Dentin bonding: effect of tubule orientation on hybrid-layer formation

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Abstract
In an attempt to compare the morphology of the resin-dentin interface in areas where the dentinal tubules run perpendicularly or at an angle to the cavity surface with that of areas where they run parallel to it, we studied a dentin adhesive system using transmission electron microscopy and fluorescence confocal laser scanning microscopy. The design of the study included the simulation of the normal hydrostatic pressure within the pulp and the dentinal tubules. Following acid etching of the dentinal surface with maleic acid/HEMA, the smear layer was removed, and a superficial zone was demineralized in such a way that the exposed collagenous dentin matrix retained its integrity. Confocal laser scanning microscopical investigations using primer labeled with rhodamine B showed that the penetration of the primer occurred not only vertically via surface porosities, but mainly laterally, via the dentinal tubules. The adhesive resin labeled with fluorescein completely infiltrated the demineralized layer, thereby forming a hybrid layer. The orientation of the dentinal tubules had a profound effect on the formation of the hybrid [...]
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Adhesive systems for dentin bonding have been developed to adhere to dentin either via a smear-layer, or by modifying or totally removing it (1, 2). Recent studies strongly suggest that the nature of a bond is micromechanical for adhesive systems that remove the smear-layer by acid etching and demineralize superficial dentin (3, 4). Bonding in this case may result in the enmeshment of the exposed collagen fibrils by hydrophilic adhesive resin monomers in superficial dentin, thereby forming a resin-dentin hybrid layer located between solid dentin and the covering restorative material (5, 6).

The formation of the hybrid layer depends on tooth, patient and material factors (1), but may also depend on microstructural features of dentin such as dentinal sclerosis (7-10). Harnirattisai et al. (7) and Van Meerbeek et al. (10), using SEM of ion etched, polished cross-sections of the resin-dentin interface to enhance structural differences, found the hybrid layer to be wider in the area of empty tubules compared with that of sclerotic, obliterated tubules, and described the hybrid layer as thinnest at the lateral walls of the cavity where the tubules run parallel to the cavity surface. However, although the argon-beam etching technique can give information on the depth of the demineralization, this method is not suitable for determination of resin infiltration and subsequent hybrid-layer formation at the dentin surface (11). Two alternative techniques for the analysis of the dentin adhesive interfaces are fluorescence confocal microscopy of fluorochrome-labeled adhesive resins (9) or scanning transmission electron microscopy in combination with energy-dispersive spectroscopy (4). In addition, the studies of Harnirattisai et al. (7) and Van Meerbeek et al. (10) were carried out using extracted teeth and did not take into account
the presence of dentinal tubule fluid under physiological pulpal pressure, which may adversely affect dentin bonding (11). Thus, the design of the present study includes the simulation of the normal hydrostatic pressure within the pulp and the dentinal tubules according to the method of Krecji et al. (12).

Morphological analyses of the hybrid layer have been performed using scanning electron microscope (3, 6, 13–19), transmission electron microscopy (3, 6, 16, 20–22), scanning transmission electron microscopy/energy-dispersive spectroscopy (4), and confocal microscopy (9, 23). Although these studies have contributed to clarify single steps of dentin bonding, it seems clear that the complex nature of the dentin adhesive bond cannot be elucidated with one single method. In the present study, confocal scanning light microscopy (CLSM) was combined with transmission electron microscopy (TEM). The main advantage of CLSM is that it offers the possibility of subsurface imaging under near normal conditions, i.e., without prior dehybridation of the specimens. Fluorescent dyes incorporated in the components of adhesive systems can be used to trace their distribution within interfaces. Primer-labeled with rhodamine B was used to demonstrate its distribution in the dentin surface. Furthermore, CLSM was used to trace the distribution of fluorescein-labeled adhesive resin in the resin-dentin hybrid layer and to demonstrate the formation of resin tags in both the dentinal tubules and their side-branches. The CLSM results were compared with those from TEM of undecalcified ultrathin sections obtained from the same restoration.

The hypothesis tested in this study was that the thickness of the hybrid layer depends upon the orientation of the dentinal tubules.

Material and methods

In 10 teeth, class-V cavities (2 mm × 1 mm) with 90° cavosurface margins were prepared at the cemento-enamel junction of extracted caries-free human premolars using cylindrical diamond burs (Amalgam Prep Set; Intensiv, Lugano, Switzerland) under continuous water cooling. In another 10 teeth, v-shaped cavities were prepared in sclerotic dentin of extracted teeth showing v-shaped defects.

For the simulation of the normal hydrostatic pressure of about 25 mm Hg within the pulp and the dentinal tubules within the tooth (24), the teeth were prepared according to the method of Krecji et al. (12). Briefly, a metal tube was glued in a cylindrical cavity extending from the pulpal chamber to the tooth surface. The pulpal chamber was evacuated with a vacuum pump, filled with horse serum and finally connected with a silicone tube to a serum infusion bottle. The bottle was placed vertically 34 cm above the sample.

A.R.T. Composite Bonding System (Coltène, Altstätten, Switzerland) was selected as an adhesive system. With two exceptions, the adhesive system was applied according to the manufacturer’s instruction. Part A of the self-conditioning primer (1.6% maleic acid; 0.1% sodium fluoride, 98.3% water) was labeled with 0.1% rhodamine B. Dentin conditioning occurred with a one-to-one mixture of part A with part B [47% hydroxyethylmethacrylate; 36% hydroxypropylmethacrylate; 6.2% polymethacryloylgomalic acid, 9.8% water] for 30 s and air-dried for 10 s. A.R.T. Bond [44% Isopropylidenbis, 49% 3,6 dioxacontamethylendimethacrylate; 7% polymethacryloylgomalic acid] was labeled with 0.37% sodium fluorescein. The adhesive was applied for 20 s and light cured for 60 s before composite (Brilliant EL; Coltène) insertion and curing. Two teeth of each type of prepared cavity (class-V and v-shaped, respectively) were collected following application of the primer. The remaining teeth were treated with primer, bond and composite.

The teeth were cut longitudinally through the center line of the restoration using a bandsaw equipped with a diamond-coated band (Exakt-Cutting-Grinding System; Exakt Apparatebau, Norderstedt, Germany). One half of a restoration was prepared for TEM and the other half for CLSM.

Transmission electron microscopy (TEM)

Immediately following treatment, the specimens selected to demonstrate the effect of the primer on dentin were immersed in half-strength Karnovský’s fixative (25), buffered (pH 7.4) with 0.02 M sodium cacodylate for 48 h at 4°C. The specimens were then washed in 0.185 M sodium cacodylate buffer (pH 7.4; 350 mOsM/kg). The blocks were post-fixed with 1.33% osmium tetroxide, buffered in 0.067 M S-collidine for 2 h at 4°C and embedded non-decalcified in Epon 812. Following polymerization, pyramids were trimmed with microtomes. Non-decalcified ultrathin sections were obtained with an ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria). They were either stained with neutral uranyl acetate or left unstained. The sections were examined with a Phillips 200 TEM (Phillips, Eindhoven, The Netherlands). Specimens selected to demonstrate the resin-dentin hybrid layer were fixed as described above but were not embedded in resin. Sections from the resin-dentin interface were obtained as described above.
Confocal laser scanning microscopy (CLSM)

The specimens were mounted on glass slides and kept at 100% humidity until and during examination with a confocal scanning microscope. Fluorescence imaging was performed with a Bio-Rad MRC 600 confocal scanning microscope equipped with a crypton-argon laser. Either water ($\times 25$) or oil immersion objectives ($\times 40$, $\times 63$) were used. Image processing was performed with Comos software and storage of the images was in digital form. A Sony UP 500 color video printer was used for the preparation of black-and-white and color prints. Images were stored in digital form at 15 focus levels, with 0.5 µm intervals between each level. The first level was located at the surface which was identified by scratches created by the bandsaw. At each level the thickness of the labeled hybrid dentin-bonding agent layer was measured using Comos software. Finally, computer-assisted 3-dimensional reconstructions of the serial optical sections were carried out.

Fig. 1. TEM micrographs of non-decalcified sections through the dentinal surface following conditioning with A.R.T Primer. (a) Note the partially demineralized surface layer with exposed collagen fibrils (between arrows) and the gradually increased degree of mineralization (star) towards the unaltered dentin (D). (b) Note the exposed collagen fibrils which have retained their cross-banding (arrows). Also note areas of remaining mineralization (star); (c) Remaining hydroxyapatite crystals (arrows) scattered between collagen fibrils. (d) Non-decalcified section which has remained unstained by heavy metals. Note the distribution of residual hydroxyapatite crystals in the demineralized surface layer. Also note the gradual increase of crystals towards the unaltered dentin. The white areas represent unstained collagen fibrils. Bars: (a) 4 µm, (b) 1 µm, (c) 0.5 µm, (d) 1.5 µm.
Results

Non-decalcified ultrathin sections through surfaces treated with the self-conditioning primer only disclosed that the smear layer was completely removed (Fig. 1a). Furthermore, these sections revealed a demineralized superficial dentin layer (Fig. 1a). The thickness of this layer varied between 2 and 4 μm in areas where the dentinal tubules ran perpendicular or at an angle to the surface, between 1–3 μm in areas where they ran parallel to the surface, and approximately 0.5 μm in sclerotic dentin. These layers were characterized by a loose meshwork of demineralized collagenous fibrils and remaining hydroxyapatite crystals scattered between (Fig. 1b, c). At the surface of the layer, there was no evidence of residual pieces of demineralized dentin matrix from the original smear layer. The collagenous matrix was not collapsed, and the single collagen fibrils seemed not to be denatured as they retained their typical cross-banding (Fig. 1c). Remaining hydroxyapatite crystals became even more pronounced in non-stained ultrathin sections (Fig. 1d).

In the absence of a stain with heavy metals, the collagenous fibrils appeared as white dots and fibers, and the distribution of fine, electron-dense crystals was visible (Fig. 1d). Such sections also showed a gradual increase of mineralization towards unaltered dentin. The transition between partially-demineralized superficial dentin and unaltered fully mineralized dentin was smooth rather than sharply demarcated (Fig. 1d).

Fluorescence CLSM allowed the recording of rhodamine labeled primer at different depths of the partially demineralized superficial dentin layer. Optical sections recorded at a plane parallel to the etched dentin surface were carried out at a depth of 2, 4, 6 and 8 μm below the dentin surface. At a depth of 2 μm, all intertubular dentin was labeled. Only the lumen of the dentinal tubes remained unlabeled (Fig. 2a). At a depth of 4 μm, labeling occurred particularly around the dentinal tubules only, while the remaining intertubular dentin was unlabeled (Fig. 2b). This was even more pronounced at a depth of 6 μm, where most of the intertubular dentin remained unlabeled (Fig. 2c).

The ultrastructure of non-decalcified sections through the resin-dentin hybrid layer is presented in Fig. 3. The hybrid layer was clearly evident and distinguishable from unaltered dentin by its loss of mineral (Fig. 3a). The structural features of the intertubular dentin of the hybrid layer differed from the observations described following application of the self-conditioning primer, as the loose meshwork of collagenous fibrils was masked by the resin (Fig. 3b, c). Higher magnifications of the hybrid layer disclosed that the typical cross-banding of the collagenous fibrils was still present but, in particular
near the surface, was less distinct than before the application of the adhesive resin (Fig. 3b).

Low-power examinations with confocal scanning laser microscopy of sections through the dentin surface following application of fluorescein-labeled adhesive resin made visible its penetration into the dentin (Fig. 4). A labeled, bright band was observed along the cavity walls. In areas where the dentinal tubules ran parallel to the cavity, the hybrid layer was thin. In contrast, in areas where the dentinal tubules ran at an angle or perpendicular to the dentin surface, additional labeling of the dentinal tubules was observed (Fig. 4).

Higher magnifications of the hybrid layer revealed that in areas where the dentinal tubules were oriented at an angle or perpendicular to the dentin surface, the hybrid layer was 3.2 ± 0.8 μm thick (Table 1) and strongly labeled (Fig. 5a). The layer was characterized by labeled adhesive which had penetrated into intertubular dentin (Fig. 5a) and labeled by resin tags in the dentinal tubules to a depth of 27 ± 9 μm \( (n = 8) \) (Fig. 5d, e). The assignment of false colors to the various degrees of labeling intensity showed that the highest intensity was reached in the intertubular dentin (red color and white dots in Fig. 5a), while the walls of the dentinal tubules (yellow color) and the lumen of the tubules (green color) were labeled less strongly. Fluorescence CLSM and computer-assisted 3-dimensional reconstructions of serial optical sections showed the presence of labeled adhesive resin also in lateral side branches of the dentinal tubules (Fig. 5d).

In areas where the dentinal tubules ran parallel to the cavity surface, this pattern was characterized by a significantly \( (P < 0.01) \) thinner \( (1.3 ± 0.6 \mu m; n = 8) \) labeled hybrid layer (Table 1). Labeling of adhesive resin was restricted to the intertubular dentin. Resin tags in the dentinal tubules were absent (Fig. 5b). The intensity of labeling in intertubular dentin was comparable to that observed in areas showing perpendicular tubule orientation.

In areas where the cavity was prepared in sclerotic dentin (Fig. 5c), the hybrid layer remained thin \( (0.5 ± 0.2 \mu m; n = 8) \) and was only weakly labeled. Labeled resin tags were absent. TEM examinations revealed that the surface dentin was still

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Fig. 5. Transmission electron micrographs of a non-decalcified ultrathin section through the interface between adhesive (A) and dentin (D). Note the adhesive layer (A) and the demineralized dentin of the hybrid layer (HL). Also note the widened lumen of the dentinal tubules (arrows in a). Crossbanding of collagen fibrils was absent in superficial areas of the hybrid layer (b), while it was evident in deeper layers (arrows in c). A thin, electron-dense layer was evident at the transition between hybrid layer and adhesive (arrow in b). Bars: (a) 4 μm, (b) 0.5 μm, (c) 0.5 μm.
Resin-dentin hybrid layer

**Discussion**

The formation of a hybrid layer requires that the dentin surface is suitably prepared by acidic primers/conditioners to facilitate the diffusion and permeation of resin monomers (6, 16, 20, 21, 26, 27). Ideally, such an acidic agent should (i) remove both the smear layer and smear plugs, and (ii) demineralize superficial dentin in such a way that the exposed collagenous dentin matrix retains its integrity. The present study shows that following acid etching of the dentin surface with maleic acid/HEMA, these requirements were fulfilled. The smear-layer was completely removed (Fig. 1a), and there was no evidence of the so-called “collagen smear layer” (17). The formation of such a layer consisting of residual pieces of demineralized dentin matrix from the original smear layer would mean that the overlying resin layer is not well united with the underlying resin-infiltrated layer (27). The collagen framework created at the dentin surface was stable, and a porous substrate for resin infiltration was formed (Fig. 1b). Stabilization of the exposed dentin matrix was achieved by remaining crystals, most probably hydroxyapatite, scattered between the collagenous fibrils (Fig. 1c, d). Similar phenomena were described by Van Meerbeek et al. (3).

The present study shows that following treatment with maleic acid/HEMA, the typical cross-banding of collagen fibrils was retained and the fibrils thus most probably not denatured (Fig. 1c). This may have a significant influence on adhesive strength, as a stable layer would be tougher and more rigid than a collapsed and impermeable gel-like dentin matrix (17, 28). The investigation of non-decalcified and non-stained ultrathin sections demonstrated an increasing degree of mineralization towards the unaltered dentin (Fig. 1d). This is desirable, as a rather abrupt demarcation between the demineralized surface layer and the underlying unaltered dentin may result in a weak link between dentin and the restoration.

Our CLSM investigations using primer labeled

![Image](https://via.placeholder.com/150)

**Fig. 4.** Low-power CLSM showing the distribution of fluorescein along the cavity margin. Note the small hybrid layer in areas where tubules run parallel to the cavity surface (arrowheads) and the broad layer, due to labeled resin tags in the tubules, in areas where tubules run towards the surface (large arrows). Pooling of adhesive was evident in the corners of the cavity (small arrows). Bar: 20 μm.

sclerotic following preparation, i.e., the dentinal tubules were obliterated by hydroxyapatite crystals.

Three-dimensional reconstructions of serial optical sections through the hybrid layer revealed clearly the presence of resin tags in the dentinal tubules and their side-branches (Fig. 5d). These reconstructions allowed us to exclude the possibility that the observed labeling was created by light reflections along the restoration margins (Fig. 5e).

<table>
<thead>
<tr>
<th>Type of dentin</th>
<th>Hybrid layer (μm)</th>
<th>Resin tags (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal dentin/box-shaped cavities – tubule orientation: perpendicular</td>
<td>3.2 ± 0.8</td>
<td>27.2 ± 9.2</td>
</tr>
<tr>
<td>normal dentin/box-shaped cavities – tubule orientation: parallel</td>
<td>1.3 ± 0.6*</td>
<td>–</td>
</tr>
<tr>
<td>sclerosed dentin/Y-shaped cavities – tubule orientation: parallel</td>
<td>0.5 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>sclerosed dentin/Y-shaped cavities – tubule orientation: perpendicular</td>
<td>0.4 ± 0.2</td>
<td>–</td>
</tr>
</tbody>
</table>

* Significantly different in the Student t-test at *P*<0.01 from box-shaped cavities with a tubule orientation perpendicular to the cavity margins.
Fig. 5. CLSM images of the hybrid layer in areas where tubules run perpendicularly or in an angle towards the surface (a), in areas where tubules run parallel to the surface (b) and in areas of sclerotic dentin (c). (a) Note red-labeled intertubular dentin and green-labeled lumen of dentinal tubules (large arrows) in the hybrid layer. Also note labeled projections along the dentinal tubules (arrowheads) and less strongly labeled dentinal tubules (small arrows). (b) Note the unlabeled lumen of dentinal tubules (arrows) running parallel to the dentin surface. (d, e) CLSM view of a projection of 15 optical sections with 2 μm intervals between each section demonstrating the dentinal tubules, their side-branches (arrows in d) and the hybrid layer (e). Note the yellow orifices of dentinal tubules in the hybrid layer surface (arrows) and labeled dentinal tubules (arrowheads). Bars: (a) 10 μm, (b) 10 μm, (c) 10 μm, (d) 12 μm, (e) 10 μm.
Resin-dentin hybrid layer

with rhodamine B showed that the penetration of the primer occurred laterally, via the dentinal tubules, into intertubular dentin (Fig 2a–c). This was indicated by the presence of labeled primer forming rings around the dentinal tubules as previously described by Griffiths & Watson (23). Obviously, this lateral diffusion can only occur in areas where open dentinal tubules are available for a fast penetration of the acids and following dissolution of the peritubular dentin lining the tubule walls. This may explain the thinner demineralized layer in areas where the tubules run parallel to the surface and in sclerotic dentin, where the dentinal tubules are obliterated and the intertubular dentin hypermineralized.

The orientation of the dentinal tubules had a profound effect on the depth of demineralization. Once the depth of demineralization was established, the adhesive penetrated to the depth of the demineralization. This was most evident in overview images showing a broad hybrid layer and resin tags in the dentinal tubules in areas where the latter communicated with the cavity surface, and a thin hybrid layer without impregnation of the tubules in areas where they ran parallel to the cavity surface (Fig. 4). However, we would like to make it clear that the distribution of fluorescein may not perfectly represent the distribution of the adhesive monomers, because we used a simple mixture rather than a covalent linkage between the fluorescein molecule and one of the adhesive monomers.

In the present study, the resin tags reached a length of 27 ± 9 µm despite the simulation of a normal hydrostatic pressure within the pulp and the outward serum flow within the dentinal tubules. This is not surprising since Shimada et al. (11), in an in vivo experiment under clinical conditions and without application of local anesthesia, recently found resin tags to a depth of 20 µm. Other in vivo experiments (14, 19) described long tag formation in the dentinal tubules, but in these studies local anesthesia was used, which may suppress intrapulpal blood circulation (29). However, long resin tags may only contribute to the bond strength if the resin actually bonds to the wall of the dentinal tubules. Recently, dentinal tubules anastomosis has been suggested as a potential factor in adhesive bonding (30). Those authors described lateral side-branches of dentinal tubules, filled with polymerized adhesive resin.

Bonding strength of a dentin adhesive does not depend solely on the thickness of the hybrid layer. Indeed, if the demineralized layer is too deep, the short length of time permitted for resin diffusion before light curing may not be sufficient to allow complete infiltration of the hybrid layer. Recently, lateral subsurface diffusion via dentinal tubules of resin in demineralized dentin was suggested (27, 31). Such lateral diffusion is only possible in areas where dentinal tubules communicate with the cavity surface.

In conclusion, this study demonstrates structurally completely different hybrid layers which depend on tubule orientation and/or the presence of sclerotic dentin. It is reasonable to predict that the above discussed differences as (i) a thicker hybrid layer, (ii) the presence of solid resin tags in the hybrid layer, (iii) the presence of resin tags in the network of the dentinal tubules and their side-branches in deeper dentin may contribute to a higher dentinal/resin bond in areas with perpendicular tubule orientation.

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References


