©Operative Dentistry, 2015, **40-1,** E28-E39

Ultramorphological Assessment of Dentin-Resin Interface After Use of Simplified Adhesives

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Clinical Relevance

Knowing the details of ultramorphological features of the dentin-resin interface can enhance the proper selection of simplified self-etch adhesives when used in clinical practice.

SUMMARY

This study assessed dentin-resin interface integration in Class I cavities restored with simplified adhesives by using a focused ion-beam milling (FIB) and transmission electron microscope (TEM). Class I cavities (1.5-mm depth with dentin thickness of ~0.5 mm, 4-mm length, and 2-mm width) were prepared on freshly extracted, sound human molars. Two all-in-one adhesive systems (Scotchbond/Single Bond Universal [SUD] and Xeno-V$^+$ [X5D]) were used and compared with a two-step etch-and-rinse system (Prime&Bond NT [NTD]). The adhesives were applied according to the manufacturers' guidelines. A universal resin composite (Filtek Z350 XT Universal) was used to restore the cavities in one bulk filling and was irradiated at 550 mW/cm^2 for 40 seconds by a quartz-tungsten-halogen light (Optilux 501). After exposure to liquid nitrogen coolant, the specimens were milled to nanoscale thickness by FIB to view and then assess the area of dentin-resin interface by TEM. Unlike the unfilled X5D, a noticeably smooth transition zone at the dentin-resin interface was shown for the SUD and NTD adhesives. The SUD demonstrated an uneven hybrid layer with clearly demineralized collagen bundles. Ultramorphologically, dispersed needlelike apatite crystals were detected within the partially demineralized dentin or the hybrid layer of both compositionally different all-in-one simplified adhesives. Conversely, these crystals were entirely absent from the hybrid layer of the etch-and-rinse NTD adhesive. In the X5D group, a bright band was noted beneath the hybrid layer. The methacryloxydecyl dihydro-

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DOI: 10.2341/13-373-L

gen phosphate monomer containing ultramild self-etch adhesive (SUD) was still validated in terms of its capability in dentin adhesion.

INTRODUCTION

The efficiency of a direct composite restoration is affected mainly by the good sealing ability of adhesive resins.[1] Bonding to dentin is more difficult because it is a highly organic structure made of collagen and smear deposits as well as water.[2] Therefore, effective bonding of resin-based restorations to dentin is more challenging compared with that of enamel.[3] Adhesion to dentin can be attained by micromechanical hybridization, which involves infiltration of the collagen fibrils and dentinal tubules by the adhesive resin, resulting in formation of a homogeneous and strong hybrid layer.[3] The hybrid layer is a mixed area of conditioned collagenous dentin impregnated with an adhesive resin monomer.[3] The main reason that inadequate sealing develops following dental composite restoration is poor bonding between the resin and dentinal tissue, which can be evidenced by rapid marginal degradation and early debonding.[4,5] This may arise from inadequate entanglement of dentinal collagen by the resin, resulting in microgaps.[6] To maximize the dentinal hybridization process, different types of adhesive resins have been developed to allow better wettability of dentin and complete resin infiltration, thus yielding a superior adhesion.

Dental adhesive resins have been improved with fast advancement in chemical components and mode of adhesion. Simplified two-step etch-and-rinse systems were developed in the late 1990s to overcome the technique sensitivity of three-step etch-and-rinse systems.[2] The two-step etch-and-rinse bonding strategy combines hydrophilic primer and hydrophobic adhesive resin into one bottle, maintaining an initial step of a separate total etch. Etching is necessary to remove the smear layer, demineralize the dentinal tissue, and expose dentinal tubules for infiltration by the resin.[2,6] Self-etch adhesives were introduced by incorporating all three components of the adhesive (etching, priming, and bonding) in one bottle.[1] These are called all-in-one simplified adhesives, which is the most common, recent formulation for bonding agents.[6] Nowadays, they are used widely in the dental profession due to their easy, fast application; lesser risk of technique sensitivity; and acceptable clinical performance.[7] This approach to adhesives is based on the use of acidic monomers that concurrently condition and prime dental tissues without rinsing.[8] It is performed by dissolving and entangling the smear and the dentin layers as substrates for bonding according to the potency of the acidic monomer's pH.[1,9] These functional acidic monomers can dissolve the smear layer by demineralizing the superficial dentin and then incorporating adhesive resin into that mixture, which will be polymerized after exposure to light irradiation.[9] It is claimed that this approach is clinically efficient, in essence reducing the working time and technique sensitivity.[10]

Even though self-etch adhesive systems minimize technique-sensitivity during their clinical application, some of them did not absolutely enhance bonding effectiveness to dentin.[11] This could be related to an increase in their hydrophilicity or to the presence of a combined blend of hydrophilic and hydrophobic monomers that may compromise each other's function, resulting in inadequate clinical durability.[10,12] Notwithstanding a considerable improvement in adhesive resins, the bonded interface between two compositionally different substances remains the weakest area of tooth restoration. Therefore, studying the nanomorphological features of the dentin-resin interface zone by an accurate device is essential to knowing the side effects of the debonding, and it can be valuable in the innovation of dentin bonding agents.

An ultramicrotomy is commonly used to cut hard-substance samples such as dental tissues and dental composite materials for such characterization by either transmission electron microscope (TEM) or scanning electron microscope (SEM).[13,14] However, these microscopic examinations require a special set of sample-preparation procedures in order to prepare ultrathin sections. An accurate and more specific milling technique based on the ion beam has been developed for ultrathin sample preparation and structural pattern fabrication: focused ion-beam (FIB) milling.[15] This milling technique is efficient and fast and can possibly minimize some present drawbacks of the ultramicrotomy technique. It is associated with the great energy density of ions originating from a gallium source.[16] The resultant ion beam allows thinning of a homogeneous or heterogeneous structured sample into an ultrascale thickness with definite clean cutting and an artifact-free, less-distorted section.[17] Nanoscale sections of dentinal tissue samples can be prepared by FIB, and such sections can enhance imaging of structural surfaces and interfaces by a TEM to reveal the bond effectiveness of adhesive resin to dentin.[18] It is a perceptive high-quality imaging that is most com-

Table 1: *Manufacturers' Composition of Dentin Bonding Agents*

Material	Code	Type	Composition	Batch No.	Manufacturer
Filtek Z350 XT (Filtek Supreme Ultra or Filtek Supreme XTE)	Z35	Nanofill	Bis-GMA, TEGDMA, Bis-EMA, UDMA, PEGDMA, silane treated ceramic, nonagglomerated/ nonaggregated silica and zirconium dioxide nanofiller (MPS = 20 and 4-11 nm, respectively), nanocluster (MPS = 6-10 μm) and filler content of 72.5% by wt.	N234311	3M ESPE Dental Products, Seefeld, Germany
Scotchbond Universal (Single Bond Universal)	SUD	All-in-one self-etch	10-MDP, phosphate monomer, dimethacrylate resins, HEMA, methacrylate-modified polyalkenoic acid copolymer, initiators, filler, silane, initiators, ethanol, water	476114	
Xeno-V$^+$	X5D	All-in-one self-etch	Bifunctional acrylic amides, acrylamide alkylsulfonic acid, "inverse" functionalized phosphoric acid ester, acrylic acid, camphorquinone coinitiator, butylated benzenediol, water, tertiary butanol, stabilizers	1111002727	Dentsply DeTrey GmbH, Konstanz, Germany
Prime&Bond NT	NTD	Two-step etch-and-rinse	Di- and trimethacrylate resins, functionalized amorphous silica, PENTA, photoinitiators, stabilizers, cetylamine hydrofluoride, acetone	0911001257	

Abbreviations: Bis-EMA, bisphenol A diglycidyl ether dimethacrylate; Bis-GMA, bisphenol A diglycidylmethacrylate; HEMA, 2-hydroxyethylmetacrylate; MPS, mean particle size; PENTA, dipentaerythritol penta acrylate monophosphate; TEGDMA, triethylene glycol dimethacrylate; 10-MDP, 10-methacryloxydecyl dihydrogen phosphate; UDMA, urethane dimethacrylate.

monly used in investigating biological water-based tissues and nonbiological materials.[18,19]

Studying the nanomorphological feature at the dentin-resin interface after treating the dentin with bonding agents is critical to understanding the quality of their adhesion. Therefore, the objective of this study was to assess ultramorphological features of the dentin-resin interfacial area by the FIB-TEM technique following the use of two different compositionally based, simplified all-in-one self-etch adhesives and compare them with a two-step etch-and-rinse adhesive.

METHODS AND MATERIALS

Materials Used

Three dental adhesive systems (multi-mode or universal adhesive, Scotchbond/Single Bond Universal Adhesive [SUD; 3M ESPE Dental Products, Seefeld, Germany]; all-in-one self-etch, Xeno-V$^+$ [X5D; Dentsply DeTrey GmbH, Konstanz, Germany]; and two-step etch-and-rinse, Prime&Bond NT (NTD; Dentsply DeTrey]) as well as a nanofill universal composite (Filtek Z350 XT Universal, 3M ESPE) were used in this study. Table 1 describes the materials used and their composition.

Specimen Preparation

Freshly extracted, sound human third molars were used in this study after patients signed an informed consent in the oral surgery department. The extraction was done for candidates between 19 and 24 years old at one month before the launch of the study. The selected teeth were initially and thoroughly scaled of any organic debris using ultrasonic and periodontal hand scalers and then cleaned by damp-pumice slurry and a rotary bristle-brush mounted on a low-speed handpiece. The teeth were then stored in a 0.02% sodium azide–containing solution at 4°C until the time of their use to avoid bacterial growth and dehydration. Then, they were immersed in distilled water for one week at 23°C and examined for the existence of any microstructural defects or cracks that would require them to be disposed.

A conventional Class I cavity 1.5 mm deep with enough remaining dentin thickness, 4 mm long, and 2 mm wide was prepared on the occlusal surface of the tooth by a coarse flat-ended cylindrical diamond bur (837, Komet, Brasseler Lemgo, Germany) using a high-speed handpiece with copious water coolant and then finished with a fine diamond bur (8830L, Komet). The depth of the cavity into dentin was

Table 2: *Mode of Dentin Bonding Agent's Application*

Adhesive System	Mode of Adhesive's Application
Scotchbond Universal (Single Bond Universal)	1. Rub one coat of the adhesive onto the entire prepared cavity for 20 s with a disposable applicator.
	2. Subsequently dry the cavity for 5 s with a soft airstream till evaporation of solvent is indicated by no movement of the adhesive film.
	3. Irradiate with light for 10 s.
Xeno V$^+$	1. Apply one ample coat to wet the cavity with gentle agitation for 20 s.
	2. Air dry thoroughly with relatively strong air pressure for 5 s to evaporate solvent until there is no movement of the film.
	3. Light cure for 20 s.
Prime&Bond NT	1. Apply the acid etching (Conditioner 36 etching gel, Dentsply DeTrey) by a syringe needle tip for 15 s on the prepared surface.
	2. Rinse the gel and the conditioned surface with copious water spray and/or aspirator tube for 15 s and then dry slightly for 5 s, leaving a visibly moist surface.
	3. Apply one ample coat of the adhesive to wet the prepared tooth surface and leave undisturbed for 20 s.
	4. Gently dry with the mild oil and moisture-free air spray syringe for 5 s to have a uniform shiny surface and to avoid dentin dehydration and collagen collapse.
	5. Light cure for 10 s.

verified by a graded periodontal-probe. The prepared cavity was thoroughly cleaned with pumice and water and flushed gently with air to receive the restoration.

The Restorative Treatment

The prepared teeth were randomly distributed according to the brand of adhesive bonding agent used. The application of these adhesive bonding agents according to their respective manufacturer's instructions are displayed in Table 2. The light irradiation of the adhesive layer was carried out by a visible quartz-tungsten-halogen light (Optilux 501, Demetron/Kerr Corp., Danbury, USA) at 550 mW/cm^2 as verified with an external radiometer (Demetron/Kerr Corp., Danbury, USA).

All prepared occlusal cavities treated with the respective adhesive system were restored with a nanocomposite (Filtek Z350 XT Universal [Filtek Supreme Ultra or Filtek Supreme XTE], 3M ESPE) in one bulk filling and then irradiated by the Optilux 501 light (Demetron/Kerr) at 550 mW/cm^2 for 40 seconds. Subsequently, the polymerized resin composite was finished with a diamond finishing bur (8368.016, Komet) and polished with Astropol points (Ivoclar Vivadent, Schaan, Liechtenstein) mounted on a low-speed handpiece at 12,000 rpm under copious water coolant. Thereafter, the restored samples were stored in a humid state at room temperature for one week preceding the milling process.

FIB Milling and TEM Evaluation

The roots of the prepared teeth were cut away and the specimens were trimmed from the buccal side longitudinally parallel to the opposing wall to expose the dentin-resin interface. The trimming was performed manually on 600-grit silicon carbide papers under water coolant and then sequentially finished with a series of these abrasive papers (800-, 1000-, 1500-, and 2000-grit). The specimens were cleaned in an ultrasonic water bath for three minutes between each abrasive paper grit and then polished in a descending series with diamond pastes (6, 3, 1, 0.25 μm) under running water. After polishing, the interfacial area of the specimen was selected by direct observation with a confocal laser scanning microscope (1LM21H/W, Lasertec Co, Yokohama, Japan) and then imaged at 100× magnification.

Each sample was sputter-coated with a 500-nm layer of tungsten in order to permit enhanced exposure of the resin-dentin interface to the FIB milling procedure (FB2200, Hitachi, Tokyo, Japan) and thereafter for microscopic and morphologic feature patterns evaluation. The FIB milling procedure was conducted in a cryogenic state (Cryo-FIB) using a liquid nitrogen coolant reservoir attached to a specimen milling stage. FIB milling is based on the impact interaction between profound ions produced mainly by a gallium source that is accelerated in a high electrical voltage field of 10 kV, and the tooth surface's sample yielding a fine beam. The initial milling current by the FIB for bulk slicing was obtained at 4 nA, yielding a 3-μm thick specimen. On

the other hand, the current beam applied to attain fine milling of a specimen slice to a nanoscale thickness of 100 nm was 10 pA at 10 kV. This high-energy beam can precisely mill and cleanly cut both the tooth-tissue surface and the resin filler of the composite into nanofoils. Moreover, this current is needed to view this ultrathin slice and to characterize the sectioned area at the dentin-resin interface by subsequent TEM for better assessment.

The ultrathin FIB-milled specimens (100 nm thick) were examined by the TEM (TEM HT7700, Hitachi) operating at 80 kV. The TEM samples were produced *in situ*, which involved a micromanipulator to transfer the thinned section to a TEM grid inside the vacuum chamber.[17] The resin-dentin zone of interest was identified at a range of magnification between 10,000× to 80,000×.

RESULTS

The FIB cross-sectioning technique clearly disclosed typical representative micrographs that were obtained from the X5D, SUD, and NTD nondemineralized and nonstained sample groups (Figures 1, 2, and 3, respectively). Unlike conventional microtomes, all FIB-sliced specimens had a limited range of view and uniform sample thickness (~100 nm). Moreover, preparing the specimens in a cryogenic state had preserved the morphology of the interface with no signs of beam damage.

Generally, the obtained sections showed a homogeneous adhesive layer over the treated dentin surface. Excluding the unfilled resin in X5D (Figure 1), the interface between the filler particles and resin matrix of the other adhesives was markedly smooth (Figures 2 and 3). We found it interesting that although the nanofillers within the adhesive layer were clearly perceived in SUD and NTD, these clusters were not perceived to be infiltrating the interfibrillar spaces of the hybrid layer. Furthermore, similar ultramorphologies were observed among the self-etch adhesive groups. The transitional zone was different in each self-etch group, and it was filled with dispersed needlelike apatite (Ap) crystals within the partially demineralized dentin or the hybrid layer (200-800 nm thick) (Figures 1d and 2d,f). However, these Ap crystals were completely removed from the hybrid layer of the etch-and-rinse adhesive NTD group (Figure 3d).

In the X5D group, the adhesive layer did not show any sign of fillers; however, it was associated with variable round voids within the adhesive layer (Figure 1a,b). The actual hybrid layer and the resin-infiltrated smear layer were difficult to distinguish from each other (Figure 1b-d). The ultramorphological examination of this layer demonstrated a mixture of remnants of smear layer and traces of demineralized Ap crystals that had lost their needlelike characteristics (Figure 1e). Moreover, this layer had an uneven thickness ranging from 500 to 800 nm with consistent underlying dentin surface to some extent. A submicron hiatus (clear bright band) was found between the hybrid layer and dentin surface (Figure 1d,e). The underlying resin-infiltrated dentin layer presented a complex of microstructure intermingled with Ap crystals, micropores, and nested demineralized, banded collagen bundles with gap and overlap zones (Figure 1f). It is important to mention there was no sign of artifact due to beam damage related to preparation with a high-energy ion beam.

For the SUD group, there appeared to be a uniform distribution of the inorganic nanofillers within the light-cured adhesive resin matrix and a homogeneous interfacial adhesion between the dentinal surface and the bonding resin layer (Figure 2a). Furthermore, there was a smooth transition from the adhesive layer to the hybrid layer without separation or microporosities. However, the hybrid layer was uneven and showed a small remnant of the smear layer, and the treated dentin surface was markedly smooth (Figure 2b,c). At higher magnification, an abundance of crisscross needlelike Ap crystals can be clearly observed above the treated dentin surface and within the hybrid layer, which ranged from 200 to 500 nm in thickness (Figure 2c,d,f). Moreover, banded demineralized collagen fibrils with gap and overlap zones were clearly observed along with Ap crystals and other dental microstructures in the dentin complex (Figure 2c-e). However, this group did not show any hiatus space beneath the hybrid layer as was noted in Figure 1.

In the NTD group, it was clearly detected that the nonaggregated nanofiller-sized particles were highly and homogeneously distributed within the adhesive resin matrix (Figure 3). It was evident that the phosphoric acid treatment had completely removed the smear layer, resulting in a grossly etched and dented underlying dentin surface in the form of nano-irregularities or "shredded" appearance of the dentin. This can produce a layer of mineral-depleted collagen fibrils that are infiltrated with resin monomer (Figure 3a-e). Although the obtained sections revealed an intimate interaction with a relatively sharp transitional zone between dentin-resin substrates, the interaction zone was filled with

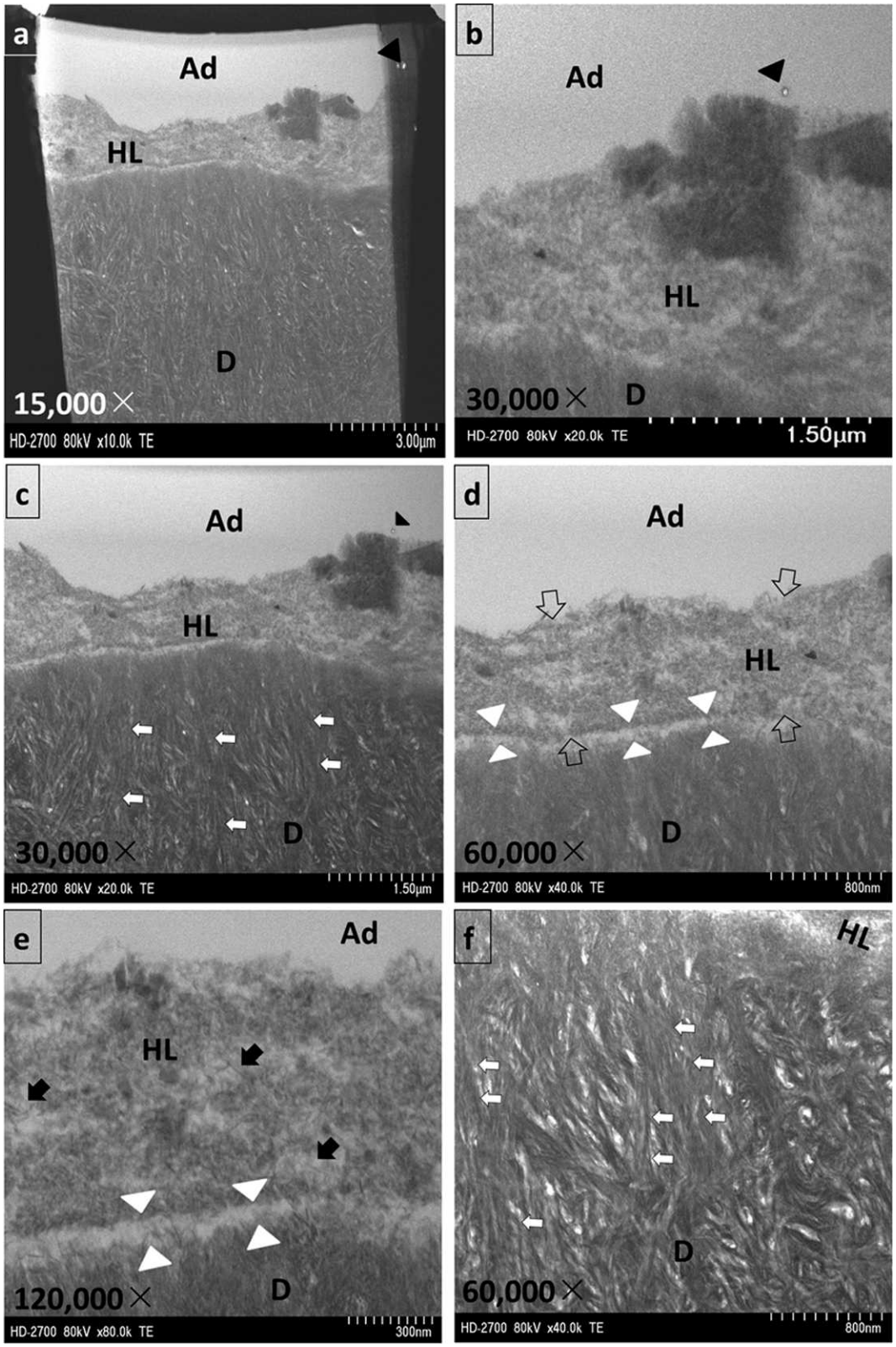

Figure 1. *Representative unstained TEM micrographs of the X5D group. (a): Low magnification of the sectioned adhesive-resin interface. The black triangles are pointing to representative round voids of phase-separation phenomena within the adhesive. (b): The round void indicated by the black triangle is representing phase separation within the adhesive. (c): The adhesive resin layer was clear (electron-lucent) and lacked inorganic filler. The underlying dentin complex showed the contrast of collagen and polymer-based phases (white arrows). The black triangle is showing the phase-*

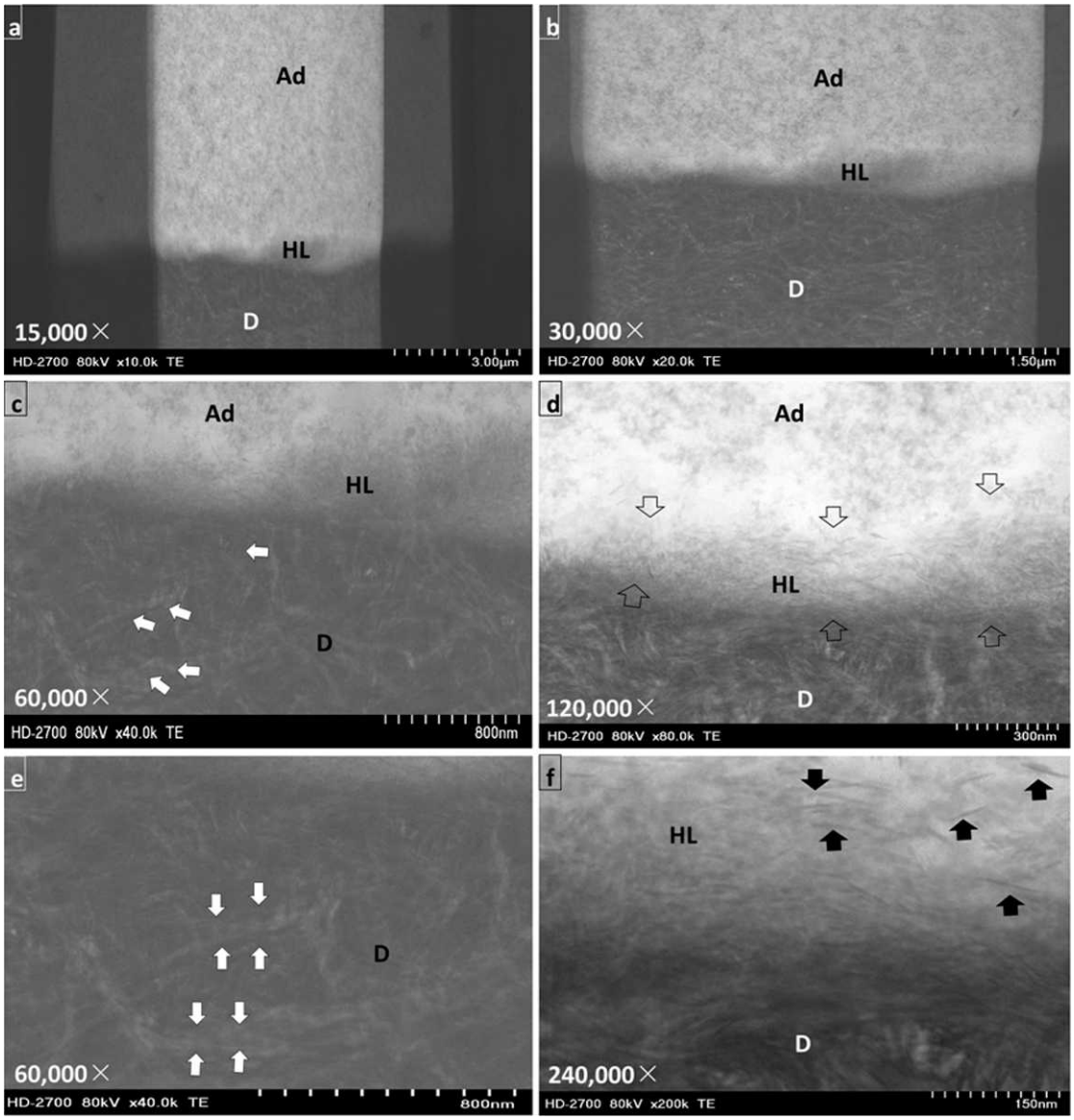

Figure 2. *Typical representative TEM micrographs of the SUD group. (a): Low magnification image showed the resin infiltrating dentin with hybrid layer formation. (b): TEM observation revealed the adhesive layer with homogeneous scattered nanofillers within the adhesive matrix. (c): A gradient transitional zone was detected from the adhesive resin to the dentin surface, ranging from 200 to 500 nm. The dentin organic phase with demineralized collagen bundles (white arrows) were clearly detected superficially and were not affected during FIB preparation. (d): At higher magnification, crisscross needlelike hydroxyapatite crystals within the hybrid layer with some remnants of the smear layer were clearly disclosed between the pointed hollow arrows. (e): The demineralized collagen bundles are indicated between the white arrows. (f): At high magnification of the hybrid layer, the black arrows are pointing to the needlelike hydroxyapatite crystals. Abbreviations: Ad, Adhesive resin; HL, hybrid layer; D, dentin.*

←
separation phenomena. (d): The transitional zone from the adhesive layer to the dentin complex through the hybrid layer (500~800 nm) with undefined contents was clearly distinguished between the illustrated hollow arrows. A bright band of interfacial space presented between the white triangles. (e): Higher magnification of the hybrid layer revealed some scattered Ap crystals (black arrows) that had lost their needlelike shape with the peculiar appearance of the remnants of the smear layer. The bright band beneath the hybrid layer (white triangles) could be attributed to the water movement into the space. (f): The microstructures of the dentin complex presented micropores, scattered Ap crystals, and nested resin-infiltrated collagen bundles. Preparing the specimens in a cryogenic state had preserved the resin-infiltrated dentin organic phase. The effect of moderate self-etch adhesive in demineralizing the mineralized collagen bundles with a gap and overlap zones (white arrows) was clearly perceived at a deeper level. Abbreviations: Ad, adhesive resin; HL, hybrid layer; and D, dentin.

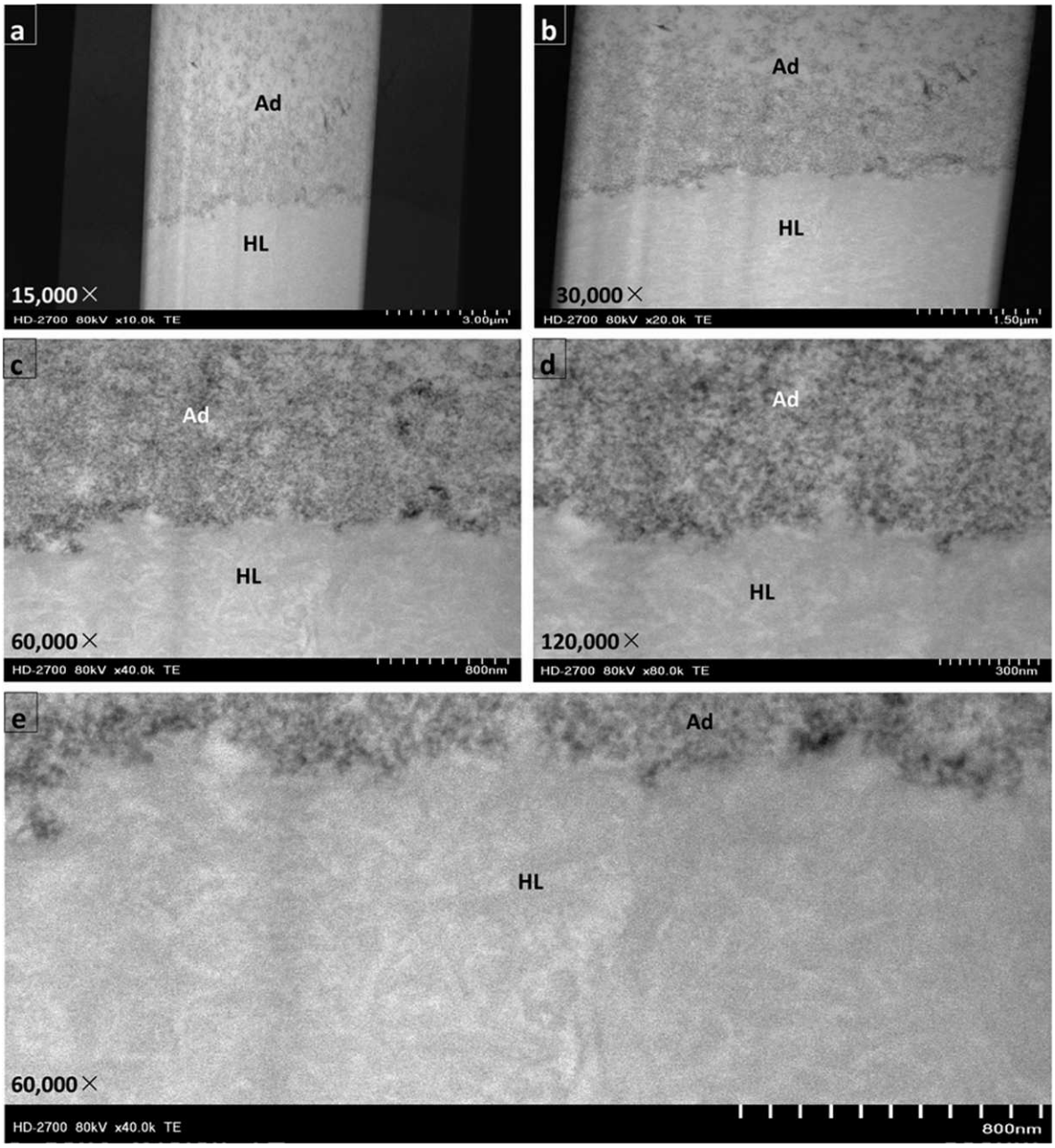

Figure 3. *Unstained TEM micrographs of the interfacial area of the NTD group. (a): The low magnification of the obtained microsample showed adhesive infiltrated dentin complex with thick hybrid layer formation (>3μm). (b): The unstained images revealed homogeneously scattered nonaggregated nanofillers throughout the adhesive layer intermixed with remnants of demineralized dentin microstructures and without penetration of the underlying hybrid layer. The sharp transitional zone from adhesive layer to dentin complex was markedly perceived. (c): The underlying hybridized dentin was mineral depleted with an electron-lucent appearance, and no remnants of the smear layer were distinguished. (d) Phosphoric acid conditioning had washed out the apatite crystals and produced nano-irregularities or shredded dentin surface with indistinguishable appearance of the underlying denuded dentin collagen. (e): The unstained images revealed homogeneously scattered nonaggregated nanofillers throughout the adhesive layer mixed with remnants of demineralized dentin microstructures and without penetration of the underlying mineral-depleted hybridized dentin layer. Phosphoric acid conditioning had washed out the Ap crystals and produced nano-irregularities or a shredded dentin surface with indistinguishable appearance of the underlying denuded dentin collagen. Abbreviations: Ad, adhesive resin; HL, hybrid layer.*

fillers and undefined remnants of demineralized dentinal microstructure, and it was very difficult to define or detect the demineralized Ap crystals (Figure 3b,c). It is worth mentioning that the hybrid layer was electron-lucent and thicker than that in other groups, with an appearance of radical demineralization and undefined dentin collagen complex (Figure 3d,e).

DISCUSSION

Ultramicrotomy has been routinely applied as a conventional technique for preparation of TEM samples. More recently, cryogenic ultramicrotomy was introduced to limit the need for sample fixation. However, both preparation techniques are unable to maintain true structural features, which hampers their broad applications. Moreover, in these techniques, the cellular structures are generally compressed in the direction of cutting. Some investigators have overcome this distortion by applying the FIB preparation method.[20] Other hindrances to ultramicrotomy preparation relate to nonuniform slice thickness and the inability to choose specific areas of interest.[21,22]

FIB is a site-specific milling tool that had been used in monitoring and manipulating the structure of tissues and materials on a nanoscale.[21,22] The main advantages of this technique are the accuracy in site selection and the cutting and thinning directions for the sample that enable precise investigation when FIB is combined with TEM. Formerly, FIB had been implemented using a conventional cross-sectioning technique, which later was modified and simplified with the introduction of the *in situ* microsampling method.[17,23,24] For dental application, it has been reported in the literature that use of the latter method resulted in a much higher sample yield during the preparation of ivory dentin for ultramorphological examination.[19,21] Moreover, combining the FIB modality with TEM facilitated the investigation of the hard materials (ie, composite fillers, as well as brittle phases; enamel and dentin) and the tooth-resin interaction for clinically relevant cavity setups, in contrast to conventional ultramicrotomy.[21]

During cavity preparation, a coarse diamond bur was used for cavity outlining and extension followed by finishing with a fine diamond bur. Apart from the aggressiveness of the diamond burs, the reported studies in the literature showed no difference in the created smear layer between carbide bur and fine-diamond bur, with comparable bonding strength.[24] It is noteworthy that the same resin composite was used to restore the cavities for the purpose of standardizing the experiment.[25]

Bonding to dentin is more challenging than bonding to enamel because it requires great attention. This is mostly attributed to the complex hydrated structure of dentin[26,27] and to the water that can diffuse from hydrated dentin through water pathways in the hybrid layer to reach the adhesive-composite interface.[28] The characteristics of the dentin smear layer are dependent upon the surface-conditioning treatment. Moreover, preparation of the dentin cavities using diamond burs resulted in undulated surfaces that promoted debris stagnation.[29,30] Additionally, the pH values of the conditioning agents have a great influence on the remaining smear layer covering the dentinal surfaces.[29,30] Therefore, the pH values of the self-etch adhesive and the method of dentin cutting might possibly contribute to the detected variation in the smear layer thickness as reported in the literature.[29,30] Moreover, different adhesive formulae were selected with different monomers and solvents to characterize the hybridized dentin-adhesive interaction zone.

The clustered nanofillers within the adhesive layer that neither infiltrated the hybrid layer in SUD nor the hybridized dentin complex in NTD group were clearly perceived (Figures 2 and 3). These findings correspond with a previously reported study in the literature that the formed filler clusters are too bulky to infiltrate the spaces of the hybrid layer.[31] Furthermore, retention of the ground substance within the demineralized intertubular collagen matrix can hamper the infiltration of the nanofillers.[31] However, these nanofillers may contribute to the higher viscosity of the filled adhesive resin. This in turn will produce a sufficient adhesive thickness over the hybrid layer that may stabilize it.[32-34]

For the two all-in-one adhesive systems studied (X5D and SUD), FIB-TEM images revealed a variable transitional zone between the adhesive resin and the underlying treated dentin representing their respective hybrid layers. Moreover, the detected micropores in the hybridized dentin were similar to those with comparable dimensions and dispersion between Ap crystals in the dentin sections that were found by ultramicrotomy-TEM.[22,35] The vivid appearance of the organic components of the dentin could be attributed to the minimized beam-damaging effect achieved by conducting FIB in a cryogenic state, which enabled visualization of the resin-infiltrated demineralized collagen with the characteristic gap and overlap zones (Figures 1 and 2).[21,36] However, strong phosphoric-acid etching in the NTD group completely or predominantly deprived dentin collagen of Ap crystals, leaving the organic component exposed for the infiltrating adhesive resin (Figure 3).

X5D is a 2-hydroxyethyl methacrylate (HEMA)-free all-in-one self-etch adhesive that has an im-

proved chemical formula from its predecessors and is optimized without acrylic acid inclusion. The absence of HEMA in this category may reduce water uptake and thus hydrolysis at the adhesive interface.[37,38] However, it may induce phase separation between hydrophilic and hydrophobic components, given that it was spotted at some areas within the adhesives above the hybrid layer (Figure 1a,b). According to the manufacturer, the adhesive matrix lacks nanofillers, which explains the clear appearance of the adhesive layer and relative reduced viscosity. Moreover, the increased acidity of the functional monomers (ie, functionalized phosphoric acid ester and acrylamide-alkylsulfonic acid) that reduced the pH value (pH = 1.3) can explain the undistinguished content of the hybrid layer, which had the vague appearance of partially demineralized Ap crystals in shallow and deep dentin collagen (Figure 1b-f). Another point to be mentioned is that most of the reported TEM studies on HEMA-free adhesives displayed variable degrees of submicron hiatus space beneath and within the hybrid layer, which agreed with the results obtained in the X5D group (Figure 1d,e).[29,39,40] This space could be ascribed to the water movement from the underlying hydrated dentin complex toward the adhesive resin[28] and/or to the incomplete removal of the solvent (tertiary butanol), which requires relatively more pressure (vapor pressure = 4.1 kPa at 20°C) to evaporate than the solvent in SUD (ethanol; vapor pressure = 5.8 kPa at 20°C) and NTD (acetone; vapor pressure = 24 kPa at 20°C) during the drying phase.[41]

The prepared cavities in the SUD group were treated using one-step self-etch adhesive (Scotchbond Universal adhesive) that had shown a gradient transitional zone from the adhesive resin to the dentinal surface through the hybrid layer, with some nanofillers dispersed within the adhesive matrix. Unlike in X5D, this adhesive formula contains HEMA that may inhibit phase separation by homogenizing the hydrophilic and hydrophobic phases.[38] Also, the high pH used in SUD (pH = 2.7) comprised of methacryloxydecyl dihydrogen phosphate (MDP) functional monomer, which has a high affinity to bond ionically to partially demineralized Ap crystals, can preserve their needlelike appearance without complete removal of the smear layer at the interdiffusion zone.[42] Although it was not the aim of this study, the author speculates this MDP-apatite interaction would result in formation of MDP-calcium salts in the form of nano-layering assemblies at higher magnifications, as was reported by other studies.[43,44] A point of interest in this category is the ability to maintain the uniformity of the dentin surface with limited demineralization depth and a shallow resin-infiltrated collagen demineralization process, which is attributed to the mild acidity of this adhesive system and may account for dentin bonding stability.[45]

In NTD, the obtained TEM sections confirmed the claims of the high dispersion of the nonaggregated nanofillers within the adhesive resin matrix that were clearly perceived in the interaction zone mixed with remnants of demineralized dentin microstructures. This adhesive is an acetone-based two-step etch-and-rinse adhesive system and uses strong phosphoric acid as a surface conditioning, before adhesive application, that can completely eliminate the smear layer as well as demineralize the underlying dental tissues, leaving denuded collagen behind. It contains acidic phosphonated monomer (dipentaerythritol penta acrylate monophosphate) that has the ability to interact with calcium ions left on the dentin surface after collagen demineralization.[46] The inclusion of acetone in its chemical formula will ensure adhesive infiltration by displacing water remnants from the dentin surface and act as a "water-chaser."[33] However, this volatile solvent has a relative high vapor pressure, which makes this system more technique sensitive during the restorative procedure. The effect of phosphoric acid conditioning was apparent in the form of nano-irregularities or shredded dentin surface with pronounced hybridized resin–infiltrated dentin complex that appeared electron-lucent in the TEM sections, indicating the absence of a crystalline phase within these regions. These findings coincide with other reports in the literature.[42,47] Taking into consideration the adhesive viscosity and deep dentin demineralization in etch-and-rinse systems in general, the enzyme activation of proteolytic matrix metalloproteinases is expected to be induced and long-term bond durability would be affected.[48] However, this is unlikely to be seen in self-etch adhesives that have the ability to etch and penetrate simultaneously, which would result in resin-collagen fixation and consequent long-term bond stability.[45,48,49]

Within the limitations of this study, including time consumption, reduced field of view, and technique sensitivity that requires skilled personnel to operate the FIB system, the ultrastructural characterization of the changes in adhesive resins over the long-term should be considered in future FIB-TEM studies.

CONCLUSIONS

It can be concluded that FIB preparation methodology is a unique technique for preparation of hard materials and brittle phases and can substitute for conventional methods. Although this technique is very sensitive and may be prone to some artifacts, it showed a lot of potential for future research when performed in a cryogenic state. Interfacial assessments showed the etching pattern differences of the self-etch adhesives in comparison with an etch-and-rinse adhesive system and their marked influence on the organic phase of dentin. Incorporating MDP as a functional monomer to the newly introduced self-etch adhesive confirmed its competence as a dentin bonding mechanism. The overall results of this study provided a valuable insight that should be considered during adhesive selection in routine dental practice.

Acknowledgments

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU), Jeddah, Saudi Arabia, under grant no. (3-33-GP). The authors, therefore, acknowledge with thanks DSR technical and financial support.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

(Accepted 7 June 2014)

REFERENCES

1. Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, & De Stefano Dorigo E (2008) Dental adhesion review: Aging and stability of the bonded interface *Dental Materials* **24(1)** 90-101.
2. Van Meerbeek B, De Munck J, Yoshida Y, Inoue S, Vargas M, Vijay P, Van Landuyt K, Lambrechts P, & Vanherle G (2003) Buonocore Memorial Lecture. Adhesion to enamel and dentin: current status and future challenges *Operative Dentistry* **28(3)** 215-235.
3. Nakabayashi N, & Saimi Y (1996) Bonding to intact dentin *Journal of Dental Research* **75(9)** 1706-1715.
4. Hashimoto M, Ohno H, Kaga M, Endo K, Sano H, & Oguchi H (2000) *In vivo* degradation of resin-dentin bonds in humans over 1 to 3 years *Journal of Dental Research* **79(6)** 1385-1391.
5. Hashimoto M, Ohno H, Sano H, Tay FR, Kaga M, Kudou Y, Oguchi H, Araki Y, & Kubota M (2002) Micromorphological changes in resin-dentin bonds after 1 year of water storage *Journal of Biomedical Materials Research* **63(3)** 306-311.
6. Tay FR, & Pashley DH (2002) Dental adhesives of the future *Journal of Adhesive Dentistry* **4(2)** 91-103.
7. van Dijken JW, & Pallesen U (2011) Four-year clinical evaluation of Class II nano-hybrid resin composite restorations bonded with a one-step self-etch and a two-step etch-and-rinse adhesive *Journal of Dentistry* **39(1)** 16-25.
8. Pashley EL, Agee KA, Pashley DH, & Tay FR (2002) Effects of one versus two applications of an unfilled, all-in-one adhesive on dentine bonding *Journal of Dentistry* **30(2-3)** 83-90.
9. Tay FR, & Pashley DH (2001) Aggressiveness of contemporary self-etching systems. I: Depth of penetration beyond dentin smear layers *Dental Materials* **17(4)** 296-308.
10. Peumans M, Kanumilli P, De Munck J, Van Landuyt K, Lambrechts P, & Van Meerbeek B (2005) Clinical effectiveness of contemporary adhesives: A systematic review of current clinical trials *Dental Materials* **21(9)** 864-881.
11. Tay FR, & Pashley DH (2003) Water treeing—A potential mechanism for degradation of dentin adhesives *American Journal of Dentistry* **16(1)** 6-12.
12. Tay FR, Pashley DH, Suh BI, Carvalho RM, & Itthagarun A (2002) Single-step adhesives are permeable membranes *Journal of Dentistry* **30(7-8)** 371-382.
13. Earl J, Leary R, Perrin J, Brydson R, Harrington J, Markowitz K, & Milne S (2010) Characterization of dentine structure in three dimensions using FIB-SEM *Journal of Microscopy* **240(1)** 1-5.
14. Botton G, & Phaneuf M (1999) Imaging, spectroscopy and spectroscopic imaging with an energy filtered field emission TEM *Micron* **30(2)** 109-119.
15. Giannuzzi L, & Stevie F (2004) *Introduction to Focused Ion Beams: Instrumentation, Theory, Techniques and Practice*. Springer, New York **1st edition.**
16. Yonehara K, Baba N, & Kanaya K (1989) Application of ion-beam etching techniques to the fine structure of biological specimens as examined with a field emission SEM at low voltage *Journal of Electron Microscopy Technique* **12(1)** 71-77.
17. Phaneuf M (1999) Applications of focused ion beam microscopy to materials science specimens *Micron* **30(3)** 277-288.
18. Van Meerbeek B, Yoshida Y, Lambrechts P, Vanherle G, Duke ES, Eick JD, & Robinson SJ (1998) A TEM study of two water-based adhesive systems bonded to dry and wet dentin *Journal of Dental Research* **77(1)** 50-59.
19. Jantou V, Turmaine M, West GD, Horton MA, & McComb DW (2009) Focused ion beam milling and ultramicrotomy of mineralised ivory dentine for analytical transmission electron microscopy *Micron* **40(4)** 495-501.
20. Marko M, Hsieh C, Schalek R, Frank J, & Mannella C (2007) Focused-ion-beam thinning of frozen-hydrated biological specimens for cryo-electron microscopy *Nature Methods* **4(3)** 215-217.
21. Coutinho E, Jarmar T, Svahn F, Neves AA, Verlinden B, Van Meerbeek B, & Engqvist H (2009) Ultrastructural characterization of tooth-biomaterial interfaces prepared

with broad and focused ion beams *Dental Materials* **25(11)** 1325-1337.

22. Van Meerbeek B, Conn LJ, Duke ES, Schraub D, & Ghafghaichi F (1995) Demonstration of a focused ion-beam cross-sectioning technique for ultrastructural examination of resin-dentin interfaces *Dental Materials* **11(2)** 87-92.
23. Li J, Malis T, & Dionne S (2006) Recent advances in FIB-TEM specimen preparation techniques *Materials Characterization* **57(1)** 64-70.
24. Marques MS, Kenshima S, Muench A, Ballester RY, & Rodrigues Filho LE (2009) Effect of the C-factor and dentin preparation method in the bond strength of a mild self-etch adhesive *Operative Dentistry* **34(4)** 452-459.
25. Pashley DH, Carvalho RM, Sano H, Nakajima M, Yoshiyama M, Shono Y, Fernandes CA, & Tay F (1999) The microtensile bond test: A review *Journal of Adhesive Dentistry* **1(4)** 299-309.
26. Marshall GW, Marshall SJ, Kinney JH, & Balooch M (1997) The dentin substrate: Structure and properties related to bonding *Journal of Dentistry* **25(6)** 441-458.
27. Itthagarun A, & Tay FR (2000) Self-contamination of deep dentin by dentin fluid *American Journal of Dentistry* **13(4)** 195-200.
28. Chersoni S, Suppa P, Breschi L, Ferrari M, Tay FR, Pashley DH, & Prati C (2004) Water movement in the hybrid layer after different dentin treatments *Dental Materials* **20(9)** 796-803.
29. Shinoda Y, Nakajima M, Hosaka K, Otsuki M, Foxton RM, & Tagami J (2011) Effect of smear layer characteristics on dentin bonding durability of HEMA-free and HEMA-containing one-step self-etch adhesives *Dental Materials Journal* **30(4)** 501-510.
30. Barros JA, Myaki SI, Nör JE, & Peters MC (2005) Effect of bur type and conditioning on the surface and interface of dentine *Journal of Oral Rehabilitation* **32(11)** 849-856.
31. Tay FR, Moulding KM, & Pashley DH (1999) Distribution of nanofillers from a simplified-step adhesive in acid-conditioned dentin *Journal of Adhesive Dentistry* **1(2)** 103-117.
32. Van Landuyt KL, Snauwaert J, De Munck J, Peumans M, Yoshida Y, Poitevin A, Coutinho E, Suzuki K, Lambrechts P, & Van Meerbeek B (2007) Systematic review of the chemical composition of contemporary dental adhesives *Biomaterials* **28(26)** 3757-3785.
33. Aguilera FS, Osorio R, Osorio E, Moura P, & Toledano M (2012) Bonding efficacy of an acetone/based etch-and-rinse adhesive after dentin deproteinization *Medicina Oral, Patología Oral y Cirugía Bucal* **17(4)** e649-e654.
34. Gallo JR, Comeaux R, Haines B, Xu X, & Burgess JO (2001) Shear bond strength of four filled dentin bonding systems *Operative Dentistry* **26(1)** 44-47.
35. Nakabayashi N, Ashizawa M, & Nakamura M (1992) Identification of a resin-dentin hybrid layer in vital human dentin created *in vivo*: Durable bonding to vital dentin *Quintessence International* **23(2)** 135-141.
36. Balooch M, Habelitz S, Kinney JH, Marshall SJ, & Marshall GW (2008) Mechanical properties of mineralized collagen fibrils as influenced by demineralization *Journal of Structural Bioliology* **162(3)** 404-410.
37. Yoshida Y, Yoshihara K, Hayakawa S, Nagaoka N, Okihara T, Matsumoto T, Minagi S, Osaka A, Van Landuyt K, & Van Meerbeek B (2012) HEMA inhibits interfacial nano-layering of the functional monomer MDP *Journal of Dental Research* **91(11)** 1060-1065.
38. Van Landuyt KL, Snauwaert J, Peumans M, De Munck J, Lambrechts P, & Van Meerbeek B (2008) The role of HEMA in one-step self-etch adhesives *Dental Materials* **24(10)** 1412-1419.
39. Mine A, De Munck J, Van Landuyt KL, Poitevin A, Kuboki T, Yoshida Y, Suzuki K, Lambrechts P, & Van Meerbeek B (2008) Bonding effectiveness and interfacial characterization of a HEMA/TEGDMA-free three-step etch & rinse adhesive *Journal of Dentistry* **36(10)** 767-773.
40. Agee KL, Pashley EL, Itthagarun A, Sano H, Tay FR, & Pashley DH (2003) Submicron hiati in acid-etched dentin are artifacts of desiccation *Dental Materials* **19(1)** 60-68.
41. Bakhsh TA, Sadr A, Shimada Y, Mandurah MM, Hariri I, Alsayed EZ, Tagami J, & Sumi Y (2013) Concurrent evaluation of composite internal adaptation and bond strength in a Class-I cavity *Journal of Dentistry* **41(1)** 60-70.
42. Nurrohman H, Nikaido T, Takagaki T, Sadr A, Ichinose S, & Tagami J (2012) Apatite crystal protection against acid-attack beneath resin-dentin interface with four adhesives: TEM and crystallography evidence *Dental Materials* **28(7)** e89-e98.
43. Yoshida Y, Yoshihara K, Nagaoka N, Hayakawa S, Torii Y, Ogawa T, Osaka A, & Van Meerbeek B (2012) Self-assembled nano-layering at the adhesive interface *Journal of Dental Research* **91(4)** 376-381.
44. Yoshihara K, Yoshida Y, Nagaoka N, Fukegawa D, Hayakawa S, Mine A, Nakamura M, Minagi S, Osaka A, Suzuki K, & Van Meerbeek B (2010) Nano-controlled molecular interaction at adhesive interfaces for hard tissue reconstruction *Acta Biomaterialia* **6(9)** 3573-3582.
45. Hiraishi N, Tochio N, Kigawa T, Otsuki M, & Tagami J (2013) Monomer-collagen interactions studied by saturation transfer difference NMR *Journal of Dental Research* **92(3)** 284-288.
46. Inai N, Kanemura N, Tagami J, Watanabe LG, Marshall SJ, & Marshall GW (1998) Adhesion between collagen depleted dentin and dentin adhesives *American Journal of Dentistry* **11(3)** 123-127.
47. Breschi L, Perdigao J, Lopes MM, Gobbi P, & Mazzotti G (2003) Morphological study of resin-dentin bonding with TEM and in-lens FESEM *American Journal of Dentistry* **16(4)** 267-274.
48. De Munck J, Van den Steen PE, Mine A, Van Landuyt KL, Poitevin A, Opdenakker G, & Van Meerbeek B (2009) Inhibition of enzymatic degradation of adhesive-dentin interfaces *Journal of Dental Research* **88(12)** 1101-1106.
49. Pashley DH, Tay FR, & Imazato S (2011) How to increase the durability of resin-dentin bonds. *Compendium of Continuing Education in Dentistry* **32(7)** 60-64.