Review

# Dentin bonding systems: From dentin collagen structure to bond preservation and clinical applications

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ABSTRACT

*Objectives.* Efforts towards achieving durable resin–dentin bonds have been made for decades, including the understanding of the mechanisms underlying hybrid layer (HL) degradation, manufacturing of improved adhesive systems, as well as developing strategies for the preservation of the HL.

*Methods.* This study critically discusses the available peer-reviewed research concerning the formation and preservation of the HL, the mechanisms that lead to the degradation of the HL as well as the strategies to prevent it.

*Results.* The degradation of the HL occurs through two main mechanisms: the enzymatic degradation of its collagen fibrils, and the leaching of the resin from the HL. They are enabled by residual unbound water between the denuded collagen fibrils, trapped at the bottom of the HL. Consequently, endogenous dentinal enzymes, such as the matrix metalloproteinases (MMPs) and cysteine cathepsins are activated and can degrade the denuded collagen matrix. Strategies for the preservation of the HL over time have been developed, and they entail the removal of the unbound water from the gaps between the collagen fibrils as well as different modes of silencing endogenous enzymatic activity.

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*Significance.* Although there are many more hurdles to be crossed in the field of adhesive dentistry, impressive progress has been achieved so far, and the vast amount of available research on the topic is an indicator of the importance of this matter and of the great efforts of researchers and dental material companies to reach a new level in the quality and longevity of resin–dentin bonds.

## 1. Introduction

Adhesive systems can be considered revolutionary in many aspects of conservative dentistry, making possible previously inconceivable clinical maneuvers. Current adhesive systems allow clinicians to bond to tooth structure without the need of a retentive cavity since they provide immediate bond strength.

Two different strategies can presently be employed in resin bonding procedures: the etch-and-rinse technique (E&R) and the self-etch (SE) or etch-and-dry technique. Regardless of the strategy used, dentin bonding relies on the formation of the "hybrid layer" (HL), a structure composed of demineralized collagen fibrils reinforced by the resin matrix [1,2].

The goal of adhesive procedures is to form and maintain a tight adhesive-dentin interface that is stable for a number of years, providing retentive strength, marginal seal, and clinical durability [3]. However, regardless of the advances in dental materials, the HL created on the variable and dynamic organic dentin phase is not perfect, and may fail over time, inducing marginal discolorations, marginal leakage and subsequent loss of retention of the composite restoration [3–8].

The aim of this review is to analyze and critically assess the available research on the factors that influence the stability of the resin–dentin bonds and the strategies for preservation of the adhesive interface over time.

## 2. Adhesive systems and adhesion strategies to dentin

Since resin monomers themselves cannot infiltrate mineralized tissues, traditionally, adhesive bonding systems consist of an acid, primer and adhesive. Acid is used for the removal of mineral crystals and exposure of the collagen fibrils. Primer is a hydrophilic solution of resinous monomers, which allows the infiltration of the resinous monomers, especially in demineralized dentin. The adhesive itself contains mixtures of monomers that penetrate the surfaces treated with the primer, creating a mechanical adhesion to dentin [9]. These components can be presented in separate bottles or together, being carried out in one, two or three clinical application steps.

Van Meerbeek et al., in 2003, suggested a classification of the adhesive systems according to the way they interact with the dental substrate, dividing it into two categories: E&R and SE technique (Fig. 1) [10]. In the E&R strategy, an acid etchant is used to remove the smear layer and create a superficial layer of demineralized dentin approximately 5–10 μm deep. The exposed mineral-free network of collagen is suspended in the rinse-water. That water must be completely replaced by adhesive blends if one wishes to achieve a stable bond [4,10,11]. However, complete infiltration of monomers into the wet and demineralized dentin is not consistently achieved,

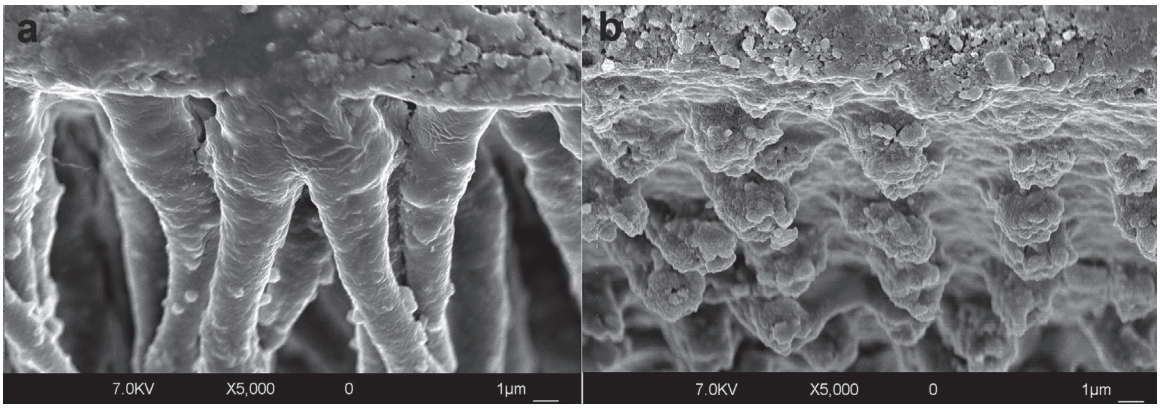


**Fig. 1 – FEISEM micrographs of an (a) E&R and (b) SE adhesive system. Bonded interfaces were created with Scotchbond 1 (3M ESPE) and Protect Bond (Kuraray) in deep dentin tissue. HLs were then exposed with a slow speed diamond saw and dentin was dissolved by sequential rinses in hydrochloric acid and sodium hypochlorite to reveal resin penetration. Resin tags are clearly detectable in the E&R adhesive systems (a) since they infiltrated dentin tubules funneled by the etching agent. SE adhesives often infiltrate no further than the smear layer and smear plugs, revealing a more homogenous morphology that is devoid of long resin tags. Reprinted from Breschi et al. [6], with permission. *FEISEM* field emission in-lens scanning electron microscopy, *E&R* etch and rinse technique, *SE* self-etch technique, *HL* hybrid layer.**

leaving incompletely infiltrated zones along the bottom of the HL containing denuded collagen fibrils [12–14] surrounded by rinse-water. This has been confirmed by immunohistochemical labeling of acid-etched, resin-infiltrated dentin, after staining with anti-type I collagen antibodies. This revealed a weak labeling of collagen fibrils at the top half of the HL, but an intense labeling of collagen fibrils in the deepest part of the HL [15]. These results suggest that resin encapsulation of acid-etched collagen fibrils is more complete in the top half of the HL, but not in the bottom half.

In the SE strategy, a separate acid-etching step is not required since the adhesive co-monomers simultaneously demineralize and infiltrate the dentinal substrate, decreasing the discrepancy between the depth of demineralization and the depth of resin infiltration, creating a more homogenous resin infiltration of demineralized collagen fibrils when compared to E&R systems [15–17]. The stability of the SE adhesive bonding technique depends on the effectiveness of the coupling between the collagen fibril substrate and the co-monomers [7]. Some studies reported a reduced amount of porosities and more homogenous resin infiltration and a better protection of collagen fibrils in SE adhesives compared to the E&R technique [18]. Immunohistochemical labeling with anti-type I collagen antibodies bound to colloidal gold nanoparticles confirmed these findings by presenting a weak, uniform gold labeling of the HL [15]. However, the efficacy of SE bonding on enamel without separate acid etching is still questionable [19,20].

Currently, there is an ongoing trend in adhesive dentistry that tends to simplify bonding procedures by reducing application steps and shortening clinical application time. Thus, a new adhesive system has been created, the Universal Adhesive (UA). These latest adhesives can be used either in the E&R or the SE mode with an additional chemical bonding agent. There is relatively little information on the clinical performance of UA, but it has been confirmed that this new type of adhesives cannot infiltrate to the full depth of demineralized dentin created by phosphoric acid in the E&R strategy [21]. In contrast, the HL of UA with the SE technique seems to be shallower and more durable, since this adhesive contains functional monomers capable of chemically interacting with hydroxyapatite and maintaining the collagen fibrils protected over time [21–24].

## 3. Dentin collagen structure

In order to better understand the resin infiltration processes underlying collagen impregnation during HL formation and degradation over time, it is necessary to understand the basic structure and composition of dentin, with special attention to the organic matrix, and the changes that occur in the structure during adhesive procedures.

Dentin is a mineralized collagen matrix that contains approx. 30–50 vol% organic material and approx. 20 vol% of water [25]. The composition of dentin can vary in different areas of the tooth, depending on its proximity to the pulp tissue, as well as whether the matrix is demineralized or caries affected/infected. These differences can greatly influence the mechanical properties of dentin, as well as the success of bonding to dentin [26].

The extracellular organic dentinal matrix (ECM) has been studied by means of transmission electron microscopy, field-emission scanning electron microscopy and atomic force microscopy, revealing it as a complex 3-dimensional network of fibrillar collagen and globular entities that become mineralized by nanoscopic apatite crystallites during the process of dentinogenesis [27].

Collagen type I comprises the majority of the collagen structure (90 wt%), that also contains traces of collagen V and III [25]. The remaining constituents of the ECM are the non-collagenous proteins, among which the proteoglycans are the most prominent. Others include dentin sialoproteins,

phosphoproteins, bone morphogenic proteins, enzymes and growth factors [28,29].

A collagen molecule consists of three α chains, two α1 and one α2 chain intertwined into a left-handed triple helix [30–32]. Collagen molecules are further intertwined into a right-handed helix (300 kDa, 300 nm of length, 1.5 nm diameter, containing around 1000 amino acids). Collagen chains consist of three domains: a central triple helical region (>95%), a non-helical aminoterminal (N-telopeptide) region and a carboxyterminal (C-telopeptide) region [33]. These peptide chains spontaneously form insoluble collagen fibrils by aggregating and stacking in parallel. These collagen fibrils contain a 67 nm gap between the neighboring collagen molecules, and are further organized in bundles [27]. During the process of dentin maturation, apatitic mineral crystallites precipitate and first fill the 67 nm gaps between the collagen molecules and then the interfibrillar spaces [34], thereby inactivating enzymes that are present in the ECM and were active during the dentinogenesis [35].

Unlike insoluble collagen in other bodily systems, dentinal collagen does not metabolically [25] turn over, meaning that it is not easily degraded, but once it is, it cannot be replaced. This stability is due to the slow formation of covalent inter- and intramolecular cross-links, which occur between the C-terminal of one collagen molecule and the N-terminal of the adjacent collagen molecule [33]. Hydrogen bonds also play a role in the stabilization of the triple helix by bridging the water-filled gaps between the collagen molecules, thereby bringing them closer together and facilitating intra- and intermolecular reactions [36]. Because dentin collagen does not turn over, the natural cross-links accumulate over time [37] and can influence the mechanical properties of collagen fibrils [37–39]. Dentin collagen is the most cross-linked collagen in the body. These cross-links are responsible for the ability of dentin collagen to be acid-etched during bonding procedures without denaturing its collagen [40].

Type I collagen fibrils represent the pillar of the collagen structure, perpendicularly connected by non-collagenous proteins [41,42]. Of the non-collagenous dentinal proteins, the most prominent ones are the proteoglycans (PGs), which consist of a core protein, glycosaminoglycans (GAGs) and linkage proteins [43]. PGs are involved in the process of dentin mineralization and the maintenance of the structural three-dimensional integrity of collagen fibrillar alignment [43,44]. Moreover, these proteins can bind and organize water molecules, regulating the affinity of collagen to water and can affect the substitution of water during the formation of the HL [45–48]. Sensitive selective immunolabeling protocols reveal the three-dimensional relationships between the different structural elements of the ECM with great precision [17,48–50].

There have been conflicting reports on the influence of proteoglycans on collagen degradation. Bedran-Russo et al. [51] reported a decrease in bond strength after the removal of GAGs from the demineralized surface of dentin. Mazzoni et al. [52], on the other hand, demonstrated an increase in resin–dentin bond strength after the removal of PGs. Perhaps this discrepancy can be attributed to their ability to form a hydrophilic interfibrillar nano-network, supporting the collagen structure, while simultaneously facilitating the penetration of water, thus contributing to the hydrolysis of the collagen matrix.

Dentinal collagen can withstand adhesive procedures that would destroy the structure of the dermal collagen [40]. If the acid etching step is limited to the recommended 15 s, the structural integrity of the ECM is preserved [17,48,50]. Over-etching could, however, induce structural changes in the collagen molecules [17] as well as PGs [45,47], introducing a damaged organic part into the HL, and should therefore be avoided (Fig. 2).

## 4. Resin degradation

Two general patterns of degradation of the HL have been described: the disorganization and solubilization of collagen fibrils and the hydrolysis and leaching of the adhesive resin from the interfibrillar spaces [53,54].

Hydrolytic degradation occurs only in the presence of water and is a chemical reaction capable of breaking covalent bonds between polymers causing loss of the resin mass [6,55]. Hydrolysis is considered the primary reason for resin degradation within the hybrid layer [56]. Dentin is a naturally moist substrate, therefore intrinsically hydrophilic. Hence, contemporary adhesives contain mixtures of hydrophilic resin monomers, such as two-hydroxyethyl methacrylate (HEMA), in diluents and organic solvents, usually water, ethanol or acetone. These hydrophilic resin monomers are essential for the infiltration of the adhesives through the wet and demineralized dentin causing the hybridization of the adhesive with the substrate [9,57]. However, these hydrophilic resin monomers in adhesives formulations cause high sorption of water by the resin systems and generate a HL that behaves as a permeable membrane after polymerization, permitting water movement throughout the bonded interface [58,59]. The water movement begins as a diffusion mechanism, followed by the creation of large water-filled channels that rapidly degrade the hydrophilic phase of the adhesive [60,61]. Water penetration into the hydrophilic domains of the adhesive facilitates the leaching of the solubilized resin. As the previously resin-infiltrated collagen matrix is solubilized and is slowly extracted, the underlying insoluble collagen fibrils are exposed and become vulnerable to attack by proteolytic enzymes [62]. Matrix proteases are hydrolases. They add water across specific peptide bonds to break the collagen “polymer” into smaller units.

The presence of residual water in acid-etched dentin and/or adhesives may additionally decrease the polymerization of the adhesive monomers, contributing to increased permeability within the adhesive layer [60]. Irrespective of the bonding system and the number of steps required for its application, all adhesive systems exhibit variable degrees of incomplete polymerization that can be correlated to the extent of fluid movement throughout the adhesive layer [6,62]. Besides the presence of water [63], other factors have been investigated as potential causes of incomplete polymerization of adhesive resin blends, in which both hydrophilic and hydrophobic domains coexist: adhesive hydrophilicity [64], monomer structure and functionality [65,66] and solvent type and concentration [64,67,68]. Nano-phase sepa-

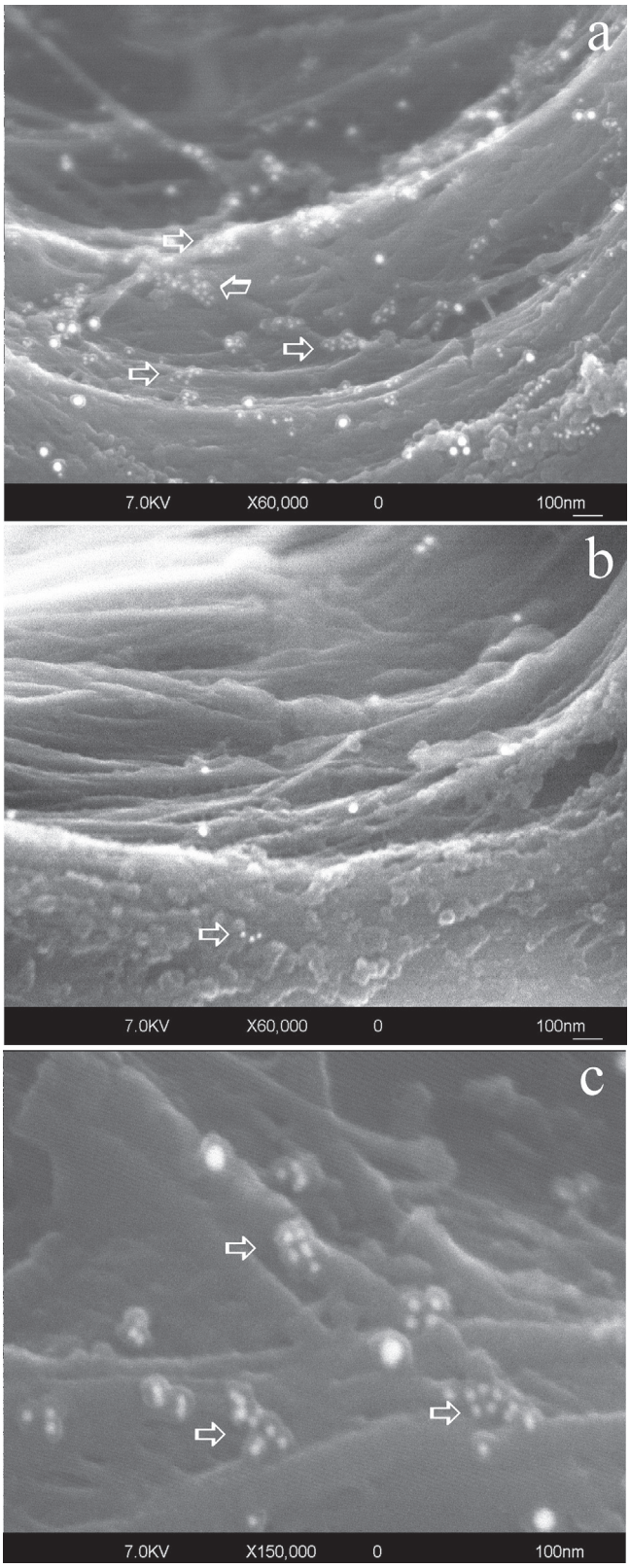


**Fig. 2 – FEISEM images of dentin etched with 35% phosphoric acid. Smear layer removal, funneling, and collar formation around the tubular orifices are observable regardless of the etching time. (a) Dentin treated with phosphoric acid for 15 s. CF labeling (30-nm white spots) is localized mostly on major fibrils while CS labeling (15-nm white spots) is observable on the peritubular dentin matrix. CS assumed mostly a globular aspect (wide arrows). (b) The use of phosphoric acid for 30 s reduced both CF (larger bright white spots) and CS (smaller white spots labeled by arrows) labeling. (c) High magnification images reveal the complex network of major CF and globular CS (arrows) on the intertubular dentin after 15 s of etching with phosphoric acid. Reprinted from Breschi et al. [50], with permission. *FEISEM* field emission in-lens scanning electron microscopy, *CF* collagen fibril, *CS* chondroitin sulfate.**

ration phenomena have been observed in these amphiphilic resin blends, mainly within simplified adhesives [69–71], leading to the formation of heterogeneous resin layers. Camphorquinone (CQ), the most common photo-initiator, is hydrophobic, and may cause suboptimal degree of cure (DC) of hydrophilic monomers [71]. The use of alternative hydrophilic photo-initiators such as TPO (ethyl 4-dimethylaminobenzoate and diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide), in addition to conventional CQ, has been proposed to improve the DC of hydrophilic adhesive systems [71,72]. The addition of water-compatible photo-initiators promotes improved polymerization of both hydrophilic and hydrophobic domains, increasing the DC [73] and reducing the detrimental effect of phase separation [70].

Long-term exposure of restorations to the occlusal forces and repeated changes in temperature cause contraction and expansion of restorative materials, affecting interfacial stability by allowing oral fluids and water to penetrate the resin [74]. Water can penetrate by diffusion into the hydrophilic domains and the resin-infiltrated collagen matrix, or may become trapped within the matrix during photopolymerization [75]. This residual water fuels the hydrolysis of collagen and resin polymers, accelerating matrix degradation by abrading the surface, increasing the surface area and allowing greater entrance of both water and soluble salivary enzymes, which can greatly accelerate ester bond hydrolysis, contributing to the failure of the adhesive interface [62].

## 5. Degradation of the collagen scaffold/fibrils

The collagenolytic activity in dentin was first reported by Dayan et al. [76], whereas Tjäderhane et al. [77] provided further clarification and attributed this activity to the matrix metalloproteinases (MMPs). Pashley et al. [78] demonstrated that collagen can degrade over time in aseptic conditions, indicating that it could be caused by intrinsic matrix proteases. Interestingly, the intrinsic dentin gelatinolytic and collagenolytic activity reported was significantly lower in specimens treated with a MMPs inhibitor [78]. Ever since that report, efforts have been made to elucidate the type of enzymes involved, their localization within the dentin ECM, the significance and the implications of this endogenous proteolytic activity in the degradation of the HL, as well as to find strategies to silence this activity. The most prominent groups of endogenous enzymes in dentin are the MMPs and cysteine cathepsins.

### 5.1. MMPs

MMPs are $Zn^{2+}$ and $Ca^{2+}$-dependent endogenous proteases that are common to both bone and dentin. The $Zn^{2+}$ ion plays a part in the activation of the enzyme, while $Ca^{2+}$ is involved in the preservation of their tertiary structure. These proteases consist of a prodomain with a cysteine residue, a hemopexin domain, catalytic domain containing the $Zn^{2+}$ ion and a hinge region [79]. MMPs play an important role during dentin maturation, but they become trapped and inactive after the collagen matrix becomes mineralized [35]. They are secreted in the form of pro-enzymes with a bridge between the $Zn^{2+}$ ion and the cysteine residue in the pro-peptide, creating a "cysteine switch" which, when intact, prevents water binding to the $Zn^{2+}$ ion, thereby preventing the activation of the enzyme [79].

The most abundant MMP in dentin is MMP-2, followed by MMP-9. Mazzoni et al. measured the presence and concentration of all molecular forms of both MMP-2 and MMP-9 in demineralized dentin quantifying enzyme concentrations by immunoassay, form distribution by gelatin zymography and immunological characterization by western blotting [80]. Interestingly, both endogenous MMP-2 and -9 are present in latent forms (as proenzymes) in mature sound mineralized human teeth. When the matrix was demineralized, zymography identified proMMP-2 migrating at 72-kDa, the zymogen activated form of MMP-2 and, for the first time, the presence of several forms of both zymogen and activated MMP-9. Dentin extracts showed zymogen proMMP-9 (92-kDa), activated MMP-9 (86-kDa), and a high-molecular weight enzyme polymer (225-kDa, probably disulfide-bonded dimers of MMP-9) that were separated and, enzymatically and immunologically detected [81]. MMP-2 and -9 were then three-dimensionally identified by means of an immunohistochemical approach with monoclonal antibodies in partially decalcified human dentin under field emission in-lens-scanning electron microscopy and in demineralized human dentin by transmission electron microscopy, demonstrating that MMP-2 and MMP-9 are intrinsic constituents of the fibrillar network of the human dentin organic matrix (Fig. 3) [81]. The study also showed that the amount and distribution patterns of MMPs in partially decalcified dentin are different from those in undemineralized dentin, in which lower labeling was observed, thus suggesting that demineralization affects MMPs bioavailability and further activation.

The gelatinases MMP-2 and -9 are not true collagenases. However, they are very important for the process of collagenolysis and the endogenous degradation of dentin collagen. Other studies reported the presence of additional enzymes such as collagenase MMP-8, stromelysin-1, MMP-3 and MMP-20 which have been shown in dentin using different methodologies [61,82–87].

In fact, the true collagenases (MMP-1, -8, -13, -18) cannot cleave intact collagen molecule at the cleavage site, due to collagen molecule orientation and the position of the C-terminal end, which sterically blocks access to the peptide bonds [88]. Gelatinases, which are also telopeptidases, remove the blocking C-terminal telopeptides [88], thus allowing access to the true collagenases. Collagenases can only approach the collagen at the cleavage site, fragmenting it into a 3/4 N-terminal and a 1/4 C-terminal fragment [79]. Removal of the telopeptides also eliminates the C-terminal cross-links, possibly rendering the collagen more susceptible to non-specific degradation [89].

During adhesive procedures, the MMPs of the collagen matrix are exposed and can become active, irrespective of the E&R or SE procedure employed [90–93]. The very first studies on this topic were performed mixing human dentin powder with the adhesive blends and then evaluating their relative proteolytic activities before and after the sequential applications of the phosphoric acid-etchant and different E&R [90] or

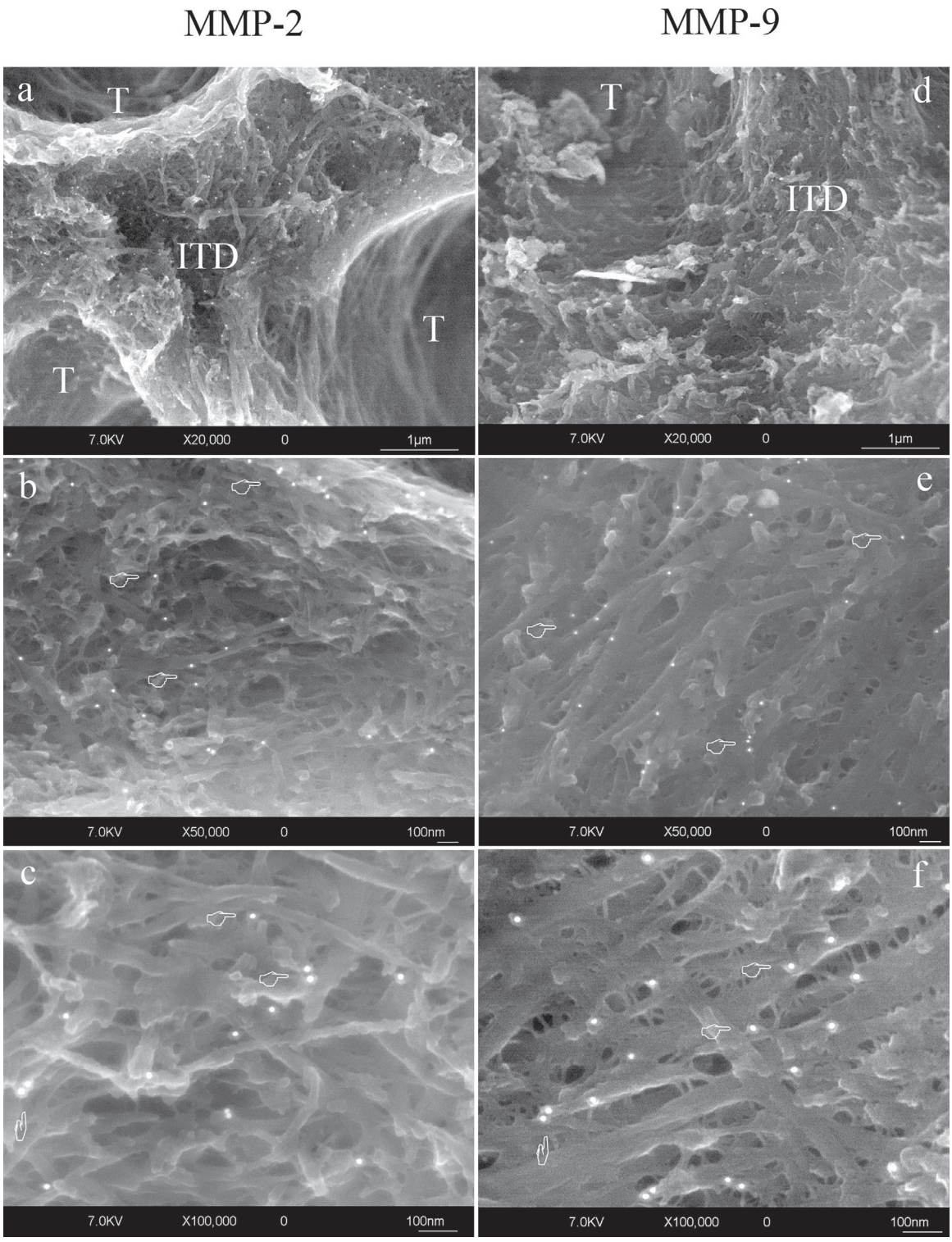


**Fig. 3 – FEISEM micrographs of unfixed partially decalcified dentin after a preembedding immunolabeling procedure with monoclonal antibodies for MMP-2 or MMP-9. The images were obtained by a combination of secondary electron and backscattered electron signals to simultaneously reveal immunogold labeling white spots and related substrate morphology. Labeling can be identified as electron-dense white spots under the electron beam. (a,d) Low magnification view (20,000) of the partially decalcified dentin surface showing open T surrounded by a thick collar of organic matrix and ITD. MMP-2 and -9 labeling can be identified as mainly localized in peritubular dentin. (b,e) A higher magnification view (350,000) of the partially decalcified surface: positive immunohistochemical staining identifying MMP-2 (b) and -9 (e) antibodies are located along the collagen fibrils. (c,f) High magnification FEI-SEM micrographs (100,000), revealing the relationship between MMP-2 and -9 and the collagen meshwork. The specimen shows a moderate labeling for MMP-9 (e) uniformly weaker than MMP-2 staining (c). Reprinted from Mazzoni et al. [81], with permission. *FEISEM* field emission in-lens scanning electron microscopy, *T* tubular orifices, *ITD* intertubular porous dentin.**

SE [93] adhesives. These studies were based on the use of a heavily-labelled but quenched fluorescein-labelled substrate that yields fluorescent peptides upon enzymatic cleavage. When dentin powder mixed with an adhesive system was placed in contact with the substrate, it generated fluorescent signals that were proportional to the amount of active enzymes present in the specimen, allowing screening of the relative proteolytic activity for each adhesive tested. Interestingly, a correlation between the pH values of the tested adhesives [90,93] and their rejuvenated proteolytic activities was found due to the increase in the quantity of activated, non-denatured enzymes, when these adhesives were applied to acid-etched dentin [94].

However, although the existence of an intrinsic proteolytic activity was clearly revealed, such activity could not be related to a specific enzyme. For this reason, a different innovative specific assay was used to selectively relate the activity of the tested specific dentinal MMPs to a specific adhesive application compared to nonspecific gelatin zymography [91,94]. The assay confirmed that E&R adhesives contribute to the activation process due to their acidity, but also revealed that activation is adhesive-dependent [90]. Similarly, with SE adhesives, the exposure of matrix-bound MMPs is accompanied with increased activity, but sometimes showed reduced extent of activation [93].

All the above-mentioned studies were performed on dentin powder, while Mazzoni et al. also demonstrated the three-dimensional localization of dentin proteolytic activity at the adhesive interface, by means of *in situ* zymography [94]. *In situ* zymography is a technique that provides direct evidence of the activity of endogenous enzymes in the tissue using a quenched fluorescein-labeled gelatin substrate that is incubated directly on the specimen surface. The enzyme activity located in the inner tubular walls and at the bottom of the HL (Fig. 4). This correlated well with the layer of demineralized, uninfiltrated collagen previously detected with a highly sensitive immunogold labeling technique [15], and by confocal microscopy of adhesive interfaces created by simplified E&R adhesives. Interestingly, the areas of exposed collagen also seem to correlate well with the morphological characteristic of nanoporosities of the hybrid layer defined as interfacial silver nanoleakage expression [95]. These are locations of the initial areas of degradation of the HL over time.

### 5.2. *Cysteine cathepsins*

Another prominent group of endogenous proteolytic enzymes in dentin matrices with implications in dentin degradation are cysteine cathepsins. Their presence in dentin was recently reported [96,97]. They were shown to play a part in the progression of caries, as well as in the degradation of the HL [97–99]. Cathepsin (CT)-K comprises 98% of cathepsin activity against collagen and differs from the MMPs and the other cathepsins in its ability to cleave helical collagen at multiple sites and generate multiple collagen fragments [100]. Conversely, other CTs can only cleave the non-helical telopeptide part of collagen [101].

It was speculated that CTs and MMPs may work synergistically and that these two groups of enzymes are localized very close together and in the vicinities of their target substrates [97]. This is supported by studies showing the existence of at least some MMPs and CTs members distributed in the same space occupied by collagen, both in sound and carious teeth [99], indicating a possible enzymatic cascade between these different classes of proteases [96,97]. More recently an *in situ* co-occurrence/distribution of CTs and MMPs in human dentin matrix by immunogold labeling correlative FEI-SEM and TEM microscopies was reported, showing the presence of CT-B and CT-K on the collagen fibrils after demineralization (Fig. 5) [102]. Additionally, MMPs and CTs activities were observed spectrofluorometrically using fluorogenic substrates showing that these two families of proteases play different coordinated roles in matrix degradation. Interestingly, in other tissues, MMPs and CTs are known to be able to regulate the activities of each other, and possible CT-MMP interactions have also been suggested to occur in dentin [97].

## 6. Strategies to reduce HL degradation

Endogenous proteases are actually hydrolases, since they require unbound water to cleave collagen peptides. During dentin bonding procedures, it is very difficult, if not impossible, to fully envelope the deepest portion of the demineralized collagen fibrils within the HL with resin. The gaps between the exposed collagen fibrils are filled with water, enabling the activation of the endogenous enzymes and collagen degradation, leading to plasticization of the adhesive resin, and mechanical strain on the exposed fibrils. Even though it is not perfectly clear whether the loss of collagen or plasticization occurs first, electron microscopy studies indicate that collagen degradation may precede the loss of resin. Inhibition of collagen degradation also results with the improved overall HL integrity, lower increase of nanoleakage and improved bond strength durability [103–106]. Loss of collagen allows increased water flow in and under the hybrid layer, which may accelerate resin plasticization. Hence, there are several bonding strategies designed to reduce adhesive interface degradation, aiming either to prevent the loss of collagen either directly by collagenolytic enzyme inhibition or increasing collagen resistance to degradation, or indirectly by eliminating water between the exposed collagen fibrils, thereby preventing their degradation.

### 6.1. *Inhibition of the enzymatic activity*

The inhibition of endogenous collagenolytic activity can occur by a chelating mechanism, since the activity of the MMPs is dependent on the metal ions that can be chelated.

Of the numerous MMPs inhibitors, *chlorhexidine* (*CHX*) has been studied the most. It has been used in dentistry as an antimicrobial agent, and is therefore suitable for use as a primer. Apart from its antimicrobial properties, CHX was shown to inhibit the activity of the MMPs [107] and cysteine cathepsins [108]. As low as 0.2% solutions of CHX have been shown to increase the longevity of the HL [105,106,109–115]. After the discovery of CHX as an efficient enzyme inhibitor when used as a primer, there have been efforts to incorporate it into the components of dental adhesive systems with the intention of shortening chair time, with promising results

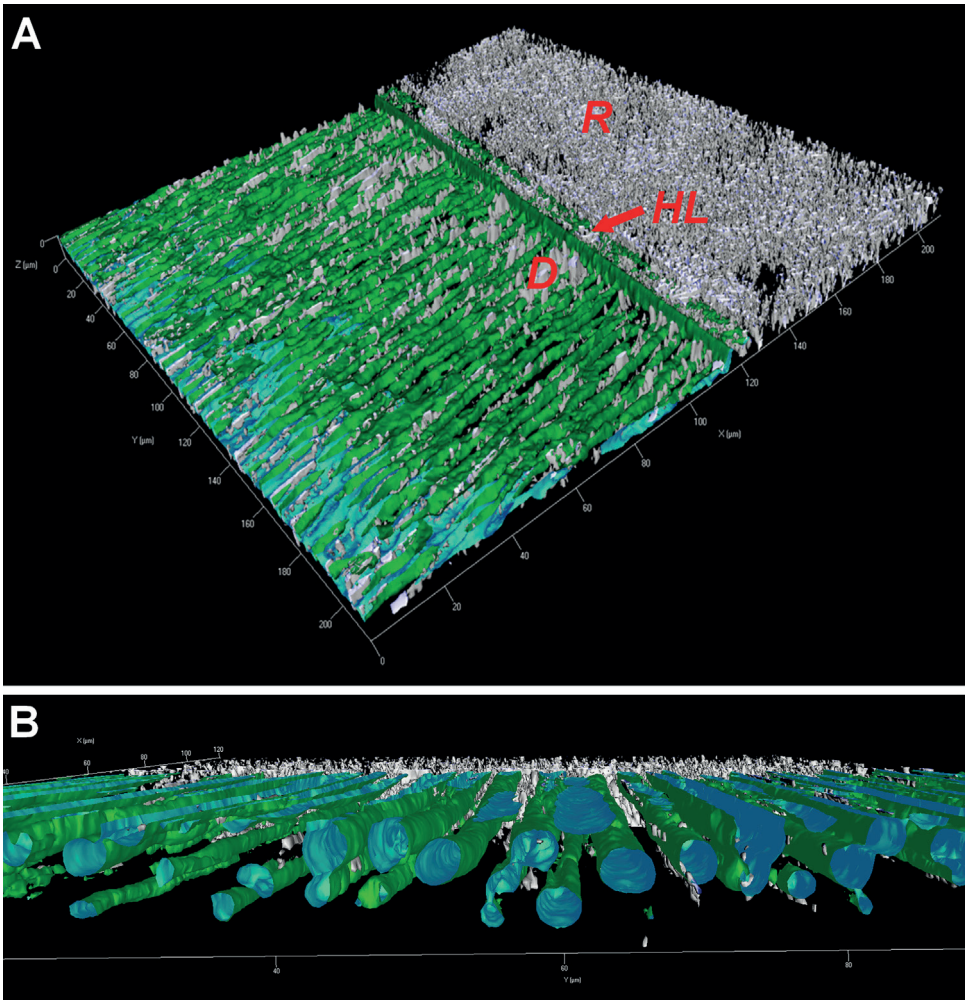


**Fig. 4 – (a,b) Three-dimensional model of the acquired image. (a) The intense fluorescence, showing gelatin hydrolysis, throughout the entire extension of hybrid layer. (b) Higher magnification image model shows gelatinolytic activity inside dentinal tubules, shown as cylindrical tubes, in deep dentin. The high tubule density reveals this to be very deep dentin. Reprinted from Mazzoni et al. [94], with permission. *R* resin composite, *HL* hybrid layer, *D* dentin.**

[116–119]. However, CHX binding was shown to be a reversible mechanism, and probably due to leaching, the resin–dentin interfaces treated with CHX were shown to be unstable after 18 months [120,121]. *In vitro* studies demonstrated that cationic CHX binds to anionic sites in mineralized and demineralized dentin [122,123]. However, sodium chloride was able to displace CHX from both mineralized and demineralized dentin, indicating that CHX binding to dentin is only electrostatic [122]. Despite possible leaching, the use of CHX as additional therapeutic primer is still the most commonly used *in vitro* and *in vivo* tested method to stabilize the adhesive interface with clinical applicability due to the large availability of CHX in dental offices and the limited clinical application time (as low as 30 s showed efficacy in inhibiting the enzymatic activity of dentin) [122].

*Ethylenediaminetetraacetic acid* (*EDTA*) has been used for decades in the endodontic therapy for the enlargement of the root canals due to its chelating properties. EDTA removes the $Ca^{2+}$ from the collagen matrices, and binds the $Zn^{2+}$ ions from the catalytic site of the MMPs [124,125]. However, several downsides of this agent have been reported, such as long application time and reversibility caused by water solubility [126].

*Quaternary ammonium compounds*, positively charged at physiological pH, can inhibit the endogenous enzymatic activity of dentin using a cationic mechanism, similar to CHX. One of the quaternary ammonium compounds already tested as an MMPs inhibitor is *benzalkonium chloride* (*BAC*), which is a mixture of alkylbenzyl-dimethylammonium chlorides of various alkyl chains that strongly binds to demineralized dentin and that was shown to have an immediate inhibiting effect comparable to that of CHX [127–129], with promising results in bond strength preservation over time [128,129]. Integrating methacrylates into these compounds (quaternary ammonium methacrylates – QAMs) seems to improve their efficiency. 12-methacryloyloxydodecylpyridinium bromide (MDPB), a quaternary ammonium methacrylate well-known for its antimicrobial properties, has been incorporated into a commercially available adhesive system [130,131], showing a good ability to inhibit the MMPs activity [132,133]. Follow-

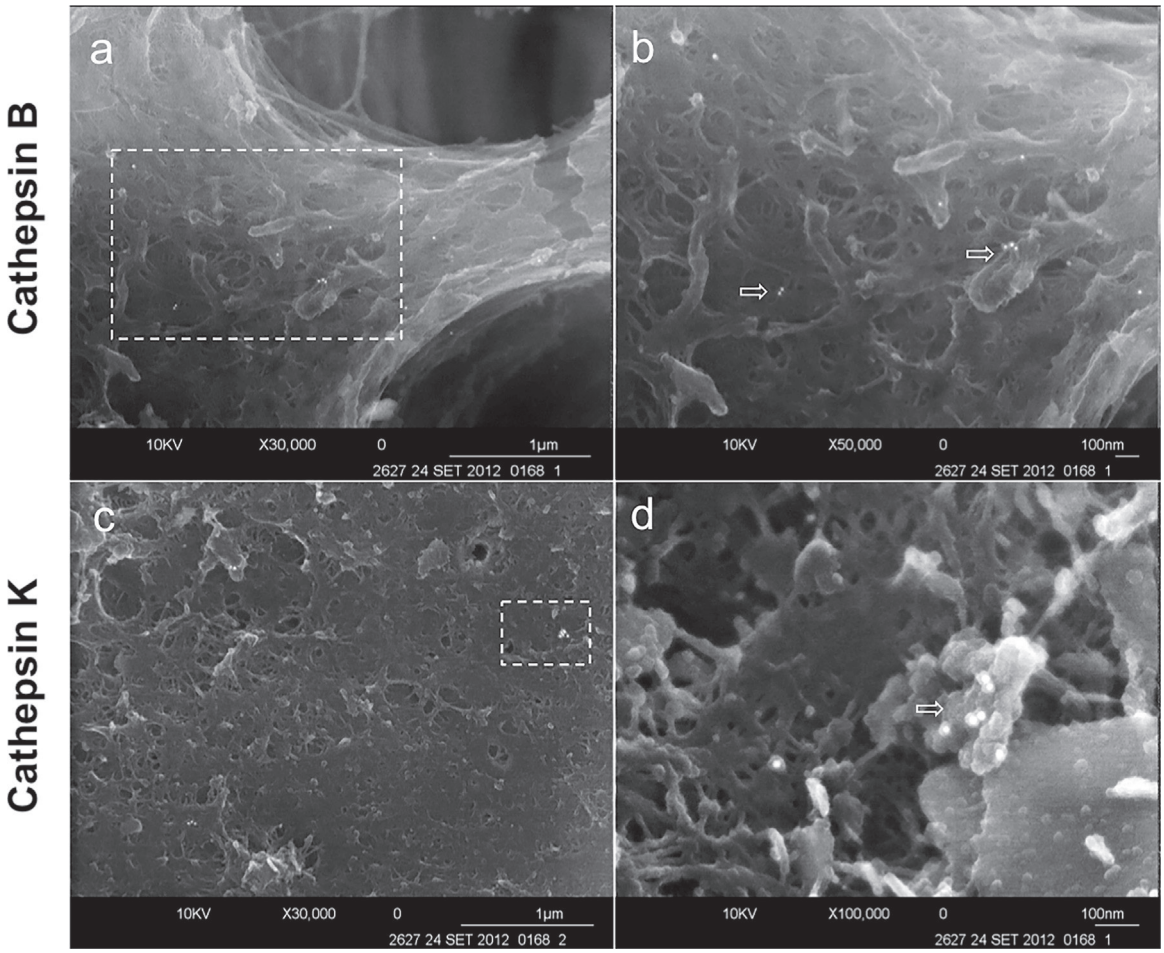


**Fig. 5 – FEISEM micrographs of partially demineralized dentin after a pre-embedding immunolabeling procedure. Labeling can be identified as electron-dense white spots under the electron beam (arrows in b and d). (a,c) Low magnification view (×30,000) of the partially demineralized dentin surface showing a mild CT-B and CT-K labeling signal. (b) A higher magnification view (×50,000) of the partially demineralized surface: positive immunohistochemical staining identifying CT-B (15 nm white spots) located along the collagen fibrils. (d) High magnification FEISEM micrographs (×100,000) revealing a moderate labeling for CT-K (30 nm white spots) staining. Reprinted from Scaffa et al. [102], with permission. *FEISEM* field emission in-lens scanning electron microscopy, *CT-B* cathepsin B, *CT-K* cathepsin K.**

ing the good results obtained with MDPB, other QAMs were investigated as MMPs inhibitors with encouraging results, comparable to CHX [134]. Research is still focused on the development of new monomers with quaternary ammonium functionalities [135], and further data are needed to demonstrate that adhesives based on QAM compounds can effectively counteract the MMPs activity at the adhesive interface and thus, increase the durability of resin–dentin bonds.

Pharmaceutical agents used for various medical conditions have also been shown to have an inhibiting effect on the MMPs through a chelating mechanism. For instance, *bisphosphonates*, in particular, polyvinylphosphonic acid, has shown good immediate results, but with questionable longevity [136]. Further, *tetracycline* and its analogs (doxycycline and minocycline) have shown collagenases- and gelatinases-inhibitory properties [137–139]. The longevity of the resin–dentin bond preservation effect of these antibiotics has not been studied, and special caution in use of these agents in dentistry should be taken due to the dark photo-oxidation stains it can cause on dental tissue.

Moreover, specific inhibitors of the MMPs and cysteine cathepsins have been produced and tested in terms of bond strength preservation. *Galardin* incorporated within a primer of the E&R adhesive has shown a reduction in the degradation of the HL after one year [104]. Similarly, *SB-3CT*, a specific MMP-2 and -9 inhibitor showed a positive influence on HL preservation [140]. Specific inhibitors for cysteine cathepsins, such as *E-64* and *odanacatib* express their inhibiting effect through binding to the enzyme at the cleavage site, mimicking the substrate [141], but the data on effect on dentin bond durability is very limited [142].

### 6.2. *Cross-linking agents*

As previously mentioned, cross-linking of dentin matrix collagen is a naturally occurring mechanism in dentin, and therefore, researchers have attempted to enhance this mecha-

nism using chemical substances with cross-linking properties. This causes biomodification of the collagen scaffold enhancing the biomechanical properties of dentin, and making it less prone to proteolytic attack [143–145].

*Aldehydes*, such as *glutaraldehyde* (*GD*), are used as tissue fixatives, but have been proposed as potential cross-linking agents for the use in dentistry, since they form covalent bonds between the amino groups of proteins and the two aldehyde groups of glutaraldehyde. In particular, GD binds the ε-amino groups of peptidyl lysine and hydroxylysine residues within the collagen scaffold. GD has been extensively studied as a cross-linking agent in dentistry [143,144,146–153]. Similarly, acrolein (2-propenal), the simplest unsaturated aldehyde, was used as an additional primer to improve the mechanical durability of resin-bonded dentin and was as effective in cross-linking dentin as was GD, which involves much longer application time. Even when acrolein was applied for only 1 min, at concentration as low as 0.01 wt%, it showed bond strength preservation after one year (Maravic et al., unpublished results), confirming the validity of the cross-linking approach [154,155].

Despite the effectiveness of aldehydes in the preservation of the HL, their cytotoxicity makes them inadequate for everyday clinical practice. Therefore, less toxic agents, such as *carbodiimides* and *plant-derived cross-linkers* have been recently studied [145].

The most studied carbodiimide in dentistry is *1-ethyl-3-(3-dimethylamino-propyl) carbodiimide* (*EDC*), which was shown to achieve a similar bond strength preservation to that of GD [156,157] with the advantage of a much lower cytotoxicity. EDC contains a functional group with the formula RN=C=NR and can react 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. EDC has a two-fold activity being able to cross-link both helical and telopeptide domains in collagen and also preventing telopeptidase activity that would normally remove telopeptides [158]. Its ability to increase the stiffness of demineralized dentin matrix [156,159] and the HL [160] has been well documented. Recent zymographic assays on dentin powder [157] and *in situ* zymographic three-dimensional images obtained by confocal microscopy [161,162], clearly demonstrated that when EDC is used as additional therapeutic primer on acid-etched dentin, it also has the ability to silence dentinal MMPs, even after a 1-year storage in artificial saliva [162].

As previously mentioned, plant-derived cross-linking agents are another prominent group, gaining much interest in the scientific community. They are characterized by high potency and low cytotoxicity, and are therefore suitable for use in dentistry. The most studied of these agents are *genipin*, *polyphenolic compounds*, such as *tannins*, *proanthocyanidins* and *curcumin* [148,150,151,163–166].

Apart from chemical compounds, the cross-linking effect can also be achieved through physical and photochemical treatment, such as ultraviolet A (UVA) [167] or gamma irradiation, heating and drying [168].

Another important feature of cross-linking agents is their ability to react with the collagen-degrading enzymes [158,161,169]. To date, GD [170], EDC [94,158,161] and plant-derived cross-linking agents [171,172] have shown this ability.

### 6.3. *Removal of the unbound/residual water within the HL*

Most dimethacrylates that are capable of creating strong, highly, crosslinked resin polymers, are so poorly soluble in water that they undergo phase changes when mixed with water that interferes with monomer infiltration of acid-etched dentin [173]. Manufacturers market their adhesive formulations in ethanol solvents to ensure the mixture is in a single phase. Most dentists place ethanol-solvated adhesives on water-saturated acid-etched dentin, a procedure fraught with the danger of creating microscopic phase changes in the applied adhesive. To avoid such dangers, Pashley et al. [174] altered the wet-bonding technique by replacing rinse-water with ethanol, so that the dentin was saturated by ethanol rather than water. That same year, Tay et al. [175] reported excellent resin–dentin bonding by applying bis-GMA, the least soluble dimethacrylate, to ethanol-saturated dentin. Applying ethanol-solvated adhesives to ethanol-saturated dentin avoids any risk of phase separations. These approaches reduce the presence of residual water in the resin–dentin bonds [176]. In the absence of water, matrix proteases cannot cleave collagen.

Using ethanol wet-bonding permits infiltration of more hydrophobic resins [177,178] than is possible with water wet-bonding. Hydrophobic resins absorb less water than hydrophilic resins.

It is well-known that ethanol can remove and replace unbound water from demineralized dentin [179]. To determine whether ethanol can replace bound water from collagen matrices, Jee et al. [180] used molecular dynamic simulations to recreate the three layers of bound water in collagen matrices. Using this computer simulation, they confirmed that the first and second layers of tightly-bound water in collagen could not be replaced by ethanol. However, half of the bound water in the outermost layer of bound water could be replaced by ethanol. Thus, ethanol wet-bonding brings ethanol-solvated resin closer to collagen than is possible during water wet-bonding. It is predicted to occur more in the gap regions of collagen. The resin infiltration of the gap regions may also inactivate collagen-bound proteases.

A second approach to removal of residual water from hybrid layers was the development of SE primer adhesives. First of all, exposed collagen fibers are better embedded into SE adhesives [15–17,181] as described above. Manufacturers also selected acidic monomers like 10-methacryloyloxydecanethylene phosphoric acid (MDP), and added them in higher concentrations than were used before for wet-bonding adhesives (*i.e.* 20–25 vol%). They only added sufficient water to allow the acidic monomers to become ionized, and to solubilize the mineral phase of dentin. These water concentrations are proprietary, but are generally in the range of 20–25 vol% [177], unlike the 70 vol% [4] water that is found in acid-etched dentin just prior to adhesive application. When using SE primer/adhesives, one is performing "dry bonding". That is, one dries an unetched tooth before applying a SE primer that contains 20–25 vol% water. No water rinsing

is employed. After "self-etching" for 10 s, one dries the primed dentin and then seals it with a solvent-free adhesive before light-curing. Such hybrid layers are only 1 μm thick and contain much of the smear layer including the smear plugs that prevent dentinal fluid from wetting the surface during bonding procedures. In general, these bonds show good durability, but are still prone to loss of bond strength with time [182].

Another approach to control rinse-water or dentinal fluid flow involves the use of dimethyl sulfoxide (DMSO) as a primer or a solvent. DMSO has low surface energy and can be used as a solvent to facilitate radical polymerization reactions such as are used in dental adhesion. It also possesses the polarity needed to break down water's self-association and forms stable complexes with water to create "hydrophobic water" [183,184]. Recent studies have shown increase both in immediate and long-term bond strength [185,181,186–188]. The increase in immediate bod strength may be due to DMSO-induced improvement in dentin wettability [189], which may be the reason for the better adhesive penetration [181]. Improved penetration may also contribute to the durability of the bond strength, but DMSO may also have MMP-inhibitory effects [185].

### 6.4. *Remineralization of hybrid layers*

A novel, exciting approach to HL preservation is *biomimetic remineralization*. It entails ion-releasing materials, which are intended to mimic the natural mineralization process. Since infiltration of resin is incomplete, there are numerous water-filled regions in HLs that can slowly solubilize the insoluble collagen fibrils. Is it possible to coax hydroxyapatite into those water-filled spaces? The infiltration of apatite can remineralize the collagen fibrils, and simultaneously fossilize the MMPs. Tay and Pashley [190] showed that Portland cement in a phosphoprotein analog-containing fluid can form a meta-stable amorphous calcium-phosphate, which can deposit inside the collagen organic network in the form of apatite crystallites. Further studies confirmed this concept [191–193] and validated the importance of the presence of biomimetic analogs, since the mineralization achieved without the analogs was in the form of extrafibrillar mineral deposits. In order to facilitate the transition of this proof-of-concept into a clinically applicable system, the authors performed a follow-up study, binding the phosphoprotein analog directly to collagen, instead of having it in the solution [194,195]. The study showed a complete remineralization of dentinal collagen after 3–4 months. It should be possible to cover resin-bonded dentin with "therapeutic" resin composite containing slow-release remineralization reagents that can diffuse through the adhesive layer, into water-filled regions of the hybrid layers to remineralize naked collagen fibrils. The demineralized regions can be "back-filled" with nanoscopic apatite crystallites. This results in physical displacement of free water, thereby preventing protease hydrolysis of collagen. It is also very important that the remineralizing reagents contain an inhibitor of matrix proteases. If the demineralized collagen matrix degrades before it can remineralize, then remineralization cannot occur. Recently, certain studies investigated the efficiency of E&R [194] and SE [195] experimental adhesives and primers doped with phosphoprotein biomimetic analogs to preserve the HL longevity. Microtensile bond strength was preserved in the experimental groups after 3- and 6-month storage in the dentin specimens bonded with E&R and SE adhesive systems, respectively. Although very interesting, this strategy has not yet become applicable in everyday clinical practice.

### 6.5. *Calcium-chelation dry bonding*

Can interfibrillar minerals be selectively removed from between collagen fibrils, without demineralizing the fibrils? Recent research has revealed that collagen fibrils behave as if they were size-exclusion beads of Sephadex in column chromatography. While molecules with molecular weights smaller than 600 Da can enter collagen, molecules larger than 40 KDa are excluded [196–198]. Phosphoric acid, with a molecular weight of 100 Da, is small enough to permeate throughout collagen fibrils, solubilizing both extra- and intrafibrillar mineral, leaving dentin completely demineralized and soft.

If, instead of acid-etching dentin with phosphoric acid, one used 15 wt% of a large molecular weight (*i.e.* 225,000 Da) calcium chelator, like sodium polyacrylate, then the chelator would be too large to permeate collagen. Application of such a chelator would only remove apatite mineral from the extrafibrillar space, creating interfibrillar spaces for inward monomer diffusion and uptake into the hybrid layer. After 30 s of chelation, the reaction is stopped by water rinsing and air-drying [199]. Because the collagen fibrils remain completely mineralized, they are too stiff to shrink or collapse when the residual rinse water is evaporated with strong air blasts. These procedures allow for "dry bonding", using hydrophobic resins much like pit-and-fissure sealants [199].

The matrix proteases are not activated by these processes, and there is no residual water to fuel proteases if they ever become activated. This is an exciting new example of dry bonding.

## 7. Conclusions

Although there are many more hurdles to be overcome in the field of adhesive dentistry, impressive progress in the understanding of the processes underlying HL degradation, as well as in the development of strategies for the preservation of the adhesive interface have been achieved. The removal of the unbound water from the hybrid layer and the silencing of the endogenous enzymatic activity, have been achieved using chemical agents and physical approaches, which are increasing in number, effectiveness, biocompatibility and clinical applicability. This large amount of available research on the topic is an indicator of the importance of these issues, and of the significant efforts of researchers and dental material companies to reach a new level of quality and longevity of resin–dentin bonds.

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