CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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Limitations in Bonding to Dentin and Experimental Strategies to Prevent Bond Degradation

ABSTRACT

The limited durability of resin-dentin bonds severely compromises the lifetime of tooth-colored restorations. Bond degradation occurs *via* hydrolysis of suboptimally polymerized hydrophilic resin components and degradation of water-rich, resin-sparse collagen matrices by matrix metalloproteinases (MMPs) and cysteine cathepsins. This review examined data generated over the past three years on five experimental strategies developed by different research groups for extending the longevity of resin-dentin bonds. They include: (1) increasing the degree of conversion and esterase resistance of hydrophilic adhesives; (2) the use of broad-spectrum inhibitors of collagenolytic enzymes, including novel inhibitor functional groups grafted to methacrylate resins monomers to produce anti-MMP adhesives; (3) the use of cross-linking agents for silencing the activities of MMP and cathepsins that irreversibly alter the 3-D structures of their catalytic/allosteric domains; (4) ethanol wet-bonding with hydrophobic resins to completely replace water from the extrafibrillar and intrafibrillar collagen compartments and immobilize the collagenolytic enzymes; and (5) biomimetic remineralization of the water-filled collagen matrix using analogs of matrix proteins to progressively replace water with intrafibrillar and extrafibrillar apatites to exclude exogenous collagenolytic enzymes and fossilize endogenous collagenolytic enzymes. A combination of several of these strategies should result in overcoming the critical barriers to progress currently encountered in dentin bonding.

KEY WORDS: bottom-up, biomimetic remineralization, chlorhexidine, collagen, cross-linking agents, cysteine cathepsins, degradation, esterase, ethanol wet-bonding, extracellular matrix proteins, hydrolysis, matrix metalloproteinases, resin monomers, top-down, water sorption.

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INTRODUCTION

Over the past two decades, bonding of resin-based composite restorations has been revolutionized by continuing advances in dentin bonding technology. Within the history of contemporary dentin adhesives, minimally invasive tooth preparations have become possible in daily clinical practice. Esthetic tooth-colored restorations have become very popular worldwide. However, resin-dentin bonds are less durable than resin-enamel bonds (Loguercio *et al*., 2008), because dentin bonding relies on organic components. Even though moisture is essential for successful bonding, it also adversely affects long-term bonding results. Although the immediate bond strengths of contemporary adhesives have been shown to be quite high, substantial decreases in resin-dentin bond strength occurred after aging (Breschi *et al*., 2008), with continuous loss of bonded restorations over time for both etch-and-rinse and self-etch adhesive systems (van Dijken *et al*., 2007). Bond durability is critical for the longevity of restoratives, because degradation can weaken adhesion and lead to gaps between teeth and restoratives (Amaral *et al*., 2007). The NIDCR 2009-2013 strategic plan on tooth-colored resin restorations reported that the average replacement time of these restorations is only 5.7 years (NIDCR, 2009-2013). Replacing defective dental fillings costs about five billion dollars *per* year in the US alone (Jokstad *et al*., 2001).

Resin-dentin bonding is a unique form of tissue engineering in which a demineralized dentin collagen matrix is used as the scaffold for resin infiltration, to produce a hybrid layer that couples adhesives/resin composites to the underlying mineralized dentin. To date, resin-dentin bonds created by infiltration of hydrophilic resin monomers into demineralized dentin matrix are imperfect (Breschi *et al*., 2008; Vaidyanathan and Vaidyanathan, 2009). One of the reasons for incomplete permeation of resin monomers within these hybrid layers is fluid movement within the dentinal tubule anastomosis complex during resin infiltration (Hashimoto *et al*., 2009). Thus, the exposed collagen fibrils resulting from incomplete resin infiltration cannot be protected against denaturation challenges and are susceptible to creep (Pashley *et al*., 2003) or cyclic fatigue rupture (Fung *et al*., 2009) after prolonged function. These denuded collagen matrices are also filled with water, which serves as a functional medium for the hydrolysis of resin matrices by esterases and collagen by endogenous and exogenous collagenolytic

enzymes. Even when water-rich, resin-sparse collagen fibrils can be prevented from degradation, the mechanical properties of these denuded collagen fibrils are far inferior to those of resin-infiltrated collagen or mineralized collagen (Sano *et al*., 1995). These issues are the critical barriers to future progress in dentin bonding.

The limited durability of dentin bonding severely shortens the lifespan of tooth-colored resin composites. There is a compelling need to explore the underlying mechanisms involved in preventing the degradation of resin-dentin bonds and extend their longevity. This paper reviews the factors compromising dentin bonding and the experimental strategies available to prevent degradation of resin-dentin bonds.

FACTORS THAT COMPROMISE THE DURABILITY OF RESIN-DENTIN BONDS

Hydrolytic Degradation by Water Sorption

The water "wet bonding" technique was introduced in the early 1990s to prevent the problem of collagen collapse after acid-etching and resulted in improved resin infiltration into acid-etched dentin. In this bonding technique, acid-etched dentin is kept fully hydrated throughout the bonding procedure. Two-hydroxyethyl methacrylate (HEMA) was incorporated into many dentin adhesives to serve as a solvent for non-water-compatible resin monomers, to reduce phase separation of those monomers after evaporation of the volatile solvents (Spencer and Wang, 2002), to enhance the wetting properties of those adhesives on acid-etched dentin (Marshall *et al*., 2010), and for its purported affinity to demineralized collagen (Sharrock and Grégoire, 2010).

In the evolution of dentin adhesives, manufacturers have incorporated increasing concentrations of hydrophilic and ionic monomers to make these adhesives more compatible for bonding to intrinsically moist, acid-etched dentin (Van Landuyt *et al*., 2007). Increasing the hydrophilic nature of the adhesive-dentin interface has several disadvantages (Tay and Pashley, 2003a) and creates a weak link in the bonded assembly (Spencer *et al*., 2010). Hydrophilic and ionic resin monomers are vulnerable to hydrolysis, due to the presence of ester linkages (Ferracane, 2006). Inclusion of high concentrations of monomers in these adhesives lowers the vapor pressure of bonding solvents, including water. High concentrations of resin monomers in an adhesive may therefore hamper water evaporation, the entrapment of which deteriorates mechanical properties of the resulting polymer (Van Landuyt *et al*., 2008; Ye *et al*., 2009a). Previous studies have correlated the incorporation of hydrophilic and acidic resin monomers in adhesive blends with decreased longevity of resin-dentin bonds (Peumans *et al*., 2005), because these hydrophilic resins expedite water sorption (Malacarne *et al*., 2006). This, in turn, results in plasticization of polymers, which decreases the mechanical properties of resins (Ito *et al*., 2005; Chiaraputt *et al*., 2008). Even the inclusion of small amounts of water may result in nano-phase separation of the adhesive components in the form of nanoscopic worm-like structures between the polymerized hydrophilic and hydrophobic resin phases (Ye *et al*., 2008, 2009b). Nano-phase separation decreases the dynamic mechanical properties of the polymerized adhesives (Park *et al*., 2010) and increases their susceptibility to esterase-catalyzed hydrolysis (Kostoryz *et al*., 2009).

Dentin bonds created by contemporary hydrophilic adhesives are also susceptible to fluid permeation induced by pulpal pressure (Tay *et al*., 2005; Cadenaro *et al*., 2005; Sauro *et al*., 2006), which further promotes their hydrolysis and decreases bond durability (Armstrong *et al*., 2004; De Munck *et al*., 2004). Adhesive hydrophilicity, water sorption, and subsequent hydrolytic degradation have been considered as highly correlative, because hydrolytic degradation occurs only in the presence of water (Carrilho *et al*., 2005). Hydrolysis of methacrylate ester bonds caused either by the increase in acidity of monomer components (Aida *et al*., 2009) or by salivary esterases (Shokati *et al*., 2010) can break covalent bonds between the polymers by the addition of water to the ester bonds. Moreover, problems associated with water-based, strongly acidic single-bottle self-etching adhesives arise from both the hydrolytic instability of the methacrylate monomers used in those systems and the side-reaction of the applied initiator components (Moszner *et al*., 2005). These resin formulation issues raise concerns on whether current adhesives have become too hydrophilic (Tay and Pashley, 2003a).

Incomplete Infiltration of Resin Monomers

There is common consensus that the final goal of dentin bonding is complete infiltration of resin monomers into demineralized collagen fibrils exposed by acid-etching or self-etch adhesives (Breschi *et al*., 2008). The discrepancy between dentin demineralization and resin infiltration results in silver-nitrate- or fluorescent-dye-detectable nanoleakage within the water-rich zones of the hybrid layer (Reis *et al*., 2007; Sauro *et al*., 2009a) and the adhesive layer (Tay and Pashley, 2003b). The critical barrier to progress in contemporary dentin bonding is that both etch-and-rinse and self-etch approaches in establishing retention in dentin are hampered by their inability to completely replace free and loosely bound water from the internal and external water compartments of collagen fibrils (Kim YK *et al*., 2010a).

Apart from water, the interfibrillar spaces in acid-etched dentin also contain highly hydrated negatively charged proteoglycans that form a hydrogel within that space (Scott and Thomlinson, 1998). If these hydrogels remain hydrated in interfibrillar spaces, they may be responsible for "molecular sieving" of larger dimethacrylates like BisGMA, allowing only smaller molecules such as HEMA to penetrate the base of the hybrid layers. Since HEMA forms a linear polymer that does not cross-link, HEMA-rich regions of hybrid layers may experience large strains during function that result in fatigue failure of collagen fibrils. To date, complete replacement of lost apatites by resin within the intrafibrillar spaces has never been demonstrated. Thus, apatite-depleted, resin-sparse collagen fibrils within the hybrid layers become susceptible to degradation, compromising the longevity of resin-dentin bonds (Breschi *et al*., 2008; Hashimoto, 2010).

Collagenolysis by Endogenous MMPs and Cysteine Cathepsins

Mineralized dentin contains matrix metalloproteinases (MMPs) such as MMP-2, -3, -8, and -9 (Sulkala *et al*., 2002, 2007; Mazzoni *et al*., 2007, 2009; Boukpessi *et al*., 2008; Santos *et al*.,

2009; Toledano *et al*., 2010). These host-derived proteases are a group of zinc- and calcium-dependent enzymes that contribute to the breakdown of collagen matrices in the pathogenesis of dentinal caries (Tjäderhane *et al*., 1998; van Strijp *et al*., 2003; Chaussain-Miller *et al*., 2006) and periodontal disease (Hannas *et al*., 2007). In addition, non-collagen-bound MMPs are also present in saliva (Tjäderhane *et al*., 1998; Sulkala *et al*., 2001), in dentinal tubules, and, presumably, in dentinal fluid (Sulkala *et al*., 2002; Boushell *et al*., 2008). Degradation of resin-sparse collagen fibrils in aged bonded dentin *in vivo* has also been correlated with the activation of collagen-bound MMPs and/or salivary MMPs by application of components of etch-and-rinse adhesives (Mazzoni *et al*., 2006; De Munck *et al*., 2009). The acid-etching procedure was initially thought to release and activate pro-MMPs trapped within the mineralized dentin (Pashley *et al*., 2004), resulting in collagenolytic and gelatinolytic activities identified within the hybridized dentin. However, phosphoric acid used in the etch-and-rinse technique is highly acidic (pH 0.7 to -1) and denatured MMPs due to its low acidity (Pashley *et al*., 2004). The acidic resin components incorporated into etch-and-rinse adhesives (Mazzoni *et al*., 2006) and self-etch adhesives (Nishitani *et al*., 2006a) have subsequently been shown to increase the collagenolytic and gelatinolytic activities of completely or partially demineralized collagen matrices. These studies, which utilized dentin powder and an internally quenched fluorescent collagen/gelatin substrate, could identify only collagen degradation activities, but were non-specific in terms of whether MMPs, or which type of MMP, are involved in the degradation process. The results were more recently confirmed with MMP-2 and MMP-9 specific assays for both etch-and-rinse and self-etch adhesives (Breschi and Mazzoni, unpublished observations). In addition, a self-etch adhesive has been shown to increase MMP-2 synthesis in human odontoblasts (Lehmann *et al*., 2009), possibly increasing MMP-2 penetration into the hybrid layer *via* dentinal fluid. Mildly acidic resin monomers can activate MMPs by inhibiting tissue inhibitor of metalloproteinases-1 (TIMP-1, Ishiguro *et al*., 1994) in TIMP-MMP complexes, thereby producing active MMPs (Tjäderhane *et al*., 1998; Sulkala *et al*., 2001). Alternatively, these acidic resin monomers may activate latent forms of MMPs (pro-MMPs) *via* the cysteine-switch mechanism that exposes the catalytic domain of these enzymes that were blocked by propeptides (Tallant *et al*., 2010).

Cysteine cathepsins are papain-like endopeptidases that participate in intracellular proteolysis within the lysosomal compartments of living cells (Dickinson, 2002). However, they also exist as exopeptidases and participate in extracellular matrix degradation involving the breakdown of type I collagen and proteoglycans (Obermajer *et al*., 2008). Cathepsins B, L, and S cleave the non-helical telopeptide extensions of collagen molecules, while cathepsin K cleaves the collagen molecules along their triple helix region. Unlike the collagenolytic MMPs (MMP-1, -2, -8, and -13) that cleave type I collagen within the triple helix at a single site (between amino acids 775 and 776 from the first GXY triplet of the triple helix domain) into a ¾ N-terminal fragment and ¼ C-terminal fragment, cathepsin K cleaves collagen molecules at multiple sites within the triple helix, thereby generating fragments of various sizes (Garnero *et al*., 1998). Cysteine cathepsins have recently been reported to be expressed by mature human odontoblasts and present in intact dentin (Tersariol *et al*., 2010), but were more abundantly (approximately 10-fold) identified from carious dentin (Nascimento and Tjäderhane, unpublished observations). Similar to MMPs, cysteine cathepsins may be activated in mildly acidic environments. Acid activation of dentin-bound cathepsins may further result in activation of matrix-bound MMPs. Moreover, glycosaminoglycans (GAGs) can accelerate conversion of the latent forms of the cathepsin enzyme family into their mature forms at neutral pH (Obermajer *et al*., 2008). Thus, GAG-cathepsin activation permits active cathepsins to be functional even in neutral pH environments. The presence of cysteine cathepsins in dentinal tubules (Tersariol *et al*., 2010) indicates that they are derived from the dental pulp *via* the dentinal fluid and may be activated by mildly acidic adhesive resin monomers. They may also interact with GAGs in the dentinal fluid or the collagen matrix after bonding and neutralization of the acidic monomers and participate with salivary MMPs in the degradation of resin-dentin bonds.

Etch-and-Rinse *vs.* Self-etch Adhesives

Analysis of data from a recent systematic review on Class V clinical data indicated that the annual failure rates of these bonded restorations are in decreasing order: 1-step self-etch > 2-step etch-and-rinse > 2-step self-etch (aggressive) > 3-step etch-and-rinse > 2-step self-etch (mild and moderately aggressive) (Van Meerbeek *et al*., 2010). The better durability of the mild 2-step self-etch adhesives may be attributed to the use of a separate, relatively more hydrophobic resin layer on top of the hydrophilic self-etch primer, which renders the interface less susceptible to water sorption, and partial dissolution of the apatite minerals which appear to exert a protective effect on collagen degradation. The latter was demonstrated recently by the minimal involvement of endogenous MMP-2 and MMP-9 in interfaces bonded by the mild two-step self-etch adhesives (De Munck *et al*., 2010). Nevertheless, mild two-step self-etch adhesives are not totally immune to bond degradation, since they are still hydrophilic, and enzymatic hydrolysis of ester bonds may still occur over time. Failure of water to be completely removed is probably another reason why bond degradation may occur in this class of adhesives, as shown by the presence of silver nanoleakage in the interface of these adhesives. Also, there is no evidence showing that water in the intrafibrillar compartments of these adhesives is completely replaced by resin.

EXPERIMENTAL STRATEGIES TO PREVENT DEGRADATION OF RESIN-DENTIN BONDS

Increasing the Degree of Conversion and Esterase Resistance of Hydrophilic Adhesives

Resin degradation is directly related to water sorption, since hydrolytic attack on ester linkages by increased acidity/basicity of resin components or by salivary esterases can both occur in the presence of water. The use of a hydrophobic photoinitiator such as camphorquinone for hydrophilic adhesives in the presence of

water also does not result in optimal polymerization of those adhesives (Ye *et al*., 2009a). Human saliva contains sufficient amounts of cholesterol esterase and pseudocholinesterase, which act synergistically to degrade dimethacrylates (Finer *et al*., 2004). These challenges provide the rationale for the development of water-compatible and esterase-resistant dentin adhesives (Spencer *et al*., 2010)

The degrees of conversion of hydrophilic adhesive components may be improved by the use of hydrophilic photoinitiators such as QTX [2-hydroxy-3-(3, 4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-*N*,*N*,*N*-trimethyl-1-propanaminium chloride] and compatible accelerators, as well as novel bulky/branched esterase-resistant hydrophilic urethane-modified resin monomers (Hayakawa *et al*., 2005; Ye *et al*., 2009a; Park *et al*., 2008, 2010). Other groups have also used water-soluble photoinitiators such as TPO [diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide; Cadenaro *et al*., 2010] and sodium acylphosphine oxide containing crown ether (Ikemura *et al*., 2009) to improve the polymerization of hydrophilic adhesives in the presence of water. Secondary cross-linking of polar functional groups on methacrylate side-chains may increase the hydrophobic nature of the hybrid layer after initial infiltration of the hydrophilic resin monomers into the partially or completely demineralized collagen matrix. Increasing the degree of conversion of mono- and dimethacrylate resins may also reduce the susceptibility of the polymerized adhesive layer and resins that infiltrated the hybrid layer to esterase hydrolysis by reducing the number of unreacted pendant functional groups.

The use of these novel photoinitiators, accelerators, and cross-linking resin monomers is likely to increase the initial dynamic mechanical properties of the resin-dentin interface, as well as reducing the heterogeneity and nanophase separation of polymer matrices when the latter is polymerized in the presence of water (Park *et al*., 2010). These strategies, however, will not result in a quantum increase in the ability of adhesive resin monomers to infiltrate a demineralized collagen matrix completely, particularly when water is present within the internal water compartments of the collagen fibrils (Fig. 1A).

Inhibitors of Collagenolytic Enzymes

Matrix metalloproteinases contribute to the degradation of collagen fibrils within incompletely resin-infiltrated hybrid layers (Zhang and Kern, 2009) and the loss of *quasi*-static mechanical properties of the collagen matrix (Carrilho *et al*., 2009; Tezvergil-Mutluay *et al*., 2010a). Thus, application of MMP inhibitors to the demineralized collagen matrix prior to the application of dentin adhesives appears to be a rational approach for extending the longevity of resin-dentin bonds (Pashley *et al*., 2004) (Fig. 1B). There is a major difference between the selection of appropriate non-specific MMP inhibitors for preventing the degradation of hybrid layers and the design of specific MMP inhibitors for the treatment of diseases – the issue of inhibitor selectivity.

MMPs are associated with a variety of pathological conditions such as cancer, arthritis, and other diseases associated with tissue remodeling. Despite intensive interests to develop synthetic MMP inhibitors capable of potently and selectively blocking the uncontrolled activity of these enzymes under disease conditions, only a few selective and effective drugs with the desired properties have emerged for inhibiting individual MMPs (Li and Wu, 2010). The structural homology of the catalytic domains among members of the MMP family becomes an advantage in the quest for non-specific inhibitors for dentin matrix-bound MMPs and salivary MMPs. Since the application of such inhibitors to acid-etched dentin is analogous to a topical application method and involves nanogram quantities, the issue of systemic toxicity becomes less critical, albeit still important. There are unique problems associated with this use of MMP inhibitors – the issues of substantivity and its release from polymerized resin matrices, as will be discussed below. Chlorhexidine (CHX), a biguanide antimicrobial agent, has been successfully applied to inhibit the activities of MMP-2, MMP-8, and MMP-9 (Gendron *et al*., 1999). Investigators have used CHX as a non-specific MMP-inhibitor during adhesive application (Pashley *et al*., 2004; Hebling *et al*., 2005; Carrilho *et al*., 2007a,b; De Munck *et al*., 2009). For etch-and-rinse adhesives, CHX may be applied to the demineralized dentin directly or incorporated into an acid conditioner prior to the application of adhesives, which has been shown to be effective for reducing degradation of resin-dentin bonds after *in vivo* aging (Hebling *et al*., 2005; Carrilho *et al*., 2007b; Brackett *et al*., 2009; Ricci *et al*., 2010). Another non-specific MMP inhibitor, GM 6001 (galardin), is often used as a reference inhibitor in generic MMP assay kits. Galardin has been used as an experimental primer on acid-etched dentin prior to the application of an etch-and-rinse adhesive and prevented degradation of the hybrid layer after 12 months (Breschi *et al*., 2010). Others have shown that polyvinylphosphonic acid (Tezvergil-Mutluay *et al*., 2010b) and benzalkonium chloride (Pashley, unpublished observations) also possess generic anti-MMP activities. Chemically modified tetracyclines (*i.e*., tetracyclines that lack antibiotic activities but retain their anti-MMP activities) are effective non-specific MMP inhibitors and have been used as MMP inhibitors in experimental caries (Sulkala *et al*., 2001) and periodontal disease models (Tjäderhane *et al*., 2007). They have not been used to prevent the degradation of hybrid layers, since they may stain teeth with a purple hue after photo-oxidation of the tetracycline.

One of the benefits of using CHX as an antimicrobial agent is that it possesses substantivity by binding to mineralized dentin for at least 12 weeks (Mohammadi and Abbott, 2009). It has been recently shown that demineralized dentin can bind more CHX than mineralized dentin (Kim J, 2010b). Although the binding is electrostatic in nature and is reversible, CHX is not de-bound by HEMA and may remain bound to demineralized dentin after bonding (Kim J, 2010b). This may be the reason for the long-term efficacy of CHX as a MMP inhibitor in resin-dentin bonds. Relatively large amounts of CHX remain bound to partially and completely demineralized dentin incubated in phosphate-buffered saline for at least 8 weeks, and no de-binding occurred after the first half-hour of incubation (Carrilho *et al*., 2010). Since the binding mechanism is only electrostatic (Blackburn *et al*., 2007), there is concern that CHX may eventually be displaced by competing cations derived from dentinal fluid or saliva. This may account for the recent observation that bonds made to CHX pre-treated acid-etched dentin with commercial adhesives and water-wet bonding were preserved after 9 months but not after 18 months, with severe hybrid layer

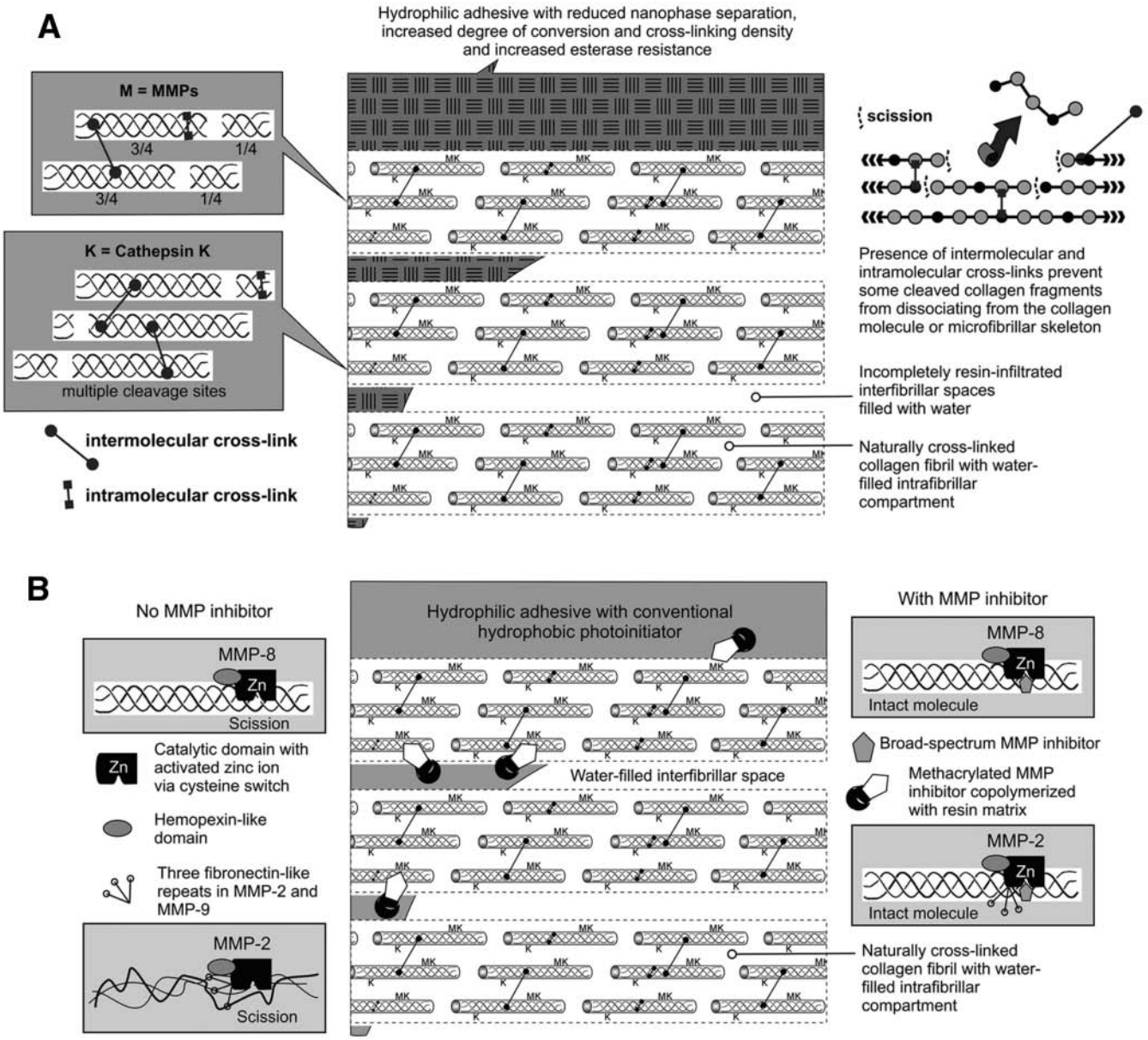

Figure 1. Schematics on the use of experimental adhesives or MMP inhibitors to prevent the degradation of resin-dentin bonds. **(A)** A schematic depicting the use of experimental adhesives with increased degree of conversion and esterase resistance for bonding to acid-etched dentin. However, such adhesives may not completely infiltrate the entire depth of the hybrid layer. Left side depicts denuded collagen fibrils being degraded by matrix metalloproteinases (MMPs) and cathepsins. Right side depicts cleaved, degraded collagen fragments prevented from dissociating from the collagen molecule *via* intermolecular and intramolecular cross-links. Because of these cross-links, measuring the amount of hydroxyproline from a degraded dentin collagen matrix is likely to underestimate the extent of collagen degradation. M, MMP; K, cathepsin K. **(B)** A schematic depicting the use of MMP inhibitors or MMP-inhibitor-conjugated adhesives for bonding to acid-etched dentin. Left side: Unlike MMP-8, MMP-2 is thought to function by unwinding the triple collagen helix prior to scission of the tropocollagen molecules. Right side: The catalytic domain of MMPs is blocked in the presence of a broad-spectrum MMP inhibitor. M, MMP; K, cathepsin K.

degradation at 18 months (Sadek *et al*., 2010b). Such a concern provides the rationale for chemically grafting CHX to resin monomers to create CHX-methacrylates (Luthra and Sandhu, 2005) and incorporating these resin monomers with anti-MMP potentials into dentin adhesives.

Another group of antimicrobial agents, the quaternary ammonium salts, also possesses anti-MMP activities. One of the best-known commercially available quaternary ammonium methacrylates, 12-methacryloyloxydodecylpyridinium bromide (MDPB; Imazato *et al*., 2007), was also found to possess anti-MMP activities (Tezvergil-Mutluay *et al*., unpublished observations). This could have accounted, in hindsight, for the *in vitro* and *in vivo* observations that resin-dentin bonds degraded after one year when Clearfil SE Bond (Kuraray Medical Inc., Tokyo, Japan) was used as the self-etching primer, while bonds created in the same study with the MDPB-containing self-etching

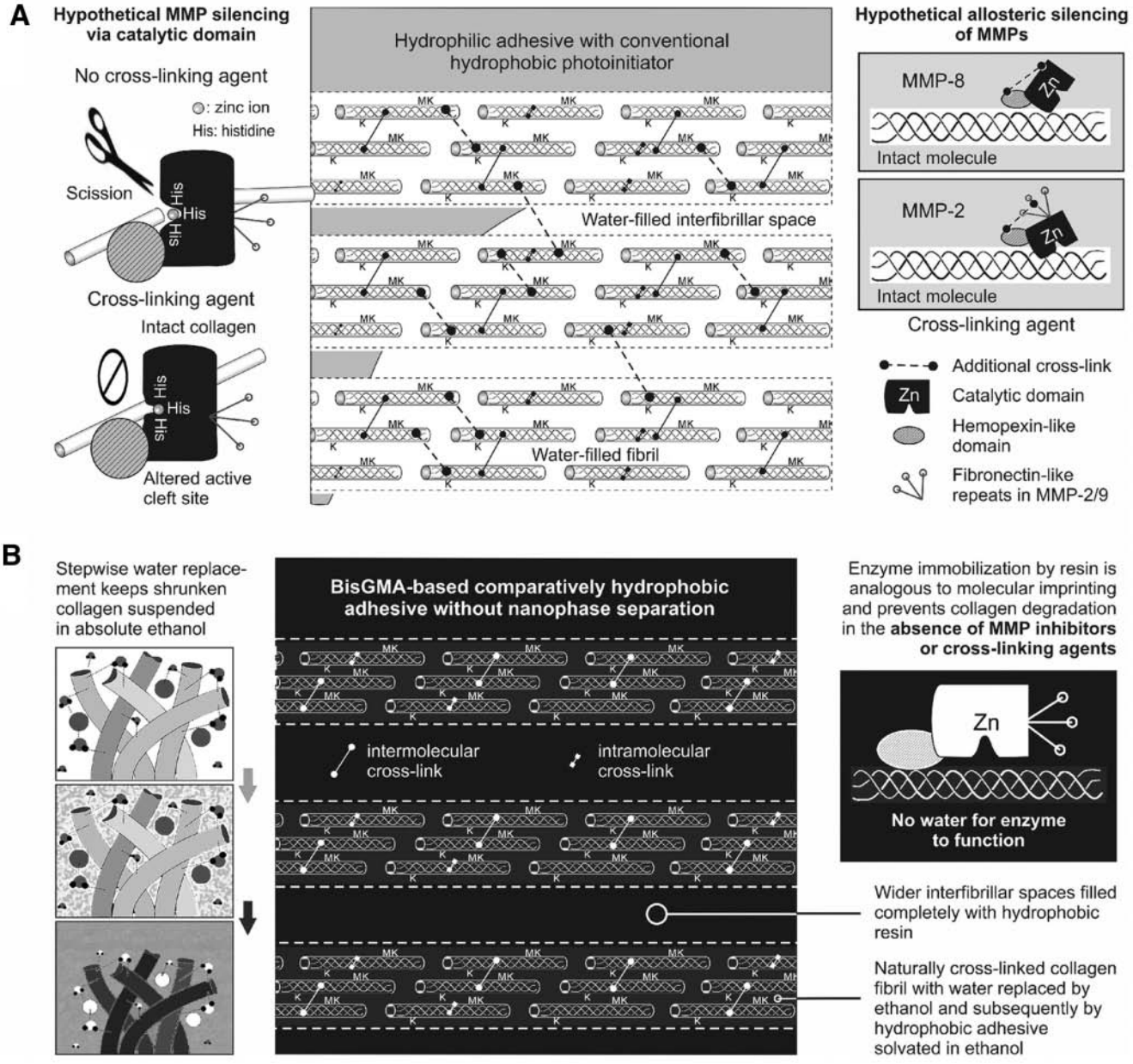

Figure 2. Schematics on the use of cross-linking agents or ethanol wet-bonding to prevent the degradation of resin-dentin bonds. **(A)** A schematic depicting the use of cross-linking agents for silencing the collagenolytic activities in bonded acid-etched dentin. Cross-linking may cause conformational changes in the active site, catalytic domain, and/or binding site of MMPs, eliminating the enzymes' ability to degrade the substrate. Left side depicts hypothetical MMP *via* its catalytic domain. Right side depicts allosteric inhibition of MMPs *via* their other non-catalytic domains. M, MMP; K, cathepsin K. **(B)** A schematic depicting the use of the ethanol wet-bonding technique for bonding hydrophobic adhesives to acid-etched dentin. Both apatite-depleted extrafibrillar and intrafibrillar spaces are infiltrated by hydrophobic adhesive without nanophase separation. Left side depicts progressive water replacement of collagen matrix by ethanol, with the shrunken fibrils suspended in ethanol. Right side depicts immobilization of MMP by resin that is analogous to "molecular printing" but without removal of the enzyme. M, matrix metalloproteinase; K, cathepsin K.

primer Clearfil Protect Bond (Kuraray) were well preserved after one year (Donmez *et al*., 2005). The recent discovery of more quaternary ammonium methacrylate resin monomers with various degrees of anti-MMP activities (Tezvergil-Mutluay *et al*., unpublished observations) has brought on a new fervor for incorporating these resin monomers into dentin adhesives to circumvent the displacement of electrostatically bound CHX and other potential non-resin-conjugated MMP inhibitors from denuded collagen matrices of the hybrid layers during aging (Fig. 2A). Copolymerization of CHX-methacrylates or quaternary ammonium methacrylates with other dimethacrylate resin monomers prevents CHX from de-binding and diffusing out of the incompletely resin-infiltrated hybrid layers.

For self-etch adhesives, chlorhexidine was incorporated directly into primers (De Munck *et al*., 2009; Zhou *et al*., 2010). However, there are severe limitations when CHX is directly

incorporated into polymerizable resin monomer formulations. Although incorporation of 1-2% CHX directly into resin blends does not affect their degree of conversion significantly, such a process adversely affects the mechanical properties of the polymerized resins. For example, addition of even 1% CHX to a variety of resin blends with different hydrophilicity resulted in reducing the modulus of elasticity (*i.e.*, stiffness) of the polymerized resins by 27-48% (Cadenaro *et al*., 2009a). This inhibition effect may also be weakened over time as CHX release from the resins is related to water-induced swelling (Hiraishi *et al*., 2008). Release of CHX from a polymer matrix is also pH-dependent, with more being released at lower pH values (Anusavice *et al*., 2006). Chlorhexidine also interacts with calcium fluoride that may be simultaneously incorporated into the resin matrix (Shen *et al*., 2010).

Since cathepsin K is highly expressed in osteoclasts and is the primary enzyme involved in type I collagen degradation in bone resorption, much work has been done on designing drugs that mimic natural cystatin inhibitors of cathepsins (Liu *et al*., 2010) as well as specific nitrile-based orally applicable cathepsin K inhibitors (Teno and Masuya, 2010). Little is known about whether these drugs that are targeted at inhibiting specific cathepsins are useful for direct application to acid-etched dentin or incorporation into self-etch adhesives for preventing the degradation of type I dentin collagen scaffolds. Conversely, CHX has been reported to be effective against cysteine proteinases produced by *Porphyromonas gingivalis* (Houle *et al*., 2003; Sela *et al*., 2009) and human recombinant cysteine cathepsins (Scaffa *et al*., 2010). Thus, it is worth examining if CHX will serve as a non-specific inhibitor for both MMPs and cysteine cathepsins associated with intact and carious dentin.

MMP and Cathepsin Silencing *via* the Use of Cross-linking Agents

Over the last few years, the experimental use of cross-linking agents to increase the longevity of resin-dentin bonds has taken on a life of its own, with various attempts to use agents such as glutaraldehyde, genipin, proanthrocyanidin, and carbodiimide for long time periods (generally > 1 hr) to introduce additional cross-links to acid-demineralized dentin collagen (Al-Ammar *et al*., 2009; Macedo *et al*., 2009; Bedran-Russo *et al*., 2009, 2010; Castellan *et al*., 2010a,b). These *in vitro* studies demonstrated that the use of cross-linking agents improved the short-term mechanical properties of dentin collagen, reduced the susceptibility of additionally cross-linked dentin collagen to enzymatic degradation by collagenases, and increased the stability of the resin-dentin interface.

It is beyond doubt that the use of cross-linking agents will improve the resistance of uncross-linked or mildly cross-linked collagen matrices to degradation by bacterial collagenases (Avila and Navia, 2010; Ma *et al*., 2010). Dentin collagen is strengthened by native inter- and intramolecular cross-links, which increase its resistance to thermal denaturing and enzymatic degradation (Kuboki and Mechanic, 1982). Since dentin collagen is already highly cross-linked, it is doubtful if the increase in resin-dentin bond longevity can be explained by augmentation in cross-linking density alone. Proanthrocyanidin, for example, is a potential inhibitor of MMP-2 and MMP-9 (Matchett *et al*., 2005). Current literature suggests that this MMP resistance may be attributed to an alternative mechanism – silencing of MMPs and probably other exogenous collagen degradation enzymes *via* conformational changes in the enzyme 3-D structure (Busenlehner and Armstrong, 2005). Theoretically, this may be achieved *via* irreversible changes induced within the catalytic domain or allosteric inhibition of other modular domains that co-participate in collagen degradation (Sela-Passwell *et al*., 2010) (Fig. 2A).

Natural TIMPs inhibit MMPs by recapitulating the thiol-zinc interaction between the pro-peptide and catalytic domains of pro-MMPs *via* insertion of a conserved peptide anchor into the catalytic domain. The molecular anchor coordinates a catalytic zinc ion with a cysteine residue along the N-terminal of the TIMP molecule (Gomis-Ruth *et al*., 1997). Many non-specific, small-molecule MMP-inhibitors exert their inhibitory function by coordinating with the histidine-coupled zinc ion to produce MMP-inhibitor complexes (Tallant *et al*., 2010). Conversely, the use of cross-linking agents may create multiple cross-links between amino acids within the catalytic site that irreversibly alter the 3-D conformation or flexibility of the cleft-like catalytic domain and prevent its optimal recognition and complexing with the type I collagen substrate (O'Farrell *et al*., 2006). Although there is no evidence that the catalytic domain of collagenolytic MMPs can be cross-linked to inactivate their functions, oxidative cross-linking of adjacent tryptophan and glycine residues in the catalytic domain of Matrilysin (MMP-7) by hypochlorous acid released by myeloperoxidase resulted in silencing of the catabolic activity of this enzyme (Fu *et al*., 2004).

The use of cross-linking agents may also contribute to MMP silencing *via* allosteric control of non-catalytic domains. For example, the catalytic domains in collagenolytic MMPs can cleave non-collagen substrates, but the hemopexin-like domain of these enzymes is essential for them to initially unwind and subsequently cleave the three triple-helical fibrillar elements of the collagen molecule in succession (Lauer-Fields *et al*., 2009). For MMP-8, binding and orientation of the triple helix through the hemopexin-like domain results in cleavage of the α1(1) and β2(1) chains without discrimination, while the gross conformation of the triple helix is preserved (Gioia *et al*., 2007). For MMP-2, there are three fibronectin-like repeats that form a domain for binding to collagen or gelatin substrates. This collagen-binding domain binds preferentially to the α1(1) chain and mediates local unwinding and gross alteration of the triple helix prior to the cleavage of the β2(1) chain (Gioia *et al*., 2007). Regardless of which of the two collagen-binding mechanisms is involved, cross-linking of either the hemopexin-like or fibronectin-like domains may contribute to inactivation of the associated MMPs and reduction in their collagenolytic efficacy. This hypothesis appears to be supported by the results of a study in which MMP-2 and -9 activities of native bovine and porcine pericardium were completely abolished by cross-linking with glutaraldehyde or diphenylphosphorylazide. The use of carbodiimide was less effective, but was more efficacious in reducing MMP-9 than MMP-2 (Calero *et al*., 2002). These results have recently been confirmed in our laboratory; the use of carbodiimide for 1 min on a thiopeptolide from a generic MMP assay

kit completely abolished the lytic activity of purified human recombinant MMP-9. Moreover, treating completely demineralized dentin with carbodiimide for only 1 min resulted in a significant decrease in the amount of free hydroxyproline derived from the degraded collagen after 30 days when compared with untreated demineralized dentin (Tezvergil-Mutluay *et al*., unpublished observations). Analysis of these data indicates that cross-linking agents can inactivate MMP, which, in turn, is responsible for the reduction of collagen degradation.

Cathepsin K, which breaks down the non-telopeptide triple helix of the collagen molecules, is also allosterically regulated by modifiers such as sulphated glycosaminoglycans (GAGs) outside of its active catalytic site (Novinec *et al*., 2010). Binding of heparin to cathepsin K induces allosteric conformational changes that increase the collagenolytic activity of the enzyme. Thus, GAGs act as natural allosteric modifiers of cathepsin K. It is possible that the use of cross-linking agents may alter the GAG-cathepsin allosteric interaction and "trap" the enzyme in a certain conformation that inactivates its collagenolytic activity. Cross-linking may also affect MMP activities known to be modified by non-collagenous proteins (Malla *et al*., 2008). In dentin, MMP activities and resistance to degradation may be regulated by fetuin-A (Ray *et al*., 2003) and the SIBLINGs Bone Sialoprotein (BSP) and Dentin Matrix Protein-1 (DMP-1) (Fedarko *et al*., 2004), all of them being present in dentin. Thus, cross-linking of these non-collagenous proteins may indirectly silence MMPs *via* inactivation of the functional domains of these glycoproteins.

Similar to the use of non-specific inhibitors, the major drawback in the use of cross-linking agents to inactivate MMPs and cysteine cathepsins is that a water-rich, resin-sparse collagen matrix with poor mechanical properties is retained within the hybrid layer. Demineralized dentin collagen has a modulus of elasticity of less than 8 MPa (Carrilho *et al*., 2009). Even if these demineralized collagen fibrils can be stiffened 50X with cross-linking agents, the resulting modulus of elasticity (*ca*. 0.4 GPa) is still far inferior to that of resin-infiltrated dentin (*ca*. 3-5 GPa; Ito *et al*., 2005; Chiaraputt *et al*., 2008) and mineralized dentin (*ca*. 20 GPa; Kinney *et al*., 2003). Moreover, chemical cross-linking of collagen does not alter the intrinsic collagen molecular stiffness (Liao *et al*., 2005). Thus, these very flaccid collagen fibrils are susceptible to creep and subsequent fatigue rupture after prolonged function (Fung *et al*., 2009). This highlights the need for additional strategies that enable the dynamic mechanical properties of denuded collagen matrices to be improved or regained as a mechanism to prevent the degradation of resin-dentin bonds.

Ethanol Wet-bonding with Hydrophobic Resins

During dentin bonding with contemporary hydrophilic etch-and-rinse adhesives, the polar solvents used in these adhesives remove water and cause shrinkage of the collagen matrix (Pashley *et al*., 2007). After solvent evaporation, the matrix will further shrink, with a higher solvent concentration resulting in greater matrix shrinkage upon evaporation of the solvent. Pre-treatment of water-saturated collagen matrix with 100% alcohol achieves similar matrix shrinkage but prevents phase separation of hydrophobic resin monomers such as BisGMA. That is, wet-bonding with ethanol instead of water provides an opportunity for coaxing hydrophobic monomers into a demineralized collagen matrix without sacrificing any additional matrix shrinkage. Infiltration of hydrophobic monomers into a collagen matrix decreases water sorption/solubility, resin plasticization, and enzyme-catalyzed hydrolytic cleavage of collagen (Hosaka *et al*., 2009; Sadek *et al*., 2010b), thereby creating more durable resin bonds.

A demineralized collagen matrix may be rendered more hydrophobic by replacing the water with ethanol, while keeping the collagen matrix suspended in absolute ethanol. Likewise, a hydrophobic resin blend consisting of BisGMA and TEGDMA may be rendered more hydrophilic by dissolving these comonomers in ethanol. These sequential steps allow for improved miscibility of the solvated adhesive and the collagen matrix (Tay *et al*., 2007), thereby enabling the ethanol-solvated hydrophobic resin blend to infiltrate an ethanol-saturated collagen matrix. Whereas infiltration of resin monomers invariably results in a diffusion gradient of resin infiltration within the collagen matrix, recent studies with two-photon laser confocal microscopy (Sauro *et al*., 2009a) and micro-Raman spectral analysis (Shin *et al*., 2009) indicated that a relatively homogeneous distribution of hydrophobic resins within the hybrid layer could be achieved with ethanol wet-bonding (Fig. 2B).

There are two versions of the ethanol wet-bonding technique. In the simplified technique, absolute ethanol is applied to water-saturated acid-etched dentin for 1 min prior to the application of ethanol-solvated hydrophobic resin comonomer blends (Nishitani *et al*., 2006b; Sauro *et al*., 2010). The original rationale for this simplified technique was to provide a method of application of hydrophobic resin comonomers to acid-etched dentin within a clinically relevant time frame. However, this technique is extremely technique-sensitive and does not completely reduce dentin permeability or replace the water caused by outward fluid flow without the use of adjunctive tubular occlusion agents (Sadek *et al*., 2007; Cadenaro *et al*., 2009b; Sauro *et al*., 2009b), even with three absolute ethanol applications (Sadek *et al*., 2010a). In the progressive ethanol replacement version of the technique, water is gradually removed from the collagen matrix *via* a series of ascending ethanol concentrations (Sadek *et al*., 2007, 2010b). However, this technique version is time-consuming and impractical for clinical application. For both techniques, when ethanol replacement is not meticulously performed to prevent water-saturated collagen from exposure to air, the surface tension present along the air-collagen interface can easily result in collapse of the collagen matrix and prevent optimal infiltration of the adhesive monomers (Osorio *et al*., 2010).

Since hydrophobic resins are considerably difficult to apply to dentin, an alternative version of the ethanol wet-bonding technique is to apply hydrophilic adhesives to ethanol-saturated demineralized dentin. Wet-bonding with ethanol achieved higher bond strengths even with hydrophilic resins than were possible with water-saturated matrices (Nishitani *et al*., 2006b). When hybrid layers created with commercially available etch-and-rinse adhesive using water wet-bonding or ethanol wet-bonding were compared, significantly less micropermeability of

the fluorescent tracer could be seen in hybrid layers created with ethanol wet-bonding (Sauro *et al*., 2009a). The results suggest that ethanol wet-bonding is capable of increasing resin uptake and producing better sealing of the collagen matrix, even with the use of hydrophilic adhesives. The presence of ethanol probably also increases the degree of conversion of the hydrophilic adhesives. Nevertheless, incomplete removal of ethanol from the hydrophilic adhesives may render the polymerized matrix more susceptible to water sorption when compared with the use of hydrophobic adhesives (Malacarne-Zanon *et al*., 2009). Thus, the use of less-hydrophilic resins in dental adhesives may create more reliable and durable bonds to dentin.

Ethanol wet-bonding is generally conceived to be a bonding philosophy rather than a bonding technique, due to its clinical impracticality. However, it represents a major contribution to adhesive density, since the philosophy behind it reveals the critical barrier to progress in dentin bonding with etch-and-rinse and self-etch adhesives. Hybrid layers created by ethanol wet-bonding were found to contain collagen fibrils with reduced fibrillar diameter and increased interfibrillar spaces (Tay *et al*., 2007; Hosaka *et al*., 2009) (Fig. 3). While this permits high resin uptake, such a phenomenon has never been observed in hybrid layers created during water-wet-bonding with hydrophilic etch-and-rinse adhesives, or bonded with water-containing self-etch adhesives. Milder versions of self-etch adhesives are generally more capable of infiltrating the interfibrillar spaces of a partially demineralized collagen matrix *via* the mechanism of simultaneous etching and resin infiltration. Nevertheless, both self-etch and etch-and-rinse adhesives are incapable of removing water from the intrafibrillar compartments of the collagen fibrils. Hence, the phenomena of fibrillar shrinkage and widened interfibrillar spaces could not be detected in hybrid layers created by these two classes of commercially available adhesives when they were bonded according to the manufacturers' instructions. With the use of these adhesive techniques, apatite crystallites are removed from both the extrafibrillar and intrafibrillar compartments and subsequently replaced by water. If intrafibrillar spaces are replaced by resin during infiltration, there should be no additional room for filling with additional material. Conversely, intrafibrillar spaces created by both classes of adhesives are amenable to remineralization by apatite crystallites (Kim J *et al*., 2010c). Such an observation provides indirect evidence that these water-filled intrafibrillar spaces are not completely replaced by resins. However, when ethanol wet-bonding was meticulously performed, neither nanoleakage (Sadek *et al*., 2008) nor intrafibrillar remineralization (Kim J *et al*., 2010a) could be detected. This indicates that resin can enter the intrafibrillar compartment if it contains ethanol, but not if it contains water.

A recent study showed that resin-dentin bonds created by ethanol wet-bonding with an experimental hydrophobic adhesive did not degrade after 18 months of water storage in the absence of MMP inhibitors (Sadek *et al*., 2010b). This is an important observation, since it addresses the issue that MMPs are not capable of collagenolysis in the absence of water as a functional medium. During collagen mineralization, bulk water and loosely bound water are progressively removed from the internal compartments of the collagen fibrils and replaced by apatite crystallites (Chesnick *et al*., 2008). Encapsulating MMPs and cathepsins with hydrophobic resins represents another innovative way of immobilizing the catalytic and allosteric domains of these enzymes to inactivate their functional activities. Such a method is similar to the technique of molecular imprinting to produce abiotic polymers with enzymatic functions (Takeuchi and Hishiya, 2008), with the exception that the enzyme substrate is not removed to expose their functional sites (Fig. 2B). Even if hydrophilic adhesives may infiltrate a collagen matrix to immobilize collagenolytic enzymes initially, resin hydrolysis *via* esterases and water sorption may subsequently result in re-activation of those immobilized enzymes. Hard tissue fossils from the late Cretaceous era (65 million years ago) were well-preserved at the microstructural and molecular levels and responded to collagenase digestion (Avci *et al*., 2005). Analysis of these data suggests that MMP-bound collagen must have been functionally immobilized by apatite crystallites for the integrity of those collagen fibrils to be preserved. Conversely, when apatites are replaced by water in dentin bonding, the longevity of these resin-dentin bonds has seldom been shown to last for more than 10 years (van Dijken *et al*., 2007). Thus, recapitulating the mechanism of molecular immobilization of the functional activity of collagenolytic enzymes created during evolution appears to be a logical biomimetic strategy for extending the longevity of man-made resin-dentin bonds. This may be achieved with a biomimetic mineralization strategy for remineralizing resin-dentin bonds.

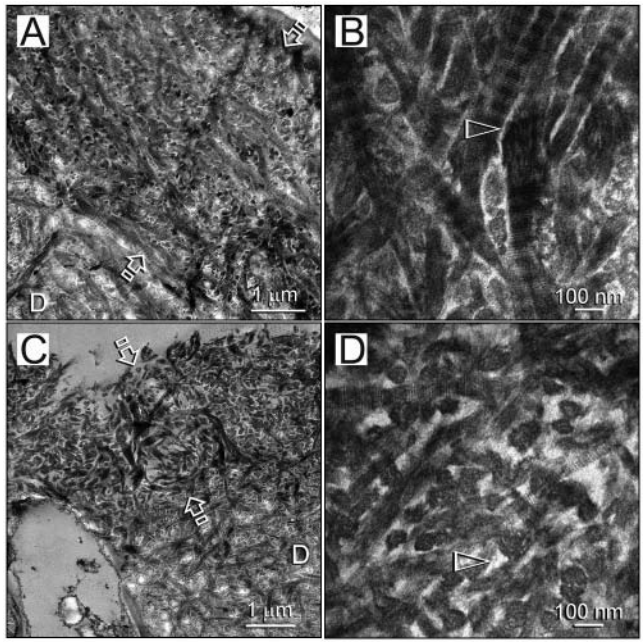

Figure 3. Stained transmission electron micrographs comparing the thickness of hybrid layers (low magnification, between open arrows) and dimensions of the collagen fibrils and interfibrillar spaces (high magnification, open arrowheads). **(A,B)** Resin-dentin interface bonded with a hydrophilic adhesive by the water wet-bonding technique. **(C,D)** Resin-dentin interface bonded with an experimental hydrophobic adhesive by the ethanol wet-bonding technique. D, laboratory-demineralized intact dentin.

Biomimetic Remineralization of Resin-Dentin Bonds

Apart from increases in mechanical properties (Kinney *et al*., 2003; Balooch *et al*., 2008), a major role played by the hierarchical deposition of apatite in mineralized collagen is the exclusion of molecules larger than water (*ca*. 18 Da) from the mineral-protein biocomposite (Lees and Page, 1992). Whereas even ethanol (*ca*. 46 Da) cannot solvate air-dried mineralized dentin, demineralized, water-filled collagen is susceptible to penetration by larger molecules. Molecules larger than 40 kDa are completely excluded from the internal water compartments of type I collagen, while molecules smaller than 6 kDa can diffuse into all of the water compartments within the collagen fibril (Toroian *et al*., 2007). The physical exclusion of exogenous collagenolytic enzymes (bacterial collagenase, 68-130 kDa; activated MMP-2, 67 kDa; activated MMP-9, 85 kDa; activated cathepsin K, 27 kDa) by apatite forms the tenet of the "enzyme exclusion" mechanism that protects archeological collagen from degradation (Nielsen-Marsh *et al*., 2000). Experiments on collagenase hydrolysis of dentin have also confirmed the protective role played by the mineral phase on collagen degradation (Klont and ten Cate, 1991). Similar to a host of growth factors and signaling molecules, endogenous MMPs and cathepsins become "fossilized" (Smith, 2003) within dentin by the apatite minerals, but retain their biologic activities after they are incorporated into the mineralized dentin matrix. These molecules assume their biologic activities upon removal of the mineral phase, provided that the demineralization agent is not strong enough to denature these molecules. As collagen mineralizes, free and loosely bound water is progressively replaced by apatite. This physiologic dehydration mechanism (Chesnick *et al*., 2008) ensures that the internal environment of the mineralized fibril remains relatively dry to preserve the integrity of the entrapped bioactive molecules.

In dentin bonding, the mineral phase of dentin is intentionally removed by acids, chelating agents, or acidic resin monomers to expose the collagen for creating micromechanical retention of resins. Unfortunately, it seems that contemporary etch-and-rinse and self-etch adhesives are incapable of completely replacing water from the extrafibrillar and intrafibrillar collagen compartments with resin monomers. This can be seen by the number of publications on nanoleakage within hybrid layers. Biomimetic mineralization is a proof-of-concept strategy that utilizes nanotechnology principles to mimic what occurs in biomineralization (Tay and Pashley, 2008). This strategy replaces water from resin-sparse regions of the hybrid layer with apatite crystallites that are small enough to occupy the extrafibrillar and intrafibrillar compartments of the collagen matrix, and has been adopted for remineralization of resin-dentin bonds (Tay and Pashley, 2009). By restoring the enzyme exclusion and fossilization properties of mineralized dentin, this proof-of-concept strategy is capable of preserving the longevity of resin-dentin bonds (Kim YK *et al*., 2010a).

Remineralization of apatite-depleted, partially demineralized dentin is not new. Reports on remineralization of carious dentin appeared in the dental literature more than half a century ago. The dental literature abounds with reports on the use of fluoride-releasing glass-ionomer cements (Ngo, 2010) and calcium-phosphate-based composites (Peters *et al*., 2010) for remineralizing partially demineralized carious dentin. Intact, non-denatured collagen is remineralizable as long as seed crystallites are present as nidi for heterogeneous nucleation of calcium phosphate phases (Koutsoukos and Nancollas, 1981). Remineralization by epitaxial growth is a thermodynamically favorable process that overcomes the energy barrier of homogeneous nucleation (Jiang and Liu, 2004). In nanotechnology terminology, remineralization techniques currently used in dentistry represent a top-down approach (Wong *et al*., 2009). This approach creates materials using scaled down versions of a bulk material that incorporates nanoscale details of the original material. Partial demineralization of mineralized collagen matrix by acids derived from bacteria or dentin-bonding procedures creates the seed crystallites necessary for this top-down remineralization approach. The orientation of those remineralized crystalline lattices is pre-determined by the lattice of the original seed crystallites. However, remineralization does not occur in locations where seed crystallites are absent, as demonstrated with the use of a strontium-based glass-ionomer cement (Kim YK *et al*., 2010b). This limitation severely restricts the apatite-sparse hybrid layers created by etch-and-rinse and moderately aggressive self-etch adhesives to be remineralized with a conventional top-down approach.

During biomineralization, there are no apatite seed crystallites present in an organic scaffold. Consequently, biomineralization has to proceed *via* an alternative pathway that involves homogeneous nucleation. One of the mechanisms of homogeneous nucleation involves sequestration of the amorphous mineral phase by polyanionic extracellular matrix protein molecules. Additional acidic matrix phosphoproteins are utilized as templates to induce mineral nucleation and growth within the organic scaffold (George and Veis, 2008). Fluoride, which has been shown to enhance conventional remineralization *via* the top-down approach, is not a functional motif for biomineralization. Homogeneous nucleation is not as thermodynamically favorable and involves alternative kinetically driven protein/polymer-modulated pathways for lowering the activation energy barrier for crystal nucleation *via* sequential steps of phase transformation (Wang and Nancollas, 2009). In nanotechnology terminology, such pathways are examples of a bottom-up approach (Wong *et al*., 2009), wherein nanoscale materials are created by a particle-based self-assembly process (Xu *et al*., 2007; Gower, 2008). This process is compatible with the observation of amorphous calcium phosphate phases in the biomineralization of hard tissues (Mahamid *et al*., 2010).

Although it is possible to utilize recombinant matrix proteins as nucleating agents for *in situ* mineralization, the cost of production of these molecules renders their use in restorative dentistry prohibitive. Accordingly, scientists have resorted to the use of poly(anionic) acid molecules to mimic the functional domains of naturally occurring mineralization-promoting proteins. Biomimetic remineralization of resin-dentin bonds adopts the bottom-up biomineralization approach by using two polyanionic analogs of acidic matrix proteins to separately mimic the sequestration and templating functional motifs that are present in naturally occurring matrix protein molecules (Fig. 4A). This approach does not rely on the presence of remnant dentin matrix proteins within a demineralized collagen matrix and can mineralize reconstituted collagen that is devoid of mineralization-promoting proteins (Kim YK *et al*., 2010c). A polycarboxylic acid such as polyacrylic acid (Girija *et al*., 2004) was used as a sequestration

agent to simulate the aspartic acid and serine-rich mineralization-promoting C-terminal domain (ASARM) of cleaved DMP-1 (Gericke *et al*., 2010) to stabilize amorphous calcium phosphate into fluidic nanoprecursor particles (Gower, 2008; Nudelman *et al*., 2010). The latter has the ability to infiltrate the microfibrillar spaces within collagen fibrils and produce initially amorphous, intertwining mineral strands within the fibril (Kim J *et al*., 2010c). A phosphorus-based analog of matrix phosphoproteins (George and Veis, 2008), such as polyvinylphosphonic acid, is used as a collagen-binding template to induce the nucleation and growth of apatite from the initial amorphous mineral phase (Gajjeraman *et al*., 2008). In the past, sequestration or templating analogs had been separately used for biomineralization, without success in producing intrafibrillar apatites. For example, when only polyaspartic acid was used as the sequestration analog, infiltration of amorphous calcium phosphate nanoprecursors into reconstituted collagen fibrils produced intrafibrillar mineralization that apparently lacked the hierarchical order of apatite arrangement in natural mineralized collagen (Deshpande and Beniash, 2008). Conversely, when only a templating analog was used, only large extrafibrillar mineral spheres were deposited around the collagen matrix (Li and Chang, 2008). Thus, two biomimetic analogs (sequestration and templating) must be utilized in concert to reproduce the dimension and hierarchy of the apatite crystallites that are found in natural mineralized dentin.

Both hybrid layers created by etch-and-rinse adhesives (Mai *et al*., 2009, 2010) and self-etch adhesives (Kim J *et al*., 2010c,d) have been shown to be remineralizable with a biomimetic mineralization approach. For etch-and-rinse adhesives, apatite crystallites can be detected in both extrafibrillar and intrafibrillar spaces, since denuded collagen matrices are present within those hybrid layers. It is amazing how strikingly similar the remineralized part of the hybrid layer is when compared with hybrid layers that have undergone degradation after aging (Figs. 4B, 4C). For self-etch adhesives, apatite deposition is almost exclusively identified from the intrafibrillar spaces, indicating that the extrafibrillar spaces are better infiltrated by adhesive resins. In addition, water-filled spaces within the adhesive layers of some adhesives are also filled by apatite

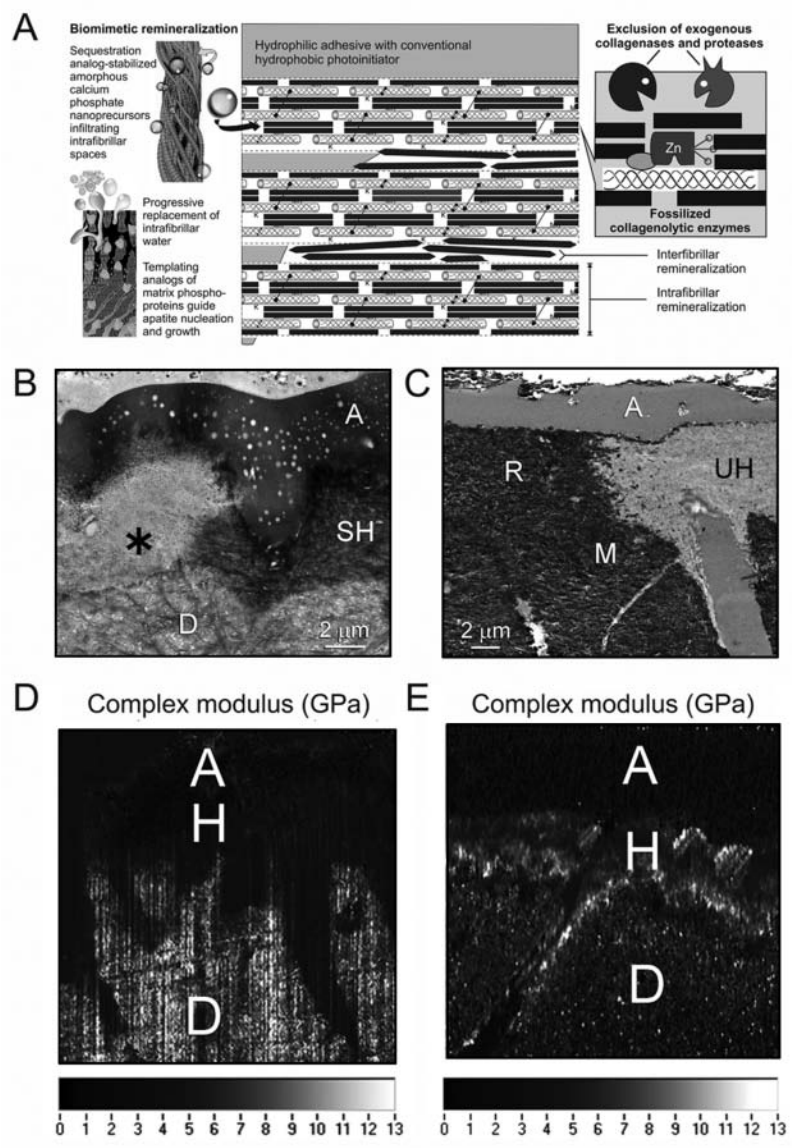

Figure 4. Biomimetic remineralization of resin-dentin bonds as a potential means of preventing the degradation of resin-dentin bonds. **(A)** A schematic depicting the use of a biomimetic remineralization mechanism for refilling water-rich, resin-sparse regions of the hybrid layer with apatite for exclusion of exogenous collagenolytic enzymes and fossilization of endogenous collagenolytic enzymes. Left side depicts the action of a sequestration analog such as polyaspartic acid or polyacrylic acid in stabilizing amorphous calcium phosphate nanoprecursors, and the use of a templating analog such as sodium trimetaphosphate to initiate the nucleation and growth of apatite within the intrafibrillar spaces of a collagen fibril. Right side depicts apatite crystallites fossilizing the collagen molecules, protecting them from exogenous and endogenous collagen-degrading enzymes. M, MMP; K, cathepsin K. **(B)** Stained transmission electron micrograph showing the degradation of collagen fibrils within the hybrid layer (asterisk) after aging. SH, stained, intact hybrid layer; A, dentin adhesive; D, laboratory-demineralized intact dentin. **(C)** Unstained transmission electron micrograph of the resin-dentin interface that has undergone 3 months of biomimetic remineralization with a remineralizing composite and in the presence of sequestration and templating biomimetic analogs present in the incubation medium. Note the striking similarity between the remineralized part of the hybrid layer (R) and the degraded part of the hybrid layer shown in Fig. 4B. UH, unstained, non-degraded part of the hybrid layer; A, adhesive; M, mineralized intact dentin. **(D)** Nanoscopic dynamic mechanical analysis showing the distribution of complex modulus (*i.e.*, loss modulus/storage modulus) across the resin-dentin interface after 3 months of immersion in a control medium. A, adhesive; H, hybrid layer; D, intact dentin. Note the complete lack of high modulus (*i.e.*, light-colored material within the hybrid layer or resin tag). **(E)** Nanoscopic dynamic mechanical analysis showing the distribution of complex modulus (*i.e.*, loss modulus/storage modulus) across the resin-dentin interface after 3 months of biomimetic remineralization. A, adhesive; H, hybrid layer; D, intact dentin. Note the presence of high-modulus material throughout the hybrid layer and resin tag.

nanocrystals (Kim J *et al*., 2010a; Kim YK *et al*., 2010a). Unlike the conventional top-down remineralization approach that proceeds rapidly *via* epitaxial growth over existing seed crystallites, biomimetic remineralization is a slower process, since it involves at least two kinetically driven pathways and usually takes 3-4 months to complete. Thus, it is important to prevent the collagen matrix from degrading while it is being remineralized. In a proof-of-concept biomimetic remineralization strategy, polyvinylphosphonic acid has been shown to possess MMP-inhibiting properties that prevent collagen degradation during remineralization (Tezvergil-Mutluay *et al*., 2010b).

A critical problem encountered with the use of the experimental strategies described in the previous four sections is that the water-filled denuded collagen fibrils remain flaccid and exhibit weak mechanical properties, even with preservation of their integrity (Bertassoni *et al*., 2009). The modulus of elasticity of resin-infiltrated dentin beams increased by 55-118% after biomimetic remineralization as a result of intrafibrillar remineralization of the collagen matrices (Gu *et al*., 2010). A limitation of that study, however, is that the resin-infiltrated dentin beams (macro-hybrid layers) were evaluated *en masse* by three-point bending. Since remineralization does not occur in locations of the collagen matrices that are occupied by resins, three-point bending is insensitive for evaluating the localized increases in modulus of elasticity of the remineralized collagen. Due to the complexity of these regions, we have resorted to the use of nanoscopic dynamic mechanical analysis (nano-DMA) for characterizing the viscoplastic mechanical behavior of resin-infiltrated dentin before and after biomimetic remineralization. This is achieved with scanning probe microscopy (SPM) attached to a tribo-indentor to perform rasterized imaging and nanoindentations across an area of interest (Arola *et al*., unpublished observations). We have developed a method of scanning these hybrid layers under hydrated conditions, preventing water from evaporating. Analysis of our preliminary data from use of the combined nanoDMA and SPM approach indicates that biomimetic remineralization is capable of restoring the storage modulus, loss modulus, and complex modulus of water-rich, resin-sparse regions of the hybrid layer to those values characteristic of intact mineralized dentin (Figs. 4D, 4E).

To date, studies published on biomimetic remineralization of resin-dentin bonds represent a proof-of-concept for that approach. Remineralization was performed *via* a lateral diffusion mechanism by the immersion of specimen slabs in a remineralizing medium containing dissolved biomimetic analogs. Experiments are under development for translating this proof-of-concept strategy into a clinically applicable technique. For example, an experimental calcium-silicate-based hydrophilic composite has been developed for the sustained release of calcium and hydroxyl ions (Kim YK *et al*., 2010c). While this method is useful for remineralizing resin-dentin interfaces with thin adhesive layers (Fig. 4C), remineralization is hampered when a thick adhesive layer is encountered (Dickens and Flaim, 2008). Thus, alternative strategies are being developed to deliver calcium, hydroxyl, and phosphate ions to the base of the hybrid layers created by etch-and-rinse adhesives, where remineralization is most needed. Additional work is also needed to deliver sequestration and templating analogs to the collagen matrix. It is at the nanotechnology level that one would expect the greatest development of translational strategies for biomimetic remineralization as a means to preserve the longevity of resin-dentin bonds.

CONCLUSIONS

Contemporary dentin bonding is not as durable as has previously been assumed. Two major mechanisms are involved in the degradation of resin-dentin bonds over time. One mechanism is the slow hydrolysis of resin components caused by water sorption and/or esterases, which are promoted by the increased hydrophilicity of contemporary adhesive formulations. The other is degradation of water-rich, resin-sparse collagen fibrils within hybrid layers by the activation of host-derived MMPs and possibly cysteine cathepsins during bonding procedures. Complete replacement of free and loosely bound water within the collagen water compartments and inactivation or silencing of collagenolytic enzymes appear to be the ultimate goals for improving the durability of resin-dentin bonds. During the past five years, the knowledge acquired from experimental techniques to increase the service life of resin-based bonding procedures has been nothing short of phenomenal. This pool of knowledge is summarized into five experimental strategies that attempt to address different problems encountered in dentin bonding. While each strategy has its merits and limitations, it is probably the amalgamation and translation of several strategies into a single treatment approach that may overcome the critical barriers currently encountered in bonding to dentin.

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