Title: Two modes of nanoleakage expression in single-step adhesives

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Citation: Journal of Dental Research, 2002, v. 81 n. 7, p. 472-476

Issued Date: 2002

URL: http://hdl.handle.net/10722/53249

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Two Modes of Nanoleakage Expression in Single-step Adhesives

ABSTRACT
Self-etch adhesives that etch, prime, and bond simultaneously should not exhibit incomplete resin infiltration within hybrid layers. We hypothesized that nanoleakage patterns in these systems are artifacts caused by mineral dissolution in mildly acidic silver nitrate. Resin-dentin interfaces bonded with four single-step, self-etch adhesives were examined for nanoleakage by conventional (pH 4.2) and basic ammoniacal (pH 9.5) silver nitrate and prepared for transmission electron microscopy. All adhesives exhibited a reticular mode of nanoleakage within hybrid layers when conventional silver nitrate was used. With ammoniacal silver nitrate, an additional spotted pattern of nanoleakage was observed within adhesive and hybrid layers. The reticular mode of nanoleakage in self-etch adhesives probably represents sites of incomplete water removal that leads to regional suboptimal polymerization. The spotted pattern identified with the use of ammoniacal silver nitrate probably represents potentially permeable regions in the adhesive and hybrid layers that result from the interaction of the basic amine silver ions with acidic/hydrophilic resin components.

KEY WORDS: self-etch, single-step adhesives, conventional silver nitrate, ammoniacal silver nitrate, permeability, water tree.

INTRODUCTION
Dentin adhesives are currently available as three-step, two-step, and single-step systems, depending on how the three cardinal steps of etching, priming, and bonding to tooth substrates are accomplished or simplified (Inoue et al., 2000). Two-step systems are subdivided into single-bottle self-priming adhesives that require a separate etching step, and the two-bottle self-etching primers that require an additional bonding step (Haller, 2000). The recently introduced single-step, self-etch adhesives further combine these three bonding procedures into a single-step application.

Self-etch adhesives are attractive in that prior removal of the smear layer and smear plugs is not required. This reduces the potential for post-operative sensitivity (Brunton et al., 1999) and bonding problems associated with movement of dentinal fluid through patent dentinal tubules (Ithagarun and Tay, 2000). The technique-sensitivity associated with bonding to a dehydrated collagen matrix is also eliminated (Perdigao et al., 1999), since water is an essential component in these systems (Tay and Pashley, 2001). With the use of ethanol as a co-solvent, the variability of bonding results associated with the use of acetone-based adhesives is also reduced (Finger and Balkenhol, 1999).

Since they do not require a separate acid-etching step, they are less likely to result in a discrepancy between the depth of demineralization and the depth of resin infiltration (Spencer et al., 2000), since both processes occur simultaneously (Watanabe et al., 1994).

Nanoleakage was originally used to describe microporous zones beneath or within hybrid layers that permitted tracer penetration to occur in the absence of interfacial gaps (Sano et al., 1995). It occurs through submicrometer-sized spaces within dentin hybrid layers where disparities existed between the depths of demineralization and monomer diffusion (Pioch et al., 2001). Studies of resin-dentin interfaces bonded by self-etching primers demonstrated the presence of a fine network of silver deposits within thin hybrid layers formed by these systems (Sano et al., 1995). There is a possibility, however, that remnant dentin apatites and amorphous calcium phosphates that are re-precipitated in the bonded interfaces of these non-rinsing adhesives may be dissolved after immersion in conventional, mildly acidic silver nitrate (Li et al., 2001). This may produce artifactual microporosities that give rise to false-positive results. We hypothesize that nanoleakage observed in the hybrid layers created by self-etch adhesives with the use of conventional acidic silver nitrate may be eliminated by the use of a basic version of ammoniacal silver nitrate (Kusanagi and Yanagibashi, 1975). Thus, the null hypothesis of this study was that there is no difference in expression of nanoleakage in self-etch adhesives when resin-dentin interfaces are challenged with a conventional or an ammoniacal version of silver nitrate.
MATERIALS & METHODS

Bonding was performed on the occlusal surfaces of deep coronal dentin from extracted, human third molars. The teeth were collected after the patients’ informed consent was obtained under a protocol reviewed and approved by the institutional review board of the Medical College of Georgia, USA. They were stored in a 1% chloramine T solution at 4°C and used within one month following extraction. The occlusal enamel and half of the dentin were removed by means of a slow-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling/lubrication. The tooth surfaces were polished with 180-grit silicon carbide papers to create bonding surfaces with thick, clinically relevant smear layers (Koibuchi et al., 2001).

Experimental Design

Four single-step, self-etch adhesives were used in this study, each consisting of 2 experimental groups of 3 teeth each. The compositions of these adhesives—Prompt L-Pop (3M-ESPE, St. Paul, MN, USA), Etch&Prime 3.0 (Dentsply Degussa, Hanau, Germany), One-Up Bond F (Tokuyama Corporation, Tokyo, Japan), and Reactmer Bond (Shofu Inc., Kyoto, Japan)—are shown in the Table. The teeth were bonded with non-rinsing adhesives according to the manufacturers’ instructions. The adhesives were light-cured (ca. 600 mW/cm²) prior to the incremental placement of a microfilled lining resin composite (Protect Liner F, Kuraray Medical Inc., Tokyo, Japan) to facilitate incremental placement of a microfilled lining resin composite (Protect Liner F, Kuraray Medical Inc., Tokyo, Japan) to facilitate sectioning occlusally into 0.9-mm-thick serial slabs. Slabs from each adhesive were randomly divided into 2 experimental groups for evaluation in the Table. The teeth were bonded with these non-rinsing adhesives according to the manufacturers’ instructions. The adhesives were light-cured (ca. 600 mW/cm²) prior to the incremental placement of a microfilled lining resin composite (Protect Liner F, Kuraray Medical Inc., Tokyo, Japan) to facilitate sectioning occlusally into 0.9-mm-thick serial slabs. Slabs from each adhesive were randomly divided into 2 experimental groups for evaluation.

Nanoleakage Evaluation

After storage in distilled water at 37°C for 24 hrs, the bonded teeth covered with the lining composite were sectioned occlusogingivally into 0.9-mm-thick serial slabs. Slabs from each adhesive were randomly divided into 2 experimental groups for evaluation of nanoleakage by transmission electron microscopy:

1. The conventional silver nitrate group. These tooth slabs were coated with 2 layers of fast-setting nail varnish applied up to within 1 mm of the bonded interfaces. Before the slabs could become dehydrated, they were immersed in a 50 wt% silver nitrate solution (pH = 4.2) for 24 hrs.

(2) Ammoniacal silver nitrate group. Ammoniacal silver nitrate was prepared by the dissolution of 25 g of silver nitrate crystals (Sigma Chemical Co., St. Louis, MO, USA) in 25 mL of distilled water. Concentrated (28%) ammonium hydroxide (Sigma) was used to titrate the black solution until it became clear as ammonium ions complexed the silver into diamine silver ions ([Ag(NH3)2]+). This solution was diluted to 50 mL with distilled water, yielding a 50 wt% solution (pH = 9.5). Tooth slabs were varnish-coated and immersed in the tracer solution for 24 hrs as previously described.

Transmission Electron Microscopy (TEM)

The silver-impregnated slabs were then rinsed thoroughly in distilled water and placed in photodeveloping solution for 8 hrs under a fluorescent light to reduce the silver or diamine silver ions into metallic silver grains. Undemineralized, epoxy resin-embedded, 90-mm-thick ultrathin sections were prepared according to the TEM protocol of Tay et al. (1999). The unstained sections were examined by means of a TEM (Philips EM208S, Philips, Eindhoven, The Netherlands) operated at 80 kV.

RESULTS

When examined by TEM, the self-etch adhesives exhibited widely differing degrees of acidity that were reflected by the depth to which they etched into the smear-layer-covered intact dentin. Prompt L-Pop (Figs. 1A, 2A) and Etch&Prime 3.0 (Figs. 1B, 2B) etched more deeply and formed completely demineralized zones in intact dentin that were 4 to 5 μm thick. Conversely, One-Up Bond F (Figs. 1C, 2C) and Reactmer Bond (Figs. 1D, 2D) etched less deeply, forming partially demineralized zones in intact dentin that were between 0.75 and 1 μm thick. Since the sections were unstained, collagen fibrils in the hybrid layer created by these adhesives appeared electron-lucent.

Regardless of the acidity of the self-etch adhesives and the thickness of the resultant hybrid layers, characteristic nanoleakage patterns were invariably observed from specimens that were immersed in both types of silver nitrate. When conventional silver nitrate was used (Fig. 1), a reticular mode

Table: Composition of the Single-step, Self-etch Adhesives Used in this Study

<table>
<thead>
<tr>
<th>Self-etch Adhesive</th>
<th>Manufacturer</th>
<th>Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prompt L-Pop</td>
<td>3M-ESPE, Seefeld, Germany</td>
<td>Unfilled</td>
<td>Watera, stabilizer, parabenes; methacrylated phosphoric acid esters; fluoride complex; photoinitiator (BAPO).</td>
</tr>
<tr>
<td>One-Up Bond F</td>
<td>Tokuyama Corp., Tokyo, Japan</td>
<td>Filled</td>
<td>Reactmer Bond A: Watera, acetone, F-PRG fillers, FASG fillers, initiators (TMBA, p-TSNa). Reactmer Bond B: 4-AET, 4-AETA, UDMA, photoinitiator.</td>
</tr>
<tr>
<td>Reactmer Bond</td>
<td>Shofu Inc., Kyoto, Japan</td>
<td>Filled</td>
<td>Reactmer Bond A: Watera, acetone, F-PRG fillers, FASG fillers, initiators (TMBA, p-TSNa). Reactmer Bond B: 4-AET, 4-AETA, UDMA, photoinitiator.</td>
</tr>
</tbody>
</table>

Abbreviations: 4-AET, 4-acryloxyethyltrimellitic acid; 4-AETA, 4-acryloxyethyltrimellitic anhydride; BAPO, bis-acryl phosphate oxide; F-PRG, full-reaction-type pre-reacted glass-ionomer filler; FASG, fluoroaluminoasilicate glass; HEMA, 2-hydroxyethyl methacrylate; MMA, methyl methacrylate; p-TSNa, p-toluenesulfonic acid sodium salt; TMBA, trimethyl borate catalyst; UDMA, urethane dimethacrylate.

a Water is a common component in self-etch adhesives for demineralization of dental hard tissues.
of nanoleakage expression could be identified along the resin-dentin interfaces. It consisted of a horizontal or oblique network of silver deposits around the unstained collagen fibrils, and a series of vertical streaks of dendritic silver deposits that extended almost perpendicularly from the surface of the hybrid layers into the overlying adhesive layers. For the Reactmer Bond adhesive, dense silver deposits had also accumulated within the siliceous hydrogel layer around the periphery of the fluoroaluminosilicate glass fillers (not shown).

The reticular mode of nanoleakage expression could also be identified in specimens that were challenged with ammoniacal silver nitrate (Fig. 2). An additional unique pattern of nanoleakage expression was observed, consisting of very small \((ca. ~5-10 \text{ nm})\) isolated silver grains dispersed randomly throughout both the adhesive and the hybrid layers.

**DISCUSSION**

The reticular mode of nanoleakage identified with the use of conventional silver nitrate was not eliminated when specimens were immersed in basic ammoniacal silver nitrate. In addition, a new, second, spotted pattern of nanoleakage was observed in all adhesives examined. In the case of ammoniacal silver nitrate, this silver uptake cannot be attributed to dissolution of remnant calcium phosphate phases within hybrid layers by a mildly acidic tracer solution. Thus, the results support the null hypothesis that there is no difference in expression of nanoleakage in self-etch adhesives when resin-dentin interfaces are challenged with conventional or ammoniacal silver nitrate. The results require rejection of the alternate hypothesis that nanoleakage is an artifact caused by mineral dissolution from the mildly acidic silver nitrate.

In the absence of the vertical streaks of silver deposits directly above the hybrid layer, it would have been difficult to explain why a reticular mode of nanoleakage occurred within hybrid layers formed by self-etch adhesives, since etching and infiltration of resin occur simultaneously in these systems. The reticular pattern of silver deposits within the hybrid layer was similar to that previously reported with the use of a self-etching primer (Sano et al., 1995). However, nanoleakage within the adhesive layers (i.e., outside the hybrid layer) has been observed only recently, by field emission scanning electron microscopy (Li et al., 2000). The occurrence of the reticular mode of nanoleakage, thus, cannot be attributed to incomplete resin infiltration. These microvoids more likely represent areas in which water was incompletely removed from the resin-dentin interfaces. All self-etch systems contain water to ionize the acidic monomers for effective
demineralization of dental hard tissues. Whereas water is comparatively easy to remove with the use of acetone-based adhesives, because its azeotrope contains more water and it has a higher vapor pressure, it is difficult to remove the last traces of water from ethanol-based adhesives, due to the increased capacity of ethanol to form hydrogen bonds with water [Hansen’s solubility parameter for hydrogen bonding $\delta_h$ of ethanol is 19.4 MPa$^{1/2}$ vs. 7.0 for acetone vs. 37.3 for water; Hansen and Skaarup, 1967]. The presence of residual water within the adhesive may lead to domains of incomplete polymerization of the adhesive or sequestrations of more hydrophilic oligomers in these particular regions.

The reticular mode of the nanoleakage pattern—in particular, the silver deposits that were oriented perpendicular to the surface of the hybrid layer—is the morphological manifestation of water-treeing (Miyashita, 1969; Raharimalala et al., 1994), a well-known phenomenon in the dielectric insulation cable industry that is responsible for water-induced deterioration of polymer insulation of electrical cables after aging. Water trees in polyethylene-coated cables are submicroscopic, self-propagating, water-filled tracks that are formed electrochemically by the oxidation of the hydrophobic polymer into more hydrophilic moieties, resulting in the condensation of moisture from the hydrophobic polymer into the hydrophilic, electro-oxidized regions (Moreau et al., 1993). The increase in water conductivity results in self-propagation along these tracks and the growth of a microscopic tree-like pattern of water channels. In the context of dentin bonding, an electrochemical process is not required for water tree formation, since both hydrophilic resin monomers and water are present in single-step, self-etch adhesives. The typical morphology of a water tree is illustrated in Fig. 1D. We speculate that it represents a region within which bulk water (Mohsen et al., 2001) is retained within the adhesive-dentin interface. The horizontal/oblique reticular silver patterns within the hybrid layers may also represent the retention of bulk water. However, their morphological manifestation is pre-determined by the orientation of the interfibrillar spaces within the demineralized collagen fibril matrices. We speculate that the heat of polymerization generated during light-activation of these adhesives results in the upward convective movement of bulk water from the dentin, resulting in the more vertically oriented water trees that were identified within the base of the adhesive layers. In regions over dentinal tubules, there may be osmotically induced outward movement of water during etching and convective water movement if movement of monomers into the tubules displaces additional water.

Whereas the reticular mode of nanoleakage expression was observed after immersion in both conventional (i.e., acidic) and basic ammoniacal silver nitrate, the spotted pattern of nanoleakage expression could be identified only in the latter. We hypothesize that the silver is taken up into microdomains in the resin matrices of these adhesives that contain mainly hydrophilic and/or acidic functional groups compared with the adjacent, more hydrophobic, domains. Even if a water-containing adhesive is air-dried to remove all visible signs of water moisture, water molecules that exist in the form of thermodynamically stabilized water clusters (Tanaka, 2000) may form hydrogen bonds with hydrophilic functional groups of resin monomers. This is comparable with the presence of bound water in resin composites after water sorption (Mohsen et al., 2001) and in soft tissues such as collagen fibrils, that cannot be removed with simple air-drying. Diamine silver ions may compete with hydronium ions in water for hydrogen bonding with the carboxyl and phosphate functional groups in the polymerized adhesive resin matrices. Alternatively, these basic ions may react with the acidic functional groups via acid-base reactions. Regardless of the mechanism of formation, the spaces identified by ammoniacal silver nitrate are not nanoleakage in its original definition (Sano et al., 1995), but more likely represent microphase separations of copolymers containing hydrophobic and hydrophilic resin domains into the more hydrophobic “hard-chain segments” and the more hydrophilic “soft-chain segments” (Kanapitsas et al., 1999). The occurrence and characteristics of these microdomains in adhesive polymer blends must be further characterized by dielectric relaxation analysis (Georgoussis et al., 1999). A recent study of a polymer blend of polymethyl acrylate and polyethyl acrylate showed that a higher diffusion coefficient of water was obtained for this interpenetrating polymer network compared with the pure polymethyl acrylate polymer (Gomez Ribelles et al., 1999), indicating that resin matrices that contain hydrophilic monomers have non-uniform chain mobility and are more permeable to water movement. This probably explains why all the single-step, self-etch systems examined in this study are permeable to fluid movement, even when resin-dentin interfaces are optimally sealed (Tay et al., 2001).

Water sorption by hydrophilic resin monomers within both the hybrid layer and the adhesive layer has been thought to contribute to the degradation of resin-dentin bond strengths over time (Hashimoto et al., 2000). This phenomenon is aggravated by the incorporation of increased concentrations of hydrophilic resin components into contemporary self-etch adhesives (Tanaka et al., 1999), since hydrophilicity and hydrolytic stability of resin monomers are generally antagonistic properties. Water-rich domains, as represented by the reticular mode of nanoleakage expression and manifested as self-propagating water trees along the adhesive-dentin junction, may result in a rapid deterioration of the mechanical properties of the adhesive along this region, resulting in adhesive failure along the surface of the hybrid layer. A recent in vivo study showed that deterioration of resin-dentin bonds occurs predominantly via the leaching of resins instead of by degradation of collagen fibrils within the hybrid layer (Sano et al., 1999). The spotted pattern of nanoleakage expression exhibited by specimens immersed in ammoniacal silver nitrate probably represents regional hydrophilic phases within these adhesive matrices that are more prone to water sorption. In preliminary aging studies of dentin adhesives, the spotted type of nanoleakage that was observed before aging increased tremendously in magnitude as early as one month after accelerated aging in artificial saliva (Tay and Pashley, unpublished results). Although contemporary single-step, self-etch adhesives are user-friendly because of their reduction in the number of bonding steps, the benefit of saving time may be achieved at the expense of compromising the durability of resin-dentin bonds. This is a critical issue in dentin bonding and has to be further substantiated by comparing the permeability and nanoleakage in a series of polymer blends with increasing hydrophilicity.
ACKNOWLEDGMENTS

We thank Amy Wong of the Electron Microscopy Unit, The University of Hong Kong, for technical support, and Michelle Barnes of the Medical College of Georgia for secretarial support. This study was supported by grant DE 06427 from the National Institute of Dental and Craniofacial Research, USA. The materials used in this study were generously sponsored by 3M-ESPE, Dentsply-DeGussa, Tokuyama Corp., Shofu Inc., and Kuraray Medical Inc. No other financial support or consultation fees were received by any of the authors for this project.

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