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Clinical performance of resin composite restorations: the value of accelerated in-vitro testing

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Publication date
2012

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Citation for published version (APA):

Garcia-Godoy, F. (2012). *Clinical performance of resin composite restorations: the value of accelerated in-vitro testing*. [Thesis, externally prepared, Universiteit van Amsterdam].

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CHAPTER 2

Degradation of resin-bonded human dentin after 3 years of storage

Introduction

Considerable evidence in adhesive dentistry has accumulated over the past decade, based on *in vitro* and *in vivo* work. Dentin bonds created by resin-based adhesives may not be as durable as previously conjectured.¹ Although the current strategies of incorporating ionic and hydrophilic resinous components in etch-and-rinse and self-etch adhesives arise from the need to bond to an intrinsically wet substrate,² they create potentially unstable resin matrices that slowly degrade *via* water sorption.³ This is particularly so when resin-dentin bonds are not protected by enamel, and when the durability of these bonds was challenged by reducing adhesive interfaces into smaller portions, to expedite aging effects and increasing their interactions with water.⁴⁻⁶ Another mechanism of bond degradation is the potential instability of the demineralized dentin collagen matrix, that was manifested as the thinning or disappearance of collagen fibrils from aged, bonded dentin,^{7,8} or the failure of aged hybrid layers to take up heavy metal stains.⁴ This issue of collagen instability has caused concern, with the demonstration of the potential involvement of host-derived matrix metalloproteinases (MMPs), a class of independent endopeptidases, in the breakdown of the collagen matrices in dentin caries⁹⁻¹¹ and the periodontium.¹²⁻¹⁴ In the context of dentin bonding, residual collagenolytic activity was observed in mineralized dentin of extracted teeth that accounted for the disintegration of collagen fibrils from unbonded, aged acid-etched dentin, in the absence of the contribution from bacterial or salivary MMPs. This low but persistent endogenous collagenolytic activity was strongly inhibited by the use of protease inhibitors, the incorporation of which preserved the structural integrity of the collagen fibrils.¹ In that study, the demineralized collagen matrices were not protected by adhesives. Thus, it remains to be resolved whether these endogenous enzymatic activities can result in the proteolysis of the resin-infiltrated collagen network in aged, adhesive-bonded dentin.

Using a model that consisted of soluble fluorescein-labeled Type I collagen and gelatin in a previous study, both collagenolytic and gelatinolytic activities were identified in powdered mineralized human dentin. Retention of these activities was evident even upon autoclaving of the mineralized dentin powder in water. Conversely, these activities were sufficiently suppressed after treatment of the powder with phosphoric acid. The results derived from this model suggested that if resin-bonded dentin is permeable to endogenous enzymes released from the underlying mineralized dentin, complete cleavage of the collagen fibrils within the hybrid layer is possible,

first into $\frac{3}{4}$ - and $\frac{1}{4}$ -length fragments,¹⁵ and subsequently into smaller peptides.¹⁶ Thus, the objective of the present study was to investigate the morphologic correlations of these endogenous enzymatic activities in aged, adhesive-bonded dentin. As water molecules are putatively required for the activity of zinc-dependent MMPs,^{17,18} the null hypothesis tested was that there was no difference in the ultrastructure of resin-dentin bonds that were aged in mineral oil or artificial saliva.

Materials and Methods

Twenty-one non-carious human third molars were collected after the subject's informed consent had been obtained under a protocol reviewed and approved by the Human Assurance Committee of the Medical College of Georgia, USA. Within 1 month of extraction, the occlusal enamel and roots of these teeth were removed using a slow-speed saw (Isomet^a) under water-cooling. The exposed dentin surfaces were polished with wet 180-grit silicon carbide papers.

Experimental design - Three total-etch adhesives were examined. They included two two-step systems (Prime&Bond NT^b and Excite^c), and a multi-step system (All-Bond 2^d). Six teeth were used for each adhesive. Each tooth was etched with 32-37% phosphoric acid gel for 15 seconds, and rinsed with water for 20 seconds. The teeth were then bonded with the respective adhesives according to the manufacturers' instructions, and restored with a microfilled resin composite (EPIC-TMPT^e). After allowing the bonds to mature for 24 hours, each tooth was sectioned longitudinally into 0.9 mm-thick serial slabs. Two slabs from the center part of each tooth were further sectioned into 0.9 x 0.9 mm beams. The composite-dentin beams from each adhesive group were randomly divided into two equal portions and stored respectively in 5 ml aliquots of artificial saliva *or* mineral oil at 55°C for 3 years, according to the accelerated aging protocol described by Tay *et al.*¹⁹ The artificial saliva contained (mmoles/L): CaCl₂ (0.7), MgCl₂ • 6H₂O (0.2), KH₂PO₄ (4.0), KCl (30), NaN₃ (0.3) and HEPES buffer (20). The rationale for using artificial saliva was to prevent demineralization of the mineralized dentin during aging. The artificial saliva was replaced every month during the 3-year period. Sodium azide was added to prevent bacterial growth, and to ensure that the only available source of MMPs was derived from the dentin substrate.¹ The resin-bonded dentin beams that were to be aged in mineral oil (Dow-Coming 200 Fluid^f), were wiped with lint-free gauze and briefly air-dried to remove excess water prior to immersion in oil. They served as controls in that collagenolytic and gelatinolytic activity cannot occur in a non-aqueous medium.

To further ensure that degenerative changes observed after accelerated aging were not caused by the increased aging temperature (55°C), an "extreme" control was performed for each adhesive, using an additional tooth that was bonded in the same manner. Composite-dentin beams prepared from these teeth were subjected to an autoclave cycle at 121°C and 103 kPa for 30 minutes in water before further laboratory processing.

Transmission electron microscopy (TEM) - For each adhesive, 10 specimens were randomly retrieved from those aged in mineral oil, and another 10 from the artificial saliva at time zero and after 3 years of incubation. Half of the specimens were immersed in a 50 wt% ammoniacal silver nitrate solution for 24 hours, according to the tracer protocol for nanoleakage examination.² These specimens were processed for TEM examination without further laboratory demineralization. The remaining specimens were completely demineralized in ethylene diamine tetra-acetic acid. Both undemineralized and demineralized, epoxy resin-embedded, 90 µm-thick sections were prepared according a TEM protocol.²⁰ Undemineralized sections were examined without further staining. Demineralized sections were stained with 2% uranyl acetate and Reynold's lead citrate for examining the characteristics of the resin-dentin interfaces, and with a specific collagen staining technique (1% phosphotungstic acid and 2% uranyl acetate) for examination of the status of the collagen fibrils. The sections were examined using a TEM (Philips EM208S^g) operating at 80 kV.

Results

The "extreme" control specimens that were autoclaved at 121°C showed that collagen fibrils were not denatured at this temperature when they were protected by adhesive resin or apatite minerals (not shown). Their ultrastructural features were similar to those observed in control specimens that were aged in mineral oil at 55°C for 3 years. For example, in undemineralized sections of Prime&Bond NT that were aged in mineral oil, a 5-7 µm thick zone of demineralized dentin with sparse silver deposits was observed (Fig. 2.1A), that corresponded with the electron-dense hybrid layer in stained, demineralized sections (Fig. 2.1B). Collagen fibrils with normal dimensions and organization could be identified both within the hybrid layer (Fig. 2.1C) and the underlying dentin (Fig. 2.1D).

By contrast, extensive nanoleakage was observed in Prime&Bond NT specimens that were aged in artificial saliva (Fig. 2.2A). The corresponding hybrid layer was

abnormal and only discontinuous patches of stained fibrillar remnants were observed (Fig. 2.2B). They consisted of grossly disintegrated, short microfibrillar fragments (Fig. 2.2C). The collagen matrix from the underlying mineralized dentin was also denatured, but to a lesser extent, and appeared as swollen, partially unraveled fibrils that lacked cross banding (Fig. 2.2D).

Whereas the Excite specimens that were aged in mineral oil exhibited minimal nanoleakage (Fig. 2.3A) and a highly electrondense hybrid layer (Fig. 2.3B), those that were aged in artificial saliva demonstrated substantial degenerative changes. Extensive patches of nanoleakage could be seen in the zone of demineralized dentin that extended into the underlying mineralized dentin (Fig. 2.3C). In such regions, stained fibrillar components were absent from the hybrid layer. Collagen fibrils from the underlying dentin were sparsely distributed among abnormally wide interfibrillar spaces (Fig. 2.3D).

Stained fibrillar components were evident throughout the entire hybrid layer in All-Bond 2 specimens that were aged in mineral oil (Fig. 2.4A). These banded collagen fibrils exhibited the characteristic unraveling of their severed ends along the dentin surface (Fig. 2.4B). In specimens that were aged in artificial saliva, crystalline deposits (Fig. 2.4C) derived from the supersaturated artificial saliva (Pashley *et al.*¹) were seen along the adhesive-hybrid layer interface. Although nanoleakage was only identified in discrete parts of the demineralized collagen matrix (Fig. 2.4C), the entire hybrid layer was completely devoid of stained fibrillar components (Fig. 2.4D).

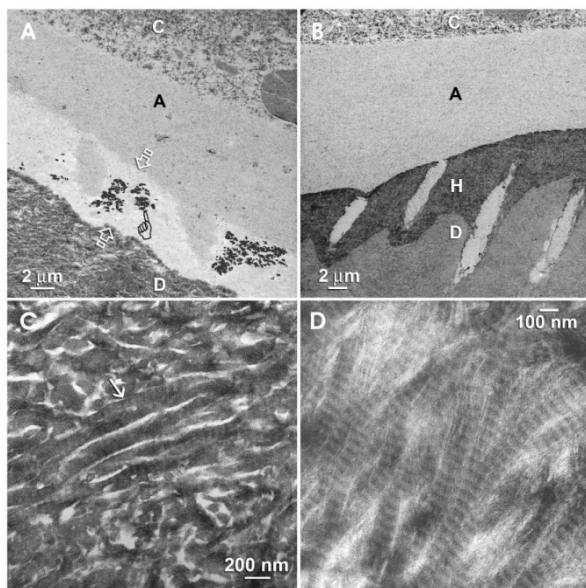


Figure 2.1: Control resin-bonded dentin beams of Prime&Bond NT that were aged in mineral oil for 3 years. C: resin composite; A: adhesive; D: intertubular dentin. **A.** Unstained, undemineralized section of a specimen that has been immersed in ammoniacal silver nitrate. The electron-lucent zone of demineralized dentin (between open arrows) corresponded with the stained hybrid layer in Fig.2. **B.** Areas of incomplete resin infiltration within this zone are represented by reticular patterns of silver deposits (nanoleakage; pointer). **B.** The corresponding image of the electron-dense hybrid layer (H) from a demineralized section that was stained with uranyl acetate and lead citrate. **C.** A demineralized section that was stained with phosphotungstic acid and uranyl acetate. Although the hybrid layer was electron-dense due LO the intense mordanting effect of the adhesive solution, cross banding could be identified in longitudinaHy-oriented collagen fibrils (arrow). **D.** Normal collagen dimensions and organization could

be identified from the laboratory demineralized, epoxy resin-infiltrated dentin beneath the hybrid layer.

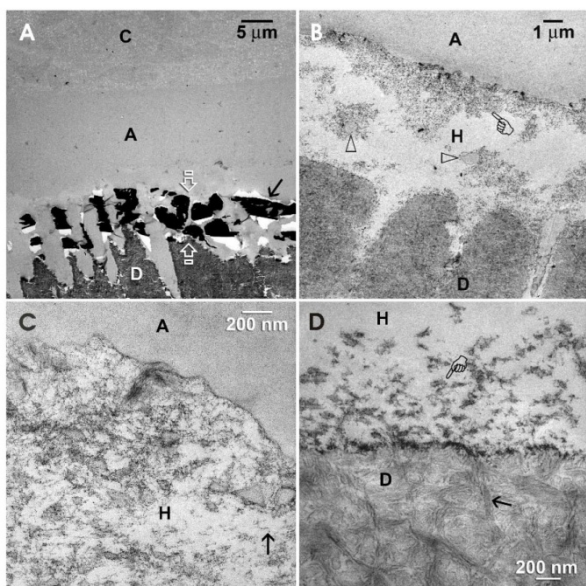


Figure 2.2: Experimental resin-bonded beams of Prime&Bond NT that were aged in artificial saliva for 3 years. C: resin composite; A: adhesive; D: intertubular dentin. **A.** Unstained, undemineralized section after immersion in ammoniacal silver nitrate. Extensive silver impregnation (arrow) was present in the hybrid layer (between open arrows). The intertubular dentin appeared normal in the undemineralized section. **B.** Phosphotungstic acid and uranyl acetate stained, demineralized section showing the breaking down of collagen fibrils (pointer) within the hybrid layer (H). A substantial part of the hybrid layer was devoid of stainable fibrillar components. Oblique sections of resin tags (open arrowheads) could also be seen in the disintegrated hybrid layer. No bacteria was observed in the section. **C.** A high magnification view of the adhesive-hybrid layer interface, showing the stainable fibrillar remnants that were present within the hybrid layer (H). Collagenolysis resulted in the appearance of loose

strands of gelatin microfibrils (arrow). **D.** A high magnification view of the hybrid layer dentin junction where grossly disintegrated fibrillar remnants (pointer) were observed at the base of the hybrid layer (H). Further gelatinolysis by matrix metalloproteinases that diffused from the underlying dentin probably resulted in the breakdown of the gelatin strands into smaller peptides. This may account for the absence of stainable fibrillar components from the rest of the hybrid layer. Collagen fibrils in the laboratory demineralized dentin were denatured to a lesser extent, probably due to the protection rendered by the mineral phases. There was a loss of cross banding from these partially denatured and swollen fibrils, in which only the microfibrillar architecture could be identified (arrow).

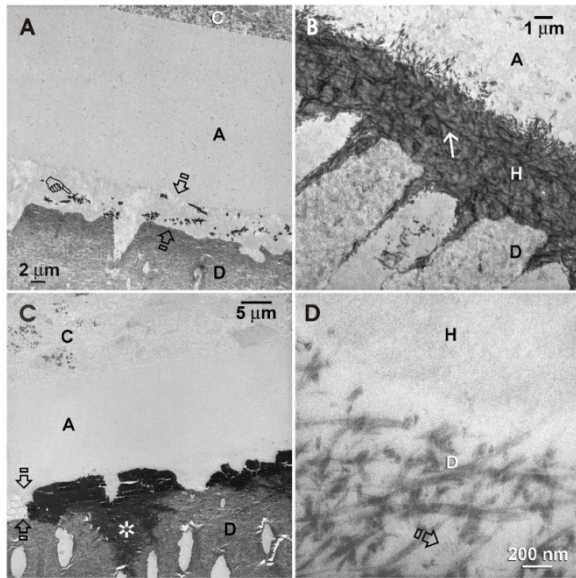


Figure 2.3: Control (Figs. A, B) and experimental (Figs. C, D) resin-bonded dentin beams of Excite after 3 years of accelerated aging. C: resin composite; A: adhesive; D: intertubular dentin. **A.** Unstained, undemineralized section following immersion in ammoniacal silver nitrate. The control specimen that was aged in mineral oil exhibited relatively little silver uptake (pointer) within the demineralized dentin zone (between open arrows). **B.** The corresponding uranyl acetate and lead citrate-stained, demineralized section showing the presence of a highly electron dense hybrid layer (H) in which collagen fibrils could be clearly distinguished (arrow). **C.** Unstained, undemineralized section following immersion in ammoniacal silver nitrate. The experimental specimen that was aged in artificial saliva exhibited extensive areas of heavy silver deposits within the demineralized dentin zone (between open arrows) that extended into the underlying mineralized dentin (asterisk). The rest of the mineralized dentin appeared normal when

undemineralized sections were examined. **D.** The corresponding uranyl acetate and lead citrate-stained, demineralized section demineralized section showing a palely-stained hybrid layer (H) in which fibrillar components could not be identified. Stained collagen fibrils in the underlying intertubular dentin were sparse and separated by abnormally wide interfibrillar spaces (open arrow).

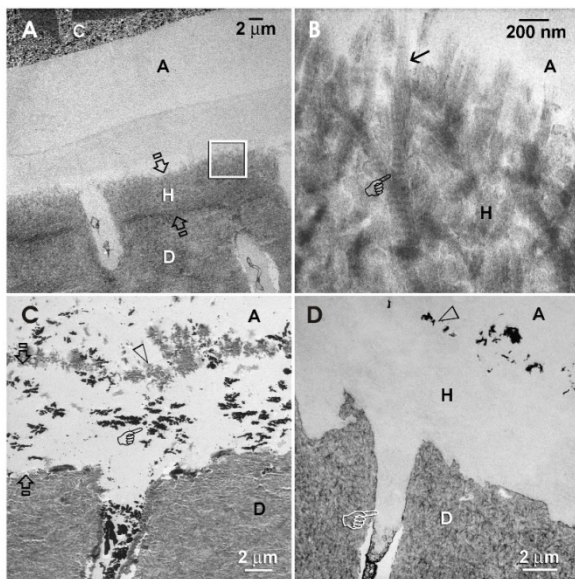


Figure 2.4: Control (Figs. A, B) and experimental (Figs. C, D) resin-bonded dentin beams of All-Bond 2 after 3 years of accelerated aging. C: resin composite; A: adhesive; D: intertubular dentin. **A.** Phosphotungstic acid and uranyl acetate stained, demineralized section of a control specimen that was aged in mineral oil, showing the presence of stained fibrillar components within the 5-6 μm thick hybrid layer (H and between open arrows). **B.** A high magnification view of the area depicted by the box in Fig.2. 4A. Collagen fibrils within the hybrid layer (H) were partially obscured by the infiltrated adhesive resins. Nevertheless, banded collagen fibrils could be identified (pointer), with unraveling of microfibrillar strands along the surface of the cut dentin (arrow). **C.** Unstained, undemineralized section of an experimental specimen that was aged in artificial saliva and examined after immersion in ammoniacal silver nitrate. Crystalline deposits (open arrowhead) derived from the supersaturated artificial saliva were

present between the adhesive and the surface of the cut dentin. The presence of segregated silver deposits within demineralized dentin zone (between open arrows) indicated that the latter was an intact layer, despite the absence of stainable fibrillar components (Fig 2.4D). **D.** The corresponding phosphotungstic acid and uranyl acetate-stained, demineralized section of a specimen that was aged in artificial saliva. The hybrid layer (H) was completely devoid of stainable fibrillar components, and exhibited a similar staining characteristic as the underlying resin tag (pointer) that is supposed to consist predominantly of adhesive resin. The activity of the phosphotungstic acid dissolved the crystalline deposits along the cut dentin surface (open arrowhead). At higher magnification (not shown), collagen fibrils within the underlying dentin were denatured and appeared as microfibrillar strands that were devoid of cross banding (Fig. 2.2D).

Discussion

The null hypothesis had to be rejected as pronounced differences were present between the mineral oil and artificial saliva specimens. The extent of nanoleakage, interfacial staining characteristics, and the conditions of the collagen fibrils in both the hybrid layers and the underlying dentin from all the adhesives were examined. As the glass transition temperature of mineralized dentin is 166.7°C,²¹ it is not surprising that the temperature of superheated steam (121°C) was insufficient to denature collagen in mineralized or resin-infiltrated dentin. As the structural integrity of the collagen fibrils were also preserved in all control specimens that were aged at 55°C in mineral oil, one may confidently assert that the degenerative changes that occurred after accelerated aging in artificial saliva was not caused by the increase in aging temperature. In the absence of salivary and bacterial MMP activities, these changes must have been initiated by the endogenous collagenolytic and gelatinolytic activities of the mineralized dentin, confirming the results of our previous long-term study.¹

The inability to take up heavy metal stains from water-aged hybrid layers has previously been reported by DeMunck *et al.*⁴ The authors surmised that such a phenomenon was due to the decline in polar groups along the surfaces of degrading collagen fibrils. Using a specific collagen stain, we demonstrated the existence of discontinuous patches of grossly disintegrated microfibrillar fragments in some of the artificial saliva-aged interfaces (Fig. 2.2C). These fragments probably represented remnant $\frac{3}{4}$ - and $\frac{1}{4}$ -length fragments that resulted from collagenolysis,²² but were retained by the adhesive resins within the hybrid layer. The existence of mild to moderate nanoleakage (*i.e.* silver uptake) in the hybrid layers that were aged in oil suggested that these hybrid layers were initially permeable to water that was utilized by residual MMP hydrolases to degrade the collagen matrix of the hybrid layer and the underlying mineralized dentin. In resin-bonded specimens incubated in artificial saliva for 3 years, the coexistence of areas that did and did not take up specific collagen stains and areas exhibiting a complete lack of stainable fibrillar components from other specimens (Figs. 2.3D, 2.4D) indicates that the degenerated microfibrillar fragments have further been degraded beyond detection. Such a process may occur via gelatinolytic MMPs released from the mineralized dentin, with the gelatin breaking down into peptides of lower molecular weight (kDa). Such a phenomenon is analogous to the appearance of clear bands in Coomassie bluestained gels, when the cleavage products of gelatin were subjected to Western blotting after treatment with MMP-2 (Gelatinase A) or MMP-9 (Gelatinase B).^{23,24}

Although the collagenase MMP-8 has been shown to exist in carious human dentin,⁹ its endogenous origin has not been established. Conversely, the gelatinase MMP-2 has been shown to be present in human dentin.²⁵ Apart from its gelatinolytic activity via fibronectin-like domains, MMP-2 is also capable of collagenolysis,²⁶ albeit at a slower rate, via its hemopexin domain.²⁷ Thus, even in absence of an endogenous collagenase source, endogenous MMP-2 from the dentin matrix can apparently result in the slow but complete cleavage of the entire resin-infiltrated collagen network over 3 years. Further immunolabeling of MMP-2 within freshly-prepared and aged dentin hybrid layers should be performed to establish the role played by this proteolytic enzyme in the degradation of the demineralized collagen matrix.

It is disturbing to observe consistent partial degradation of the underlying mineralized dentin. Degradation was less severe than the hybrid layers, probably because the collagen fibrils were better protected by apatites than by adhesive resins. Nevertheless, this provides evidence that under specific circumstances, mineralized dentin is capable of self-destruction by its own matrix-bound enzymes. Such a process may require the activation of host-derived MMPs with acids, as purported in the pathogenesis of dentin caries,¹¹ A provocative clinical concern is why self-degradation and accompanying weakening of mineralized dentin has not been reported in teeth bonded *in vivo* with dentin adhesives. A possible explanation is the disturbance of the balance between MMPs and their natural inhibitors, TIMPs (tissue inhibitors of metalloproteinases) when extracted teeth are used for aging experiments. It is known that both TIMP-1 and TIMP-2 can complex with active MMP-2 and inhibit proteolytic activity.^{28,29} As these natural MMP inhibitors have shorter half-lives³⁰ than MMPs, prolonged interruption of MMP-TIMP interaction such as the cessation of dentin fluid flow (*i.e. in vitro* conditions) may prevent the replenishment of pulpal TIMPs out into peripheral dentin. While such ideas remain highly speculative, a similar degradation of collagen fibrils has recently been shown in endodontically-treated teeth that have undergone long-term clinical function.³¹ Clearly, more work is required to establish the relationship between MMPs and their natural inhibitors, or the use of synthetic inhibitors such as chlorhexidine or doxycycline, in prolonging the longevity of resin-dentin bonds and or non-vital dentin in endodontically-treated teeth.

- a. Buehler Ltd., Lake Bluff, IL, USA.
- b. Dentsply DeTrey, Konstanz, Germany.

- c. Ivoclar-Vivadent, Schaan, Liechtenstein.
- d. Bisco Inc., Schaumburg, IL, USA.
- e. Parkell Inc., Edgewood, NY, USA.
- f. Dow-Corning Corp., Midland, MI, USA.
- g. Philips, Eindhoven, The Netherlands.

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