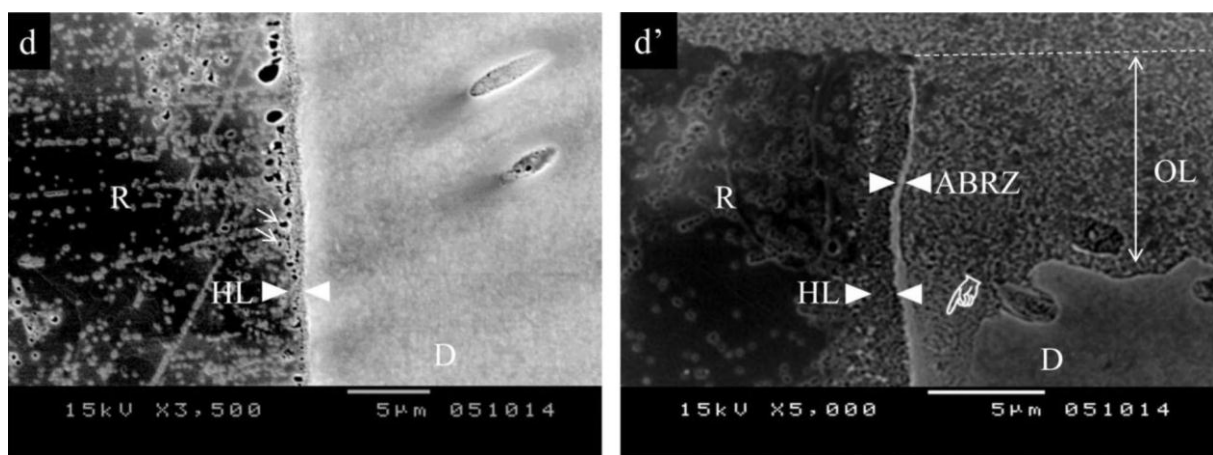


Figure 4. (d) SEM micrographs of resin-dentin interface of a control group under magnification 3500X. MB II: The adhesive interface is almost similar to that of MB group in (c) with a hybrid layer approximately 1 μm in thickness (white triangles). (d') After the acid-base challenge under magnification 5000X, a funnel-shaped area of demineralization beneath the ABRZ is observed similar to (c'). R, adhesive resin; HL, hybrid layer; ABRZ, acid-base resistant zone; D, dentin; OL, outer lesion.

For SB, a uniform and well-formed hybrid layer (HL) distinguished by argon-ion beam etching was recognized, which was approximately 1 μm thick (Figs. 4a-a'). However, an ABRZ was absent beneath the HL in the acid-base challenged specimens. The bonding interface made a right angle with the linear and flat surface of the OL.

For SBP, the thickness of the HL was less than 0.5 μm (Figs. 4b-b'). An ABRZ was recognized beneath the HL, which was approximately 1 μm thick (white triangles), the thickest observed in this study. A right angle with no loss of dentin beyond the OL was present at the junction of bonding, OL and dentin.

Figs. 4c-c' shows the interface between MB and dentin. A HL approximately 1 μm in thickness was distinguished at the interface. Also, the presence of an ABRZ approximately 0.8 μm in thickness beneath the HL was confirmed. In addition, a funnel-shaped area of demineralization at the junction of the bonding interface, dentin and OL was clearly observed (pointer).

The HL formed in the MB II was approximately 1 μm thick (Fig. 4d-d'). An ABRZ was formed approximately 0.5 μm in thickness, which was thinner than that in SBP or MB. Similar to MB, a funnel-shaped area of demineralization was observed at the dentin layer adjacent to the ABRZ. Adhesive resin layer showed multiple entrapped droplets, which were round or oval with various sizes (1-2 μm) and mostly localized close to the hybrid layer.

Discussion

In the present study, the bonding durability of the four MMA-based adhesives, including one etch-and-rinse system (SB), and three self-etching systems (SBP, MB, and MB II), was evaluated after thermal aging. Thermal cycling is a common experimental aging technique in adhesive dentistry (De Munck *et al.*, 2005; Yang *et al.*, 2005). According to a meta-analysis review (Leloup *et al.*, 2001), only 2,000 thermal cycles were not thought to affect bond strength significantly; resin-dentin interfaces were aged for 10,000 thermal cycles in the current study. The 0.9 mm x 0.9 mm bonding areas were directly exposed to water; therefore, it was expected that direct exposure to water would result in rapid degradation of the bond and accelerated aging conditions (Yang *et al.*, 2005).

In SB, dentin was etched with 10% citric acid containing and 3% ferric chloride (10-3) solution. Ferric ions adsorbed onto dentin collagen were reported to be involved in promoting polymerization of 4-META/MMA resin initiated by TBB (Imai *et al.*, 1991), as well as preventing collapse of demineralized collagen matrix on drying (Nakabayashi *et al.*, 1998; Ozaki *et al.*, 1992). The hydrophobic monomer of MMA is used as the solvent for 4-META and PMMA. The functional monomer, 4-META could increase the degree of conversion of the polymer formed (Imai *et al.*, 1994; Ozaki *et al.*, 1992). The system is water-free, and exhibits very low water absorption due to the hydrophobic nature of the matrix (Unemori *et al.*, 2003).

In SBP, the 10-3 solution is substituted by a primer containing acidic monomer, 4-META. The primer has a mild acidity with pH value around 3.1 and a reductant which promotes interfacial polymerization on the dentin surface. When the primer is applied, it partially demineralizes dentin to a depth of 0.5 μm (Fig. 4b), which is sufficient to obtain mechanical interlocking through hybridization (Van Meerbeek *et al.*, 2003). Thus, the possibility of forming a salt with the hydroxyapatite (HAp) and 4-META (Nagakane *et al.*, 2006; Yoshida *et al.*, 2004) may contribute to the long-term stability of the adhesive bond (Inoue *et al.*, 2005). Furthermore, the adhesive component (4-META/MMA-TBB resin) which is similar in composition to SB adhesive, creates a hydrophobic coat over the hydrophilic primer. This prevents the adhesive layer from behaving as a permeable membrane after polymerization (De Munck *et al.*, 2005), and might result in optimized hybridization and lower susceptibility to water degradation. Thus, the application of SBP primer is a promising alternative to the 10-3 acid etching for a tight and durable bonding of the 4-META/MMA-TBB resin to dentin. The use of self-etching primers also saves time clinically because they do not require separate acid-etching and water-rinsing steps, but are simply dried with air (Hayakawa *et al.*, 2003).

On the other hand, MB and MB II primers contain an acidic resin monomer and a catalyst capable of initiating interfacial polymerization of acidic monomers. Generally, the contents of acidic monomers and water are elevated in self-etching primers in order to increase the acidity of the primer (Tay *et al.*, 2001B, 2002A; Yiu *et al.*, 2004). The pH value of these two primers is 1.7 and 1.6 (MB and MB II, respectively) according to the manufacturer; the solutions have a higher acidity than the 4-META-containing primer of SBP. A distinct hybrid layer with thickness of about 1 μm was formed in both MB and MB II (Figs. 4c-c' and 4d-d').

Some voids were seen along the fractured interfaces of both materials in the great majority of SEM specimens (Figs. 3c and 3d). A look into the composition of MB may explain the cause of the interfacial voids. This material contains BPO and tertiary amine, and several studies have pointed out an adverse reaction between the acidic monomers in self-etching systems and the BPO in self-cured resins (Sanares *et al.*, 2001; Swift *et al.*, 2001); it was speculated that some of the voids may have originated from the production of carbon dioxide gas as a result of decomposition of the BPO initiator in the adhesive resin (Swift *et al.*, 2001).²⁵ In the composition of MB II, the BPO has been eliminated and instead a borate catalyst system has been introduced to the adhesive resin. However, it should be noted that similar to MB, several voids were also found in MB II, and the μTBS after thermal cycles was reduced in a similar manner for both materials. In addition, previous studies have raised other concerns with regard to the formulation of some self-etching materials, affecting the durability of resulting interfaces (Hiraishi *et al.*, 2009; Tay *et al.*, 2001B, 2002A; Yiu *et al.*, 2004). These findings seem to gain support from the failure distribution (Table 2) in the present study, where they shifted from cohesive failure within resin to adhesive failure at the resin-dentin interface as bond strengths decreased in MB and MB II. Moreover, after thermal cycling MB and MB II exhibited higher scattering of data (coefficient of variation) and a

significant reduction of the mean. The wide spread of the bond strength data of the materials indicated a variable bond to dentin and susceptibility of the adhesive layer to the thermal cycling challenge. It is noteworthy that MB II was the only adhesive, for which pre-testing failures were recorded. Our results thus confirmed previous report (Koishi *et al.*, 2006) that the bond strength of SB was significantly higher than MB after 100,000 thermal cycles. Therefore, the first null hypothesis that the μ TBS of the tested MMA-based adhesive resin systems were not affected by thermal cycling was partially rejected.

In the present study, the morphological integrity after acid and base challenge was analysed by exposing the resin-dentin interface to demineralisation and observation under SEM. An acid-base resistant zone (ABRZ) is a new concept reported in the recent literature (Inoue *et al.*, 2006, 2009; Nikaido *et al.*, 2009; Tsuchiya *et al.*, 2004; Waidyasekera *et al.*, 2009). The ABRZ is supposed to prevent demineralization along the hybrid layer, the sealing of restoration margins and the promotion of restoration durability. The etch-and-rinse system of SB did not show an ABRZ in this study, which confirmed the findings of the previous studies (Takagaki *et al.*, 2009; Waidyasekera *et al.*, 2009). Etching using a 10-3 solution completely demineralized dentin up to a 3 μ m depth (Fig. 4a-a').¹ A true chemical bonding is rather unlikely, because the functional groups of monomers may have only weak affinity to the HAp-depleted collagen (Ozaki *et al.*, 1992; Van Meerbeek *et al.*, 2003). On the other hand, ABRZ could be clearly detected in the three MMA-based self-etch adhesive systems. The self-etch primer agents of SBP, MB and MB II, with a mild etching capacity compared to the 10-3 solution, demineralized dentin only to depth of about 1 μ m or less (Figs. 4b-b' to d-d'). This superficial demineralization occurs only partially, and at the bottom part of the hybrid layer, some HAp crystals are preserved (Van Meerbeek *et al.*, 2003; Waidyasekera *et al.*, 2009). The formation of an ABRZ may be attributed to the functional monomer included in the primers, that bonded to HAp and remained there, thus resulting in insoluble Ca-

monomer salts with different dissolution rates that may prevent loss of calcium from the matrix against acid-base challenge (Waidyasekera *et al.*, 2009; Yoshida *et al.*, 2004). This fact has already been confirmed with some self-etching adhesives (Inoue *et al.*, 2006, 2009; Nikaido *et al.*, 2009; Tsuchiya *et al.*, 2004; Waidyasekera *et al.*, 2009). These findings lead to a rejection of the second null hypothesis that the formation of an ABRZ was not influenced by the adhesive material.

It is noteworthy that a funnel shaped area of demineralization beyond OL was formed at the junction of ABRZ, OL and dentin only in MB and MB II, indicating a weak area vulnerable to acid-base attack beneath the ABRZ (Figs. 4c and d). It was suggested that such an area of demineralization indicated that HAp crystals are not adequately enveloped by adhesive resin (Inoue *et al.*, 2009). This finding corroborates with the μ TBS result in this study; interestingly, no funnel-shaped demineralization beyond OL was observed in the resin-dentin interface of SBP that did not exhibit decreases in bond strength.

Little information is known about the chemical interaction potential of the phosphoric acid monomer in MB and MBII with HAp, or about the stability of the resultant salt in an aqueous environment. It is therefore suggested that the interaction of 'pure' monomers in a high purity with HAp should be investigated according to the protocol used by Yoshida *et al.*, 2004.

SBP group produced the thickest ABRZ with dentin-like structures. In addition to micro-mechanical hybridization in the self-etching primer, the lower thickness of the demineralized collagen layer and the presence of more residual apatite crystallites as substrates to form relatively insoluble calcium salts contribute to the long-term stability of the adhesive bond (Inoue *et al.*, 2005). It was speculated that micromechanical interlocking may provide, in particular, resistance to the debonding stress - as imposed during μ TBS testing,

whereas additional chemical bonding may be beneficial, especially in terms of ‘sealing’, resulting in interfaces less prone to demineralization by acids.

In short, results of the μ TBS test and ABRZ observation in this study suggested that besides micro-mechanical interlocking through hybridization, the potential benefit of ABRZ formation in a self-etching system may contribute to protection of the adjacent dentin against an acid-base challenge. In view of this concept, it seems reasonable not to deprive the dentin surface of all its mineral components by using etch-and-rinse approach as excessive loss of minerals will compromise such a protective capacity.

Conclusions

Dentin bonding performances after thermal cycling were adhesive material dependent in MMA-based adhesive resins. The ABRZ formation was only observed in self-etch MMA-based adhesive systems. Presence or absence of the funnel-shaped demineralization beneath the ABRZ and beyond the OL formed in the self-etching adhesive systems may indicate deterioration of dentin bond durability.

Chapter 3

Apatite crystal protection against acid-attack beneath resin-dentin interface with four adhesives: TEM and crystallography evidence

Introduction

The ultimate goal in adhesive dentistry is to achieve a durable bond to dental tissues. Bonding to dentin was conventionally achieved by three main steps namely etching, priming and bonding, each applied as a separate agent (Tay and Pashley, 2001B). Self-etching primer systems combined the etching and the priming steps into one, while the bonding agent remained as a separate agent. More recently, one-step self-etch or all-in-one systems combined the self-etching primer and the bonding agent into one application (Tay and Pashley, 2001B). Adhesion to dentin substrate has been primarily attributed to micro-mechanical hybridization which involves infiltration and subsequent *in situ* polymerization of adhesive monomers within the demineralized microporous collagen scaffold (Nakabayashi *et al.*, 1982, 1991). In addition, it was suggested that chemical interactions between carboxyl or phosphate ester groups of functional monomers in adhesives and apatite contributed to bonding (Yoshida *et al.*, 2004; Van Landuyt *et al.*, 2007).

Despite extensive morphological studies on the interface and small-scale destructive mechanical tests, the interactions of adhesive components with the structural units of hard-tissues (i.e. biological apatite crystals in dentin) *in situ*, and the properties of the complexes formed to confirm actual intermolecular interactions have been investigated to a limited extent (Yoshihara *et al.*, 2010). In addition to ultrastructural observation, application of local analytical techniques on the interface of biomaterial-hard tissue would benefit further progress of the adhesive technology. Among those analytical techniques, selected area electron diffraction (SAED) is a crystallography method that provides information on the

local crystalline structure of thin sections under transmission electron microscopy (TEM); however, only few studies to date have employed crystallography on the interface after demineralization challenge (Waidyasekera *et al.*, 2009).

Recent studies revealed an acid-base resistant zone (ABRZ) beneath the visible hybrid layer when dentin was treated with specific functional monomers as part of mild self-etch adhesives (Waidyasekera *et al.*, 2009; Tsuchiya *et al.*, 2004; Inoue *et al.*, 2006). Based on the attributes of this zone, which was more resistant against acidic and basic attack compared to the underlying normal dentin, a dentin reinforcement concept was proposed (Waidyasekera *et al.*, 2009). However, it is unknown whether apatite crystals remaining beneath the hybrid layer were acid-resistant after phosphoric-acid-etching step in the etch-and-rinse approach, with adhesives containing similar functional monomers, such as 10-methacryloyloxydecyl dihydrogen phosphate (MDP). In addition, little information is available on the acid-resistance of dentin treated with polyalkenoic acid (PAA)-based adhesives, considering the well-proven primary ionic interaction between the carboxyl groups of the PAA and the calcium (Ca) of apatite (Yoshida *et al.*, 2000; Fukuda *et al.*, 2003).

Therefore, the aim of the present study was to investigate the protection of crystals beneath the hybrid layer against acid-attack following application of commercially available PAA- or MDP-based etch-and-rinse or a self-etch adhesives. The null hypothesis tested was that the thickness of an apatite containing layer remaining after acid-base challenge was not different using various adhesive approaches containing different functional groups.

Materials and methods

Adhesives and bonding procedures

The chemical compositions and the manufacturers' instructions of the four adhesives used in this study are listed in the Table 1. The three-step etch-and-rinse system, Scotchbond Multi-

Purpose (SMP; 3M ESPE, St Paul, MN, USA) contains about 10-20 wt% carboxylic-based functional co-polymer of PAA. The two-step etch-and-rinse system, Clearfil Photo Bond (CPB; Kuraray Medical, Tokyo, Japan) contains about 5-10 wt% phosphate-based functional monomer of MDP. The two-step self-etch system, Clearfil SE Bond (CSE; Kuraray Medical) also contains about 25-30 wt% MDP. The one-step self-etch system, Adper Easy Bond (AEB; 3M ESPE) contains about 1-5 wt% PAA, and 5-15 wt% of a phosphoric acid monomer.

The study set-up is illustrated in Fig. 1. Twelve freshly extracted non-carious third molars were used following the guidelines approved by the Tokyo Medical and Dental University Ethical Committee. A 1.5-mm-thick dentin disk was prepared from mid-coronal dentin using a low-speed diamond saw (Isomet; Buehler, Lake Bluff, IL, USA) under water cooling. The disk was covered with acid-resistant nail varnish (Shiseido, Tokyo, Japan) except for a window (2.5 x 2.0 mm) in the center of the mid-coronal dentin surface. The discs were divided into two halves at the center of the window; the crosscut surface on each hemisection was wet-sanded with 600-grit SiC papers to create a standardized smear layer. Three pairs of half-disks obtained from three teeth were randomly assigned to each of the adhesives. The adhesives were then applied on the ground dentin surfaces of the teeth that were to be bonded with the etch-and-rinse adhesives were etched for 15 sec with 37-40% phosphoric acid or treated with the self-etching adhesive according to the manufacturers' instructions. The adhesive was light-cured using a visible light curing unit (Optilux 501; Demetrom, Danbury, CT, USA) at an output of 600 mW/cm² prior to the incremental placement of a hybrid composite (Z100; 3M ESPE).

Table 1 - The composition, pH, batch number and application procedure of the tested adhesives.

Adhesive systems	Components ^a	pH	Batch number	Applications ^b
Scotchbond Multi-Purpose; SMP 3-step etch-and-rinse (3M ESPE; St Paul, MN, USA)	Etchant Gel: 37% Phosphoric acid	0.6	N211706 (Primer)	a(15 s), b, e, f,
	Primer: PAA, HEMA, water	3.3		g,
	Bond: Bis-GMA, HEMA, initiators	8.2	N207729 (Bond)	h (10 s)
Clearfil Photo Bond; CPB 2-step etch-and-rinse (Kuraray Medical; Tokyo, Japan)	K-Etchant: 40% Phosphoric acid	0.6	00448A (Catalyst)	a(15 s), b, c, d,
	Catalyst liquid: MDP, HEMA, Bis-GMA, hydrophobic DMA, BPO, CQ	2.5 (mixture)	00544A (Universal)	f,
	Universal liquid: N,N-diethanol p-toluidine, sodium benzen sulfinate, ethanol			h (10 s)
Clearfil SE Bond; CSE 2-step self-etch (Kuraray Medical)	Primer: MDP, HEMA, hydrophilic DMA, CQ, water	2.0	01049A (Primer)	e (20 s), f, g,
	Bond: MDP, HEMA, Bis-GMA, hydrophobic DMA, CQ, N,N-diethanol p-toluidine, silanated colloidal silica	2.0	01566A (Bond)	h (10 s)
Adper Easy Bond; AEB 1-step self-etch (3M ESPE)	PAA, phosphoric acid-methacryloxy- hexylesters, Bis-GMA, HEMA, 1,6 hexanediol DMA, silane-treted silica, CQ, ethanol, water	2.8	415031	d (20 s), f, h (10 s)

^a Abbreviations: BPO, benzoyl peroxide; Bis-GMA, bisphenol A diglycidylmethacrylate; CQ, camphorquinone; DMA, dimethacrylate; HEMA, 2-hydroxyethyl methacrylate; MDP, 10-methacryloxydecyl dihydrogen phosphate; PAA, polyalkenoic acid co-polymer.

^b (a) acid-etch; (b) rinse and blot dry; (c) mix each drop of adhesive catalyst and universal liquid; (d) apply adhesive; (e) apply primer; (f) gently air dry; (g) apply bond; (h) light-cure.

Acid-base challenge

After 24-hour storage at 37°C in water, the specimens were either left untouched (control) or subjected to the acid-base challenge as follows. First, 100 ml buffered demineralizing solution (pH 4.5, 2.2 mmol/l CaCl₂, 2.2 mmol/l NaH₂PO₄ and 50 mmol/l acetic acid) was used for 90 min to create artificial secondary caries (Wefel *et al.*, 1995). After the acid-challenge, the specimen was subjected to 5% NaClO for 30 min with ultrasonic vibration, in an attempt to remove any denatured dentin collagen fibrils, and finally rinsed with running water for 30 sec (Inoue *et al.*, 2006). The specimens were subjected to the acidic and basic solutions (25°C) in a beaker with a magnetic stirrer to ensure complete access of acid to all of the exposed surfaces.

Transmission electron microscopy

The interface of the adhesive and dentin in the control and acid-base challenged specimens was observed under transmission electron microscope (TEM). To facilitate ultramicrotomy, the specimens were trimmed into rectangular blocks (1 x 1.5 x 2 mm) using the diamond saw. The blocks were processed for TEM according to the procedure described in detail previously (Ichinose *et al.*, 2003). Non-demineralized, epoxy-resin-embedded sections 90 nm in thickness were prepared and observed under TEM (H-7100; Hitachi, Tokyo, Japan) operating at 75 kV. Selected area electron diffraction (SAED) patterns were performed using an aperture that selected a 200-nm-diameter area of the TEM section; the *d*-spacings of the diffraction patterns were calibrated using the *d*-spacings of gold determined under identical conditions (Ichinose *et al.*, 2003). SAED provided corroborative evidence for the presence or absence of apatite crystallites at the base and beneath hybrid layer before and after acid-base challenge.

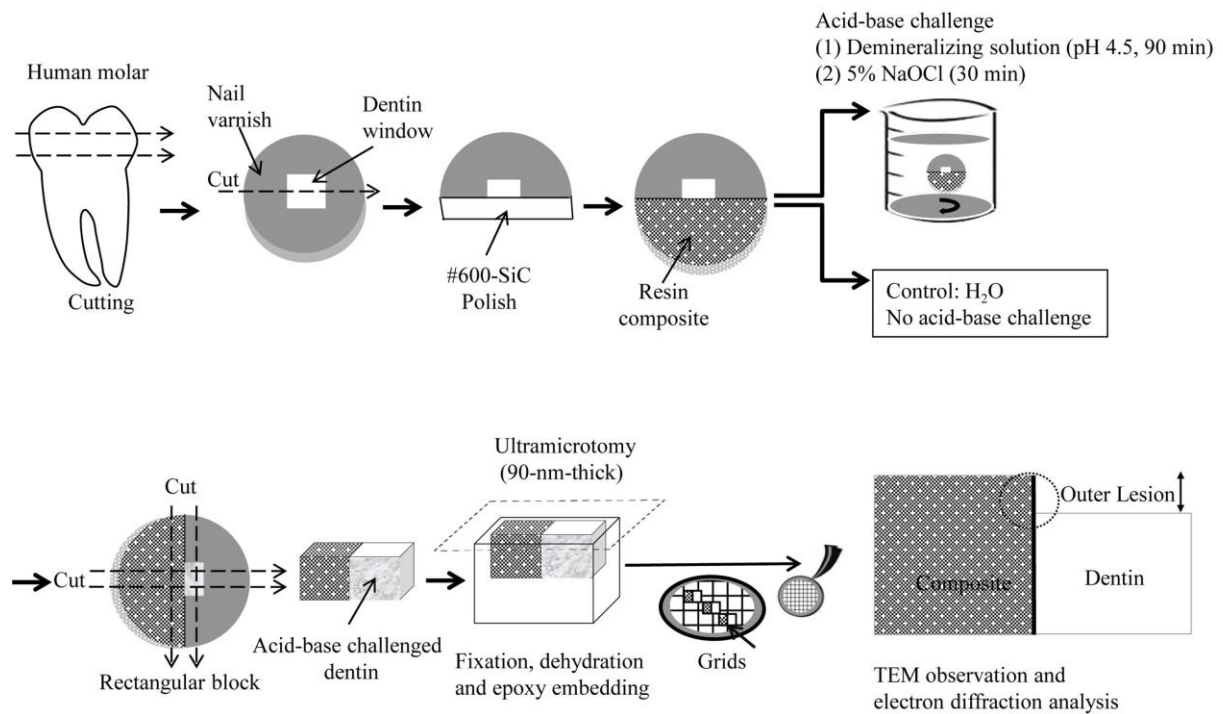


Figure 1. Schematic drawing for the sample preparation and visualization under the TEM and SAED analysis. Ultramorphological features and apatite crystallites at resin-dentin interface after acid-base challenge were studied by TEM and SAED.

Statistical analysis

The thicknesses of the hybrid layer and apatite-rich zone were measured using image analysis software (ImageJ, NIH, Bethesda, MD, USA). Ten representative images were considered for each adhesive. Data were statistically analyzed by one-way ANOVA and Tukey's multiple comparison tests with statistical significance set at $\alpha = 0.05$.

Results

The representative TEM images of the control groups are shown in Fig. 2, and those of the acid-base challenged groups are presented in Figs. 3 and 4. The ultramorphological features and their SAED patterns for each adhesive are summarized in Table 2. There were significant differences in hybrid layer (H) thickness among the adhesives ($p < 0.05$).

In the control specimens, a 4- μm -thick hybrid layer was observed for SMP (Fig. 2A). A distinct dark amorphous phase (P) was deposited at the top of the hybrid layer. Diffuse SAED patterns derived from the hybrid layer base (circle) indicated the absence of a crystalline phase within these regions (Fig. 2A-inset). For CPB, a 5- μm -thick hybrid layer (H) was observed (Fig. 2B). Within the hybrid layer, an electron-density gradient was observed at the bottom half of the hybrid layer. This region generated semi-circle SAED ring patterns along the [002] plane d -spacing of apatite (Fig. 2B-inset), indicating low density and partially dissolved apatite crystals (Zavgorodniy *et al.*, 2008). For CSE, a 1- μm -thick partially demineralized hybrid layer was observed, and continuous SAED ring patterns ascribed to the major [002] and [211] planes d -spacing of apatite (Fig. 2C-inset) were exhibited at the base of the hybrid layer, suggesting a denser overall crystallite arrangement in the base of the hybrid layer. For AEB, a partially demineralized hybrid layer, approximately 0.5 μm in thickness was created with an apatite arrangement zone along the base, as confirmed by the continuous SAED patterns (Fig. 2D-inset).

In the acid-base challenged specimens were compared against the control specimens for each adhesive system, (Figs. 3, 4), an outer lesion (OL), defined as the area at dentin surface with tissue loss due to acid-base challenge, was uniformly formed in all groups and ranged from 15 to 20 μm in depth.

In SMP (Figs. 3A, 3B), no electron-dense crystals were observed beneath the hybrid layer. A funnel-shaped lesion of dentin was extended deeper than the rest of the outer lesion into intact dentin beneath the hybrid layer (Fig. 3A). The SAED pattern at the bottom of the hybrid layer produced a diffuse pattern indicative of the absence of apatite (Fig. 3B-inset). For CPB (Figs. 3C, 3D), a layer of sparsely arranged crystallites (average 160 nm in thickness) were observed at the bottom of hybrid layer (Fig. 3D), where semi-circles SAED pattern along the [002] plane d -spacing of apatite was formed (Fig. 3D-inset). A funnel-

shaped lesion was observed beneath the hybrid layer, similar to that seen in SMP. For CSE (Figs. 4A, 4B), the hybrid layer had a lower electron density compared with that of the control group (Fig. 2C). An approximately 0.5- μm -thick electron-dense acid-resistant zone with densely arranged crystallites (Figs. 4A, 4B) formed a butt joint with the sound dentin at the border of the outer lesion with no funnel-shaped lesion in any of the specimens (Fig. 4A).

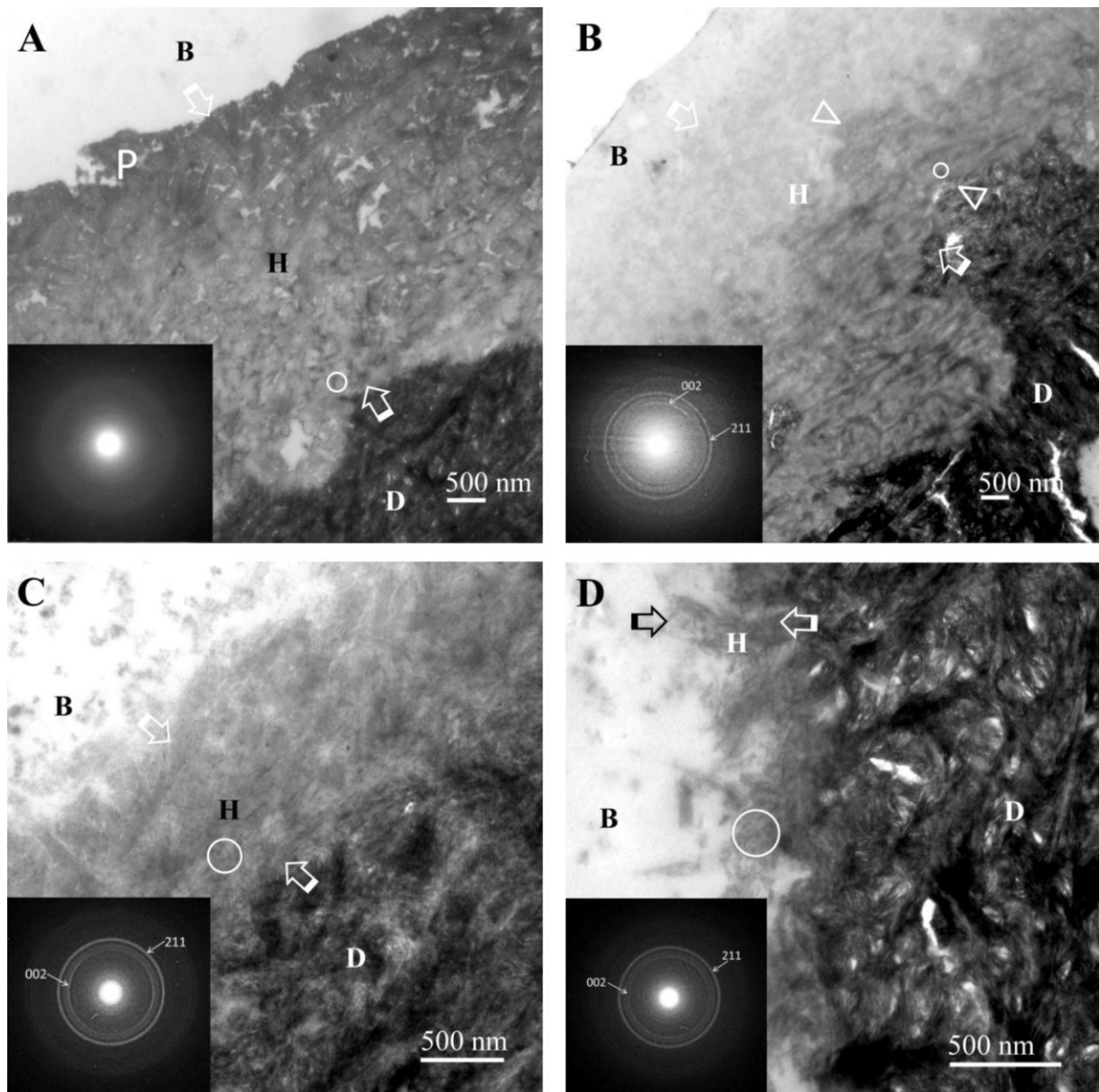


Figure 2. TEM images of the control specimens that had not been subjected to the acid-base challenge. These images provided baseline information on the depth of demineralization and extent of apatite dissolution in human dentin bonded with the four current adhesive approaches. The area between the open arrows is the layer of demineralized dentin that was infiltrated by the adhesives to produce a hybrid layer (H). SAED patterns for each material are given in the insets at the lower-left corner. The patterns were

recorded at the base of hybrid layer; the aperture selects a circular area with 200 nm diameter (circle). (A) Scotchbond Multi-Purpose created a 4- μm -thick completely demineralized hybrid layer. Diffuse SAED patterns of the completely demineralized hybrid layer confirmed the absence of apatite phases. Note the relatively dark electron-dense amorphous phase (P) deposited on top of the hybrid layer. (B) Clearfil Photo Bond created a 5- μm -thick completely demineralized hybrid layer. Note that there is electron-density gradient within the hybrid layer (between triangles). SAED patterns along the base of the hybrid layer with an indexing confirming the presence of apatite phase. The presence of semi-circle patterns corresponding to [002] crystal planes indicated a preferred orientation of apatite crystals. (C) Clearfil SE Bond, created a 1- μm -thick partially demineralized hybrid layer with a clearly discernible apatite crystallite arrangement zone along its base. SAED patterns of the hybrid layer confirmed the presence of apatite phase. The continuous circles pattern in the [002] ring suggests a dense crystallite arrangement. (D) Adper Easy Bond created a 0.5 μm -thick partially demineralized hybrid layer with an apatite crystallite arrangement zone along its base. SAED patterns of partially demineralized hybrid layer revealed the presence of apatite phase, similar to that in (C). B = bonding resin; H = hybrid layer; P = amorphous phase; D = dentin.

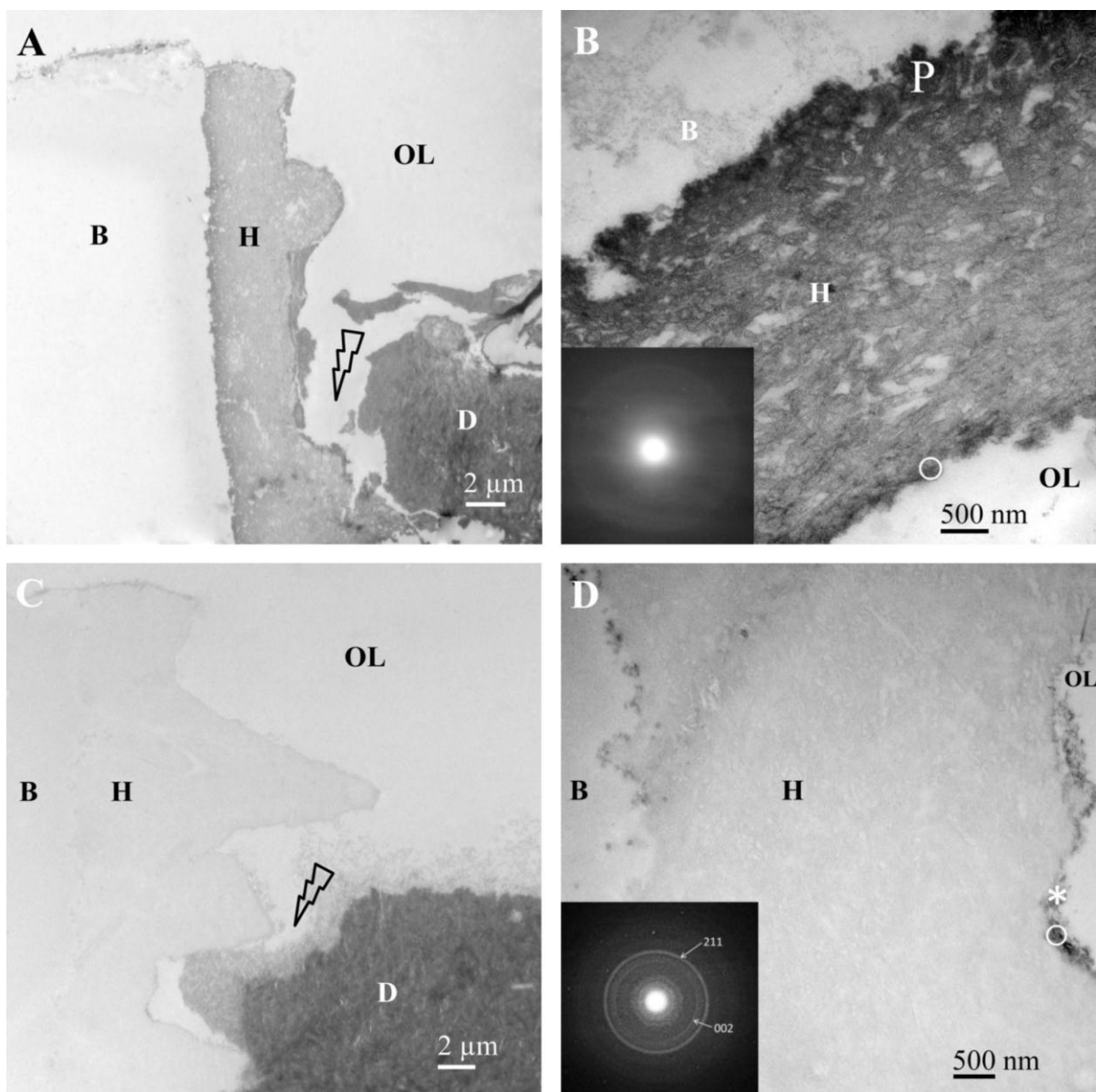


Figure 3. TEM images of the specimens in the etch-and-rinse systems Scotchbond Multi-Purpose and Clearfil Photo Bond after acid-base challenge. The insets in the lower-left corner of (B) and (D) are SAED

pattern were recorded at the bottommost part of the remaining hybrid layer in an area 200 nm in diameter (circle). (A) With Scotchbond Multi-Purpose, a hybrid layer with a distinct funnel-shaped lesion was observed (lightning bolt). (B) A high-magnification view of (A); the electron-dense apatite-rich zone was absent beneath the hybrid layer resulted in broad, diffuse electron diffraction pattern, indicative of its non-crystalline status. (C). In Clearfil Photo Bond, a hybrid layer with distinct funnel-shaped lesion was formed (lightning bolt). (D) A high-magnification view of (C); sparsely packed crystals were observed below the hybrid layer (asterisk). SAED of these sparsely packed crystals revealed the two semi-circles ring patterns ascribed to the [002] crystal planes, which indicate a preferred orientation of apatite crystals in contrast to a dense arrangement. B = bonding resin; H = hybrid layer; P = amorphous phase; D = dentin; OL = outer lesion.

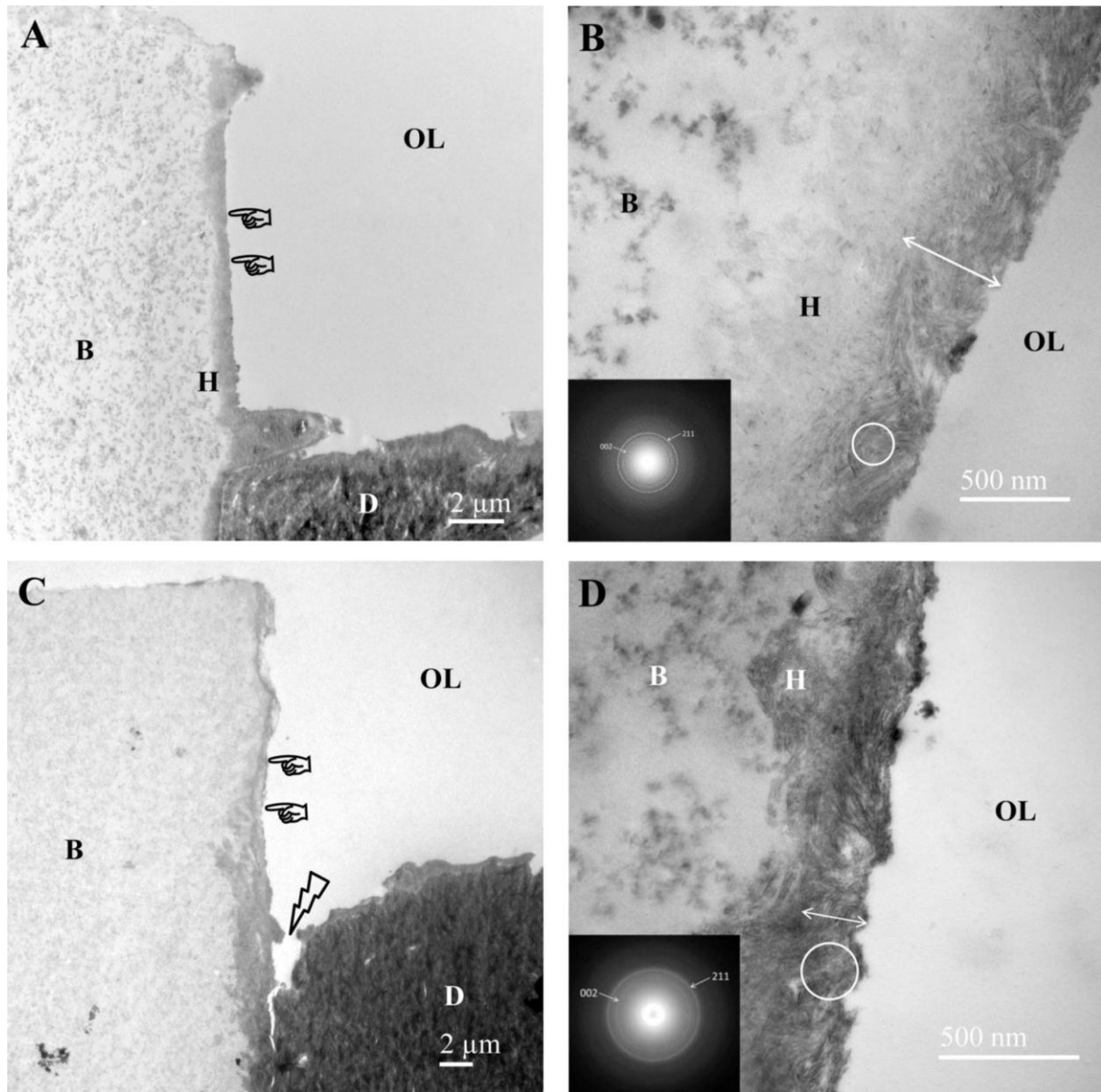


Figure 4. TEM images of the specimens of self-etching systems Clearfil SE Bond and Adper Easy Bond after the acid-base challenge. The insets in the lower-left corner of (B) and (D) are SAED patterns recorded from a small crystal cluster at the apatite-rich zone using an aperture which selected a circular area 200 nm in diameter (circle). (A) For Clearfil SE Bond, an acid-resistant apatite-rich zone was seen (designated by pointers). (B) A high-magnification view of (A); a relatively thick electron-dense apatite-rich zone are observed adjacent to the hybrid layer. Thickness of the zone was approximately 0.5 μm (indicated by

continuous white double arrow). SAED patterns of the apatite-rich zone revealed continuous ring patterns with indexing confirming the dense of apatite phase. (C) In Adper Easy Bond, the apatite-rich zone is pointed with pointers. A funnel-shaped lesion front along the apatite-rich zone extended deeper than the rest of the outer lesion was observed (lightning bolt). (D) A high-magnification view of (C); densely packed crystallites of acid-resistant apatite-rich zone was approximately 270 nm thick (white double arrow). SAED patterns of this zone revealed continuous ring patterns with indexing confirming the dense of apatite phase. B = bonding resin; H = hybrid layer; D = dentin; OL = outer lesion.

In SAED pattern, continuous rings were formed in this apatite-rich zone with an indexing confirming the high density of apatite phase (Fig. 4B-inset). For AEB, continuous rings were revealed in SAED indexing of the approximately 0.27- μm -thick electron-dense zone beneath the hybrid layer (Fig. 4D-inset). Funnel-shaped lesion was observed along the apatite-rich zone (Fig. 4C).

Discussion

Several studies to date have reported on the morphological appearance of the interface between adhesive and dentin qualitatively; however, few have attempted to employ image analyses to summarize the results quantitatively in a meaningful framework (Reis *et al.*, 2007; Tay *et al.*, 2003). In this study, the zone of apatite protected against acid-challenge at the biomaterial-dentin interface was investigated using the TEM/SAED methodology combined with digital image analysis.

Comparatively strong phosphoric-acid-etching completely or predominantly deprived dentin collagen of apatite in SMP and CPB (Figs. 2A, 2B), as a part of the etch-and-rinse procedure. However, adequate infiltration into the etched dentin and a molecular interaction of adhesive with apatite-depleted collagen turned out to be challenging in these materials. PAA could only infiltrate into the top of the demineralized dentin. A distinct dark amorphous phase in the upper part of the hybrid layer was observed in SMP (Figs. 2A, 3B), suggesting that the PAA co-polymer might have difficulty penetrating into the narrow interfibrillar spaces (10 - 30 nm wide) of the demineralized dentin. In contrast to SMP which was

completely deprived of a crystalline phase (Fig. 2A-inset), an electron-density gradient and presence of apatite in low density at the base of the hybrid layer were observed in the CPB control group (Fig. 2B-inset).

Table 2 - Summarized results of TEM and SAED patterns for the control and challenged specimens.

Adhesives	Control	Acid-base challenge			
	Hybrid layer (μm)	SAED	Apatite-rich zone (μm)	SAED	Dentin-side lesion shape
SMP	4.0 (0.8) ^a TH: dark amorphous phase BH: electron-dense without apatite phase	Diffuse	Absence ---	Diffuse	Funnel-shaped lesion
CPB	5.0 (0.3) ^b TH: without dark amorphous phase BH: electron-dense with low density of apatite crystals	Semi-circle	Unclear 0.16 (0.1) ^a	Semi-circle	Funnel-shaped lesion
CSE	1.0 (0.1) ^c TH: without dark amorphous phase BH: electron-dense with high density of apatite crystals	Continuous	Present 0.51 (0.1) ^b	Continuous	Butt joint
AEB	0.5 (0.1) ^d TH: without dark amorphous phase BH: electron-dense with high density of apatite crystals	Continuous	Present 0.27 (0.1) ^c	Continuous	Funnel-shaped lesion

TH = top of hybrid layer; BH = bottom of hybrid layer.
Values represent mean (SD), $n = 10$ per group. Different superscript letters indicate statistically significant differences in the same column (one-way ANOVA and Tukey's HSD, $p < 0.05$).

The hybrid layer was 5 μm in thickness in CPB, which was 1 μm significantly thicker than that in SMP (Table 2). Considering the thickness of hybrid layer, it should be noted that concentrations of the phosphoric acid etchants in SMP and CPB were 40% and 37%, respectively, which should have resulted in almost the same aggressiveness for acid etching of dentin. The difference in hybrid layer thickness between SMP and CPB may be related to the composition and acidity (pH) of these adhesive systems, in particular presence/or absence

of an acidic monomer (Table 1). Moreover, the hybrid layer for SMP is probably only infiltrated by water and HEMA, as PAA in the primer solution may not infiltrate. The water/HEMA mixture of primer cannot be cured well (Wang *et al.*, 2006); therefore, the demineralized collagen mesh in hybrid layer is subject to shrinkage during dehydration step of specimen preparation process (Carvalho *et al.*, 1996). CPB, on the other hand contains ethanol, which remarkably helps other components of the adhesive mixture to infiltrate (Tay *et al.*, 2007). This may result in a better infiltration/curing inside the hybrid layer and therefore, a different apparent hybrid layer thickness. In addition, MDP contained in the CPB adhesive could penetrate into the etched wet-dentin and upon ionization in the presence of water might play a role as a self-etching primer, subsequently creating further demineralized dentin. The monomer also has a strong potential to interact chemically with apatite at the bottom of the demineralized dentin. In this regard, the distinct electron-dense zone observed at the base of the hybrid layer in the CPB control group (Fig. 2B) was attributed to the effects of MDP. However, this electron-dense zone disappeared after acid-base challenge and only a few hundred-nm-thick (0.16 μm) sparsely arranged crystallites remained at the bottom of the hybrid layer (Fig. 3D). The SAED patterns at this area demonstrated semi-circle (Fig. 3D-inset) instead of a continuous ring, indicating that the crystallites had the preferred orientation of low-density apatite crystals (Zavgorodniy *et al.*, 2008). Such change in the hybrid layer after acid-base challenge suggested that the hybrid layer was permeable to acidic and basic ions, and therefore easily affected by the acid-base challenge. In line with these findings, a previous study (Van Landuyt *et al.*, 2006) reported a low-quality hybrid layer when CSE was applied after phosphoric-acid etching of dentin. The funnel-shaped lesion was observed beneath the hybrid layer in the SMP and CPB groups (Figs. 3A, 3C) confirmed suboptimal infiltration of the bottom part of the hybrid layer.

CSE and AEB were categorized as self-etching adhesive systems demineralizing dentin mildly and partially. In the current study, apatite crystals were left within the hybrid layer of these two systems (Figs. 2C, 2D). The residual apatite crystals might serve as a template for additional chemical reaction with the functional groups, such as MDP in CSE and PAA in AEB. On the chemical interaction of adhesive components with apatite, it was reported that MDP and PAA adhered to the crystals readily and intensively, forming stable Ca-MDP and Ca-PAA salts, respectively (Yoshida *et al.*, 2000, 2004). Accordingly, the acid-resistant apatite-rich zone beneath the hybrid layer was identified as ABRZ. The ABRZ below the hybrid layer should be ascribed to an effective diffusion of the reactive components (MDP and PAA) beyond the hybrid layer, and their chemical bonding potential with apatite as the substrate. In this respect, MDP is a functional monomer with usually only one or two functional chemical groups with affinity to apatite. Upon polymerization, the individual monomers become a polymer linked to apatite (Van Meerbeek *et al.*, 2011). In contrast, PAA is an already-existing polymer with multiple functional groups (Van Meerbeek *et al.*, 2011), resulting in a relatively high molecular-weight (Van Meerbeek *et al.*, 1996; De Munck *et al.*, 2005), which as mentioned earlier, has difficulty penetrating the demineralized collagen mesh.

On the other hand, AEB also contains a phosphoric acid monomer (Table 1). This functional monomer should demineralize and infiltrate dentin effectively; however, the monomer or its Ca-salts may be prone to hydrolysis in the long-term or during acid challenge in an aqueous environment, resulting in a faster demineralization in the region below the hybrid layer. The local reaction kinetics could explain thinner apatite-rich zone with funnel-shaped lesion in AEB (Figs. 4C, 4D). While, it is well-known that Ca-MDP salts are insoluble and stable (Yoshida *et al.*, 2004), there are no reports on the chemical reactivity of this phosphoric acid monomer to apatite.

The null hypothesis of this study was rejected; using SAED crystallography and quantitative TEM measurements, the current study underlined the advantage of mild self-etch adhesives; they not only keep collagen encapsulated and thus protected by apatite, but also generate chemical interaction receptiveness, rendering the capacity to reinforce the adjacent dentin against chemical (acid-base) and mechanical challenges. The latter was confirmed since ABRZ had resisted ultrasonication of the acid-base challenged samples. The interface exhibiting this property is a protected layer, which may bring up new discussions on dentin-bonding mechanisms and renew the classic hybrid layer concept (Nakabayashi *et al.*, 1991), contributing to development of improved adhesive materials in the future.

The findings may explain favorable results obtained with MDP-based two-step adhesives in laboratory as well as clinical studies (Van Meerbeek *et al.*, 2010). The resin-dentin interface is thought to be the weak-link of current restorations, and the start-point of the secondary caries process. Nevertheless, questions still remain with regard to the clinical relevance of such thin zones of reinforced dentin at the interface. Further studies are required to investigate the relationship between ABRZ and caries-protective potential in the clinical situation. With regard to the ABRZ concept, it is recommended to avoid complete demineralization of dentin to a depth of a few micrometers by using phosphoric acid, as the procedure compromises complete infiltration of monomers and reduces the chance of an effective chemical bonding and protection of apatite against acid-attack.

Conclusions

TEM/SAED evidence disclosed that the preserved dentin apatite crystals beneath the thin hybrid layer of the mild self-etch adhesives were protected against acid. The ABRZ formation was influenced not only by the functional group in the adhesive but also by the

adhesive approach; acid-base resistance of interfacial dentin may be affected by phosphoric acid etching and insufficient protection of dentin beneath hybrid layer.

Chapter 4

General conclusions

A number of factors influencing the apatite protection and bond-strength attributes of adhesives materials were identified and discussed in the presented studies.

Chapter 1 came to the conclusion that significance differences were found in the regional bond durability among MMA-based adhesives used to restore complete vertical root fracture (CVRF), which may lead to different clinical performances

Chapter 2 concluded that dentin bonding performances after thermal cycling was material dependent in MMA-based adhesive resins. The ABRZ (i.e. protected layer) formation was only observed in self-etch MMA-based adhesive systems. The presence or absence of the funnel-shaped lesion may indicate deterioration of dentin bond durability.

Chapter 3 showed that the preserved dentin apatite crystals beneath the thin hybrid layer of the mild self-etch adhesives were protected against acid. The protected layer formation was influenced not only by the functional group in the adhesive but also by the adhesive approach; acid-base resistance of interfacial dentin may be affected by phosphoric acid etching and insufficient protection of dentin beneath hybrid layer.

In the studies in chapter 1 and 2 employed long-term μ TBS technique either after aging in water or thermocycling for the investigation of material properties. It was shown that the technique had great utility in comparison between materials when a correct approach was selected based on the intrinsic behaviour of material. The studies in chapters 2 and 3 included SEM and TEM/SAED techniques for the investigation of the interactions of adhesive components with the structural units of hard-tissues in situ, and the properties of the complexes formed to confirm actual intermolecular interactions. Further studies using local reaction kinetics will be a crucial progress in this regard.

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