Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability—A literature review

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ABSTRACT

Objective. Success in adhesive dentistry means long lasting restorations. However, there is substantial evidence that this ideal objective is not always achieved. Current research in this field aims at increasing the durability of resin–dentin bonds. The objective of this paper is to examine the fundamental processes responsible for the aging mechanisms involved in the degradation of resin-bonded interfaces and the potential approaches to prevent and counteract this degradation.

Methods. PubMed searches on the hybrid layer degradation were carried out. Keywords were chosen to assess hybrid layer degradation for providing up-dated information on the basis of scientific coherence with the research objective. Approaches to prevent and counteract this degradation were also reviewed.

Results. 148 peer-review articles in the English language between 1982 and 2015 were reviewed. Literature shows that resin–dentin bond degradation is a complex process, involving the hydrolysis of both the resin and the collagen fibril phases contained within the hybrid layer. Collagen fibers become vulnerable to mechanical and hydraulic fatigue, as well as degradation by host-derived proteases with collagenolytic activity (matrix metalloproteinases and cysteine cathepsins). Inhibition of the collagenolytic activity and the use of cross-linking agents are the two main strategies to increase the resistance of the hybrid layer to enzymatic degradation.

Significance. This review analyzes the issues regarding the durability of the adhesive interface, and the techniques to create stable resin–dentin bonds able to resist the collagenolytic hydrolysis that are currently studied.

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1. Introduction

Resin–dentin bonding involves a series of topical treatments to dentin that completely changes its physical and chemical properties from being a hydrophilic, crystalline, relatively impermeable acid-labile surface, to one that is more hydrophobic, organic, highly permeable, acid-resistant surface.

When dentin is cut or ground with high-speed drills, a 1–2 μm thick layer of cutting debris is left on the surface, called the smear layer [1]. When the smear layer is removed by acid-etching with 37 wt% phosphoric acid for 15 s, all of the mineral phase of the smear layer and underlying 5 μm of mineralized dentin are solubilized, causing the smear layer to dissolve and exposing the underlying type I collagen fibril meshwork of the dentin matrix.

The space between the collagen fibrils (interfibrillar space) is about 30 ± 11 nm [2]. These spaces serve as diffusion channels for solvated adhesive comonomers to infiltrate around collagen fibrils toward the base of the 5 μm deep, demineralized layer of the collagen matrix (Fig. 1) forming the so-called hybrid layer [3] (Fig. 2).

Unlike typical tissue engineering applications where a synthetic scaffold is designed to be resorbed and replaced by the host's own tissues with 2–3 months, dentin adhesion relies on *in situ* tissue engineering, which is designed to create a resin-enveloped collagen scaffold that, ideally, will remain in place for decades. Nevertheless, the hybrid layer may be degraded over time, leading to failure of the adhesive interface [4,5]. Although the incorporation of hydrophilic and acidic resin monomers has substantially improved the initial bonding of contemporary etch-and-rinse and self-etch adhesives to intrinsically wet dental substrates, potential problems associated with these hydrophilic formulations have been reported in several *in vitro* and *in vivo* studies [6–14]. The failure of the adhesive layer leads to the formation of microgaps that are readily penetrated by pathogens. Bonding failure in the presence of bacteria, esterases [15], and dental plaque biofilm [16] provokes a cascade of events leading to deterioration of the adhesive interface and failure of the composite restoration. Recent studies demonstrated that dental composite restorations continue to show limited clinical service as a result of decay and fracture [17,18] and recurrent decay at the composite-tooth interface has consistently been the primary reason for replacement of composite restorations [19].

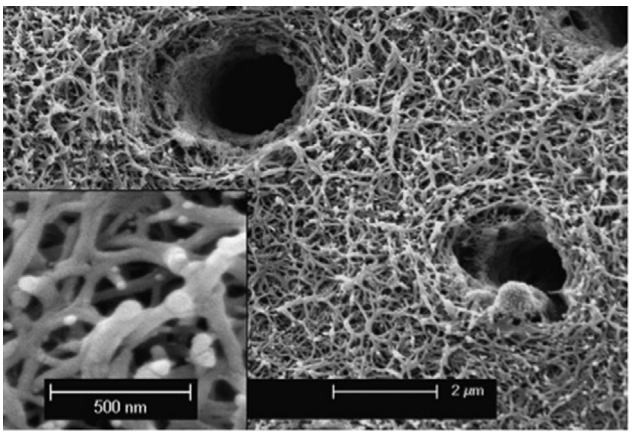

Fig. 1 – Scanning electron micrograph of acid-etched dentin showing two dentinal tubules containing remnants of peritubular dentin matrix. INSERT: High magnification of branching collagen fibrils (ca. 75 nm in diameter) separated by interfibrillar spaces that serve as channels for resin infiltrations during bonding. Reproduced from Pashley et al., Dent Mater 2011;27:1–16, with permission.

We are entering a new era in adhesive dentistry, where resin-bonding to enamel and dentin is beginning to be understood at the nanoscopic level. The long-term success and durability of resin–dentin bonds depends upon the ability of dentists to capitalize on new discoveries being made in adhesive technology.

The objective of this paper is to examine the fundamental processes responsible for the aging mechanisms involved in the degradation of resin-bonded interfaces and the potential approaches to prevent and counteract this degradation. For this purpose, an electronic search was conducted of the PubMed database with different combination of the following search terms: collagen, dentin, hybrid layer degradation, bonding agent, adhesive system, metalloproteinases, cysteine cathepsins, cross-linking agent, EDC. The search was restricted to articles written in English related to dentin collagen degradation. Only articles published in peer-reviewed journals were included. The PubMed search included literature reviews, *in vitro* and *in vivo* studies. Articles written in other languages, without available abstracts, those related to other field were excluded. 148 peer-review articles in the English language between 1982 and 2015 were reviewed.

2. Degradation of the adhesive interface

2.1. *Degradation of the adhesive resin*

Chronic deterioration of the hybrid layer involves hydrolysis and leaching of the adhesive resin that has infiltrated the demineralized dentin matrix [20–22]. Leaching is facilitated by water penetration into the loosely cross-linked or hydrophilic domains of the adhesive. The hydrophilic domains exhibit limited monomer/polymer conversion because of adhesive phase separation [23] and lack of compatibility between the hydrophobic photoinitiator and the hydrophilic phase [24]. The poorly polymerized hydrophilic phase degrades rapidly in the aqueous environment. Resin elution continues to occur while water movement along the length of the hybrid layer becomes more rapid via transport pathways form relatively large water-filled channels [25]. The previously resin-infiltrated collagen matrix is exposed and becomes vulnerable to the attack by proteolytic enzymes [6,26]. The structure of methacrylate adhesives, presenting hydrolytically susceptible groups, such as ester and urethane, as well as hydroxyl, carboxyl, and phosphate groups [27], may be hydrolyzed by chemical and enzymatic degradation in the oral environment [28].

On prolonged exposure of the restoration to oral fluids, water begins to penetrate the resin. Water initially enters the matrix by diffusion into loosely cross-linked or hydrophilic

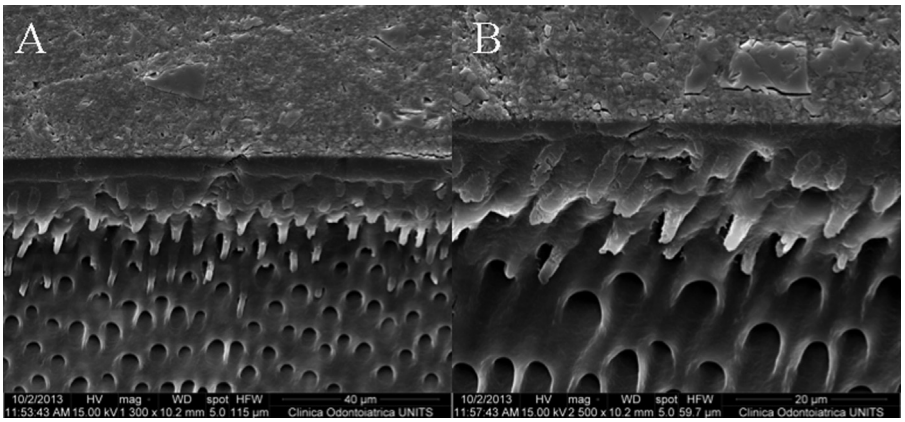

Fig. 2 – Scanning electron micrographs of resin–dentin interface formed by an etch-and-rinse adhesive system. Specimens were decalcified in 6 N HCl for 30 s, followed by deproteinization in 2% NaOCl for 10 min. (A) 1300×, (B) 2500×.

domains or may be trapped within the matrix during photopolymerization [29,30]. This water plasticizes the polymer chains by acting as a molecular lubricant. Mechanical wear of the exposed adhesive may further accelerate matrix degradation by abrading the surface, increasing the surface area and allowing greater entrance of both water and enzymes. The presence of water promotes the chemical hydrolysis of ester bonds in methacrylate materials. After exposure to salivary fluids, local domains of the methacrylate network may become sufficiently degraded and/or hydrophilic to permit access of esterases, which greatly accelerate ester bond hydrolysis.

Hydrolysis is considered a primary reason for resin degradation within the hybrid layer, contributing to the reduction of bond strength over time.

2.2. *Nanoleakage*

Many studies have shown that small ions or molecules can diffuse into the hybrid layer in absence of detectable interfacial gap formation. Sano et al. [31–33] defined nanoleakage as the presence of microporous zones beneath or within the hybrid layer that permitted tracer penetration to occur in the absence of interfacial gaps. Nanoleakage is created by the discrepancy between dentin demineralization and adhesive infiltration that occurs with total-etching adhesive systems, in the absence of evident marginal gap formation along the resin–dentin interface [34].

Self-etch procedures use acidic adhesive resin monomers to simultaneously demineralize and infiltrate the bonding substrate, theoretically avoiding incomplete infiltration. Tay et al. [35], however, reported variable degrees of silver uptake following the use of self-etching adhesives. Thus, nanoleakage is not necessarily caused by disparities between demineralization and resin-infiltration depths. Simplified one-step self-etching adhesives are highly hydrophilic and form hybrid layers that have been found to behave as permeable membranes after polymerization, permitting water movement throughout the bonded interface [35,36]. Moreover, the retention of residual water in etched dentin and/or adhesives may result in regions of incomplete polymerization or increased permeability within adhesive resin matrices [37].

Tay et al. [35] used ammoniacal silver nitrate to trace the distribution of absorbed water within the hybrid layer, and observed reticular and spotted patterns of silver tracer deposition as an indicator of nanoleakage expression. In the reticular mode, silver deposits were oriented perpendicular to the hybrid layer surface. The spotted mode of nanoleakage is thought to represent microdomains in the resin matrices that contain primarily hydrophilic and/or acidic functional groups, in contrast to adjacent, relatively hydrophobic, domains [38]. The same author reported that silver uptake likely indicated areas of increased permeability within the resin matrix. Water had been incompletely removed in these zones, resulting in regions of incomplete polymerization and/or hydrogel formation of the HEMA in the adhesive.

An immunohistochemical approach [39] that distinguishes native collagen fibrils from resin-embedded fibrils has confirmed the interpretations of nanoleakage that implicate hybrid layer porosity. This study found that collagen fibrils within the hybrid layer were not fully embedded by dentin adhesives. Thus, etch-and-rinse and self-etch adhesive systems both fail to fully infiltrate the collagen network, achieving different degrees of infiltration from the top to the bottom of the hybrid layer.

2.3. *Degradation of the collagen fibrils*

Degradation may also affect the collagen matrix of the hybrid layer. Complete coverage of the nanoscale irregularities on the collagen fibrils surface via passive monomer penetration may be difficult to achieve. Thus, fibrils that remain unprotected by hydrophobic resin coatings may be vulnerable to degradation and water is claimed to be a major cause of collagen degradation. Two degradation patterns have been observed within the hybrid layer: loss of resin from interfibrillar spaces and disorganization of collagen fibrils [40]. The degree of collagen fibril envelopment varies, depending on the type of bonding agent [25] that could lead to incompletely infiltrated zones containing denuded collagen fibrils along the bottom of the hybrid layer [41].

Collagen fibrils in the hybrid layer that have been incompletely encapsulated by resin monomer can be identified immune-histochemically [39,42]. Advances in reagent purification and the production of highly specific monoclonal antibodies have permitted the creation of reproducible and selective immune-labeling protocols to analyze collagen or proteoglycans [43]. These protocols use secondary antibodies that are conjugated with gold nanoparticles of different size. Breschi et al. [39] showed that the hybrid layer created by etch-and-rinse adhesives was characterized by different degrees of resin–collagen fibril interactions. More recently Mazzoni et al. [44], using *in situ* zymography with an ELISA assay for interrogating the functional activity of dentin proteases, quantified matrix metalloproteinases (MMPs) activity within the hybrid layer created by an etch-and-rinse adhesive, showing intense and precise fluorescent localization.

2.4. *Intrinsic collagenolytic activity of mineralized dentin*

Recent studies have examined the contribution of host-derived proteinases to the breakdown of collagen matrices in the pathogenesis of dentinal caries [45,46] and periodontal disease [47]. These findings had important implications for dental bonding, but the first evidence of an intrinsic collagenolytic activity of host-derived matrix metalloproteinases (MMPs) in human non carious mineralized dentin came when Pashley et al. [26] demonstrated that dentin demineralized by acid etching slowly degrades in absence of bacteria. These authors speculated that such proteolytic activity could be exerted by dentinal matrix metalloproteinases (MMPs), which had already been shown to be potentially expressed in the dentin-pulp complex [45,46,48].

2.5. *Matrix metalloproteinases*

MMPs are a class of zinc- and calcium-dependent endopeptidases. These endogenous enzymes are important components in many biological and pathological processes because of their ability to degrade almost all extracellular matrix components [49]. Within the oral environment, considerable interest has been devoted to the detection, distribution, and function of host-derived MMPs [50].

Several MMPs have been identified within the dentin-pulp complex compartments. Dentin matrix has been shown to contain at least four MMPs: stromelysin-1 (MMP-3) [51,52], collagenase (MMP-8) [53], and gelatinases A and B (MMP-2 and MMP-9, respectively) [54,55].

Description of the involvement of MMPs in the degradation of demineralized dentin matrix was first mentioned in a study by Tjäderhane et al. [45]. Human MMP-2, MMP-8, and MMP-9 were identified in demineralized dentinal lesions. The experiments conducted in the mentioned study provided critical evidence that bacterial acids are required for the removal of minerals in tooth decay and for the subsequent activation of host MMPs, but bacteria alone could not cause dentin matrix degradation. Therefore, it was assumed that after demineralization, activated host-MMPs would ultimately be responsible for destroying the dentin matrix in caries lesion progression [45]. As previously mentioned, more recently Pashley *et al.* [26] began discussing the likely involvement of MMPs in the degradation of poorly resin-infiltrated hybrid layers. Today, it is thought that the exposure and subsequent activation of these endogenous enzymes during dentin bonding procedures [26,55–57] are responsible for the almost complete disappearance of portions of hybrid layers from resin–dentin bonds that were aged in water [7,56].

2.6. *Cysteine cathepsins*

More recently, another group of proteases were identified in compartments of both sound and carious human dentin, cysteine proteases (CPs) [58,59]. Human CPs are best known from the ubiquitously expressed lysosomal cathepsins B, H, and L, and dipeptidyl peptidase I, which until recently were thought to mediate primarily housekeeping functions in the cell [60]. Of 15 CPs genes detected, 10 were expressed in native pulp tissue and 11 in odontoblasts.

In studies by Tersariol et al. [58] and Nascimento et al. [59], the potential correlation between the MMPs and cysteine cathepsin activities in intact or carious dentin, respectively, was also investigated. Results showed that MMPs and cysteine cathepsin activities expressed highly significant correlations between intact and carious dentin, even though the activities in carious lesions were approximately 10 times higher than in intact dentin. These data indicate that the collagenolytic/gelatinolytic activity of dentin may be due not only to the presence of MMPs but also to cysteine cathepsin synergic activities.

3. Strategies to reduce the intrinsic collagenolytic activity

As previously described, the durability of dentin bonding systems is affected by the degradation of the resin components occurring via hydrolysis of suboptimally polymerized hydrophilic resins and degradation of collagen matrices by MMPs and cysteine cathepsins [25]. MMPs and cathepsins have been shown to be present in dentin [55,58,61,62] and they seem to be responsible for the slow hydrolysis of the collagen fibrils in the hybrid layer that anchors resin composites to the underlying mineralized dentin [63]. To prolong the durability of the resin–dentin bond, inhibition of these proteases has been recommended through the use of synthetic MMPs inhibitors such as chlorhexidine [64–67], quaternary ammonium methacrylates, or benzalkonium chloride [68,69]. Alternatively, the use of collagen-cross-linkers should inactivate these enzymes to retain hybrid layer integrity and strong dentin bonding [70]. Moreover, other approaches have been proposed to reduce the hybrid layer degradation, including dentin remineralization and ethanol wet-bonding [66,71].

3.1. *Protease inhibitors*

3.1.1. *Chlorhexidine*

Most of the experiments designed to improve the durability of dentin bonds through enzyme inhibition have been performed with the use of chlorhexidine (CHX), a potent antimicrobial agent. CHX effectively inhibits MMP-2, -8 and

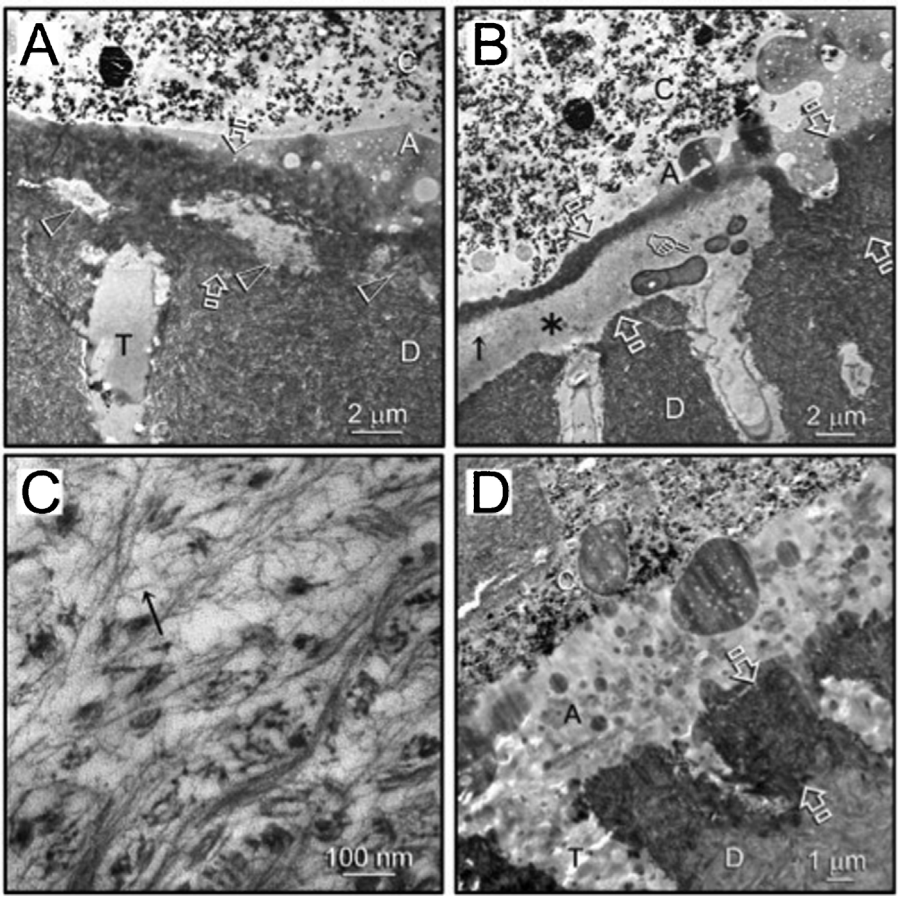

Fig. 3 – TEM images of 18-month-aged hybrid layers created by water wet-bonding in the absence/presence of chlorhexidine diacetate [CHD] and bonding with Single Bond 2. C, composite; A, adhesive. Between open arrows: hybrid layer. D, dentin; T, dentinal tubule. (A) An example of a hybrid layer with initial signs of collagen degradation (open arrowheads) in the subgroup without CHD. (B) An example of a hybrid layer with more severe degradation (asterisk) with possible microbial contamination (pointer) in the subgroup without CHD. (C) High magnification of a region indicated by a black arrow within the degraded hybrid layer in (B) (subgroup without CHD), showing the breakdown of intact collagen fibrils into microfibrillar strands (arrow). (D) An example of an intact hybrid layer that was seen in the CHD subgroup bonded by the same adhesive. Reproduced from Sadek et al., J Dent Res 2010;89:1499–1504, with permission.

-9 [72], and cysteine cathepsins [73]. In 2004, Pashley et al. [26] showed convincing evidence of its efficacy in inhibiting dentin collagenolytic enzymes. Since then, several studies have demonstrated that CHX can preserve the structural integrity of hybrid layer collagen matrix [9,14,64,74–80] and reduce time-dependent reduction in dentin bond strength both *in vivo* and *in vitro* [14,64,74,76–79,81–85].

Even though CHX binding rate to mineralized dentin is almost 80% lower that to demineralized dentin [86], low concentrations of CHX (0.05–0.2%) are sufficient to completely inhibit the collagenolytic activity of untreated dentin powder [26,87], while 0.5–2.0% concentrations are able to only partially inhibit the activity induced with acidic SE primer [87]. Since dentin [88] and MMPs require calcium to maintain their tertiary structure and zinc ions for their catalytic activity [49] and CHX loses its MMP inhibition in the presence of calcium chloride [72], CHX-related MMP inhibition may be due to its chelating property and calcium ions released by the primer may be responsible for the loss of inhibition by CHX. This is supported by the finding that treating dentin powder with Clearfil SE Bond primer for 2 min instead of 20 s not only increased the collagenolytic activity, but also caused the loss of inhibition by 0.5% and 1.0% CHX, with only 2.0% CHX showing significant inhibition [87]. Also the recent reports of chlorhexidine binding by dentin collagen [86,89] suggest that collagen may compete with MMPs for CHX binding, requiring the use of relatively high CHX concentrations.

Incorporation of CHX at reasonably low (0.2–2.0%) concentration into methacrylate comonomers has no effect on water sorption, cause a slight decrease in conversion rate, but may even increase flexural strength and modulus of elasticity of dental adhesives [90]. Chlorhexidine release from the polymerized adhesive is concentration-dependent and continues at a slow steady-state level [90,91]. Since the idea behind the incorporation of inhibitors into adhesive is their continuous release to prevent collagen degradation for a prolonged time, these results are promising (Fig. 3).

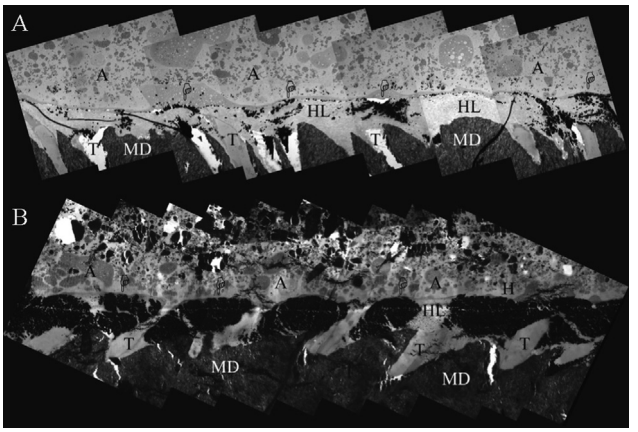

Fig. 4 – TEM images obtained combining several micrographs of (A) a representative specimen treated with galardin for 30 s, then bonded with SB1XT and (B) representative control specimen bonded with SB1XT, stored for 1 year in artificial saliva at 37 °C. In (A) the adhesive [A] interface revealed few scattered clusters (pointers) of silver nanoleakage within the hybrid layers [HL]. In (B) the adhesive interface reveals extensive interfacial silver nanoleakage due to large clusters of silver deposits (pointers) within the collagen fibrils of the hybrid layer [HL]. [MD] mineralized dentin, [T] dentinal tubules and [A] filled adhesive. Bar = 2 μm. Reproduced from Breschi et al., Dent Mater 2010;26:571–578, with permission.

3.1.2. *Galardin*

Galardin is a synthetic MMP inhibitor that exhibits strong activity against MMP-1, -2, -8, and -9. It has a collagen-like structure that binds to the active sites of MMPs. The inhibitory effect of galardin on dentinal MMPs has been confirmed by zymography and interfacial nanoleakage expression after 1 year [65]. Moreover, its incorporation in a commercial primer resulted in inhibition of dentin MMPs, with improved immediate bond strength, even if no change in bond degradation was detected after three months [67]. Galardin (0.2 mM) can inhibit the proteolytic activity of demineralized dentin at concentrations approximately 10–100 times lower than that of CHX (2.2 mM) (Fig. 4) [65].

3.1.3. *Tetracycline*

Tetracycline and their semi-synthetic analogues doxycycline and minocycline are a family of broad-spectrum antibiotics with cationic chelating proprieties that have been reported to serve as effective MMPs inhibitors [48,92,93]. In addition to zinc chelation, they can down-regulate MMPs mRNA expression [94]. Doxycycline, the only clinically available tetracycline with MMPs inhibiting properties, strongly decreases dentin matrix degradation [95]. Broad-spectrum MMP inhibitors such as chemically modified tetracyclines (CMTs) that lack antimicrobial activity, but retain their MMP-inhibiting capacity (CMT-3, Metastat) are particularly effective in inhibiting MMPs in dentinal caries lesion [48,96]. Their mechanism of action is thought to be by inhibition of the activity and secretion of the enzyme as well as calcium chelation [97]. By binding to the enzyme's active site zinc ion, CMTs can alter the conformation of the pro- enzyme molecule, thus blocking its catalytic activity in the extracellular matrix [98].

The effect of tetracyclines and their analogs on the preservation of resin–dentin bonds has not yet been investigated. Because of their MMP inhibiting potential, these compounds deserve further investigation. However the purple stain of teeth known to occur during photo-oxidation may render tetracycline-derived compounds not suitable for clinical use.

3.1.4. *Biphosphonate*

Another class of broad-spectrum proteases inhibitors, bisphosphonates, are known to be effective inhibitors of MMPs presumably by chelating the zinc and calcium ions from the enzymes [99]. In a recent study, Tezvergil-Mulutuay et al. demonstrated potent inhibition of recombinant MMP-9 with less hydroxyproline release and loss of dry mass using polyvinylphosphonic acid (PVPA) [100]. Similar to CHX, PVPA binds electrostatically to collagen. However, its use as a potential MMP inhibitor in dentin bonding may be more advantageous than CHX in that PVPA can be trapped in collagen matrices via the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [101]. These results suggest that the use of PVPA could enhance the longevity of the dentin–resin bonds. The literature in the field of bisphosphonates as MMP inhibitors is still scarce and thus future work should be performed to evaluate the long-term bond degradation of adhesive interfaces containing PVPA and bisphosphonates.

3.1.5. *Quaternary ammonium compounds*

Other cationic compounds, such as quaternary ammonium compounds, may also inhibit dentinal MMPs and thus stabilize the adhesive interface over time. Quaternary ammonium salts are positively charged at physiological pHs and have an effective antibacterial activity. Similar to CHX, these compounds are cationic, water-soluble, but unlike CHX they may not leach out of bonded interfaces. QAMs inhibit soluble MMP-9 as or more effectively than Galardin, and almost completely inhibit the demineralized dentin collagen degradation [68].

Polymerizable quaternary ammonium methacrylates (QAMs), especially 12-methacryloyloxydodecylpyridinium bromide (MDPB) have been incorporated into self-etching primers because they possess antimicrobial properties and can copolymerize with adhesive monomers [102,103]. *In vitro* and clinical experiments have also indicated that QAMs (namely MDPB in Clearfil Protect Bond) may inhibit collagenolytic enzymes in the hybrid layer [104,105]. MDPB proved to be the most effective quaternary ammonium methacrylate yet studied [68]. However, other studies have reported reductions in bond strength comparable to other adhesives [106,107], so it may be too early to make any definitive conclusions of the clinical efficacy of MDPB in the preservation of hybrid layer. Antonucci et al. recently evaluated the synthesis of two dimethacrylates with quaternary ammonium functionalities [108], demonstrating the potential of these bioactive monomers. Whether blends containing quaternary ammonium groups can also exert anti-MMP properties at the hybrid layer and thus, increase the durability of resin–dentin bonds, requires further investigation.

Benzalkonium chloride (BAC) is a mixture of alkylbenzyldimethylammonium chlorides of various alkyl chains. It is

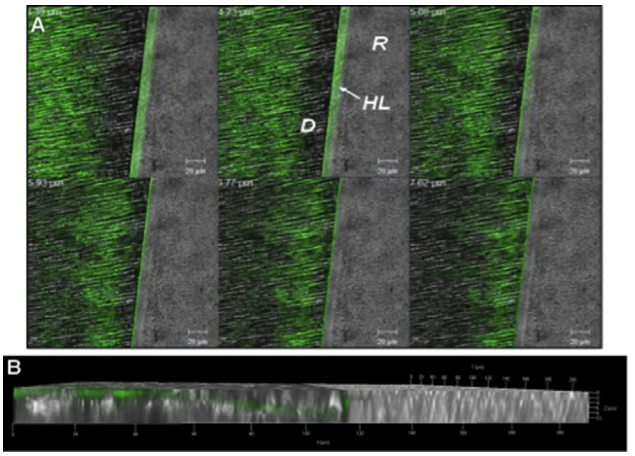

Fig. 5 – Sequence of acquired images in the green channel of the multi-photon confocal microscope superposed on images obtained with differential interference contrast (A, B). (A) An intense fluorescence was seen within the hybrid layer and within the underlying mineralized dentin at different focal depths (i.e., 3.39, 4.23, 5.08, 5.93, 6.77, 7.62 μm below the surface). The shallow depths reveal relatively uniform gelatinolytic activity, while the deeper depths show activity mainly at the base of the HL. (B) Shows the presence of hydrolyzed fluorescent-gelatin within all focal planes at Z axis. [R] resin composite, [HL] Hybrid layer, [D] Dentin. Reproduced from Mazzoni et al., J Dent Res 2012;91:467–472, with permission.

a cationic surface-acting agent with a quaternary ammonium group used as antimicrobial agent and surfactant [69]. BAC-containing etchants can be used with etch-and-rinse adhesives without affecting immediate bond strength to enamel or dentin [109]. Tezvergil-Mutluay et al. [69] demonstrated that 0.5% BAC concentrations completely inhibited soluble MMP-2, -8 or -9, and produced significant reduction in demineralized dentin collagen degradation. BAC molecule binds strongly to demineralized dentin even after rinsing increasing the amount that remains viable in the hybrid layer exerting its anti-MMP properties. The use of BAC as anti-proteolytic agent has gained attention only recently. The long-term survival of adhesive interfaces created with etch-and- rinse adhesive systems, which have been treated with various concentrations of BAC, has been investigated only recently with encouraging results [110,111].

3.2. *Collagen cross-linking agents*

Even though CHX inhibits both MMPs [72], and cysteine cathepsins [73], the potential disadvantage is that CHX may leach out of hybrid layers within 18–24 months [8,106,112–117]. Cross-linking agents are considered an interesting option for improving the stability and resistance of collagen degradation within the demineralized dentin matrix [118,119]. Covalent cross-linking agents produced with external cross-linkers are very stable, and may inactivate the active sites of dentin proteases by reducing the molecular mobility of the active site or by changing negatively charged ionized carboxyl groups into positively charged amides. Thus, an increase in the extent of cross-linking of the collagen fibrils prior to adhesive application may result in increased bonding durability.

This approach has been recently investigated by some authors with the aim to reinforce collagen fibrils through intermolecular cross-linking [120–122]. Since lower biodegradation rates and high mechanical properties of collagen are desirable, the use of collagen cross-linking agents in adhesive procedures has gained increased popularity in recent years [63,66]. The use of glutaraldehyde, proanthrocyanidin and genipin [123], riboflavin [124], as well as tannic acid [122] and carbodiimide [70] has been proposed to enhance the mechanical and structural stability of dentin collagen, leading to a stable dentin matrix network that, after resin infiltration, should provide a durable hybrid layer [125–129]. Moreover, collagen cross-linking molecules have been reported to improve the resistance of uncross-linked or mildly cross-linked collagen matrices to degradation by bacterial collagenases [130,131], potentially contributing to the stabilization of the resin–dentin interface over time.

In addition to the proven efficacy of collagen cross-linking agents in chemical or physical modification of the dentin collagen substrate, the clinical applicability of these solutions is desirable. Even though the results so far have been promising, problems remain to be solved before the clinical applications of cross-linking products become available.

The mechanism of glutaraldehyde cross-linking is known to be dependent on the formation of covalent bonds between the amino groups of proteins and the aldehyde groups of glutaraldehyde. A recent study demonstrated an increased modulus of elasticity following treatment of acid-etched dentin with 5.0% glutaraldehyde for 1 min [129]. By improving dentin's mechanical properties, it is expected that glutaraldehyde would also contribute to improving the resistance to bond degradation. However, despite its efficacy [118], glutaraldehyde is strongly toxic.

Topical treatment with 3.75 mass% proanthocyanidin for 5, 15 or 30 s of 10-μm deep demineralized dentin provided enough cross-linking to protect dentin matrix when incubated in a 0.1 mass% solution of bacterial collagenase, while untreated controls lost all their demineralized dentin matrix after one hour of incubation in the collagenase solution [132].

Grape-seed extract is also effective [118] and leads to an increase of the immediate dentin bond strength in reduced application times [133], but it stains the dentin brown and the durability of long-term bond strength remains to be examined.

Low-dose riboflavin has also been tested as dentin cross-linking agent in conjunction with UVA or dental blue light with good success [124,134]. These studies have shown effective MMP inhibition and stabilization of the adhesive interfaces [124], as well as increased mechanical properties, stability, and resistance to dentin collagen degradation [135]. Riboflavin displays additional advantages, which offer potential for application in adhesive dentistry, including its biocompatibility, easy application and activation with UVA blue light [135].

3.2.1. *1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC)*

1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), a cross-linking agent with very low cytotoxicity, has shown

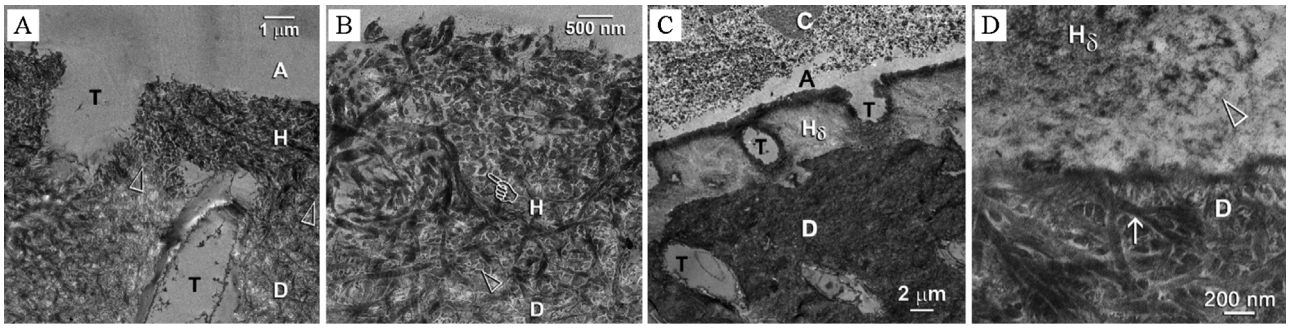

Fig. 6 – (A and B) Stained TEM of acid-etched specimen bonded with an experimental BisGMA/TEGDMA adhesive under ethanol-wet bonding conditions. Appearance of bond after 1 yr of water storage. (A) Low magnification view showing a 3 μm thick hybrid layer with an intact collagen network. Open arrowheads: base of hybrid layer. (B) High magnification view showing that the interfibrillar spaces within the hybrid layer (pointer) are wider than those in the underlying laboratory demineralized dentin (open arrowhead). The wider interfibrillar spaces created by ethanol-wet bonding are probably caused by the shrinkage of interfibrillar proteoglycans in absolute ethanol and provided a higher resin/collagen ratio within the hybrid layer. (C and D) TEM micrographs taken from stained, demineralized sections of 1yr aged composite-dentin beams bonded with Scotchbond Multi-Purpose, the control three-step adhesive using conventional water moist bonding. (C) Low magnification view of the resin–dentin interface. Intact collagen fibrils were only observed as a thin, intensely stained layer along the surface of the hybrid layer and around the periphery of the dentinal tubule orifices. The rest of the hybrid layer was grossly degraded with no identifiable banded collagen fibrils. (D) High magnification view of the base of the degraded hybrid layer depicting degradation of the collagen fibrils into short, electron-dense microfibrils (gelatin) (open arrowhead). By contrast, intact banded collagen fibrils (arrow) were present in the laboratory demineralized intertubular dentin beneath the hybrid layer. [A] adhesive resin, [H] hybrid layer, [D] intertubular dentin; [T] dentinal tubule, [C] resin composite, [H_{δ}] degraded hybrid layer. Reproduced from Sadek et al., Dent Mater 2010;26:380–386, with permission.

promising results in reducing dentin collagen degradation and preserving dentin bond strength over time with clinically acceptable application times [70,127,136].

EDC contains a functional group with the formula RN=C=NR. The carbodiimide reacts with ionized carboxyl groups in proteins to form an O-acylisourea intermediate that can react with a non-proteinated amino group and an adjacent protein chain to form a stable covalent amide bond between the two proteins, with the only by-product being urea. This category of cross-linking agent may inactivate the active sites of dentin proteases by reducing the molecular mobility of the active site or by changing negatively charged ionized carboxyl groups into positively charged amides. Additionally, EDC can induce cross-linking of both helical and especially telopeptide domains in collagen and may also prevent telopeptidase activity that would normally remove bulky telopeptides from the specific peptide bond of collagenases [136]. Increases in collagen stiffness may prevent MMPs from "unwinding" collagen peptides [137]. Since this "unwinding" is necessary to allow MMPs catalytic site to cut the peptide [25,63,66,138], it would also effectively inhibit MMPs functional activity.

In a recent study Mazzoni et al. [127], demonstrated that the use of 0.3 M EDC pre-treatment improves durability and structural integrity of resin–dentin interfaces after 1 year when bonding procedure were applied with two different total-etch adhesives. In addition, the effect of 0.3 M EDC on dentinal MMPs activity by means of zymographic analysis was tested, demonstrating its efficacy to completely inactivate dentinal gelatinases.

In a further study [128] authors showed nearly complete inactivation of MMP-2 and -9 when 0.3 M EDC pre-treatment was applied prior to bonding procedures with two different total-etch adhesives, while the application of the same two adhesives systems alone to acid-etched dentin resulted in activation of dentinal gelatinases. Moreover, as *in situ* zymography technique was performed with the aim to obtain precise localization of the MMP activity within the hybrid layer created by the tested total-etch adhesives without previous extraction of enzymes from the tissue. Gelatinolytic activity was clearly detectable within the hybrid layers and along the tubular wall dentin extending from the dentinal tubules. Furthermore, the location of the activity well correlates with the demineralized uninfiltrated collagen layer in simplified total-etch adhesives at the bottom of the hybrid layer, an area also known for nanoleakage expression and the presence of naked collagen fibrils [25], as well as with the effectiveness of a 0.3 M EDC primer applied before bonding to inactivate protease activity within the hybrid layer (Fig. 5).

Since self-etch adhesive systems do not require dentin surface demineralization before their application, a concern is that this strategy cannot be used for this kind of bonding agents. Future studies should consider incorporating cross-linking agents such as EDC into adhesive formulations. Thus, cross-linking agents could be applied in association with resin monomers as a controlled drug-delivery system to provide collagen cross-linking molecules at the interface over time and promote preservation and self-healing of the adhesive interface.

3.3. Other clinical approaches

3.3.1. Ethanol wet-bonding

To prevent the degradation of resin–dentin bonds, another approach consisting in the use of ethanol wet-bonding with hydrophobic etch-and-rinse systems has been proposed [139]. Dentin bonding with contemporary hydrophilic etch-and-rinse adhesives produced higher residual water concentrations that result in greater matrix porosity upon solvent evaporation and incomplete infiltration of hydrophobic monomers. Pretreatment of water-saturated collagen matrix with 100% alcohol removes matrix water and prevents phase separation of hydrophobic monomers (e.g., Bis-GMA) and provides an opportunity to coax hydrophobic monomers into the matrix. Hydrophobic monomers decrease water sorption/solubility and resin plasticization (Fig. 6). Recent studies using two-photon laser confocal microscopy and micro-Raman spectral analysis [140,141] found that a relatively homogenous distribution of hydrophobic resin within the hybrid layer can be achieved with ethanol wet-bonding. Comparison of the hybrid layers created with commercially available etch-and-rinse adhesives using water or ethanol wet-bonding found significantly less micropermeability of the fluorescent tracer in hybrid layers created with ethanol wet-bonding [140]. Nevertheless, incomplete removal of ethanol from hydrophilic adhesives may render the polymerized matrix more susceptible to water sorption, when compared to the use of hydrophobic resins [142,143].

3.3.2. Biomimetic remineralization

Several interesting attempts to regenerate dental tissue have been recently reported. Biomimetic mineralization is a proof-of-concept strategy that utilizes nanotechnological principles to mimic natural biomineralization [144]. This strategy replaces water from resin-sparse regions of the hybrid layer with apatite crystallites that are sufficiently small to occupy the extra- and intrafibrillar compartments of the collagen matrix, and has been adopted for the remineralization of the resin–dentin bond. Specimen slabs were immersed in a remineralizing medium containing dissolved biomimetic analogs and remineralization proceeded through a lateral-diffusion mechanism. The translation of this proof-of-concept strategy into a clinically applicable technique is currently under development.

4. Conclusions

Most dental adhesive systems currently used show favorable immediate results that reflect good retention and sealing of bonded interfaces [145]. Despite this immediate efficacy, dentin-bonded interfaces may not adequately withstand aging and may show long-term degradation. Clinical trials evaluating dental adhesives have found dramatically low bonding effectiveness for some materials, but greater bond stability in other materials [146]. Several studies have related bond failure over time to the degradation of resin polymers, initiated with the elution of unreacted monomers and leading to water sorption and polymer swelling [25]. The incorporation of hydrophilic and acidic resin monomers substantially improved the initial bonding of contemporary etch-and-rinse and self-etch adhesives to intrinsically wet dental substrates, providing quite favorable immediate results, regardless of the bonding approach used; but, in the long term, the bonding effectiveness of most simplified etch-and-rinse and self-etch adhesives drop dramatically. Experimental strategies that aim to enhance the adhesive interface, particularly improving the durability of the resin–dentin bond strength by inhibiting intrinsic collagenolytic activity and increasing the resistance of dentin collagen matrix to enzymatic degradation are needed.

Chlorhexidine (CHX) has been used as a non-specific MMP inhibitor [72] to prevent degradation of hybrid layers [9,14,64,74–79]. However, CHX is water-soluble and may leach out of hybrid layers, compromising its long-term anti-MMP effectiveness [117]. An entirely different approach is to treat the acid-etched dentin containing activated matrix-bound MMPs with cross-linking agents that inactivate the catalytic site of proteases [66]. Recent experiments [120,122,125] have sought to increase the longevity of resin–dentin bonds with various cross-linking agents, such as glutaraldehyde, genipin, proanthrocyanidin, and carbodiimide. These *in vitro* studies have demonstrated that the use of cross-linking agents improved the short-term mechanical properties of dentinal collagen, and reduced the susceptibility of additionally cross-linked dentinal collagen to enzymatic degradation by collagenases. In particular, carbodiimides have been used as alternative cross-linking agents to gluteraldehyde, since they contain no potentially cytotoxic aldehyde residuals [147,148]. Previous research successfully utilized EDC to increase the durability of resin–dentin bonds by increasing the mechanical properties of the collagen matrix [70,136].

Further improvements of existent dental materials remain necessary. New dental material types may, however, be created using nanotechnologies and other novel approaches within the fields of materials science and biomaterials. These developments could include antimicrobial properties, MMPs and cathepsins inhibition, collagen strengthening properties, and dental hard tissue regeneration. In conclusion, while there are still some unresolved problems regarding the durability of the adhesive interface, it is truly remarkable to see how far adhesive bonding has come in the past 50–60 years. Techniques able to create stable resin–dentin bonds able to resist the collagenolytic hydrolysis will be probably available in the next few years, improving the quality of dental therapies.

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