Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE E NEUROMOTORIE

Ciclo 31°

Settore Concorsuale: 06/F1
Settore Scientifico Disciplinare: MED/28

The role of collagen cross-linkers in the stability of the adhesive interface

Presentata da: **Allegra Comba**

Coordinatore Dottorato
Prof. Pietro Cortelli

Supervisore
Prof. Lorenzo Breschi

Esame finale anno 2019

CHAPTER 1
Introduction

Introduction

The subject of the present thesis is the stability of the adhesive interface created at the level of the dental substrate. The longevity of dental restorative procedures is mostly dependent on the success of the adhesive interface over time. The concept of adhesion to the toot structure has been introduced by Buonocore in 1955, and starting from that moment, continuous improvement in dental materials and bonding technologies created a great impact on the clinical practice. However, despite progression, the problem of the preservation of a tight bond to the tooth structure is still a challenge.

Adhesion to enamel is mostly micromechanical and is considered very stable. It is based on the partial demineralization of hydroxyapatite crystals produced by acid etching, followed by the interdiffusion of the resin within the porosities and the polymerization of the material to create a biocomposite, such as a substrate composed by resin-based material and tooth tissue.

In the same way, the mechanism of adhesion of resin composite restorations to dentin is based on the penetration of the resin monomers into dentin collagen matrix after the partial dissolution of the mineral (inorganic) phase. After the curing of the material, this process is finalized to produce a collagen-reinforced interdiffusion layer called hybrid layer. The connection between polymerized adhesives and the remaining mineralized dentin occurs through the collagen fibrils extending from the underlying mineralized matrix towards the hybrid layer. The collagen fibrils contain bound, non-collagenous proteins such as growth factors and matrix proteases. These proteases play an important role during dentin maturation, but they become trapped and inactive after the collagen matrix mineralized. However, during adhesive procedures, the enzymes of the collagen matrix are exposed and activated, irrespective of the E&R or SE procedure employed, resulting in the progressive degradation of the collagen fibril anchoring the restorative material to tooth structure. This leads to solubilization of collagen and loss of retention of the adhesive restoration.

Two distinct proteases – matrix metalloproteinase (MMPs) and cysteine cathepsins (CCs) – have been shown to be responsible for time-dependent enzymatic degradation of dentin collagen matrices. Therefore, the inhibition or inactivation of these enzymes is crucial to

prolong the resin-dentin bond strength over time. Current research in this field aims at increasing the longevity of the adhesive interface and this doctoral research thesis considers the fundamental processes responsible for the aging mechanisms involved in the degradation of resin-bonded interfaces, as well as some potential strategies to prevent and counteract this degradation.

CHAPTER 2
Review of literature

Review of the literature - 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 overtime.

Adhesive Systems and Adhesion Strategies to Dentin

Since resin monomers themselves cannot infiltrate mineralized tissues, traditionally, adhesive bonding systems consist of an *acid*, used for the removal of mineral crystals and exposure of the collagen fibrils, a *primer*, a hydrophilic solution of resinous monomers, which allows the infiltration of the resinous monomers, especially in demineralized dentin, and the *adhesive* itself containing 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 [10] (Figure1).

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 (Figure 2). That water must be completely replaced by adhesive blends if one wishes to achieve a stable bond [4,10,11]. However, it has been shown that complete infiltration of monomers into the wet and demineralized dentin is not consistently achieved, 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 resin-infiltrated, acid-etched 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 [16,17]. 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 is still questionable.

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 modes with an additional chemical bonding agent. Several reports indicate that these patients have a reduced incidence of post-operative sensitivity. There is relatively little information on the 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 [19].

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 [19–22].

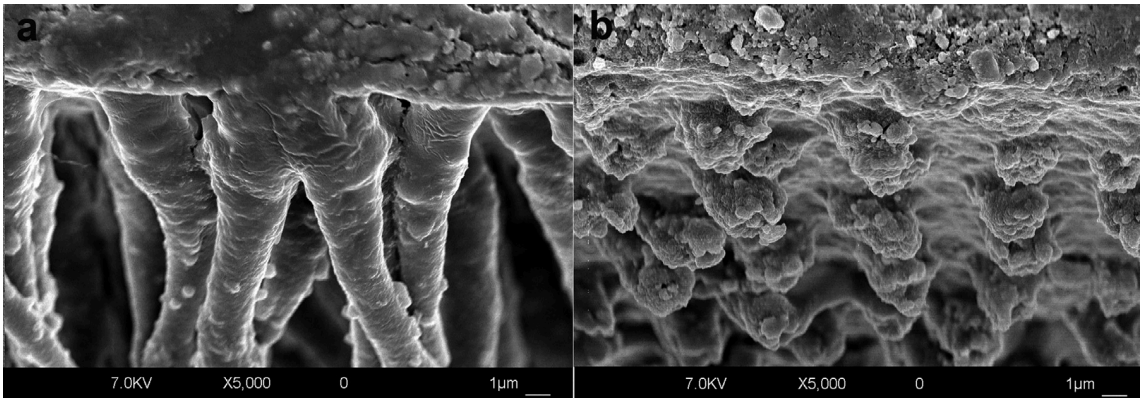


Figure 1 – FEISEM micrographs of an (a) E&R and (b) SE adhesive system. Bonded interfaces were created with Scotchbond 1(3M ESPE) and Protect Bond (Kuraray) in deep dentin tissue. HLs were then exposed with a slow speed diamond saw anddentin was dissolved by sequential rinses in hydrochloric acid and sodium hypochlorite to reveal resin penetration. Resintags are clearly detectable in the E&R adhesive systems (a) since they infiltrated dentin tubules funneled by the etchingagent. SE adhesives often infiltrate no further than the smear layer and smear plugs, revealing a more homogenousmorphology that is devoid of long resin tags. Reprinted from Breschi et al. 2008, with permission. *FEISEM* field emissionin-lens scanning electron microscopy, *E&R* etch and rinse technique, *SE* self-etch technique, *HL* hybrid layer.

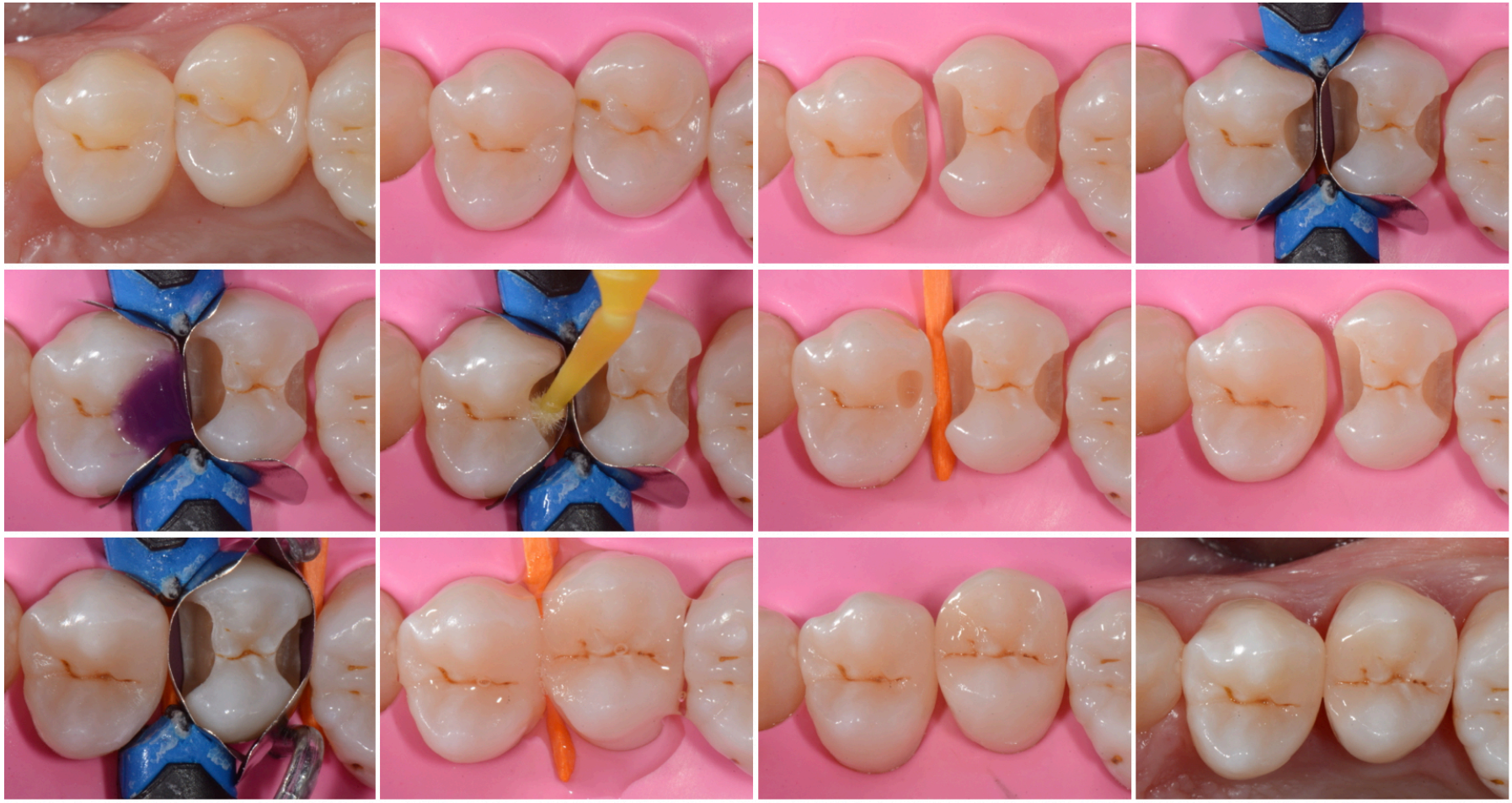


Figure 2. Step-by-step dhesive procedures on dental substrate.

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 [23]. 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 [24].

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 [25,26].

Collagen type I comprises the majority of the collagen structure (90%wt), that also contains traces of collagen V and III [23]. 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 and insulin-like growth factors 1 and 2 [27].

A collagen molecule consists of three α chains, two α1 and one α2 chain intertwined into a left-handed triple helix [28–30]. 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 [31]. 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 [32]. During the process of dentin maturation, apatitic mineral crystallites precipitate and fill the 67 nm gaps between the collagen molecules

[25,26], thereby inactivating enzymes that are present in the ECM and were active during the dentinogenesis [33].

Unlike insoluble collagen in other bodily systems, dentinal collagen does not metabolically [23] 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 [31]. Because dentin collagen does not turn over, the natural cross-links accumulate over time and improve the mechanical properties of collagen fibrils [34]. 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 [35].

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].

Type I collagen fibrils represent the pillar of the collagen structure, perpendicularly connected by non-collagenous proteins [37,38]. 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 [39]. PGs are shown to be involved in the process of dentin mineralization and the maintenance of the structural three-dimensional integrity of collagen fibrillar alignment [39,40]. 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 [41–44]. Sensitive selective immunolabeling protocols can reveal with great precision the three-dimensional relationships between the different structural elements of the ECM [17,44–46].

There have been conflicting reports on the influence of proteoglycans on collagen degradation. Bedran-Russo et al. [47] reported a decrease in bond strength after the removal of GAGs from the demineralized surface of dentin. Mazzoni et al. [48], 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 [35]. If the acid etching step is limited to the recommended 15 s, the structural integrity of the ECM is preserved [17,44,46]. Over-etching could, however, induce structural changes in the collagen molecules [17] as well as PGs [41,43], introducing a damaged organic part into the HL, and should therefore be avoided.

Pre-treatment agents of the dental substrate

Tooth whitening is a common procedure, which may be accompanied by some undesirable side effects. One of these potential drawbacks is the lower bond strength of resin-based restorative materials following the bleaching procedure [49][50]. The bond strength reduction has been associated with the residual oxygen derived from the hydrogen peroxide contained in the whitening agent that remains entrapped in the tooth surface, which can prevent the polymerization of the adhesive monomers [51]. A delayed placement of the restoration of at least 1 week following the bleaching procedure may improve the bond strength, allowing the elimination of residual oxygen. If the bonding procedure cannot be postponed, the use of antioxidant agents [52] or an increased polymerization time may partly compensate the conversion inhibition [53][54].

Hydrogen peroxide can also be used as an endodontic irrigant, to help removing residual pulp-tissue and debris produced by canal instrumentation. Similarly to tooth whitening, canal irrigation using hydrogen peroxide has been correlated to lower bond strength of resin adhesives and cements [55]. Hydrogen peroxide reacts with sodium hypochlorite, the most commonly used irrigant in endodontics, releasing water and oxygen, both of which are able to negatively affect the polymerization of resin adhesive materials.

In endodontics, RC-Prep (Premier Dental Products) is used as a lubricant and demineralizing agent containing polyethylene glycol (Carbowax, Dow Chemical). This molecule cannot be completely eliminated by canal irrigation and has also been found to inhibit resin polymerization, reducing the bond strength of resin adhesives and cements. Nevertheless, the

application of 10% ascorbic acid after RC-Prep use can neutralize the negative effect of polyethylene glycol and improve bond strength values [56].

Also eugenol, a common component of some endodontic sealants and zinc oxide–eugenol temporary materials, can negatively affect bond strength values, because it can infiltrate the dentin substrate [57]. Eugenol is a phenolic compound that can inhibit monomer polymerization, acting as a radical scavenger [58]. When eugenol-based materials are used prior to the application of resin compounds, the contaminated tooth substrate should be thoroughly cleaned both mechanically and scrubbing the surface with a detergent or alcohol to remove the oily eugenol remnants, in order to prevent polymerization inhibition and bond strength reduction [59–61].

Intraradicular polymerization of adhesives

Following root canal treatment, non-vital teeth usually demonstrate an extensive loss of dental structure and the use of fiber posts and core systems has been widely investigated and supported by clinical [62,63] and laboratory studies [62,64] to retain the final restoration [65].

However, adhesion inside the root canal can be impaired by several factors [6,66][67]. Besides the influence of endodontic irrigants and cements, already mentioned in the paper, the adverse geometric feature of root canal [68][69] the peculiar characteristics of the post space [70] and possible incompatibilities between simplified adhesives and dual-cure resin-based cements [69] are important problems that affect polymerization quality and, consequently, the bond strength within the endodontic space. Part of these factors could not be limited and attenuated by the control of clinical steps and are strongly related to the root canal itself [71].

The cavity configuration factor (C-Factor), such as the ratio of the bonded to the unbonded surface area, of an endodontic post space has been estimated to be higher than 200, while coronal restoration values range between 1 and 5 [72]. During polymerization procedure, the shrinkage stress relief of resin-based materials is possible due to the presence of unbonded areas. However, when the unbonded surface area becomes small there is insufficient stress relief and a high probability to cause debonding of the luting material from the intra-radicular dentin [69]. Previous authors showed that the geometric factors of the cavity have an important

influence on contraction stress, and post space depth has a greater influence than the measure of the diameter [73]. Thus, considering the configuration of the endodontic cavity, the development of high contraction stress during curing procedure cannot be avoided but only controlled through the rheological and viscoelastic properties of luting materials [67].

Furthermore, the narrow diameter of the post space limits the passage of the curing light necessary for a correct polymerization and the depth of the post space reduces the radiant energy proportionally to the increase of distance from the light source, thus affecting the degree of conversion of the material [74]. It has been demonstrated that a complete polymerization cannot be guaranteed when the material is placed deeper than 4 to 5 mm because of the limited transmission of light through intraradicularly placed adhesive [75,76] and bond strength of fiber posts has been proved to decrease significantly considering the root region, with lower outcomes in the apical portion of the post space compared to the coronal region [75].

For the abovementioned reasons an adhesive protocol using a light-cured resin system could not be considered ideal due to the influence of reduced light intensity produced by the shadowing of the tooth structure and the light scattering within the resin cement and the post [77,78]. Therefore, both dual-cure adhesive systems and resin cements, such as materials combining self-curing and light-curing capabilities have been suggested. Although the dual-curing systems allow use with limited light penetration, some authors reported that their activation is in any case primarily dependent on light presence [79,80]. Therefore, sufficient light penetration and intensity through the root canal is still fundamental, even when dual-cure resin cements are used for post fixation [81].

Many post systems available on the market are claimed to be ‘translucent’ and allow light to pass through the dowel leading to a higher initial polymerization of the resin cement [82]. However, even if translucent, fiber posts can limit light transmission to values lower than 40% of incident light and may not guarantee an adequate degree of conversion of the resin materials [79]. In addition, several studies reported that the light transmitted through the post was drastically decreased when the depth from the top of the post was more than 4-6 mm [76,80,83] and showed insufficient transmission of light to polymerize the resin materials [76,80]. Furthermore, several manufacturers propose to light cure both the bonding agent and the dual

cement through the translucent post, however the thickness of the resin cement could negatively affect bonding agent polymerization [66].

Some one-step self-etching adhesives are not compatible with chemically or dual-cured resin cements [84,85], due to the acidic resin monomers present in these systems [72,86]. The acidic monomers tend to react with the basic aromatic tertiary amines contained in the composite cement, inhibiting its polymerization [72]. Thus the use of one-step self-etching adhesives is discouraged in combination with these luting materials, even though a ternary catalyst might help overcoming the acid-base reaction [72,86].

On the contrary, three-step etch-and-rinse and two-step self-etching adhesive that do not include an acidic monomer in their bonding agent, are compatible with chemically or dual-cured resin cements because of the presence of the hydrophobic adhesive layer, which works as an intermediate coat preventing the contact with the acidic monomers and the adverse acid-base reaction [84].

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 [87,88]**.**

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 [6,89]. Hydrolysis is considered the primary reason for resin degradation within the hybrid layer [90]. 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 (such as ethanol, acetone, etc). 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 [9,91]. However, these hydrophilic resin monomers, in adhesives formulations cause high sorption of water by the resin systems and generates a HL that behaves as a permeable membrane after polymerization, permitting water movement throughout the bonded interface [92,93]. 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 [94,95]. 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[68]. 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 [94]. 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 [6,68]. Besides the presence of water [96], 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 [97], monomer structure and functionality [98,99] and solvent type and concentration [97,100,101]. Nano-phase separation phenomena have been observed in these amphiphilic resin blends, mainly within simplified adhesives [102–104], 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 [104]. 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 [104,105]. The addition of water-compatible photo-initiators promotes improved polymerization of both hydrophilic and hydrophobic domains, increasing the DC [106] and reducing the detrimental effect of phase separation [103].

Due to long-term exposure of restorations to the oral cavity, 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 [107]. Water can penetrate by diffusion into the hydrophilic domains and the resin-infiltrated collagen matrix, or

may become trapped within the matrix during photopolymerization [108,109]. 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, leading to the failure of the adhesive interface [68].

Degradation of the Collagen Scaffold/Fibrils

The collagenolytic activity in dentin was first reported by Dayan et al. [109], whereas Tjäderhane et al. [110] provided further clarification and attributed this activity to the MMPs. Pashley et al. [111] 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. 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 matrix metalloproteinases (MMPs) and cysteine cathepsins.

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 [112]. MMPs play an important role during dentin maturation, but they become trapped and inactive after the collagen matrix becomes mineralized [33]. 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.

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 [113]. 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 high-molecular weight enzyme polymer (225-kDa, probably disulfide-bonded dimers of MMP-9) that were separated and, enzymatically and immunologically detected [114]. 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 [114]. 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 expression 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 [95,115–120].

In fact, the true collagenases (MMP-1, -8, -13, -18) cannot cleave collagen 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 [121]. Gelatinases, which are also telopeptidases, remove the blocking C-terminal telopeptides [122], thus allowing access to the true collagenases.

Collagenases can only approach the collagen at the cleavage site, fragmenting it into a ¾ N-terminal and a ¼ C-terminal fragment [123].

During adhesive procedures, the MMPs of the collagen matrix are exposed and can become active, irrespective of the E&R or SE procedure employed [123–125]. 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 [122] or SE [124] 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 [126] 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 [127].

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 [125,127]. The assay confirmed that E&R adhesives contribute to the activation process due to their acidity, but also revealed that activation is adhesive-dependent [124]. Similarly, with self-etch adhesives, the exposure of matrix-bound MMPs is accompanied with increased activity, but sometimes showed reduced extent of activation [124].

All the above-mentioned studies were performed on dentin powder, while three-dimensional localization of the dentin proteolytic activity at the adhesive interface was achieved by Mazzoni et al., who demonstrated the activity of the MMPs at the hybrid layer by means of *in situ* zymography [127]. *In situ* zymography is a technique that provides direct evidence of the activity of endogenous dentin in the tissue using a quenched fluorescein-labeled gelatin substrate that is incubated directly on the specimen surface. The enzyme activity was found to

be located in the inner tubular walls and at the bottom of the HL. This correlated well with the layer of demineralized, uninfiltrated collagen previously detected with a highly sensitive immunogold labeling technique [126], and by confocal microscopy of adhesive interfaces created by simplified etch-and-rinse 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 [128]. These are locations of the initial areas of degradation of the hybrid layer over time.

Additionally, physical and chemical factors, such as heat, low pH, chemical reagents, etc. can remove the cysteine switch and activate the pro-enzyme [112,129], synergistically contributing to the endogenous enzyme activation.

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 [130,131] . They were shown to play a part in the progression of caries, as well as in the degradation of the HL [6,131–133]. 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 [134]. Conversely, other CTs can only cleave the non-helical telopeptide part of collagen [135].

It was speculated that CTs and MMPs work synergistically and that these two groups of enzymes are localized very close together and in the vicinities of their target substrates. 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 [133], indicating a possible enzymatic cascade between these different classes of proteases [130,131]. 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 [136]. 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 [131].

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, leading to plasticization of the adhesive resin, and mechanical strain on the exposed fibrils. Hence, there are several bonding strategies designed to reduce adhesive interface degradation, which involve the removal of water from the gaps between the exposed collagen fibrils, thereby preventing their activity.

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 [137] and cysteine cathepsins [138]. As low as 0.2% solutions of CHX have been shown to increase the longevity of the HL [138–147]. 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 [148–152]. 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 [153]. In vitro studies demonstrated that cationic CHX binds to anionic sites in mineralized and demineralized dentin [150,151]. However, sodium chloride was able to displace CHX from both mineralized and demineralized dentin, indicating that CHX binding to

dentin is only electrostatic [149,154]. 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) [149].

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 [155,156]. However, several downsides of this agent have been reported, such as long application time and reversibility caused by water solubility.

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 [157–159], with promising results in bond strength preservation over time [158,159]. 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 [154,160], showing a good ability to inhibit the MMPs activity [161,162]. Following the good results obtained with MDPB, other QAMs were investigated as MMPs inhibitors with encouraging results, comparable to CHX [163]. Researchers are still focused on the development of new monomers with quaternary ammonium functionalities [164], 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 [165,166]. Further, *tetracycline* and its analogs (doxycycline and minocycline) have shown collagenases- and gelatinases-inhibitory properties [167–169]. 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 [170]. Similarly, *SB-3CT*, a specific MMP-2 and -9 inhibitor showed a positive influence on HL preservation [171]. 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 [172].

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 mechanism 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 [173,174].

Aldehydes, such as *glutaraldehyde (GD)*, are used as tissue fixatives, but have been proposed as potential cross-linking agent 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 [173–182]. 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, confirming the validity of the cross-linking approach [183,184].

Despite the effectiveness of aldehydes in the preservation of the HL, their cytotoxicity makes them inadequate for every-day clinical practice. Therefore, less toxic agents, such as *carbodiimides* and *plant-derived cross-linkers* have been recently studied. The cross-linking capability of carbodiimides lies in the ability to form amide bonds between activated carboxylic and amino groups of the collagen molecules.

The most studied carbodiimide in dentistry is *1-**e**thyl-3-(3-dimethylamino-propyl) carbodiimide (EDC)*, which was shown to achieve a similar bond strength preservation to that of GD [15,185–187] 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 [186]. Its ability to increase the stiffness of collagen structure has been well documented by preventing the unwinding of the collagen molecule, which is needed for the MMPs to cleave the collagen. Recent zymographic assays on dentin powder [187] and in-situ zymographic three-dimensional images obtained by confocal microscopy [127], clearly demonstrated that when EDC is used as additional therapeutic primer on acid- etched dentin, it has also the ability to inhibit dentinal MMPs.

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* [177,179,180,188–191].

Apart from chemical compounds, the cross-linking effect can also be achieved through physical and photochemical treatment, such as ultraviolet A (UVA) [192] or gamma irradiation, heating and drying [193].

Another important feature of cross-linking agents is their ability to react with the other components of the ECM. In this context, they can covalently bind to the active site of the MMP, causing long-term irreversible inactivation of the enzyme [124,125,127]. The longevity of this effect is an advantage of this mechanism over the inhibition of the MMPs through chelation. In addition, the amount of active MMPs within the HL is very limited since there is no cellular activity in peripheral dentin. Hence, once deactivated, these enzymes usually do not reactivate, and due to the lack of synthetic cellular activity, there is no activation of new enzymes. To date, GD [194], EDC [127,186] and plant-derived cross-linking agents [195,196] have shown this ability.

Removal of the unbound/residual water within the HL

It is well-known that 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 [197].

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. [198] 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. [199] 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 [200]. In the absence of water, matrix poroteases cannot cleave collagen.

Using ethanol wet-bonding permits infiltration of more hydrophobic resins [201,202] 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 [203]. To determine whether ethanol can replace bound water from collagen matrices, Jee et al. [204] 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 self-etching primer adhesives. Manufacturers 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% [201], unlike the 70 vol% [4] water that is found in acid-etched dentin just prior to adhesive application. When using self-etching primer/adhesives, one is performing "dry bonding". That is, one dries an unetched tooth before applying a self-etching primer that contains 20-25 vol% water. No water rinsing is employed. After "self-etching" for 10 sec., 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. These bonds show excellent durability [205–207].

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 hybrid layers 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 [208] 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 [209–211] 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 [212,213]. The study showed a complete remineralization of dentinal collagen after 3 to 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 [212] and SE [213] 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.

Calcium-chelation dry bonding

Can interfibrillar mineral 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 Daltons can enter collagen, molecules larger than 40 KDa are excluded [214–216]. Phosphoric acid, with a MW 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 sec of chelation, the reaction is stopped by water rinsing and air-drying [217]. 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 [217].

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.

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.

References

[1] Nakabayashi N, Nakamura M, Yasuda N. Hybrid layer as a dentin-bonding mechanism. J Esthet Dent n.d.;3:133–8.

[2] Nakabayashi N. The hybrid layer: a resin-dentin composite. Proc Finn Dent Soc 1992;88 Suppl 1:321–9.

[3] Tjäderhane L. Dentin bonding: Can we make it last? Oper Dent 2015;40:4–18. doi: 10.2341/14-095-BL.

[4] Pashley DH, Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M, et al. State of the art etch-and-rinse adhesives. Dent Mater 2017;27:1–16. doi:10.1016/j.dental. 2010.10.016.

[5] Van Meerbeek B, Yoshihara K, Yoshida Y, Mine A, De Munck J, Van Landuyt KL. State of the art of self-etch adhesives. Dent Mater 2011;27:17–28. doi:10.1016/j.dental. 2010.10.023.

[6] Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano Dorigo E. Dental adhesion review: Aging and stability of the bonded interface. Dent Mater 2008;24:90–101. doi:10.1016/j.dental.2007.02.009.

[7] Maravic T, Mazzoni A, Comba A, Scotti N, Checchi V, Breschi L. How stable is dentin as a substrate for bonding? Curr Oral Heal Reports 2017;4:248–57. doi:10.1007/ s40496-017-0149-8.

[8] Mjör IA, Shen C, Eliasson ST, Richter S. Placement and replacement of restorations in general dental practice in Iceland. Oper Dent 2002;27:117–23.

[9] Swift EJ, Perdigão J, Heymann HO. Bonding to enamel and dentin: A brief history and state of the art, 1995. Quintessence Int 1995;26:95–110.

[10] Van Meerbeek B, Munck J De, Yoshida Y. Adhesion to enamel and dentin: Current status and future challenges. Oper Dent 2003;28:215–35.

[11] Pashley DH, Ciucchi B, Sano H, Horner JA. Permeability of dentin to adhesive agents. Quintessence Int 1993;24:618–31.

[12] Wang Y, Spencer P. Quantifying adhesive penetration in adhesive/dentin interface using confocal Raman microspectroscopy. J Biomed Mater Res 2002;59:46–55. doi:10.1002/jbm.1215.

[13] Frankenberger R, Pashley DH, Reich SM, Lohbauer U, Petschelt A, Tay FR. Characterisation of resin–dentine interfaces by compressive cyclic loading. Biomaterials 2005;26:2043–52. doi:10.1016/j.biomaterials.2004.07.003.

[14] Sano H, Takatsu T, Ciucchi B, Honer J, Matthews W, Pashley D. Nanoleakage: leakage within the hybrid layer. - PubMed - NCBI. Oper Dent 1995;20:18–25.

[15] Breschi L, Prati C, Gobbi P, Pashley D, Mazzotti G, Teti G, et al. Immunohistochemical analysis of collagen fibrils within the hybrid layer: A FEISEM study. Oper Dent 2004;29:538–46.

[16] Spencer P, Wang Y, Walker MP, Wieliczka DM, Swafford JR. Interfacial Chemistry of the Dentin/Adhesive Bond. J Dent Res 2000;79:1458–63. doi:10.1177/00220345000790070501.

[17] Breschi L, Perdigão J, Gobbi P, Mazzotti G, Falconi M, Lopes M. Immunocytochemical identification of type I collagen in acid-etched dentin. J Biomed Mater Res A 2003;66:764–9. doi:10.1002/jbm.a.10073.

[18] Nakabayashi N, Saimi Y. Bonding to intact dentin. J Dent Res 1996;75:1706–15. doi:10.1177/00220345960750091401.

[19] Hanabusa M, Mine A, Kuboki T, Momoi Y, Van Ende A, Van Meerbeek B, et al. Bonding effectiveness of a new ‘multi-mode’ adhesive to enamel and dentine. J Dent 2012;40:475–84. doi:10.1016/j.jdent.2012.02.012.

[20] Toledano M, Osorio R, de Leonardi G, Rosales-Leal JI, Ceballos L, Cabrerizo-Vilchez MA. Influence of self-etching primer on the resin adhesion to enamel and dentin. Am J Dent 2001;14:205–10.

[21] Yoshida Y, Van Meerbeek B, Nakayama Y, Snauwaert J, Hellemans L, Lambrechts P, et al. Evidence of chemical bonding at biomaterial-hard tissue interfaces. J Dent Res 2000;79:709–14. doi:10.1177/00220345000790020301.

[22] Inoue S, Koshiro K, Yoshida Y, De Munck J, Nagakane K, Suzuki K, et al. Hydrolytic stability of self-etch adhesives bonded to dentin. J Dent Res 2005;84:1160–4. doi: 10.1177/154405910508401213.

[23] Tjäderhane L, Carrilho MR, Breschi L, Tay FR, Pashley DH. Dentin basic structure and composition-an overview. Endod Top 2009;20:3–29. doi:10.1111/j. 1601-1546.2012.00269.x.

[24] Carvalho RM, Tjäderhane L, Manso AP, Carrilho, M. R. &, Carvalho CAR. Dentin as a bonding substrate. Endod Top 2012;21:62–88. doi:10.1111/j.1601-1546.2012.00274.x.

[25] Perdigão J, Lambrechts P, Van Meerbeek B, Tomé ÂR, Vanherle G, Lopes AB. Morphological field emission-SEM study of the effect of six phosphoric acid etching agents on human dentin. Dent Mater 1996;12:262–71. doi:10.1016/S0109-5641(96)80033-9.

[26] Van Meerbeek B, Vargas M, Inque S, Yoshida Y, Perdigão J, Lambrechts P, et al. Microscopy investigations. Techniques, results, limitations. Am J Dent 2000;13:13D–8D.

[27] Finkelman RD, Mohan S, Jennings JC, Taylor AK, Jepsen S, Baylink DJ. Quantitation of growth factors IGF-I, SGF/IGF-II, and TGF-beta in human dentin. J Bone Miner Res 1990;5:717–23. doi:10.1002/jbmr.5650050708.

[28] Okuyama K, Takayanagi M, Ashida T, Kakudo M. A new structural model for collagen. Polym J 1977;9:341–3.

[29] Kramer RZ, Bella J, Brodsky B, Berman HM. The crystal and molecular structure of a collagen-like peptide with a biologically relevant sequence. J Mol Biol 2001;311:131–47. doi: 10.1006/jmbi.2001.4849.

[30] Rainey JK, Goh MC. A statistically derived parameterization for the collagen triple-helix. Protein Sci 2009;11:2748–54. doi:10.1110/ps.0218502.

[31] Yamauchi M, Shiiba M. Lysine hydroxylation and cross-linking of collagen. Methods Mol. Biol. Post-translational Modif. proteins, Totowa, NJ: Humana Press; 2008, p. 95–108. doi: 10.1007/978-1-60327-084-7_7.

[32] Veis A. Mineralization in organic matrix frameworks. Rev Mineral Geochemistry 2003;54:249–89. doi:10.2113/0540249.

[33] Hannas AR, Pereira JC, Granjeiro JM, Tjäderhane L, Hannas AR, Pereira JC, et al. The role of matrix metalloproteinases in the oral environment. Acta Odontol Scand 2007;65:1–13. doi:10.1080/00016350600963640.

[34] Schlueter RJ, Veis A. The macromolecular organization of dentine matrix collagen. II. Periodate degradation and carbohydrate cross-linking. Biochemistry 1964;3:1657–65.

[35] Veis A, Schlueter RJ. The macromolecular organization of dentine matrix collagen I. Characterization of dentine collagen. Biochemistry 1964;3:1650–7.

[36] Brinckmann J, Notbohm H, Müller P. Collagen: primer in structure, processing and assembly. Springer-Verlag; 2005.

[37] Lin CP, Douglas WH, Erlandsen SL. Scanning electron microscopy of type I collagen at the dentin-enamel junction of human teeth. J Histochem Cytochem 1993;41:381–8. doi: 10.1177/41.3.8429200.

[38] Breschi L, Gobbi P, Mazzotti G, Ellis TH, Sacher E, Stangel I. Field emission in-lens SEM study of enamel and dentin. J Biomed Mater Res 1999;46:315–23.

[39] Goldberg M, Takagi M. Dentine proteoglycans: composition, ultrastructure and functions. Histochem J 1993;25:781–806.

[40] Cheng H, Caterson B, Yamauchi M. Identification and immunolocalization of chondroitin sulfate proteoglycans in tooth cementum. Connect Tissue Res 1999;40:37–47.

[41] Scott JE. Proteoglycan-fibrillar collagen interactions. Biochem J 1988;252:313–23.

[42] Vogel KG, Paulsson M, Heinegård D. Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. Biochem J 1984;223:587–97.

[43] Hedbom E, Heinegård D. Binding of fibromodulin and decorin to separate sites on fibrillar collagens. J Biol Chem 1993;268:27307–12.

[44] Oyarzún a, Rathkamp H, Dreyer E. Immunohistochemical and ultrastructural evaluation of the effects of phosphoric acid etching on dentin proteoglycans. Eur J Oral Sci 2000;108:546–54.

[45] Breschi L, Lopes M, Gobbi P, Mazzotti G, Falconi M, Perdigão J. Dentin proteoglycans: an immunocytochemical FEISEM study. J Biomed Mater Res 2002;61:40–46.

[46] Breschi L, Gobbi P, Lopes M, Prati C, Falconi M, Teti G, et al. Immunocytochemical analysis of dentin: a double-labeling technique. J Biomed Mater Res A 2003;67:11–7. doi: 10.1002/jbm.a.10048.

[47] Bedran-Russo AKB, Pereira PNR, Duarte WR, Okuyama K, Yamauchi M. Removal of dentin matrix proteoglycans by trypsin digestion and its effect on dentin bonding. J Biomed Mater Res Part B Appl Biomater 2008;85B:261–6. doi:10.1002/jbm.b.30944.

[48] Mazzoni A, Pashley DH, Ruggeri A, Vita F, Falconi M, Di Lenarda R, et al. Adhesion to chondroitinase ABC treated dentin. J Biomed Mater Res B Appl Biomater 2008;86:228–36. doi:10.1002/jbm.b.31010.

[49] Attin T, Hannig C, Wiegand A, Attin R. Effect of bleaching on restorative materials and restorations—a systematic review. Dent Mater 2004;20:852–61. doi:10.1016/j.dental. 2004.04.002.

[50] Lyons K, Ng B. Nightguard vital bleaching: a review and clinical study. N Z Dent J 1998;94:100–3.

[51] Arcari GM, Araújo E, Baratieri LN, Lopes GC. Microtensile bond strength of a nanofilled composite resin to human dentin after nonvital tooth bleaching. J Adhes Dent 2007;9:333–40.

[52] Feiz A, Mosleh H, Nazeri R. Evaluating the effect of antioxidant agents on shear bond strength of tooth-colored restorative materials after bleaching: A systematic review. J Mech Behav Biomed Mater 2017;71:156–64. doi:10.1016/j.jmbbm.2017.03.010.

[53] Cadenaro M, Breschi L, Antoniolli F, Mazzoni A, Di Lenarda R. Influence of whitening on the degree of conversion of dental adhesives on dentin. Eur J Oral Sci 2006;114:257–62. doi:10.1111/j.1600-0722.2006.00351.x.

[54] Breschi L, Cadenaro M, Antoniolli F, Sauro S, Biasotto M, Prati C, et al. Polymerization kinetics of dental adhesives cured with LED: Correlation between extent of conversion and permeability. Dent Mater 2007;23:1066–72. doi:10.1016/J.DENTAL. 2006.06.040.

[55] Ari H, Erdemir A, Belli S. Evaluation of the effect of endodontic irrigation solutions on the microhardness and the roughness of root canal dentin. J Endod 2004;30:792–5.

[56] Morris M, Lee K, Agee K, Bouillaguet S, Pashley D. Effects of sodium hypochlorite and RC-prep on bond strengths of resin cement to endodontic surfaces. J Endod 2001;27:753–7. doi:10.1097/00004770-200112000-00010.

[57] Kielbassa AM, Attin T, Hellwig E. Diffusion behavior of eugenol from zinc oxide-eugenol mixtures through human and bovine dentin in vitro. Oper Dent 1997;22:15–20.

[58] Carvalho CN, de Oliveira Bauer josé roberto, Loguercio AD, Reis A. Effect of ZOE temporary restoration on resin-dentin bond strength using different adhesive strategies. J Esthet Restor Dent 2007;19:144–52. doi:10.1111/j.1708-8240.2007.00087.x.

[59] Serafino C, Gallina G, Cumbo E, Ferrari M. Surface debris of canal walls after post space preparation in endodontically treated teeth: a scanning electron microscopic study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:381–7. doi:10.1016/S1079210403006061.

[60] Serafino C, Gallina G, Cumbo E, Monticelli F, Goracci C, Ferrari M. Ultrasound effects after post space preparation: An SEM study. J Endod 2006;32:549–52. doi:10.1016/J.JOEN.2005.07.003.

[61] Coniglio I, Magni E, Goracci C, Radovic I, Carvalho CA, Grandini S, et al. Post space cleaning using a new nickel titanium endodontic drill combined with different cleaning regimens. J Endod 2008;34:83–6. doi:10.1016/j.joen.2007.10.019.

[62] Bitter K, Kielbassa AM. Post-endodontic restorations with adhesively luted fiber-reinforced composite post systems: a review. Am J Dent 2007;20:353–60.

[63] Cagidiaco MC, Goracci C, Garcia-Godoy F, Ferrari M. Clinical studies of fiber posts: a literature review. Int J Prosthodont 2008;21:328–36.

[64] Goracci C, Grandini S, Bossù M, Bertelli E, Ferrari M. Laboratory assessment of the retentive potential of adhesive posts: A review. J Dent 2007;35:827–35. doi:10.1016/J.JDENT.2007.07.009.

[65] Zicari F, Coutinho E, Scotti R, Van Meerbeek B, Naert I. Mechanical properties and micro-morphology of fiber posts. Dent Mater 2013;29:e45–52. doi:10.1016/j.dental.2012.11.001.

[66] Ferrari M, Breschi L, Grandini S. Fiber posts and endodontically treated teeth : a compendium of scientific and clinical perspectives. Modern Dentistry Media; 2008.

[67] Ferrari M, Carvalho CA, Goracci C, Antoniolli F, Mazzoni A, Mazzotti G, et al. Influence of luting material filler content on post cementation. J Dent Res 2009;88:951–6. doi: 10.1177/0022034509342851.

[68] Cadenaro M, Antoniolli F, Sauro S, Tay FR, Di Lenarda R, Prati C, et al. Degree of conversion and permeability of dental adhesives. Eur J Oral Sci 2005;113:525–30. doi:10.1111/ j.1600-0722.2005.00251.x.

[69] Tay FR, Loushine RJ, Lambrechts P, Weller RN, Pashley DH. Geometric factors affecting dentin bonding in root canals: a theoretical modeling approach. J Endod 2005;31:584–9.

[70] Ferrari M, Mannocci F, Vichi A, Cagidiaco MC, Mjör IA. Bonding to root canal: structural characteristics of the substrate. Am J Dent 2000;13:255–60.

[71] Feilzer AJ, Dauvillier BS. Effect of TEGDMA/BisGMA ratio on stress development and viscoelastic properties of experimental two-paste composites. J Dent Res 2003;82:824–8. doi:10.1177/154405910308201012.

[72] Bouillaguet S, Troesch S, Wataha JC, Krejci I, Meyer JM, Pashley DH. Microtensile bond strength between adhesive cements and root canal dentin. Dent Mater 2003;19:199–205.

[73] Braga RR, Ferracane JL. Alternatives in polymerization contraction stress management. Crit Rev Oral Biol Med 2004;15:176–84.

[74] Aravamudhan K, Rakowski D, Fan PL. Variation of depth of cure and intensity with distance using LED curing lights. Dent Mater 2006;22:988–94. doi:10.1016/j.dental. 2005.11.031.

[75] Lui JL. Depth of composite polymerization within simulated root canals using light-transmitting posts. Oper Dent 1994;19:165–8.

[76] Yoldas O, Alaçam T. Microhardness of composites in simulated root canals cured with light transmitting posts and glass-fiber reinforced composite posts. J Endod 2005;31:104–6.

[77] Musanje L, Darvell B. Curing-light attenuation in filled-resin restorative materials. Dent Mater 2006;22:804–17. doi:10.1016/j.dental.2005.11.009.

[78] Faria-e-Silva A, Gallego Arias V, Soares L, Martin A, Roberto Marcondes Martins L. Influence of fiber-post translucency on the degree of conversion of a dual-cured resin cement. J Endod 2007;33:303–5.

[79] Teixeira ECN, Teixeira FB, Piasick JR, Thompson JY. An in vitro assessment of prefabricated fiber post systems. J Am Dent Assoc 2006;137:1006–12.

[80] Roberts H, Leonard D, Vandewalle K, Cohen M, Charlton D. The effect of a translucent post on resin composite depth of cure. Dent Mater 2004;20:617–22. doi:10.1016/ j.dental.2003.10.004.

[81] Kim YK, Kim SK, Kim KH, Kwon TY. Degree of conversion of dual-cured resin cement light-cured through three fibre posts within human root canals: an *ex vivo* study. Int Endod J 2009;42:667–74. doi:10.1111/j.1365-2591.2009.01565.x.

[82] Urapepon S. Degree of conversion of resin composite cured by light through a translucent fiber posts. J Adv Prosthodont 2014;6:194–9. doi:10.4047/jap.2014.6.3.194.

[83] Ozcan M, Zamboni S, Valandro F, Bottino M, Bagis B. Microhardness of dual-polymerized resin cement around a translucent fiber post in the intraradicular environment. J Conserv Dent 2011;14:370–3. doi:10.4103/0972-0707.87200.

[84] Dauvillier BS, Feilzer AJ, De Gee AJ, Davidson CL. Visco-elastic parameters of dental restorative materials during setting. J Dent Res 2000;79:818–23. doi: 10.1177/00220345000790030601.

[85] Braga R, Boaro L, Kuroe T, Azevedo C, Singer J. Influence of cavity dimensions and their derivatives (volume and 'C' factor) on shrinkage stress development and microleakage of composite restorations. Dent Mater 2006;22:818–23. doi:10.1016/j.dental.2005.11.010.

[86] Salz U, Bock T. Testing adhesion of direct restoratives to dental hard tissue - a review. J Adhes Dent 2010;12:343–71. doi:10.3290/j.jad.a19741.

[87] Hashimoto M, Ohno H, Sano H, Kaga M, Oguchi H. In vitro degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy. Biomaterials 2003;24:3795–803. doi:10.1016/S0142-9612(03)00262-X.

[88] Wang Y, Spencer P. Hybridization efficiency of the adhesive/dentin interface with wet bonding. J Dent Res 2003;82:141–5. doi:10.1177/154405910308200213.

[89] Tay FR, Pashley DH. Have dentin adhesives become too hydrophilic? J Can Dent Assoc 2003;69:726–31. doi:10.1016/S0109-5641(03)00110-6.

[90] Frassetto A, Breschi L, Turco G, Marchesi G, Di Lenarda R, Tay FR, et al. Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability - A literature review. Dent Mater 2016;32:e41–53. doi:10.1016/j.dental.2015.11.007.

[91] Van Meerbeek B, Van Landuyt K, De Munck J, Hashimoto M, Peumans M, Lambrechts P, et al. Technique-sensitivity of contemporary adhesives. Dent Mater J 2005;24:1–13.

[92] Tay FR, Pashley DH, Yoshiyama M. Two modes of nanoleakage expression in single-step adhesives. J Dent Res 2002;81:472–6. doi:10.1177/154405910208100708.

[93] Tay FR, Pashley DH, Suh BI, Carvalho RM, Itthagarun A. Single-step adhesives are permeable membranes. J Dent 2002;30:371–82. doi:10.1016/S0300-5712(02)00064-7.

[94] Jacobsen T, Söderholm KJ. Some effects of water on dentin bonding. Dent Mater 1995;11:132–6. doi:10.1016/0109-5641(95)80048-4.

[95] Mazzoni A, Papa V, Nato F, Carrilho M, Tjäderhane L, Ruggeri A, et al. Immunohistochemical and biochemical assay of MMP-3 in human dentine. J Dent 2011;39:231–7. doi:10.1016/j.jdent.2011.01.001.

[96] Paul SJ, Leach M, Rueggeberg FA, Pashley DH. Effect of water content on the physical properties of model dentine primer and bonding resins. J Dent 1999;27:209–14. doi:10.1016/S0300-5712(98)00042-6.

[97] Cadenaro M, Breschi L, Antoniolli F, Navarra CO, Mazzoni A, Tay FR, et al. Degree of conversion of resin blends in relation to ethanol content and hydrophilicity. Dent Mater 2008;24:1194–200. doi:10.1016/j.dental.2008.01.012.

[98] Anseth KS, Kline LM, Walker TA, Anderson KJ, Bowman CN. Reaction kinetics and volume relaxation during polymerizations of multiethylene glycol dimethacrylates. Macromolecules 1995;28:2491–9. doi:10.1021/ma00111a050.

[99] Shobha HK, Sankarapandian M, Sun Y, Kalachandra S, McGrath JE, Taylor DF. Effect of dilution on the kinetics of cross-linking thermal polymerization of dental composite matrix resins. J Mater Sci Mater Med 1997;8:583–6.

[100] Cadenaro M, Breschi L, Rueggeberg FA, Suchko M, Grodin E, Agee K, et al. Effects of residual ethanol on the rate and degree of conversion of five experimental resins. Dent Mater 2009;25:621–8. doi:10.1016/j.dental.2008.11.005.

[101] Malacarne-Zanon J, Pashley DH, Agee KA, Foulger S, Alves MC, Breschi L, et al. Effects of ethanol addition on the water sorption/solubility and percent conversion of comonomers in model dental adhesives. Dent Mater 2009;25:1275–84. doi:10.1016/j.dental. 2009.03.015.

[102] Ye Q, Spencer P, Wang Y. Nanoscale patterning in crosslinked methacrylate copolymer networks: An atomic force microscopy study. J Appl Polym Sci Symp 2007;106:3843–51. doi:10.1002/app.27044.

[103] Ye Q, Park JG, Topp E, Wang Y, Misra A, Spencer P. In vitro performance of nano-heterogeneous dentin adhesive. J Dent Res 2008;87:829–33. doi: 10.1177/154405910808700911.

[104] Wang Y, Spencer P, Yao X, Ye Q. Effect of coinitiator and water on the photoreactivity and photopolymerization of HEMA/camphoquinone-based reactant mixtures. J Biomed Mater Res A 2006;78:721–8. doi:10.1002/jbm.a.30733.

[105] Ilie N, Hickel R. Can CQ be completely replaced by alternative initiators in dental adhesives? Dent Mater J 2008;27:221–8. doi:10.4012/dmj.27.221.

[106] Cadenaro M, Antoniolli F, Codan B, Agee K, Tay FR, Dorigo EDS, et al. Influence of different initiators on the degree of conversion of experimental adhesive blends in relation to their hydrophilicity and solvent content. Dent Mater 2010;26:288–94. doi:10.1016/j.dental. 2009.11.078.

[107] Gale M., Darvell B. Thermal cycling procedures for laboratory testing of dental restorations. J Dent 1999;27:89–99. doi:10.1016/S0300-5712(98)00037-2.

[108] Yoshida E, Uno S, Nodasaka Y, Kaga M, Hirano S. Relationship between water status in dentin and interfacial morphology in all-in-one adhesives. Dent Mater 2007;23:556–60. doi: 10.1016/j.dental.2006.03.014.

[109] Dayan D, Binderman I, Mechanic GL. A preliminary study of activation of collagenase in carious human dentine matrix. Arch Oral Biol 1983;28:185–7. doi: 10.1016/0003-9969(83)90126-7.

[110] Tjäderhane L, Larjava H, Sorsa T, Uitto V-J, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res 1998;77:1622–9. doi:10.1177/00220345980770081001.

[111] Pashley D, Tay F, Yiu C, Hashimoto M, Breschi L, Carvalho R. Collagen degradation by host-derived enzymes during aging. J Dent Res 2004;83:216–21.

[112] Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res 2003;92:827–39. doi: 10.1161/01.RES.0000070112.80711.3D.

[113] Mazzoni A, Mannello F, Tay FR, Tonti G a M, Papa S, Mazzotti G, et al. Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. J Dent Res 2007;86:436–40. doi:10.1177/154405910708600509.

[114] Mazzoni A, Pashley DH, Tay FR, Gobbi P, Orsini G, Ruggeri A, et al. Immunohistochemical identification of MMP-2 and MMP-9 in human dentin: Correlative FEI-SEM/TEM analysis. J Biomed Mater Res - Part A 2009;88:697–703. doi:10.1002/jbm.a.31920.

[115] Santos J, Carrilho M, Tervahartiala T, Sorsa T, Breschi L, Mazzoni A, et al. Determination of matrix metalloproteinases in human radicular dentin. J Endod 2009;35:686–9. doi:10.1016/j.joen.2009.02.003.

[116] Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjäderhane L. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. Arch Oral Biol 2007;52:121–7. doi:10.1016/j.archoralbio.2006.08.009.

[117] Sulkala M, Larmas M, Sorsa T, Salo T, Tjäderhane L. The localization of matrix metalloproteinase-20 (MMP-20, enamelysin) in mature human teeth. J Dent Res 2002;81:603–7.

[118] Martin-De Las Heras S, Valenzuela A, Overall C. The matrix metalloproteinase gelatinase A in human dentine. Arch Oral Biol 2000;45:757–65. doi:10.1016/S0003-9969(00)00052-2.

[119] Boushell LW, Nagaoka H, Nagaoka H, Yamauchi M. Increased matrix metalloproteinase-2 and bone sialoprotein response to human coronal caries. Caries Res 2011;45:453–9. doi:10.1159/000330601.

[120] Boukpessi T, Menashi S, Camoin L, TenCate JM, Goldberg M, Chaussain-Miller C. The effect of stromelysin-1 (MMP-3) on non-collagenous extracellular matrix proteins of demineralized dentin and the adhesive properties of restorative resins. Biomaterials 2008;29:4367–73. doi:10.1016/j.biomaterials.2008.07.035.

[121] Garnero P, Ferreras M, Karsdal M a, Nicamhlaoibh R, Risteli J, Borel O, et al. The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. J Bone Miner Res 2003;18:859–67. doi:10.1359/jbmr.2003.18.5.859.

[122] Perumal S, Antipova O, Orgel JPRO. Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis. Proc Natl Acad Sci 2008;105:2824–9. doi:10.1073/pnas.0710588105.

[123] Mazzoni A, Carrilho M, Papa V, Tjäderhane L, Gobbi P, Nucci C, et al. MMP-2 assay within the hybrid layer created by a two-step etch-and-rinse adhesive: Biochemical and immunohistochemical analysis. J Dent 2011;39:470–7. doi:10.1016/j.jdent.2011.04.004.

[124] Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Mannello F, Tjäderhane L, et al. Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives. Biomaterials 2006;27:4470–6. doi:10.1016/j.biomaterials.2006.01.040.

[125] Mazzoni A, Scaffa P, Carrilho M, Tjäderhane L, Di Lenarda R, Polimeni A, et al. Effects of etch-and-rinse and self-etch adhesives on dentin MMP-2 and MMP-9. J Dent Res 2013;92:82–6. doi:10.1177/0022034512467034.

[126] Nishitani Y, Yoshiyama M, Wadgaonkar B, Breschi L, Mannello F, Mazzoni A, et al. Activation of gelatinolytic / collagenolytic activity in dentin by self etching adhesives. Eur J Oral Sci 2006;114:160–6.

[127] Mazzoni A, Nascimento F, Carrilho M, Tersariol I, Papa V, Tjaderhane L, et al. MMP activity in the hybrid layer detected with in situ zymography. J Dent Res 2012;91:467–72. doi: 10.1177/0022034512439210.

[128] Suppa P, Breschi L, Ruggeri A, Mazzotti G, Prati C, Chersoni S, et al. Nanoleakage within the hybrid layer: A correlative FEISEM/TEM investigation. J Biomed Mater Res - Part B Appl Biomater 2005;73:7–14. doi:10.1002/jbm.b.30217.

[129] Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci U S A 1990;87:5578–82.

[130] Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, et al. Cysteine cathepsins in human dentin-pulp complex. J Endod 2010;36:475–81. doi:10.1016/ j.joen.2009.12.034.

[131] Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, et al. Cysteine cathepsins in human carious dentin. J Dent Res 2011;90:506–11. doi: 10.1177/0022034510391906.

[132] Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, et al. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. J Dent Res 2011;90:953–68. doi:10.1177/0022034510391799.

[133] Vidal CMP, Tjäderhane L, Scaffa PM, Tersariol IL, Pashley D, Nader HB, et al. Abundance of MMPs and cysteine cathepsins in caries-affected dentin. J Dent Res 2014;93:269–74. doi:10.1177/0022034513516979.

[134] Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, et al. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 1999;273:32347–52. doi:10.1074/jbc.273.48.32347.

[135] Brömme D, Wilson S. Role of cysteine cathepsins in extracellular proteolysis. Extracell. matrix Degrad., Berlin, DE: Springer Verlag-Heidelberg; 2011, p. 23–52. doi: 10.1007/978-3-642-16861-1.

[136] Scaffa PMC, Breschi L, Mazzoni A, Vidal C de MP, Curci R, Apolonio F, et al. Co-distribution of cysteine cathepsins and matrix metalloproteases in human dentin. Arch Oral Biol 2017;74:101–7. doi:10.1016/j.archoralbio.2016.11.011.

[137] Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. Clin Diagn Lab Immunol 1999;6:437–9.

[138] Scaffa PMC, Vidal CMP, Barros N, Gesteira TF, Carmona a. K, Breschi L, et al. Chlorhexidine inhibits the activity of dental cysteine cathepsins. J Dent Res 2012;91:420–5. doi:10.1177/0022034511435329.

[139] Breschi L, Mazzoni A, Nato F, Carrilho M, Visintini E, Tjäderhane L, et al. Chlorhexidine stabilizes the adhesive interface: a 2-year in vitro study. Dent Mater 2010;26:320–5. doi:10.1016/j.dental.2009.11.153.

[140] Campos EA, Correr GM, Leonardi DP, Barato-Filho F, Gonzaga CC, Zielak JC. Chlorhexidine diminishes the loss of bond strength over time under simulated pulpal pressure and thermo-mechanical stressing. J Dent 2009;37:108–14. doi:10.1016/j.jdent.2008.10.003.

[141] Loguercio AD, Hass V, Gutierrez MF, Luque-Martinez IV, Szezs A, Stanislawczuk R, et al. Five-year effects of chlorhexidine on the in vitro durability of resin/dentin interfaces. J Adhes Dent 2016;18:35–43. doi:10.3290/j.jad.a35514.

[142] Zheng P, Zaruba M, Attin T, Wiegand A. Effect of different matrix metalloproteinase inhibitors on microtensile bond strength of an etch-and-rinse and a self-etching adhesive to dentin. Oper Dent 2014;40:80–6. doi:10.2341/13-162-L.

[143] Carrilho MRO, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, et al. In vivo preservation of the hybrid layer by chlorhexidine. J Dent Res 2007;86:529–33. doi: 10.1177/154405910708600608.

[144] Carrilho MRO, Carvalho RM, de Goes MF, di Hipólito V, Geraldeli S, Tay FR, et al. Chlorhexidine preserves dentin bond in vitro. J Dent Res 2007;86:90–4. doi: 10.1177/154405910708600115.

[145] Brackett MG, Tay FR, Brackett WW, Dib A, Dipp FA, Mai S, et al. In vivo chlorhexidine stabilization of hybrid layers of an acetone-based dentin adhesive. Oper Dent 2009;34:379–83. doi:10.2341/08-103.

[146] Mobarak EH. Effect of chlorhexidine pretreatment on bond strength durability of caries-affected dentin over 2-year aging in artificial saliva and under simulated intrapulpal pressure. Oper Dent 2011;36:649–60. doi:10.2341/11-018-L.

[147] Leitune VCB, Portella FF, Bohn PV, Collares FM, Samuel SMW. Influence of chlorhexidine application on longitudinal adhesive bond strength in deciduous teeth. Braz Oral Res 2011;25:388–92. doi:10.1590/S1806-83242011000500003.

[148] Yiu CKY, Hiraishi N, Tay FR, King NM. Effect of chlorhexidine incorporation into dental adhesive resin on durability of resin-dentin bond. J Adhes Dent 2012;14. doi:10.3290/j.jad.a25674.

[149] Kim J, Uchiyama T, Carrilho M, Agee KA, Mazzoni A, Breschi L, et al. Chlorhexidine binding to mineralized versus demineralized dentin powder. Dent Mater 2010;26:771–8. doi:10.1016/j.dental.2010.04.001.

[150] Carrilho MR, Carvalho RM, Sousa EN, Nicolau J, Breschi L, Mazzoni A, et al. Substantivity of chlorhexidine to human dentin. Dent Mater 2010;26:779–85. doi:10.1016/j.dental.2010.04.002.

[151] da Silva EM, de Sá Rodrigues CUF, de Oliveira Matos MP, de Carvalho TR, dos Santos GB, Amaral CM. Experimental etch-and-rinse adhesive systems containing MMP-inhibitors: Physicochemical characterization and resin-dentin bonding stability. J Dent 2015;43:1491–7. doi:10.1016/j.jdent.2015.10.004.

[152] Stanislawczuk R, Pereira F, Muñoz MA, Luque I, Farago PV, Reis A, et al. Effects of chlorhexidine-containing adhesives on the durability of resin-dentine interfaces. J Dent 2014;42:39–47. doi:10.1016/j.jdent.2013.11.002.

[153] Zhou J, Tan J, Chen L, Li D, Tan Y. The incorporation of chlorhexidine in a two-step self-etching adhesive preserves dentin bond in vitro. J Dent 2009;37:807–12. doi:10.1016/j.jdent.2009.06.011.

[154] Imazato S, Kinomoto Y, Tarumi H, Ebisu S, R. Tay F. Antibacterial activity and bonding characteristics of an adhesive resin containing antibacterial monomer MDPB. Dent Mater 2017;19:313–9. doi:10.1016/S0109-5641(02)00060-X.

[155] Thompson JM, Agee K, Sidow SJ, McNally K, Lindsey K, Borke J, et al. Inhibition of endogenous dentin matrix metalloproteinases by ethylenediaminetetraacetic acid. J Endod 2012;38:62–5. doi:10.1016/j.joen.2011.09.005.

[156] Carvalho RM, Tay F, Sano H, Yoshiyama M, Pashley DH. Long-term mechanical properties of EDTA-demineralized dentin matrix. J Adhes Dent 2000;2:193–9.

[157] Tezvergil-Mutluay A, Mutluay MM, Gu LS, Zhang K, Agee KA, Carvalho RM, et al. The anti-MMP activity of benzalkonium chloride. J Dent 2011;39:57–64. doi:10.1016/j.jdent. 2010.10.003.

[158] Sabatini C, Patel SK. Matrix metalloproteinase inhibitory properties of benzalkonium chloride stabilizes adhesive interfaces. Eur J Oral Sci 2013;121:610–6. doi:10.1111/eos.12089.

[159] Sabatini C, Ortiz PA, Pashley DH. Preservation of resin-dentin interfaces treated with benzalkonium chloride adhesive blends. Eur J Oral Sci 2015;123:108–15. doi:10.1111/eos. 12176.

[160] Imazato S, Tay FR, Kaneshiro A V, Takahashi Y, Ebisu S. An in vivo evaluation of bonding ability of comprehensive antibacterial adhesive system incorporating MDPB. Dent Mater 2017;23:170–6. doi:10.1016/j.dental.2006.01.005.

[161] Pashley D, Tay F, Imazato S. How to increase the durability of resin-dentin bonds. Compend Contin Educ Dent 2011;32:60–64.

[162] Tezvergil-Mutluay A, Agee KA, Mazzoni A, Carvalho RM, Carrilho M, Tersariol IL, et al. Can quaternary ammonium methacrylates inhibit matrix MMPs and cathepsins? Dent Mater 2015;31:e25–32. doi:10.1016/j.dental.2014.10.006.

[163] Tezvergil-Mutluay A, Agee KA, Uchiyama T, Imazato S, Mutluay MM, Cadenaro M, et al. The inhibitory effects of quaternary ammonium methacrylates on soluble and matrix-bound MMPs. J Dent Res 2011;90:535–40. doi:10.1177/0022034510389472.

[164] Antonucci JM, Zeiger DN, Tang K, Lin-Gibson S, Fowler BO, Lin NJ. Synthesis and characterization of dimethacrylates containing quaternary ammonium functionalities for dental applications. Dent Mater 2012;28:219–28. doi:10.1016/j.dental.2011.10.004.

[165] Heikkilä P, Teronen O, Moilanen M, Konttinen YT, Hanemaaijer R, Laitinen M, et al. Bisphosphonates inhibit stromelysin-1 (MMP-3), matrix metalloelastase (MMP-12),

collagenase-3 (MMP-13) and enamelysin (MMP-20), but not urokinase-type plasminogen activator, and diminish invasion and migration of human malignant and endothelial cell lines. Anticancer Drugs 2002;13:245–54.

[166] Tezvergil-Mutluay A, Agee KA, Hoshika T, Tay FR, Pashley DH. The inhibitory effect of polyvinylphosphonic acid on functional matrix metalloproteinase activities in human demineralized dentin. Acta Biomater 2010;6:4136–42. doi:10.1016/j.actbio.2010.05.017.

[167] Sulkala M, Wahlgren J, Larmas M, Sorsa T, Teronen O, Salo T, et al. The effects of MMP inhibitors on human salivary MMP activity and caries progression in rats. J Dent Res 2001;80:1545–9. doi:10.1177/00220345010800061301.

[168] Lauhio A, Salo T, Tjäderhane L, Lähdevirta J, Golub LM, Sorsa T. Tetracyclines in treatment of rheumatoid arthritis. Lancet (London, England) 1995;346:645–6.

[169] Sorsa T, Tjäderhane L, Konttinen YT, Lauhio A, Salo T, Lee H, et al. Matrix metalloproteinases: Contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. Ann Med 2006;38:306–21. doi:10.1080/07853890600800103.

[170] Breschi L, Martin P, Mazzoni A, Nato F, Carrilho M, Tj??derhane L, et al. Use of a specific MMP-inhibitor (galardin) for preservation of hybrid layer. Dent Mater 2010;26:571–8. doi:10.1016/j.dental.2010.02.007.

[171] De Munck J, Mine A, Van den Steen PE, Van Landuyt KL, Poitevin A, Opdenakker G, et al. Enzymatic degradation of adhesive-dentin interfaces produced by mild self-etch adhesives. Eur J Oral Sci 2010;118:494–501. doi:10.1111/j.1600-0722.2010.00758.x.

[172] Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, et al. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim Biophys Acta - Proteins Proteomics 2012;1824:68–88. doi:10.1016/j.bbapap.2011.10.002.

[173] Bedran-Russo AKB, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. J Biomed Mater Res - Part B Appl Biomater 2008;86:330–4. doi:10.1002/jbm.b.31022.

[174] Xu C, Wang Y. Cross-linked demineralized dentin maintains its mechanical stability when challenged by bacterial collagenase. J Biomed Mater Res - Part B Appl Biomater 2011;96 B:242–8. doi:10.1002/jbm.b.31759.

[175] Chen C, Mao C, Sun J, Chen Y, Wang W, Pan H, et al. Glutaraldehyde-induced remineralization improves the mechanical properties and biostability of dentin collagen. Mater Sci Eng C 2016;67:657–65. doi:10.1016/j.msec.2016.05.076.

[176] Hass V, Luque-Martinez IV, Gutierrez MF, Moreira CG, Gotti VB, Feitosa VP, et al. Collagen cross-linkers on dentin bonding: Stability of the adhesive interfaces, degree of conversion of the adhesive, cytotoxicity and in situ MMP inhibition. Dent Mater 2015;32:732–41. doi:10.1016/j.dental.2016.03.008.

[177] Bedran-Russo AKB, Pereira PNR, Duarte WR, Drummond JL, Yamauchi M. Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. J Biomed Mater Res Part B Appl Biomater 2007;80:268–72. doi:10.1002/jbm.b.30593.

[178] Liu R, Fang M, Xiao Y, Li F, Yu L, Zhao S, et al. The effect of transient proanthocyanidins preconditioning on the cross-linking and mechanical properties of demineralized dentin. J Mater Sci Mater Med 2011;22:2403–11. doi:10.1007/s10856-011-4430-4.

[179] Hiraishi N, Sono R, Sofiqul I, Yiu C, Nakamura H, Otsuki M, et al. In vitro evaluation of plant-derived agents to preserve dentin collagen. Dent Mater 2013;29:1048–54. doi:10.1016/j.dental.2013.07.015.

[180] Seseogullari-Dirihan R, Apollonio F, Mazzoni A, Tjaderhane L, Pashley D, Breschi L, et al. Use of crosslinkers to inactivate dentin MMPs. Dent Mater 2016;32:423–32. doi:10.1016/j.dental.2015.12.012.

[181] Han B, Jaurequi J, Tang BW, Nimni ME. Proanthocyanidin: a natural crosslinking reagent for stabilizing collagen matrices. J Biomed Mater Res A 2003;65:118–24. doi:10.1002/jbm.a.10460.

[182] Zhou J, Chiba A, Scheffel DLS, Hebling J, Agee K, Tagami J, et al. Cross-linked dry bonding: A new etch-and-rinse technique. Dent Mater 2016;32:1124–32. doi:10.1016/j.dental.2016.06.014.

[183] Nimni ME. The cross-linking and structure modification of the collagen matrix in the design of cardiovascular prosthesis. J Card Surg 1988;3:523–33. doi:10.1111/j.1540-8191.1988.tb00446.x.

[184] Sung H, Chang Y, CT C, Chen C, Liang H. Crosslinking characteristics and mechanical properties of a bovine pericardium fixed with a naturally occurring crosslinking agent. J Biomed Mater Res 1999;47:116–26.

[185] Bedran-Russo AKB, Vidal CMP, Dos Santos PH, Castellan CS. Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds. J Biomed Mater Res - Part B Appl Biomater 2010;94:250–5. doi:10.1002/jbm.b.31649.

[186] Tezvergil-Mutluay A, Mutluay MM, Agee KA, Seseogullari-Dirihan R, Hoshika T, Cadenaro M, et al. Carbodiimide cross-linking inactivates soluble and matrix-bound MMPs, in vitro. J Dent Res 2012;91:192–6. doi:10.1177/0022034511427705.

[187] Mazzoni A, Angeloni V, Apolonio FM, Scotti N, Tjäderhane L, Tezvergil-Mutluay A, et al. Effect of carbodiimide (EDC) on the bond stability of etch-and-rinse adhesive systems. Dent Mater 2013;29:1040–7. doi:10.1016/j.dental.2013.07.010.

[188] Al-Ammar A, Drummond JL, Bedran-Russo AK. The use of collagen cross-linking agents to enhance dentin bond strength. J Biomed Mater Res - Part B Appl Biomater 2009;91:419–24. doi:10.1002/jbm.b.31417.

[189] Liu Y, Dusevich V, Wang Y. Proanthocyanidins rapidly stabilize the demineralized dentin layer. J Dent Res 2013;92:746–52. doi:10.1177/0022034513492769.

[190] Epasinghe DJ, Yiu CKY, Burrow MF, Tay FR, King NM. Effect of proanthocyanidin incorporation into dental adhesive resin on resin–dentine bond strength. J Dent 2012;40:173–80. doi:10.1016/j.jdent.2011.11.013.

[191] Seseogullari-Dirihan R, Mutluay MM, Vallittu P, Pashley DH, Tezvergil-Mutluay A. Effect of pretreatment with collagen crosslinkers on dentin protease activity. Dent Mater 2015;31:941–7. doi:10.1016/j.dental.2015.05.002.

[192] Seseogullari-Dirihan R, Tjäderhane L, Pashley DH, Tezvergil-Mutluay A. Effect of ultraviolet A-induced crosslinking on dentin collagen matrix. Dent Mater 2015;31:1225–31. doi:10.1016/j.dental.2015.08.145.

[193] Hayashi M, Okamura K, Koychev EV, Furuya Y, Sugeta A, Ota T, et al. Effects of rehydration on dentin strengthened by heating or UV irradiation. J Dent Res 2010;89:154–8. doi:10.1177/0022034509354564.

[194] Sabatini C, Scheffel DLS, Scheffel RH, Agee KA, Takahashi M, Breschi L, et al. Inhibition of endogenous human dentin MMPs by Gluma. Dent Mater 2014;30:752–8. doi: 10.1016/j.dental.2014.04.006.Inhibition.

[195] Zhang Y, Gu Y, Lee H-M, Hambardjieva E, Vranková K, Golub LM, et al. Design, synthesis and biological activity of new polyenolic inhibitors of matrix metalloproteinases: a focus on chemically-modified curcumins. Curr Med Chem 2012;19:4348–58.

[196] Khaddam M, Salmon B, Le Denmat D, Tjaderhane L, Menashi S, Chaussain C, et al. Grape seed extracts inhibit dentin matrix degradation by MMP-3. Front Physiol 2014;5:1–8. doi:10.3389/fphys.2014.00425.

[197] Spencer P, Wang Y. Adhesive phase separation at the dentin interface under wet bonding conditions. J Biomed Mater Res 2002;62:447–56. doi:10.1002/jbm.10364.

[198] Pashley DH, Tay FR, Carvalho RM, Rueggeberg FA, Agee KA, Carrilho M, et al. From dry bonding to water-wet bonding to ethanol-wet bonding. A review of the interactions between dentin matrix and solvated resins using a macromodel of the hybrid layer. Am J Dent 2007;20.

[199] Tay FR, Pashley DH, Kapur RR, Carrilho MRO, Hur YB, Garrett L V, et al. Bonding BisGMA to dentin--a proof of concept for hydrophobic dentin bonding. J Dent Res 2007;86:1034–9. doi:10.1177/154405910708601103.

[200] Ayar MK. A review of ethanol wet-bonding: Principles and techniques. Eur J Dent 2016;10:155–9. doi:10.4103/1305-7456.175687.

[201] Hiraishi N, Nishiyama N, Ikemura K, Yau JYY, King NM, Tagami J, et al. Water concentration in self-etching primers affects their aggressiveness and bonding efficacy to dentin. J Dent Res 2005;84:653–8. doi:10.1177/154405910508400714.

[202] Shin TP, Yao X, Huenergardt R, Walker MP, Wang Y. Morphological and chemical characterization of bonding hydrophobic adhesive to dentin using ethanol wet bonding technique. Dent Mater 2009;25:1050–7. doi:10.1016/j.dental.2009.03.006.

[203] Agee KA, Prakki A, Abu-Haimed T, Naguib GH, Nawareg MA, Tezvergil-Mutluay A, et al. Water distribution in dentin matrices: Bound vs. unbound water. Dent Mater 2015;31:205–16. doi:10.1016/j.dental.2014.12.007.

[204] Jee SE, Zhou J, Tan J, Breschi L, Tay FR, Grégoire G, et al. Investigation of ethanol infiltration into demineralized dentin collagen fibrils using molecular dynamics simulations. Acta Biomater 2016;36:175–85. doi:10.1016/j.actbio.2016.03.012.

[205] Ayar MK. Bond durability of contemporary adhesive systems to pulp chamber dentin. Acta Biomater Odontol Scand 2015;1:76–80. doi:10.3109/23337931.2015.1075887.

[206] Makishi P, Thitthaweerat S, Sadr A, Giannini M. Assessment of current adhesives in class I cavity: Nondestructive imaging using optical coherence tomography and microtensile bond strength. Dent Mater 2015;31:e190–200. doi:10.1016/j.dental.2015.06.013.

[207] El-Deeb H, Badran O, Mobarak E. One-year adhesive bond durability to coronal and radicular dentin under intrapulpal pressure simulation. Oper Dent 2015;40:540–7. doi: 10.2341/14-134-L.

[208] Tay FR, Pashley DH. Guided tissue remineralisation of partially demineralised human dentine. Biomaterials 2008;29:1127–37. doi:10.1016/j.biomaterials.2007.11.001.

[209] Kim YK, Gu L, Bryan TE, Kim JR, Chen L, Liu Y, et al. Mineralisation of reconstituted collagen using polyvinylphosphonic acid/polyacrylic acid templating matrix protein analogues in the presence of calcium, phosphate and hydroxyl ions. Biomaterials 2010;31:6618–27. doi:10.1016/j.biomaterials.2010.04.060.

[210] Kim J, Arola DD, Gu L, Kim YK, Mai S, Liu Y, et al. Functional biomimetic analogs help remineralize apatite-depleted demineralized resin-infiltrated dentin via a bottom–up approach. Acta Biomater 2010;6:2740–50. doi:10.1016/j.actbio.2009.12.052.

[211] Gu L, Kim YK, Liu Y, Ryou H, Wimmer CE, Dai L, et al. Biomimetic analogs for collagen biomineralization. J Dent Res 2011;90:82–7. doi:10.1177/0022034510385241.

[212] Sauro S, Osorio R, Watson TF, Toledano M. Influence of phosphoproteins' biomimetic analogs on remineralization of mineral-depleted resin-dentin interfaces created with ion-releasing resin-based systems. Dent Mater 2015;31:759–77. doi:10.1016/j.dental.2015.03.013.

[213] Abuna G, Feitosa VP, Correr AB, Cama G, Giannini M, Sinhoreti MA, et al. Bonding performance of experimental bioactive/biomimetic self-etch adhesives doped with calcium-phosphate fillers and biomimetic analogs of phosphoproteins. J Dent 2016;52:79–86. doi: 10.1016/j.jdent.2016.07.016.

[214] Toroian D, Lim JE, Price PA. The size exclusion characteristics of type I collagen. J Biol Chem 2007;282:22437–47. doi:10.1074/jbc.M700591200.

[215] Takahashi M, Nakajima M, Tagami J, Scheffel D, Carvalho R, Mazzoni A, et al. The importance of size-exclusion characteristics of type I collagen in bonding to dentin matrices. Acta Biomater 2013;9:9522–8. doi:10.1016/j.actbio.2013.07.037.The.

[216] Chiba A, Zhou J, Nakajima M, Tan J, Tagami J, Scheffel DLS, et al. The effects of ethanol on the size-exclusion characteristics of type I dentin collagen to adhesive resin monomers. Acta Biomater 2016;33:235–41. doi:10.1016/j.actbio.2016.01.040.

[217] Mai S, Wei C, Gu L, Tian F, Arola D, Chen J, et al. Extrafibrillar collagen demineralization-based chelate-and-rinse technique bridges the gap between wet and dry dentin bonding. Acta Biomater 2017.

CHAPTER 3
Collagen Cross-Linkers

Endogenous crosslinking is a result of maturation of the tissue that occurs over time. This mechanism includes enzymatic crosslinking mediated by lysine– lysine covalent bonding between non-helical telopeptides and triple-helical fragments of collagen or/and nonenzymatic crosslinking by oxidation and glycation[1].

On the other hand, crosslinking may be stimulated by using external collagen crosslinkers to improve the biomechanical properties of the collagen against proteolytic attack and to strengthen collagen structure. This exogenous crosslinking may modify the noncollagenous component of dentin as well as collagen scaffold [2].

As a term, the 'biomodification' of dentin collagen matrices was used by Bedran-Russo to explain the structural changes in the three-dimensional scaffold of collagen treated with an exogenous crosslinking agent. The aim of modifying dentin matrices by collagen crosslinking agents is to improve the biomechanical properties of the dentin against dentin protease attacks.

Bedran-Russo et al., for the first time, showed the effect of collagen crosslinkers on dentin collagen matrices in terms of the increased durability of the hybrid layer. Further studies showed that various collagen crosslinkers, such as carbodiimides, aldehydes, phenolic compounds and photochemicals, improve biomechanical properties of the collagen structure on dentin [3–5][5] [6–8]. Collagen can be modified by using methods such as chemical, physical and photochemical crosslinking [6,8,9].

A cross-link is a bond that links one polymer chain to another one. They can be covalent bonds or ionic bonds. "Polymer chains" can refer to synthetic polymers or natural polymers (such as proteins). Proteins in nature present crosslinks generated by enzyme-catalyzed or spontaneous reactions. Such crosslinks are important in generating mechanically stable structures such as hair, skin and cartilage. Cross-linking reactions can be promoted using external cross-linking molecules.

Molecules tested

Acrolein

Acrolein (2-propenal or acrylic aldehyde) (Fig.4) is the simplest unsaturated aldehyde. It is a three-carbon α-β-unsaturated monoaldehyde that provides outstanding stabilization of proteins, similar to glutaraldehyde, but penetrates tissue more rapidly [10], providing excellent morphological preservation of fine structure for electron microscopy (EM) studies [11]. The acrolein exists as a colorless liquid with a piercing, disagreeable, acrid smell. Acrolein is ubiquitously present in foods, cooked or not, and in the environment. It is formed from carbohydrates, vegetable oils and animal fats, amino acids during heating of foods, and by combustion of petroleum fuels and biodiesel [12]. Chemical reactions responsible for release of acrolein include heat-induced dehydration of glycerol, retroaldol cleavage of dehydrated carbohydrates and lipid peroxidation of polyunsaturated fatty acids. Smoking of tobacco products equals or exceeds the total human exposure to acrolein from all other sources [13]. Acrolein is metabolized by conjugation with glutathione and excreted in the urine as mercapturic acid metabolites. The biological effects of acrolein are a consequence of its reactivity towards biological nucleophiles such as guanine in DNA and cysteine, lysine, histidine, and arginine residues in critical regions of nuclear factors,
proteases, and other proteins [14].

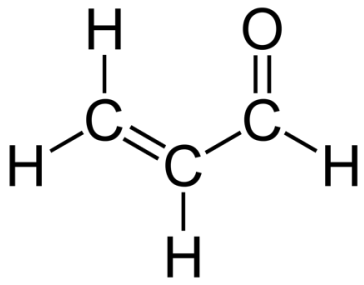


Figure 1. Acrolein molecular structure

Acrolein is commonly used as a tissue fixative although can degrade enzymatic activity or antigenicity [11] but degradation is not excessive when fixation times are short [15]. Furthermore, the rapid penetration and strong cross-linking abilities of acrolein quickly stabilize proteins, retaining even small peptides that can be successfully immunolabeled [16]. Likewise,

other aldehydes the acrolein can be classified as a cross-linker agent. Acrolein reacts preferentially with cysteine, lysine, and hystidine residues (the lysine adducts being the more stable products) via Michael-type addition reactions preserving the aldehyde functionality on the modified protein (Figure 2). The reaction of acrolein with Lys may result in β- substituted propanals (R-NH-CH2-CH2-CHO), but the major adduct formed on reaction with protein is the Ne-(3-formyl-3,4- dehydropiperidino) lysine adduct [17]. This compound is a reactive intermediate that can covalently bind to thiols, such as glutathione [18]. The amino groups (N-terminus [Phe1] or lysine29) and the histidine residues (histidine 5 or histidine 10) were identified as the main sites involved in the formation of inter and intramolecular cross-linkings adducts. These results allowed the proposal of a mechanism of protein cross-linkings by acrolein, involving inter- and intra- molecular cross-linking adducts between amino groups and the side chain of histidine through Michael addition [14].

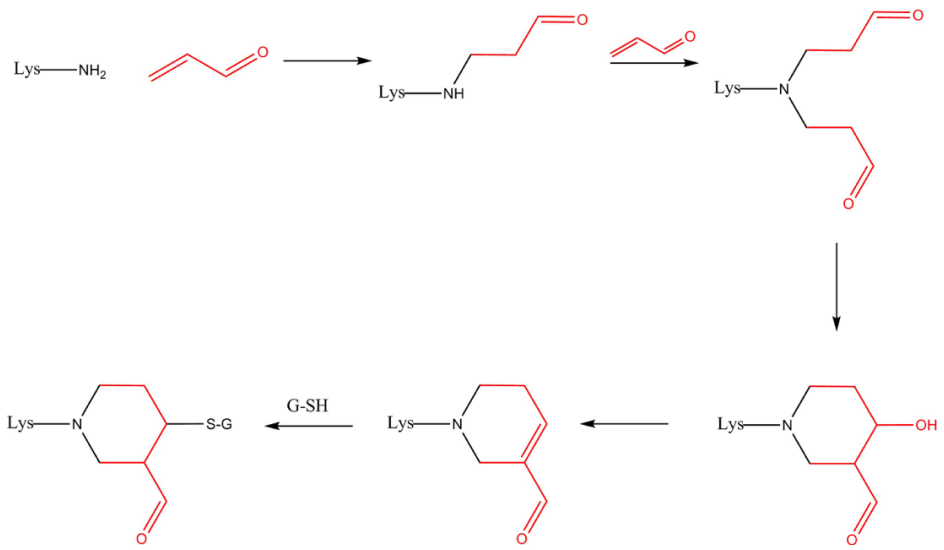


Figure 2. Acrolein cross-linking reaction: 2 acrolein molecule reacts with a N residue of lysine forming an intermediate that will bond to a thiolic residue of a glutathione of another collagen chain.

Carbodiimide

A carbodiimide or a methanediimine is a functional group consisting of the formula RN=C=NR. Carbodiimides hydrolyze to form ureas, which makes them uncommon in nature. Carbodiimides are formed by dehydration of ureas or from thioureas. They are also formed by treating organic isocyanates with suitable catalysts (generally based on phosphine oxides); in this process, carbon dioxide evolves from the isocyanate [19]. In synthetic organic chemistry, compounds containing the carbodiimide functionality are dehydration agents and are often used to activate carboxylic acids towards amide or ester formation. Additives, such as N-hydroxybenzotriazole or N- hydroxysuccinimide, are often added to increase yields and decrease side reactions. While the cross-linking potential is limited [20], carbodiimides are less toxic than aldehydes [21].

Carbodiimide hydrochloride (EDC)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Figure 3) is a water soluble carbodiimide usually obtained as the hydrochloride. It is typically employed in the 4.0-6.0 pH range [22]. It is generally used as a carboxyl activating agent for the coupling of primary amines to yield amide bonds. Additionally, EDC can also be used to activate phosphate groups in order to form phosphomonoesters and phosphodiesters. Common uses for this carbodiimide include peptide synthesis, protein cross-linkings to nucleic acids, but also in the preparation of immunoconjugates [22]. In medical filed EDC was recently studied, with promising results, as reinforced for collagen scaffold in tissue engineering [23].

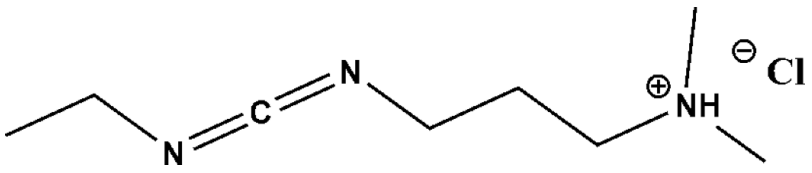


Figure 3. EDC molecular structure

EDC is often used in combination with N-hydroxysuccinimide (NHS) for the immobilization of large biomolecules.

EDC is known as a zero-length agent due to its ability to cross-link peptides without introducing additional linkage groups. The cross-linking mechanism is mediated by the activation of carboxylic acid groups of glutamic and aspartic acids to form an O-acylisourea intermediate. The latter reacts with the ε-amino groups of lysine or hydroxylysine to form an amide cross-link, leaving urea as the terminal by-product (Figure 4). The addition of N- hydroxysuccinimide to the EDC-containing solution is effective in increasing the number of induced collagen cross-linking and preventing the hydrolysis of activated carboxyl groups [24,25]. Cross-linkings with EDC are especially appealing for biological applications as the carbodiimide does not remain in the chemical bond but is released as a substituted urea molecule.

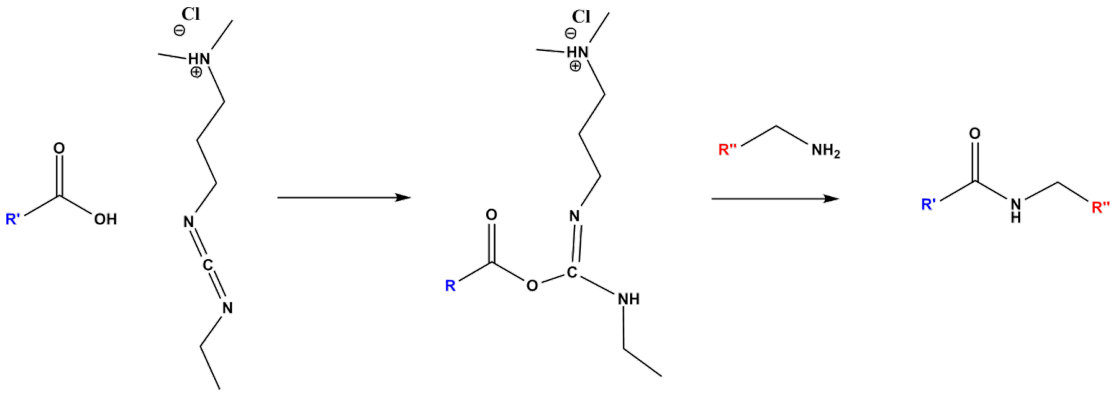


Figure 4. EDC cross-linking reaction: EDC reacts with carboxylic group of, after the elimination of O-acylisourea intermediate, the glutamic or aspartic acid bond the N residue of lysine or hydroxylysine.

Dicyclohexylcarbodiimide (DCC)

N,N'-Dicyclohexylcarbodiimide (DCC) is an organic compound with the chemical formula C13H22N2 (Figure 5) whose primary use is to couple amino acids during artificial peptide synthesis. Under standard conditions, it exists in the form of white crystals with a heavy, sweet odor; DCC is normally used as a condensation reagent in amide synthesis or esterification reactions [26]. Differently to EDC the DCC is insoluble in water but on the other hand it is well soluble in other organic compounds, such as ethanol and acetone.

During protein synthesis, the N-terminus is often used as the attachment site on which the amino acid monomers are added. To enhance the electrophilicity of carboxylate group, the negatively charged oxygen must first be "activated" into a better leaving group, and DCC is also used for this purpose. The negatively charged oxygen will act as a nucleophile, attacking the central carbon in DCC, thus it is temporarily attached to the former carboxylate group forming a highly electrophilic intermediate, making nucleophilic attack by the terminal amino group on the growing peptide more efficient [27].

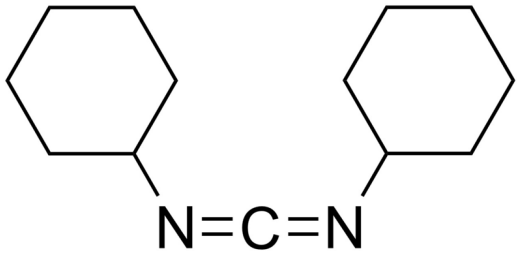


Figure 5. DCC molecular structure

Similarly to the EDC cross-linker mechanism the DCC first reacted with the carboxylic acid group of the amino acid to form the O-acylisourea intermediate, and then the DCC adduct underwent a relatively slow rearrangement process to form the final derivative that possesses higher ionization efficiency and higher molecular weight than the original amino acid [27] (Figure 6).

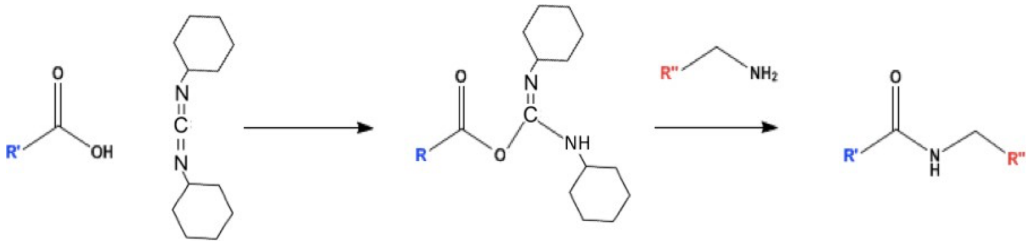


Figure 6. DCC cross-linking reaction: likewise EDC, the DCC reacts with carboxylic group of glutamic and aspartic acids, after the elimination of O-acylisourea intermediate, the glutamic or aspartic acid bond the N residue of lysine or hydroxylysine.

Aim of the Thesis

The aim of this study was to evaluate the effect of protein crosslinking agents on dentin MMPs and CCs on dentin organic matrices. The working hypotheses tested were that various collagen crosslinkers inactivate dentin MMPs and CCs, preserve microtensile and push-out bond strength and prevent degradation of dentin collagen.

The specific hypoteses were:

1. that the use of ACR as a collagen cross-linker: 1) has no effect on bond strength deterioration over time and 2) has no effect on inactivation of endogenous dentine MMPs (Experimental study 1).

2. Pre-conditioning of dentin with EDC before adhesive system application 1) does not affect immediate bond strength, 2) does not preserve adhesive interface degradation over time, and 3) does not inhibit endogenous dentin MMPs activity (Experimental study 2).

3. (1) EDC has no effect on improving post push-out strength in resin-bonded radicular dentine, (2) EDC has no effect on improving the nanoleakage expression in resin-dentine interfaces created by the two etch-and-rinse adhesives, and (3) EDC has no effect on reducing endogenous enzymatic activities within the hybrid layer created in intraradicular dentine (Experimental study 3).

4. Pre-conditioning of dentin with DCC before adhesive system application 1) does not affect immediate bond strength, 2) does not preserve adhesive interface degradation over time, and 3) does not inhibit endogenous dentin MMPs activity (Experimental study 4).

5. (1) DCC has no effect on improving post push-out strength in resin-bonded radicular dentine, (2) DCC has no effect on improving the nanoleakage expression in resin-dentine interfaces created by the two etch-and-rinse adhesives, and (3) DCC has no effect on reducing endogenous enzymatic activities within the hybrid layer created in intraradicular dentine (Experimental study 5).

References

[1] Reiser K, McCormick RJ, Rucker RB. Enzymatic and nonenzymatic cross-linking of collagen and elastin. FASEB J 1992. doi:10.1096/fasebj.6.7.1348714.

[2] Bedran-Russo AK, Pauli GF, Chen SN, McAlpine J, Castellan CS, Phansalkar RS, et al. Dentin biomodification: Strategies, renewable resources and clinical applications. Dent Mater 2014;30:62–76. doi:10.1016/j.dental.2013.10.012.

[3] Cheung D, Perelman N, Ko E, Nimni M. Mechanism of crosslinking of proteins by glutaraldehyde III: Reaction with collagen in tissues. Connect Tissue Res 1985;13:109–115.

[4] Bedran-Russo AKB, Pereira PNR, Duarte WR, Drummond JL, Yamauchi M. Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. J Biomed Mater Res Part B Appl Biomater 2007;80:268–72. doi:10.1002/jbm.b.30593.

[5] Bedran-Russo AKB, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. J Biomed Mater Res - Part B Appl Biomater 2008;86:330–4. doi:10.1002/jbm.b.31022.

[6] Hayashi M, Okamura K, Koychev EV, Furuya Y, Sugeta A, Ota T, et al. Effects of rehydration on dentin strengthened by heating or UV irradiation. J Dent Res 2010;89:154–8. doi:10.1177/0022034509354564.

[7] Cova A, Breschi L, Nato F, Ruggeri A, Carrilho M, Tjäderhane L, et al. Effect of UVA-activated riboflavin on dentin bonding. J Dent Res 2011. doi:10.1177/0022034511423397.

[8] Tezvergil-Mutluay A, Mutluay MM, Agee KA, Seseogullari-Dirihan R, Hoshika T, Cadenaro M, et al. Carbodiimide cross-linking inactivates soluble and matrix-bound MMPs, in vitro. J Dent Res 2012;91:192–6. doi:10.1177/0022034511427705.

[9] Snibson GR. Collagen cross-linking: A new treatment paradigm in corneal disease - a review. Clin Exp Ophthalmol 2010. doi:10.1111/j.1442-9071.2010.02228.x.

[10] Saito T, Keino H. Acrolein as a fixative for enzyme cytochemistry. J Histochem Cytochem 1976. doi:10.1177/24.12.187691.

[11] Sabatini DD, Bensch K, Barnett RJ. Cytochemistry and electronmicroscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyd fixation. J Cell Biol 1963. doi:10.1083/jcb.17.1.19.

[12] Stevens JF, Maier CS. Acrolein: Sources, metabolism, and biomolecular interactions relevant to human health and disease. Mol Nutr Food Res 2008;52:7–25.

[13] Bein K, Leikauf GD. Acrolein - a pulmonary hazard. Mol Nutr Food Res 2011. doi: 10.1002/mnfr.201100279.

[14] Aldini G, Orioli M, Carini M. Protein modification by acrolein: Relevance to pathological conditions and inhibition by aldehyde sequestering agents. Mol Nutr Food Res 2011;55:1301–19. doi:10.1002/mnfr.201100182.

[15] Flitney FW. The time course of the fixation of albumin by formaldehyde, glutaraldehyde, acrolein and other higher aldehydes. J R Microsc Soc 1966. doi:10.1111/j. 1365-2818.1966.tb02194.x.

[16] Pickel VM, Chan J, Park DH, Joh TH, Milner TA. Ultrastructural localization of phenylethanolamine N???methyltransferase in sensory and motor nuclei of the vagus nerve. J Neurosci Res 1986. doi:10.1002/jnr.490150402.

[17] Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N, Niki E. Acrolein is a product of lipid peroxidation reaction: Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. J Biol Chem 1998. doi:10.1074/jbc.273.26.16058.

[18] Furuhata A, Nakamura M, Osawa T, Uchida K. Thiolation of protein-bound carcinogenic aldehyde. An electrophilic acrolein-lysine adduct that covalently binds to thiols. J Biol Chem 2002. doi:10.1074/jbc.M202794200.

[19] Sheehan JC, Cruickshank P a., Boshart GL. Convenient Synthesis of Water-Soluble Carbodiimides. J Org Chem 1961. doi:10.1021/jo01351a600.

[20] Bedran-Russo AKB, Vidal CMP, Dos Santos PH, Castellan CS. Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds. J Biomed Mater Res - Part B Appl Biomater 2010;94:250–5. doi:10.1002/jbm.b.31649.

[21] Huang YP, Kimura M, Tawada K. Covalent crosslinking of myosin subfragment-1 and heavy meromyosin to actin at various molar ratios: Different correlations between ATPase activity and crosslinking extent. J Muscle Res Cell Motil 1990. doi:10.1007/BF01766669.

[22] López-Alonso JP, Diez-García F, Font J, Ribó M, Vilanova M, Scholtz JM, et al. Carbodiimide EDC induces cross-links that stabilize RNase a C-dimer against dissociation:

EDC adducts can affect protein net charge, conformation, and activity. Bioconjug Chem 2009. doi:10.1021/bc9001486.

[23] Li J, Ren N, Qiu J, Jiang H, Zhao H, Wang G, et al. Carbodiimide crosslinked collagen from porcine dermal matrix for high-strength tissue engineering scaffold. Int J Biol Macromol 2013. doi:10.1016/j.ijbiomac.2013.06.038.

[24] Staros J V., Wright RW, Swingle DM. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal Biochem 1986. doi: 10.1016/0003-2697(86)90176-4.

[25] Olde Damink LHH, Dijkstra PJ, Van Luyn MJA, Van Wachem PB, Nieuwenhuis P, Feijen J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials 1996. doi:10.1016/0142-9612(96)81413-X.

[26] Chen J, Zhang J, Zhang W, Chen Z. Sensitive determination of the potential biomarker sarcosine for prostate cancer by LC-MS with N,N’-dicyclohexylcarbodiimide derivatization. J Sep Sci 2014. doi:10.1002/jssc.201301043.

[27] Sebald W, Machleidt W, Wachter E. N,N’-dicyclohexylcarbodiimide binds specifically to a single glutamyl residue of the proteolipid subunit of the mitochondrial adenosinetriphosphatases from Neurospora crassa and Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 1980. doi:10.1073/pnas.77.2.785.

CHAPTER 4
Experimental studies

1 - Experimental use of an acrolein-based primer as collagen cross-linker for dentine bonding

Materials and methods

Microtensile bond strength

Thirty-six extracted non-carious sound human third molars were collected after the patients' informed consents were obtained under a protocol approved by the institutional review board of the University of Bologna, Italy. Tooth crowns were removed with a low-speed diamond saw under water irrigation (Micromet, Remet; Bologna, Italy) to expose coronal dentine that was devoid of occlusal enamel. A standardised smear layer was created on the middle/deep coronal dentine with 180-grit wet silicon carbide paper. The exposed dentine surfaces were etched with 37% phosphoric acid for 15 sec (etching gel, 3M ESPE; St Paul, MN, USA), rinsed with water, gently air-dried and kept moist until the adhesive was applied using the wet-bonding technique. The teeth were divided and randomly assigned to 2 treatment groups (n = 18). In group 1 (experimental), the acid-etched dentine was pre-treated with 0.01 ACR wt% aqueous solution (MilliporeSigma, St, Louis, MO, USA) for 1 min, gently air-dried and bonded with Adper Scotchbond 1XT (3M ESPE) in accordance with the manufacturer's instructions. The composition of the adhesive is shown in Table 1. In group 2 (control), Adper Scotchbond 1XT was applied directly on the etched dentine in accordance with manufacturer's instructions. In both groups, the adhesive was light-cured (Curing Light 2500; 3M ESPE) for 20 sec after solvent evaporation. Four 1-mm-thick layers of a microhybrid resin composite (Filtek Z250, 3M ESPE) were incrementally placed over the bonded dentine surface and individually polymerised for 20 sec to obtain a final 4 mm-thick composite build-up for microtensile bond strength (μTBS) testing.

Composition
Etching: 35% H_3PO_4
Adhesive: dimethacrylates, 2-hydroxyethyl methacrylate, polyalkenoic acid copolymer, 5 nm silane-treated colloidal silica, ethanol, water, photoinitiator

Table 1. Composition of Adper Scotchbond 1 XT

Resin-dentine sticks were created with a cross-sectional area of approximately 1 mm x 1 mm from each bonded tooth using the low-speed saw under water irrigation, in accordance with the protocol for the non-trimming μTBS technique. The dimension of each stick was measured with a pair of digital callipers (± 0.01 mm), and the bonded area was calculated for subsequent conversion of μTBS values into units of stress (MPa). Sticks from each tooth were randomly assigned to two storage groups. For time 0 (T0), the sticks were stored in artificial saliva [1][2] for 24 h at 37 °C; for time 1 year (T1yr), the sticks were stored for 1 year in artificial saliva at 37 °C.

After storage, the bonded beams were stressed to failure using a simplified universal testing machine (Bisco Inc., Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. Each specimen was observed under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH, Göttingen, Germany) at 50× magnification to determine the mode of failure, which was classified as adhesive (A), cohesive in composite (CC) or cohesive in dentine (CD).

Analysis was performed using the tooth as the statistical unit; bond strength data from each tooth were averaged to obtain the mean bond strength for that tooth. The acquired data (n = 18) were evaluated for compliance with the normality (Shapiro-Wilk test) and equality of variance (modified Levine test) assumptions required for parametric statistical analysis. Because these assumptions were not violated, the data were analysed with a two-factor analysis of variance, to examine the effects of “with/without ACR” and “storage time” on μTBS. Post-hoc pairwise comparisons were conducted using the Holm-Sidak method. For all analyses, statistical significance was set at $\alpha = 0.05$.

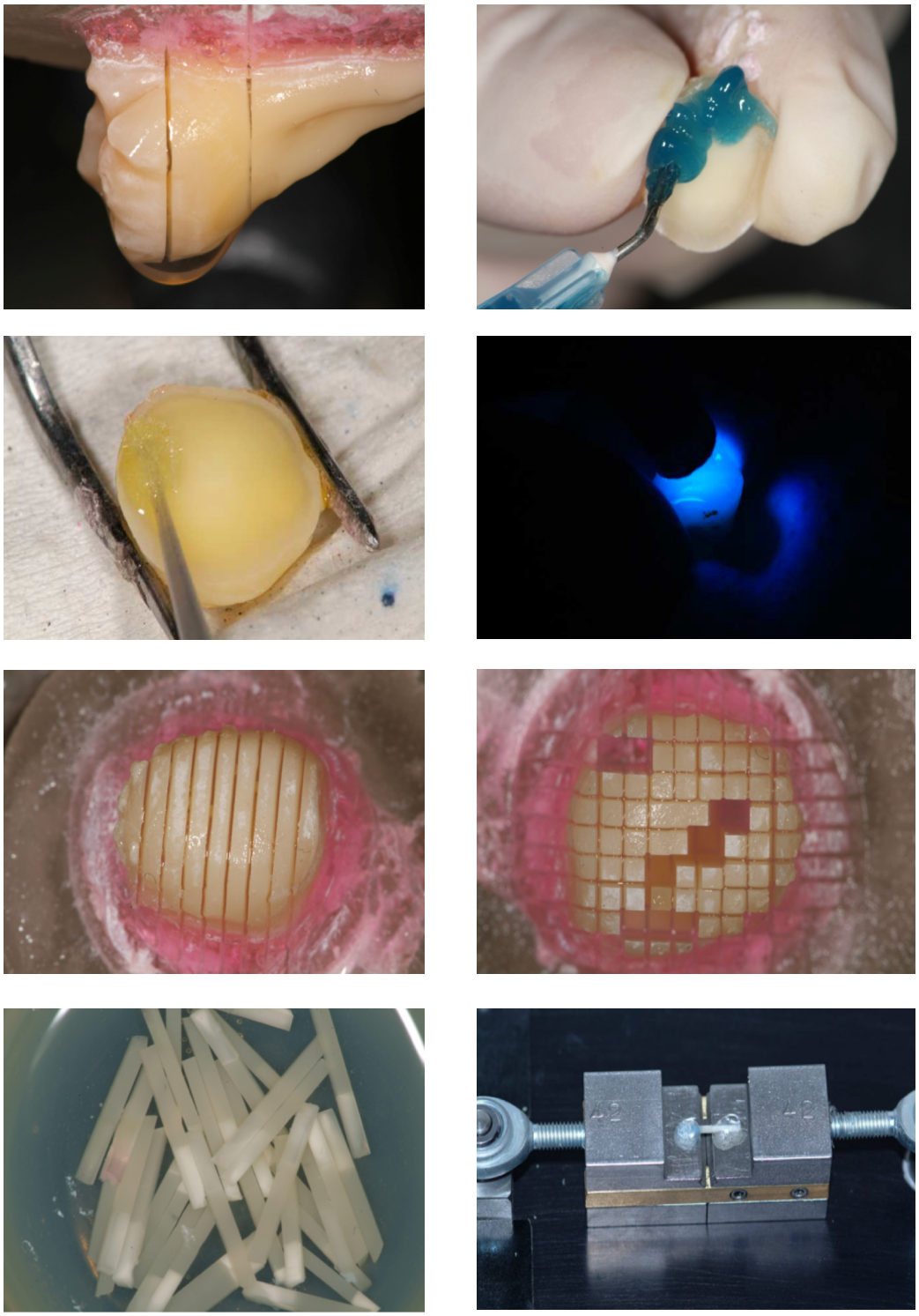


Figure 1. Step-by-step procedure for micro tensile bond strength test on dentinal substrate.

Zymography of dentine extracts

Zymography was performed using the method employed by Mazzoni *et al*. [3]. Mineralised dentin powder was obtained from additional eight human third molars by freezing the dentine in liquid nitrogen and triturating it using a Retsch mill (Model MM400, Retsch GmbH, Haan, Germany). Aliquots of mineralised dentine powder were divided in 2 groups. For group 1, the dentine powder was demineralised with 37% phosphoric acid to simulate the etching procedure used in the application of an etch-and-rinse adhesive. For group 2, the dentine powder was demineralised in the same manner as group 1 and then treated with 0.01 wt% ACR solution at 4° C for 30 min. For the control, after demineralisation with 37% phosphoric acid, the dentine powder was incubated with 2 mM of 1,10-phenanthroline at 4° C for 30 min.

After the aforementioned treatments, the dentine powder aliquots were suspended in extraction buffer (50 mM Tris-HCl, pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM $ZnCl_2$ and 0.02% NaN_3) overnight at 4 °C. The powders were subsequently sonicated for 10 min (at ≈ 30 pulses) and centrifuged for 20 min at 4 °C (20,800X G); the supernatants were retrieved and re-centrifuged. The protein content in the supernatants was concentrated using Vivaspin centrifugal concentrator (10,000 KDa cut-off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 25 ºC (15,000X G for 3 times). Total protein concentration of the dentine extracts was determined by Bradford assay. Dentine protein aliquots (60 μg) were diluted with Laemmli sample buffer in a 4:1 ratio. Electrophoresis was performed under non-reducing conditions using 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) containing 1 mg/mL fluorescent dye-labelled gelatine. Pre-stained low-range molecular weight SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as reference markers. After electrophoresis, the gels were washed for 1 h in 2% Triton X-100, and incubated in zymography activation buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.4) for 48 h. Proteolytic activity was evaluated and registered with a long-wave ultraviolet light scanner (ChemiDoc Universal Hood, Bio-Rad). Gelatinase activities in the specimens were analysed in duplicate using gelatine zymography. Densitometric evaluation of bands obtained from zymography was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

In-situ zymography of resin-dentine interfaces

One millimetre-thick slabs of middle/deep dentine were obtained from extracted human third molars using the low-speed Micromet saw with water-cooling. A standardised smear layer was created on each dentine surface using 280-grit silicon-carbide paper. One surface of each slab was treated with ACR primer and Adper Scotchbond 1XT adhesive as described for μTBS testing. This was followed by a 1-mm build-up with flowable composite (Filtek 250 flow; 3M ESPE); the composite was polymerised for 40 sec using a light-emitting diode light-curing unit (Curing Light 2500; 3M ESPE). After completion of those procedures, the bonded assemblies were sectioned vertically into 1-mm-thick slabs to expose the adhesive-dentine interface. Each slab was glued to a microscope slide and polished to the thickness of approximately 50 μm. *In-situ* zymography was performed according to the protocol reported by Mazzoni *et al*. [4], using self-quenched fluorescein-conjugated gelatine as the MMP substrate (E- 12055; Molecular Probes, Eugene, OR, USA). Briefly, the fluorescent gelatine mixture was placed over the polished slab on top of the microscopic slide, covered with a coverslip and incubated in a humidified chamber at 37 °C for 12 h. During incubation, the assemblies were prevented from direct contact with water, and were protected from exposure to light. After incubation, the microscopic slides were examined using a confocal laser scanning microscope (excitation wavelength, 488 nm; emission wavelength, 530 nm; Model A1-R; Nikon, Tokyo, Japan). For each assembly, a series of 1 μm-thick two-dimensional images were made to show the hydrolysis of the quenched fluorescein-conjugated gelatine substrate, as indicator of endogenous gelatinolytic enzyme activity. Each image series was subsequently merged into three-dimensional images, providing additional information on the changes in the gelatinolytic activity throughout the depth of the sample. Enzymatic activity was quantified on the images as the integrated density of the fluorescence signals using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The integrated density of the fluorescence signals was quantified as indicator of the dentine enzymatic activities of the tested groups. Because the data were not normally distributed even

after nonlinear transformation, they were analysed using the Mann-Whitney U-test, with $\alpha = 0.05$.

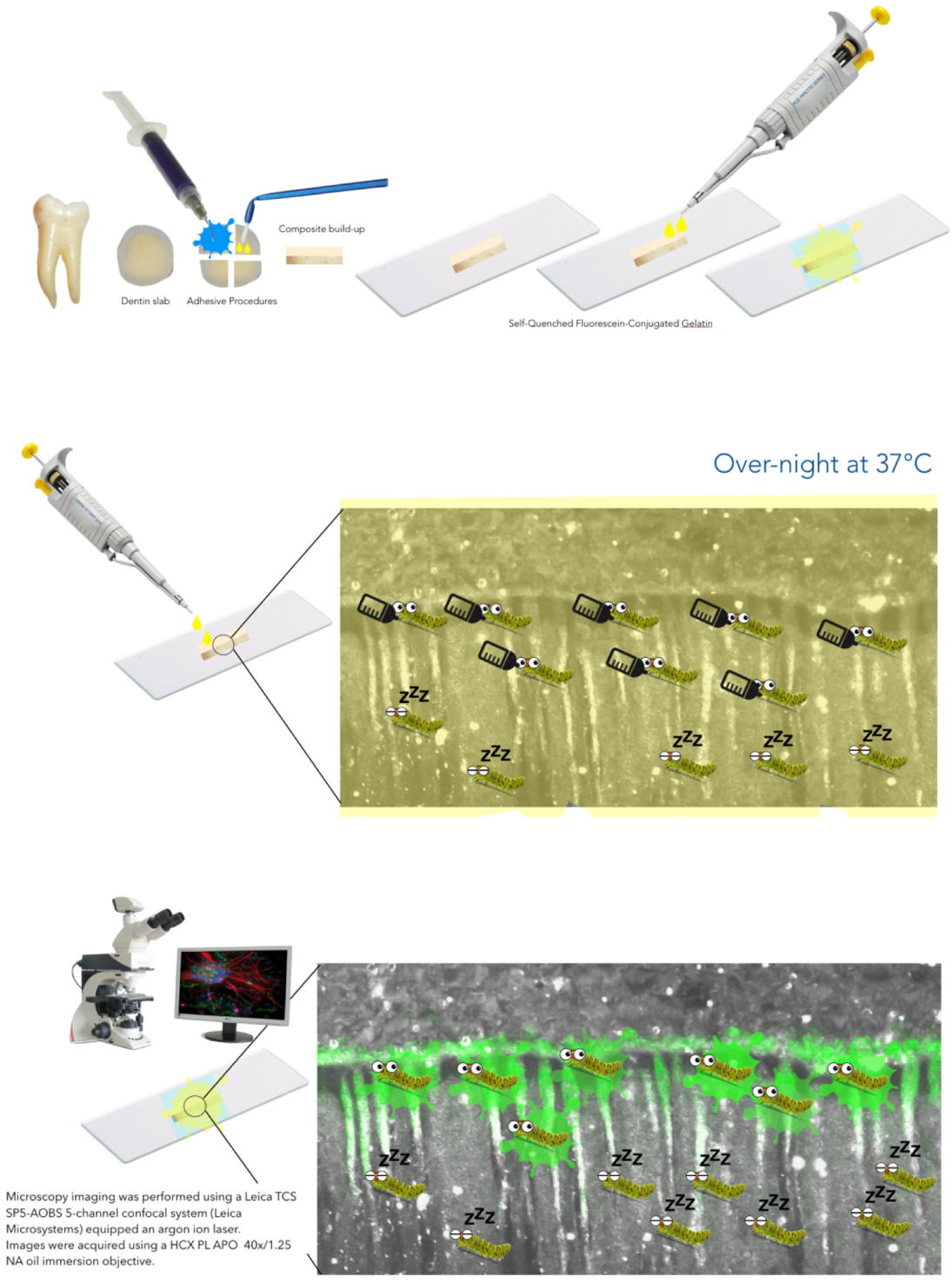


Figure 2. Step-by-step procedure for *in-situ* zymography on dentinal substrate.

Results

Microtensile bond strength

The results of μTBS are shown in Table 2 and Graph 1. The table presents the means and standard deviations of μTBS (in MPa) of groups 1 and 2 at T0 and T1yr, respectively. Two-factor analysis of variance revealed significant difference for the factor "with/without ACR" ($p < 0.001$) and the factor "storage time" ($p < 0.001$). The interaction of these two factors was also statistically significant ($p < 0.001$). Within the factor "with/without ACR", pairwise comparisons indicated no significant difference in μTBS between the two bonding methods at T0 ($p > 0.05$), and significant difference between the two bonding methods at T1 yr ($p < 0.05$). Within the factor "storage time" (T0 *vs* T1 yr), pairwise comparisons indicated significant decline in μTBS for the subgroups "adhesive only" ($p < 0.05$) and "0.01 wt% ACR + adhesive" ($p < 0.05$).

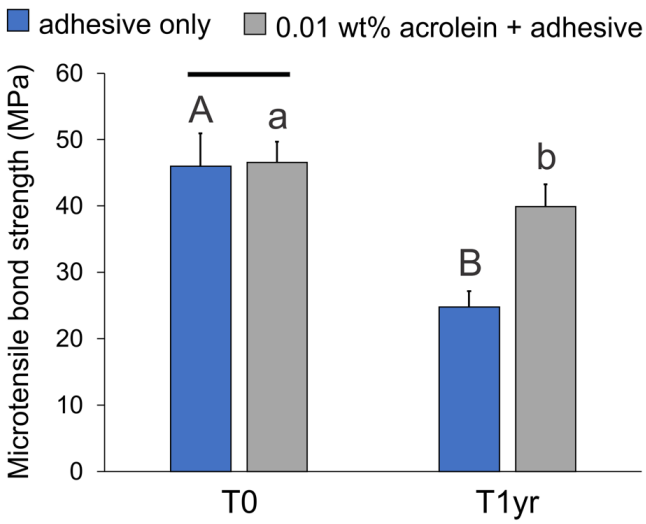
Storage time	Adhesive only	0.01 wt% ACR adhesive
T0	46.0±4.9 ^{A,1} (20A/8CC/25CD/47M)	46.6±3.1 ^{B,1} (43A/30CC/5CD/22M)
T1yr	24.8±2.4 ^{a,1} (25A/20CC/8CD/57M)	39.9±3.3 ^{b,2} (30A/10CC/0CD/60M)

Table 2. Microtensile bond strengths (mean ± SD, in MPa) of group 1 (0.01% acrolein pre-treatment) and group 2 (control) immediately after bonding (T0) and after 1 year of ageing (T1yr) in artificial saliva.

For comparisons within the factor "with/without acrolein (ACR)", subgroups in the row T0 that are labelled with different upper case superscripts (adhesive only) are significantly different ($p < 0.05$). Subgroups in the row T1yr that are labelled with different lower case superscripts (adhesive only) are significantly different ($p < 0.05$).

For comparisons within the factor "storage time", subgroups within the same column that are labelled with the same numerical designators are not significantly different ($p > 0.05$).

Percentages of the failure modes (in parentheses) were classified as: A, adhesive; CC, cohesive in resin composite; CD, cohesive in dentine and M, mixed failure.



Graph 1. Bond strength values (MPa) reported in Table 1.

Zymography of dentine extracts

The pro-form and active form of recombinant human MMP-2 and -9 for the positive control are shown in lane 1 of Figure 3a. Phosphoric acid-demineralised dentine extracts contained multiple forms of gelatinolytic enzymes, including a 72 kDa MMP-2 pro-form, a fainter 86 kDa band corresponding to the active form of MMP-9, and other minor gelatinolytic bands (Figure 2a, lane 2). Pre-treatment of demineralised dentine powder with 0.01 wt% ACR resulted in almost complete inactivation of the pro-form and active form of MMP-2 and the active form of MMP-9 (Figure 3a, lane 3). A band around 100 kDa was still detectable, which could be attributed to a complex pro-form of MMP-9. Densitometric evaluation of the zymography bands (Figure 3b) confirmed that ACR inactivated the pro-form and active form of MMP-2. A band around 100 KDa was still present, although it had a lower intensity compared to the MMP-9 active form. Control zymograms produced by incubating the phosphoric acid-demineralised dentine powder with 2 mM of 1,10-phenanthroline showed no enzymatic activity (data not shown).

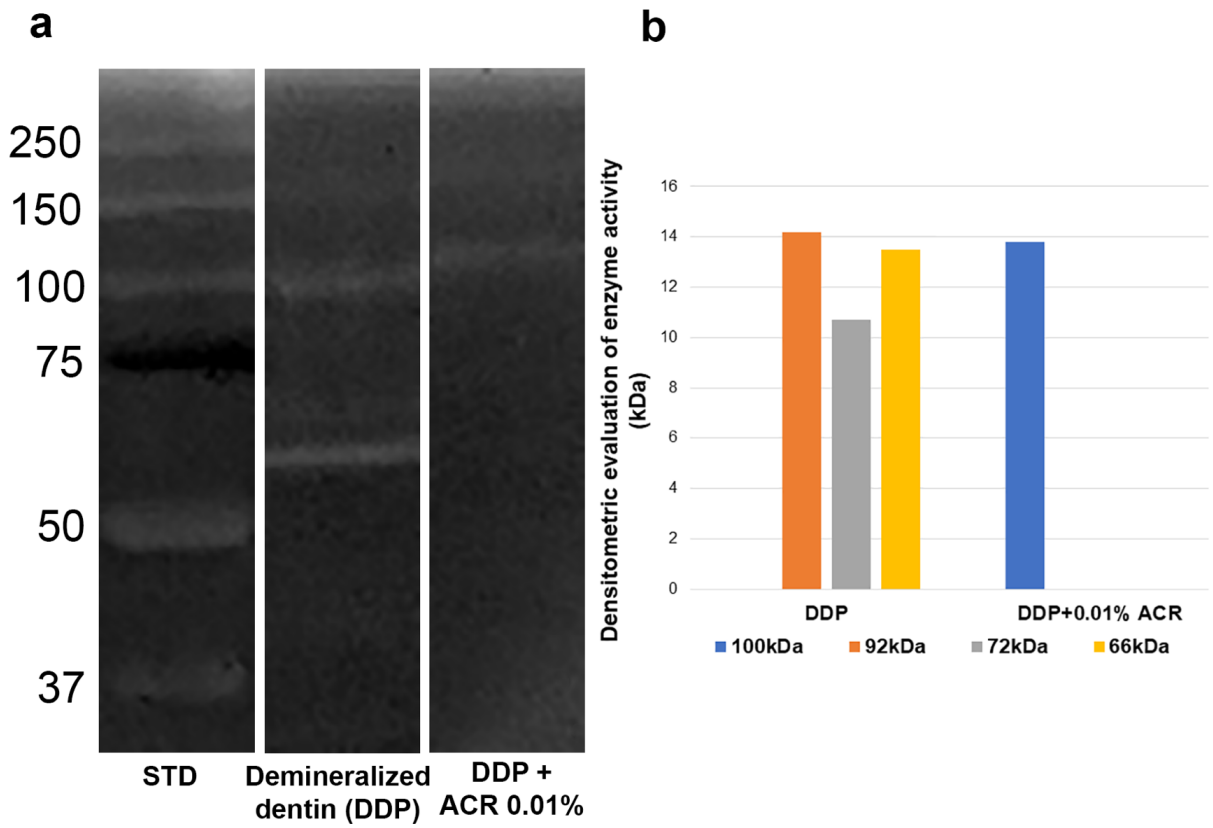


Figure 3. (a) Acrolein zymographic analysis. Lane 1: demineralized dentin powder showing activity of pro- form of MMP-9 (92 kDa) and active form of MMP-2 (66 kDa). Lane 2: demineralized dentin powder after incubation with 0.01% ACR showing complete inactivation of MMP-2, and reduced MMP-9 activity, although a complex form of MMP-9 around 100 kDa is still detectable; (b) Graph illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder**.**

In-situ zymography of resin-dentine interfaces

Confocal laser scanning microscopy images of *in-situ* zymography and superimposition of the fluorescence with light microscopy of the resin-dentine interfaces are shown in Figure 3. Specimens that were pre-treated with 0.01 wt% ACR prior to adhesive application exhibited minimal green fluorescence within the hybrid layer (Figures 4a and 4c). In contrast, specimens that were bonded using the dentine adhesive alone (control) exhibited intense green fluorescence within the hybrid layer (Figures 3b and 3d). Quantification of the fluorescence of the specimens (Figure 4e) indicates a 42.76% reduction in enzymatic activity when the ACR-

based primer was used before the bonding procedure. The difference between the control and experimental group is statistically significant ($p < 0.05$).

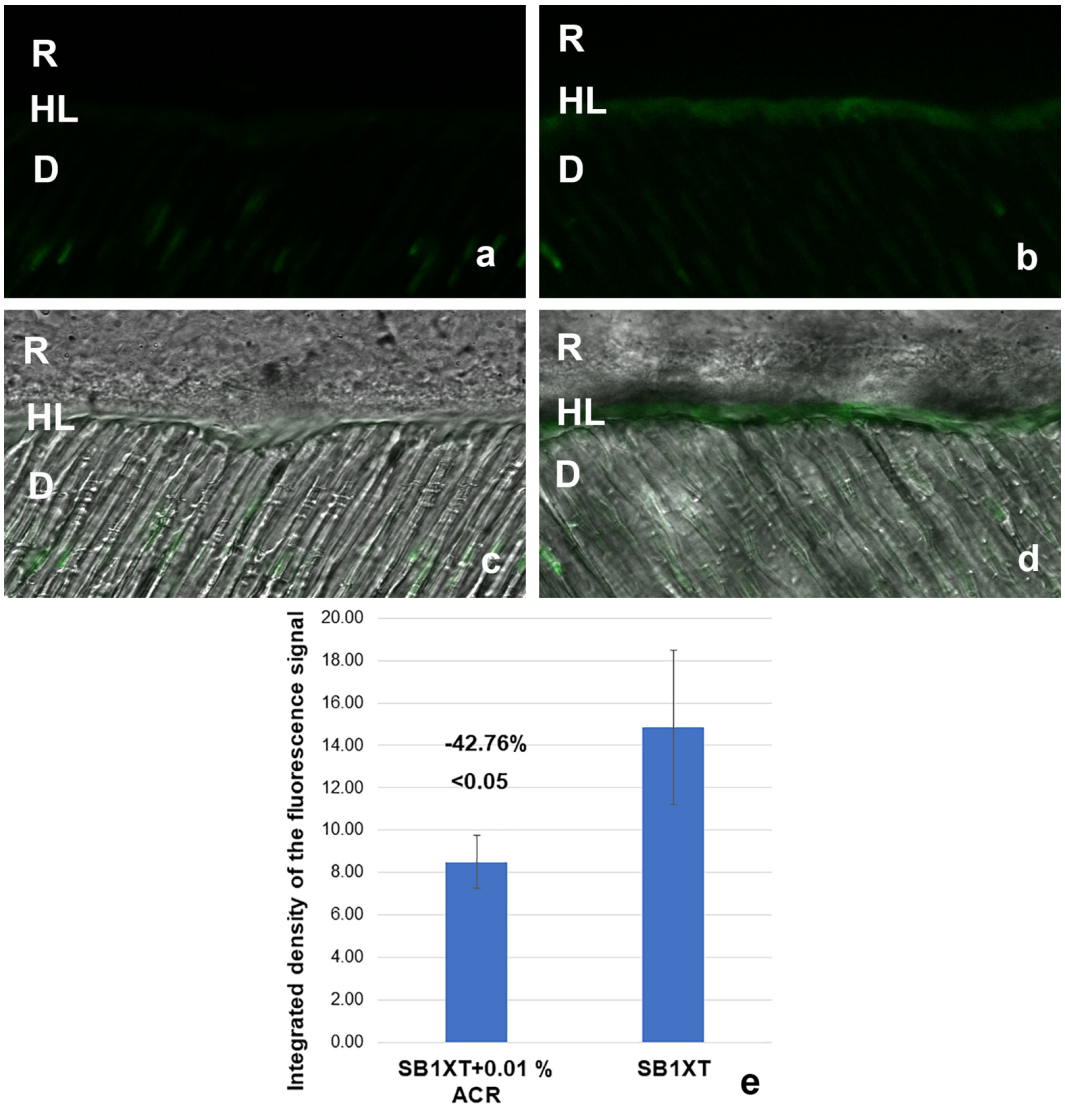


Figure 4. Resin-bonded dentin interfaces prepared with SB1XT with or without ACR pre-treatment, incubated with quenched fluorescein-labeled gelatin; (a) Image acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created with SB1XT with ACR pre-treatment; (b) Image acquired in green channel of the HL created by the application of SB1XT to acid-etched dentin without ACR pre-treatment showing higher fluorescence; (c) Image of SB1XT with ACR pre-treatment, obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity); (d) Image of HL created with SB1XT without ACR pre-treatment obtained by merging differential interference contrast image and image acquired in green channel; (e) Graph illustrating the quantification of the enzymatic activity of the tested groups**.** *SB1XT* = Adper Scotchbond 1 XT; *ACR* = Acrolein *D* = Dentin; *HL* = Hybrid Layer; *R* = Resin Composite.

2 - EDC Cross-linking effect on dentin bond strength and MMPs activity

Materials and methods

Microtensile bond strength test (μTBS)

Freshly extracted sound human third molars were obtained from anonymous individuals following their signed consent under a protocol approved by the University of Trieste (Italy). Eighty tooth crowns (n=20 for each group) were selected to conduct microtensile bond strength tests, flattened using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) under water cooling, and a standardized smear layer was created with 600-grit silicon-carbide (SiC) paper on each tooth surface.

Specimens were then randomly assigned to four different groups as according to the adhesive procedure performed:

- Group 1 (G1): Clearfil SE primer (Kuraray Dental, Osaka, Japan; abbreviation: CSE) was applied on unetched, smear layer-covered dentin according to the manufacturers' instructions. Then the dentin surface was pretreated with an aqueous solution of 0.3M EDC for 1 min, air-dried and bonded with Clearfil SE Bond (Kuraray) according to the manufacturer's instructions;
- Group 2 (G2): CSE was applied on unetched dentin without EDC pre-treatment as per manufacturer's instructions;
- Group 3 (G3): dentin was etched for 15s with 35% phosphoric-acid gel (3M ESPE, St. Paul, MN, USA) and rinsed with water. The acid-etched dentin was than pretreated with the 0.3M EDC solution for 1 min, air-dried and then bonded with XP Bond (Dentsply DeTrey GmbH, Konstanz, Deustche; abbreviation:XPB) following the manufacturer's instructions;
- Group 4 (G4): XPB was applied on etched dentin without EDC pre-treatment as per manufacturer's instructions.

Each bonded specimen was then light-cured for 20s using a LED curing light (Demi Light, Kerr). Four 1-mm-thick layers of microhybrid resin composite (Filtek Z250; 3M ESPE) were placed and polymerized individually for 20s. Specimens were serially sectioned to obtain approximately 1 mm-thick beams in accordance with the microtensile non-trimming technique.

The dimension of each stick (ca. 0.9mm×0.9mm×6mm) was recorded using a digital caliper (±0.01mm) and the bonded area was calculated for subsequent conversion of microtensile strength values into units of stress (MPa). Beams were stressed to failure after 24h (T0) or 1 year (T12) of storage in artificial saliva at 37°C [5] using a simplified universal testing machine (Bisco, Inc., Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. The number of prematurely debonded sticks in each test group was recorded, but these values were not included in the statistical analysis because all premature failures occurred during the cutting procedure and they did not exceed the 3% of the total number of tested specimens and were similarly distributed within the groups. A single observer evaluated the failure modes under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH) at magnifications up to 50× and classified them as adhesive, cohesive in dentin, cohesive in composite, or mixed failures.

As values were not normally distributed (Kolmogorov-Smirnov test), the collected data were statistically analyzed with the non-parametric Kruskal-Wallis test followed, when significant, by pair-wise comparisons using the Mann-Whitney U-test. The Chi-square test was used to analyze differences in the failure modes. For all tests, statistical significance was pre-set at p=0.05. Statistical analysis was performed using SPSS 21.0 software for Mac (SPSS Inc., Chicago, IL, USA).

Zymographic analysis

The zymographic analysis was performed according to the protocol of Mazzoni *et al*. 2014 [6]. In brief, mineralized dentin powder was obtained from additional 16 human third molars. Teeth were ground free of enamel, pulpal soft tissue, and cementum; dentin powder was obtained by freezing the dentin in liquid nitrogen and triturating it by means of a Retsch mill (Reimiller, Reggio Emilia, Italy). The fine mineralized dentin powder was pooled, dried, and kept frozen until use. Aliquots of mineralized dentin powder were divided into 6 groups as follows:

- Group 1 (Lane 1 - L1): dentin powder (DP) left untreated as mineralized control;

-Group 2 (Lane 2 - L2): DP treated with 1 mL of 10%/wt phosphoric acid for 10 min to simulate the etching procedure as the first step of the etch-and-rinse bonding technique and used as demineralized control (DDP);

- Group 3 (Lane 3 - L3): DP treated with 100 µl of 0.3M EDC for 30 min, then gently dried and treated with CSE primer for 30 min in the dark;

- Group 4 (Lane 4 - L4): DP mixed with 100 µl of CSE primer for 30 min in the dark;

- Group 5 (Lane 5 - L5): DDP treated with 0.3M EDC as for L4, followed by XPB application for 30 min in the dark;

- Group 6 (Lane 6 - L6): DDP mixed with 100 µl of XPB for 30 min in the dark.

From each group, the adhesive was extracted from the dentin-treated powder with 1 mL of acetone and centrifuged (20,800 g for 20 min), then re-suspended in acetone and re-centrifuged 2 more times for removal of additional unpolymerized comonomers [6]. For protein extraction, dentin powder aliquots were re-suspended in extraction buffer (50 mM Tris-HCl pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% non- ionic detergent P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3) for 24 hrs at 4°C and sonicated every 20 sec for 10 min (30 pulses), centrifuged for 20 min at 4°C (20.800g), after which the supernatant was removed and re-centrifuged. The protein content was further concentrated in a Vivaspin centrifugal concentrator (10,000 KDa cut-off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 4°C (15,000g, 3 times). Total protein concentration of dentin extracts was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Dentin protein aliquots (60 µg) were diluted in Laemmli sample buffer at a 4:1 ratio and subjected to electrophoresis under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/mL fluorescein-labeled gelatin. Pre-stained low-molecular-weight SDS-PAGE standards (Bio-Rad) were used as molecular-weight markers. After electrophoresis, the gels were washed for 1 hr in 2% Triton X-100, and then were incubated in zymography activation buffer (50 mmol/L Tris- HCl, 5 mmol/L CaCl2, pH 7.4) for 48 hrs. Proteolytic activity was evaluated and registered under long-wave UV light scanner (ChemiDoc Universal Hood, Bio-Rad). Gelatinase activity in the samples was analyzed in duplicate by

gelatin zymography. Zymographic bands were identified and quantified with Bio-Rad Quantity One Software (Bio-Rad).

In-situ Zymographic analysis

The procedures were performed using the method reported by Mazzoni and Nascimento, et al. [6,7]. After aging for the designated period, each bonded slab was glued to a glass slide and polished to produce an approximately 40-μm-thick section. To produce the substrate, 1.0 mg/ mL of a stock solution containing self-quenched fluorescein- conjugated gelatin (E-12055; Molecular Probes) was prepared by adding 1.0 mL deionized water to the vial containing the lyophilized gelatin. The substrate was stored at −20°C until use. The gelatin stock solution was diluted 10 times with dilution buffer (NaCl 150 mm, CaCl2 5 mm, Tris-HCl 50 mm, pH 8.0), followed by the addition of an antifading agent (Vectashield mounting medium with 4′,6-diamidino-2-phenylindole [DAPI]; Vector Laboratories). Then, 50 μL of the fluorescent gelatin mixture was placed on top of each polished dentin section and protected with a cover slip. The glass slide assemblies were light protected and incubated in a humidified chamber at 37°C for 48 h. Detection of endogenous gelatinolytic enzyme activity within the HL was based on hydrolysis of the quenched fluorescein- conjugated gelatin substrate. The process was evaluated by examining the glass slides with a multiphoton confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss), using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Sixteen to 20 optical sections (350 nm thick) were acquired from different focal planes for each specimen. The stacked images were analyzed, quantified, and processed with ZEN 2009 software (Carl Zeiss). The fluorescence intensity emitted by the hydrolyzed fluorescein-conjugated gelatin was isolated and quantified using Image J (ImageJ; National Institutes of Health). The amount of gelatinolytic activity was expressed as a percentage of the green fluorescence within the HL.

Negative control sections were similarly incubated, with the exception that 1) 250 mm ethylenediaminetetraacetic acid (EDTA) was dissolved in the mixture of quenched fluorescein-conjugated gelatin, or 2) 2 mm 1,10-phenanthroline, or 3) stan- dard nonfluorescent gelatin

instead of fluorescent-conjugated gelatin was used. The EDTA- and 1,10-phenanthroline–containing gelatin were used as negative controls.

Results

Microtensile bond strength test (μTBS)

Means and standard deviations of microtensile bond strength (in MPa) at times T0 and T12 months are reported in Table 3 and Graph 4. The use of the 0.3M EDC-containing conditioner before adhesive application did not affect the immediate bond strength of either XPB or CSE adhesive systems ($p>0.05$; Table 1). That is, both adhesives showed comparable bond strength values when employed with or without EDC pretreatment.

However, after incubating the bonded sticks for 12 months, the two control groups (2 and 4) showed significant ($p<0.05$) reduction in μTBS compared to EDC-treated experimental groups (1 and 3).

Group 2 specimens bonded with Clarefil SE Bond fell 35%, while Group 4 specimens bonded with XP Bond fell 51%.

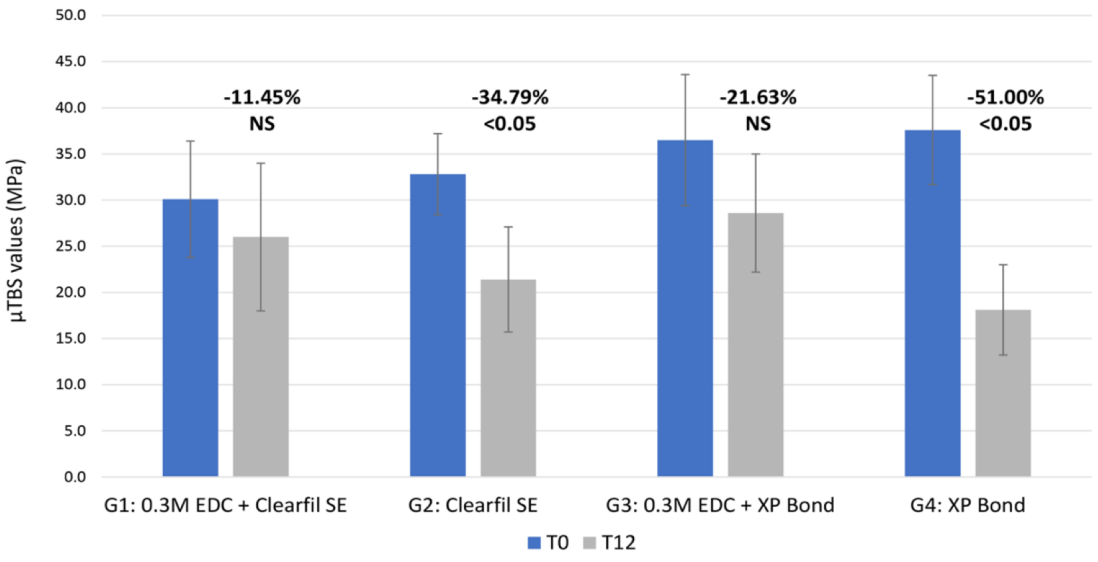
Specimens pretreated with EDC and bonded with Clarefil SE Bond showed only an 11% decrease in bond strength compared to the 35% reduction seen in Group 2. Similary, specimens pretreated with EDC before being bonded with XP Bond (Group 3) showed only 21% decrease in μTBS compared to the 51% decrease seen in non EDC-treated specimens (Group 4).

A predominance of mixed failures was detected in all groups, except for XPB control that resulted in increased adhesive fracture at the bonded interface either at T0 and T12.

Treatment Group	Storage time		Bond reduction after 1year of storage
	T_0	T_{12}	
Group 1 0.3M EDC + Clearfil SE	30.1±6.3 ^{a,A} (35A/11CC/12CD/42M)	26.±8.0 ^{a,A} (48A/5CC/7CD/36M)	-11%
Group 2 Clearfil SE	32.8±4.4 ^{a,A} (35A/0CC/10CD/55M)	21.4±5.7 ^{b,B} (42A/8CC/5CD/45M)	-35%
Group 3 0.3M EDC + XP Bond	36.5±7.1 ^{b,A} (32A/8CC/CD12/48M)	28.6±6.4 ^{a,B} (30A/4CC/7CD/59M)	-22%
Group 4 XP Bond	37.6±5.9 ^{b,A} (69A/8 CC/3CD/20M)	18.1±4.9 ^{b,B} (59A/5CC/0CD/36M)	-51%

Table 3. Means and standard deviations of microtensile bond strength (expressed as MPa) obtained by applying primer for 1 min on the etched dentin surface. T_0 and T_{12} indicate specimens that were tested after storage for 24 h or 12 months in artificial saliva, respectively. Distribution of failure mode among tested groups is also reported in square rounds and classified as: A: adhesive; CD: cohesive failure in dentin; CC: cohesive failure in resin composite; M: mixed. Bond reduction after storage report the percentage of mean bond reduction after 1 year of storage. Clearfil SE Bond (CSE) and XP Bond (XPB) with or without 0.3M EDC as additional therapeutic

†Premature failures due to preparation procedures were not included in the statistical analysis. Groups with the same superscripts are not statistically different ($p>0.05$). Different superscript lower-case letters (in rows) indicate statistical differences between storage time. Different superscript upper-case letters (in columns) indicate statistical differences between different adhesive protocol.



Graph 4. Graph summarizing the bond strength values (MPa) reported in Table 1

Zymographic analysis

Zymographic analysis and densitometric evaluation of bands, expressed as percentage of increase/decrease of MMPs activity among the different treatment groups compared with mineralized dentin, considered as baseline, are shown in Figs 5 and 6. Proteins extracted from mineralized and demineralized dentin powder (Lane1, Lane 2, Fig. 5) showed the presence of MMP-2 pro- and active-forms (72- and 66-kDa, respectively) and pro-MMP-9 (100kDa). Mineralized dentin powder treated with CSE and XPB resulted in enzymatic activation (Lane 4, Lane 6 Fig. 5), especially for the XPB where the activity of MMP-2 and -9 are clearly visible, while for CSE MMP-2 activity was almost absent. Pre-treatment with EDC followed by the application of CSE resulted in incomplete inhibition of MMPs, and the presence of a band corresponding to the active MMP-9 was still detectable (Lane 3, Fig. 5). Pre-treatment with EDC followed by the application of XPB resulted in an almost complete inactivation of dentinal gelatinases (Lane 5, Fig. 5).

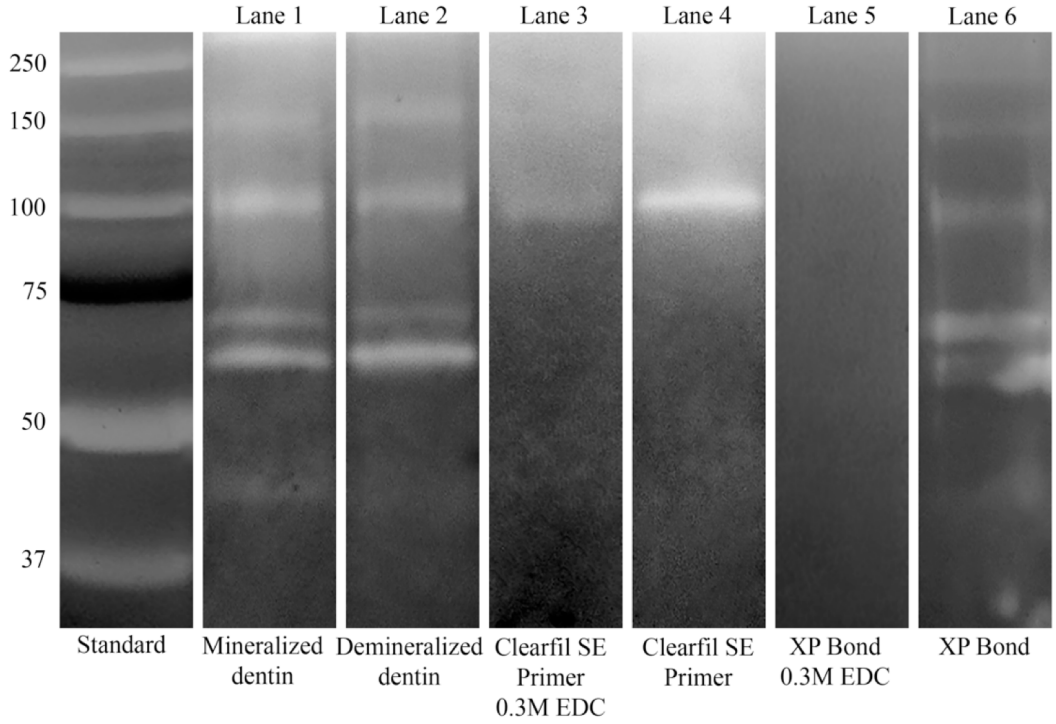


Figure 5. Zymographic analysis of proteins extracted from dentin powder. Std: Standards (Std) are reported in lane Std. Lane 1: Mineralized dentin showing the presence of MMP-9 pro-form, MMP-2 pro- and active- form (≈92, 72 and 66 kDa, respectively) and an additional band around 45 kDa. Lane 2: Proteins extracted from dentin powder demineralized with 10% phosphoric acid, showing an increase of MMP-2 active-form and a slight decrease in the expression of gelatinases pro-forms, and of the additional band at 45 kDa. Lane 3: Demineralized dentin powder after incubation with 0.3M EDC followed by CSE showing a decrease in the activity of MMP-9 pro-form and complete inactivation of dentinal MMP-2 pro- and active forms. Lane 4: Demineralized dentin powder treated with CSE showing a slight decrease in the activity of MMP-9 pro-form and complete inactivation of dentinal MMP-2 pro- and active forms. Lane 5: Proteins extracted from demineralized dentin powder pre-treated with 0.3M EDC followed by XP Bond application showing complete inactivation of dentinal gelatinases. Lane 6: Demineralized dentin powder treated with XP Bond showing enzymatic activation of both MMP-2 and -9 and of the additional band at approx. 45 kDa.

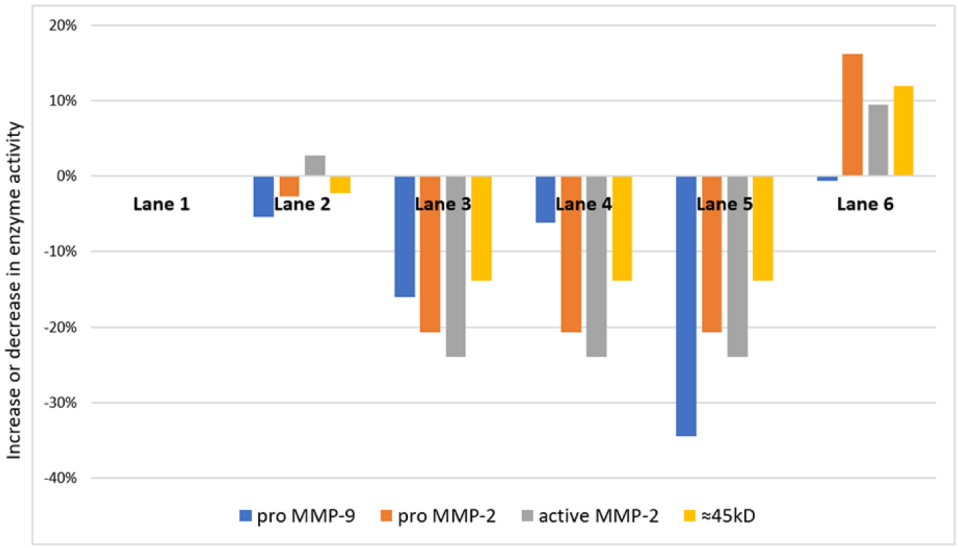


Figure 6. Graph illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder**.** The densitometric evaluation of bands is expressed as percentage increase/decrease of MMPs activity among the different treatment groups compared with mineralized dentin (considered as baseline).

In-situ Zymographic analysis

Representative images of the 4 groups are reported in Figures 1 to 4, and percentages of HLs exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin in the 4 groups (T0 and T1 y aging in artificial saliva) are shown in the Table 4.

Bonded dentin slabs at T0 revealed almost no green fluorescence within the HLs created by CSE when EDC was applied as additional primer (G1), indicating complete inhibition of gelatinase activity (Fig. 8a, c, e). In control samples without EDC pretreatment, intermittent fluorescence in the HL indicated gelatinolytic activity (Fig. 8b, d, f; Table 4). With XPB adhesive, EDC pretreatment resulted in weak gelatinolytic activity in the HL (Fig. 9a, c, e) that was about 80% less intense than in XPB control samples with relatively continuous HL gelatinolytic activity (Fig. 9b, d, f; Table 4). While the difference in activities between the adhesives was not significant with EDC pretreatment, XPB showed significantly more HL gelatinolytic activity than CSE in control samples ($P < 0.05$; Table 4).

At T1 y, EDC-pretreated specimens showed a statistically significant increase in the HL green fluorescence for both tested adhesives compared to T0 (G1 and G3; Fig. 8a, c, e; Fig. 10a, c, e; Table 4), while the 2 adhesives showed different trends of gelatinolytic expression after aging for control specimens (Fig. 8b, d, f; Fig. 10b, d, f). CSE-bonded control samples showed

significantly increased gelatinolytic activity after aging (Fig. 8b, d, f), while no difference was found for XPB (Fig. 10b, d, f; Table 4).

No fluorescence was detected in negative controls, that is, 1) EDTA-treated 2) specimens incubated with 2 mm 1,10-phenanth- roline or 3) with standard nonfluorescent gelatin (data not shown).

Adhesive System and Composition	EDC Pretreatment	% of MMP Activity of the HL	
		T_0	T_{1y}
Clearfil SE Bond	EDC	0.01 ± 0.0^{Aa1}	6.7 ± 0.8^{Aa2}
Bisphenol A diglycidylmethacrylate			
2-Hydroxyethyl methacrylate			
10-Methacryloyloxydecyl dihydrogen phosphate	Control	5.2 ± 1.7^{Ba1}	21.5 ± 2.6^{Ba2}
Hydrophobic aliphatic methacrylate			
Colloidal silica			
dl-Camphorquinone			
Initiators			
Accelerators			
XP Bond	EDC	2.8 ± 0.2^{Aa1}	7.8 ± 1.0^{Aa2}
Carboxylic acid-modified dimethacrylate (TCB resin)			
Phosphoric acid-modified acrylate resin (PENTA)	Control	14.7 ± 2.0^{Bb1}	13.6 ± 2.0^{Bb1}
Urethane dimethacrylate (UDMA)			
Triethyleneglycol dimethacrylate (TEGDMA)			
2-Hydroxyethylmethacrylate (HEMA)			
Butylated benzenediol (stabilizer)			
Ethyl-4-dimethylaminobenzoate			
Camphorquinone			
Nanofiller			
t-Butanol			

Table 4. Means of Percentage of MMP Activity (%) and Standard Deviation (SD) Expressed by HLs Created with 2 Adhesives (Compositions Are Reported as Disclosed by Manufacturers) with and without EDC Pretreatment after the 2 Time Periods, as Revealed by In Situ Gelatin Zymography.

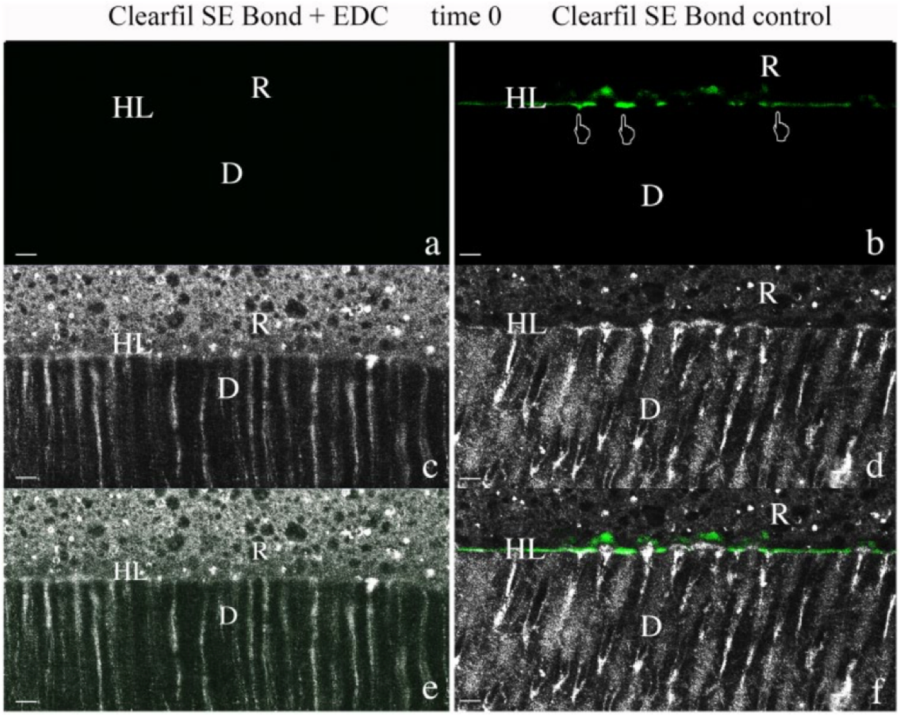


Figure 7. Resin-bonded dentin interfaces prepared with Clearfil SE (CSE) with or without 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) pretreatment and stored 24 h (T0), incubated with quenched fluorescein-labeled gelatin. D, dentin; HL, hybrid layer; R, resin composite; bars = 5 μm. (**a**) In situ zymogram confocal image acquired in green channel of HL created with CSE applied to dentin pretreated with EDC showing absence of fluorescence. (**b**) Zymogram image, showing fluorescence (identifying intense endogenous enzymatic activity) within the HL (pointers) created with CSE without EDC pretreatment. (**c**) Optical image of HL created with CSE after EDC pretreatment acquired in optical microscope showing the morphology of the HL. (**d**) Optical image of HL created with CSE without EDC pretreatment acquired as for (c). (**e**) Image of HL created with CSE after EDC pretreatment obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity). (**f**) Image of CSE without EDC pretreatment, obtained by merging differential interference contrast image as for (e) and image acquired in green channel (showing enzymatic activity).

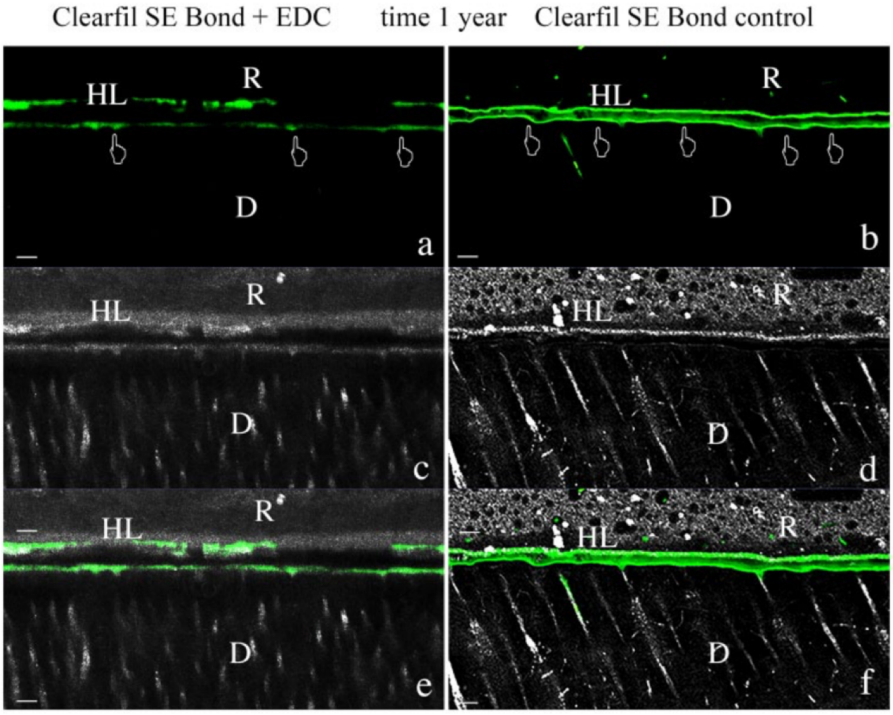


Figure 8. Resin-bonded dentin interfaces prepared with Clearfil SE (CSE) with or without 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) pretreatment after 1 y of storage (T1 y), incubated with quenched fluorescein-labeled gelatin. D, dentin; HL, hybrid layer; R, resin composite; bars = 5 μm. (**a**) Zymogram confocal image acquired in green channel of HL created with CSE applied to dentin pretreated with EDC showing weaker and discontinuous fluorescence (pointers). (**b**) Image acquired in green channel, showing intense and continuous fluorescence (identifying endogenous enzymatic activity) within the HL (pointers) created with CSE without EDC pretreatment. (**c**) Image of HL created with CSE after EDC pretreatment acquired as optical microscope showing the morphology of the HL. (**d**) Image of HL created with CSE after EDC pretreatment acquired as optical microscope. (**e**) Image of HL created with CSE after EDC pretreatment obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity). (**f**) Image of CSE without EDC pretreatment, obtained by merging differential interference contrast image as for (e) and image acquired in green channel (showing enzymatic activity).

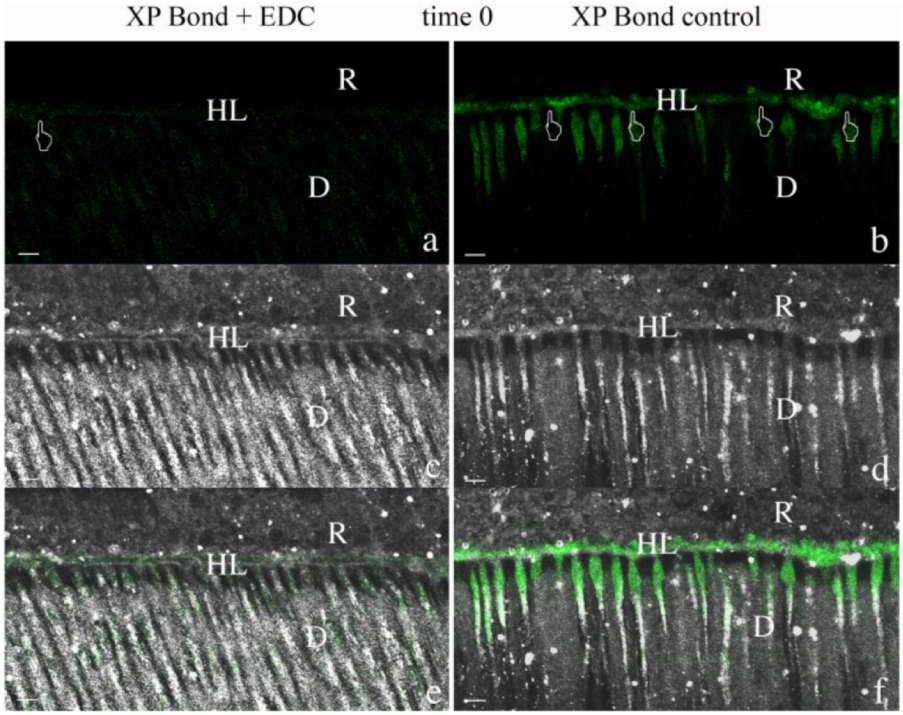


Figure 9. Resin-bonded dentin interfaces prepared with XP Bond (XPB) with or without 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) pretreatment after 24 h of storage (T0), incubated with quenched fluorescein-labeled gelatin. D, dentin; HL, hybrid layer; R, resin composite; bars = 5 μm. (a) Zymogram confocal image acquired in green channel of HL created with XPB applied to acid-etched dentin pretreated with EDC showing slight presence of fluorescence (pointers). (b) Image acquired in green channel, showing intense fluorescence (identifying endogenous enzymatic activity) in most of the dentinal tubule orifices and within the HL (pointers) created with XPB without EDC. (c) Image of HL created with XPB without EDC pretreatment acquired as optical microscope showing the morphology of the HL. (d) Image of HL created with XPB after EDC pretreatment acquired as optical microscope. (e) Image of HL created with XPB after EDC pretreatment obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity). (f) Image of XPB without EDC pretreatment, obtained by merging differential interference contrast image as for (e) and image acquired in green channel (showing enzymatic activity).

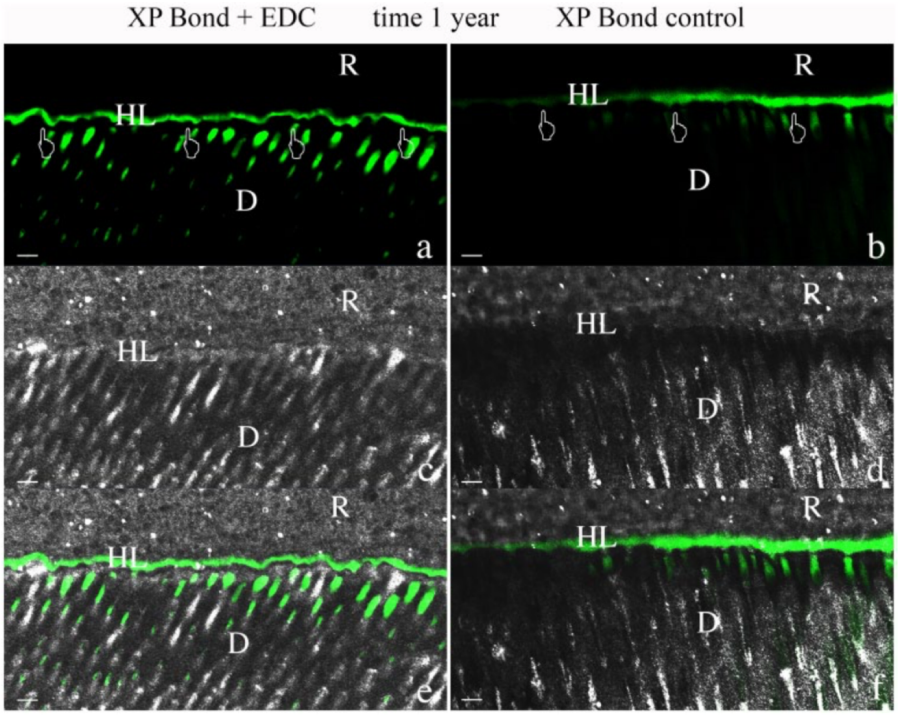


Figure 10. Resin-bonded dentin interfaces prepared with XP Bond (XPB) with or without 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) pretreatment and stored for 1 y (T1 y), incubated with quenched fluorescein-labeled gelatin. D, dentin; HL, hybrid layer; R, resin composite; bars = 5 μm. (a) Confocal image acquired in green channel of HL created with XPB applied to acid etched dentin pretreated with EDC showing fluorescence within the HL (pointers) and dentinal tubule orifices. (b) Image acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubule orifices and within the HL (pointers) created with XPB without EDC pretreatment. (c) Image of HL created with XPB after EDC pretreatment acquired as optical microscope showing the anatomy of the HL. (d) Image of HL created with XPB without EDC acquired as optical image. (e) Image of HL created with XPB after EDC pretreatment obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity). (f) Image of XPB without EDC pretreatment, obtained by merging differential interference contrast image as for (e) and image acquired in green channel (showing enzymatic activity).

3- Carbodiimide inactivation of matrix metalloproteinases in radicular dentine

Materials and methods

Specimen preparation

Extracted, caries-free, human single-rooted teeth, with similar radicular length and diameter were used for the present study (mostly upper central incisors, upper lateral incisors and upper single-rooted premolars). The specimens were stored in 0.5% chloramine at 4 °C and used within one month after harvesting. After debriding the root surface, each tooth was sectioned at the cementoenamel junction, perpendicular to the longitudinal axis of the tooth, to visualise canal morphology. Among all the collected teeth, sixty-four specimens were selected, each with a circular-shaped canal and at least 12 mm of root length. Root canal treatment was performed using Pathfiles (1-2-3) and ProTaper (S1-S2-F1-F2-F3) (Dentsply Sirona, York, PA, USA) to the working length. During instrumentation, the canals were irrigated with 10 mL of 5% sodium hypochlorite (Niclor 5; Ogna, Muggiò, Italy), alternated with 2 mL of 10% ethylenediamine tetra-acetic acid (Tubuliclean; Ogna). The clean and shaped root canals were rechecked under 20X magnification using an optimal microscope (Zeiss, Germany) to confirm the shape of the coronal part of the canal after instrumentation and to exclude the presence of visible cracks (irregular, oval and cracked canals were discarded). Canals that satisfied those criteria were obturated with gutta-percha in combination with a root canal sealer (AH-Plus; Dentsply Sirona) using warm vertical compaction.

Post-luting

Each root-treated tooth was placed in a 100% relative humidity chamber for 24 hours to facilitate setting of the sealer. A 10 mm post-space was subsequently created in each root-filled canal using fibre post drills (Rely-X Fiber Post, 3M ESPE, St. Paul, MN, USA). The post space was etched for 15 seconds with 36% phosphoric acid (Conditioner36, Dentsply Sirona), rinsed for 60 seconds with air-water spray and dried with paper points. Prior to luting, the correct length of each fibre post (RelyX Fiber Post, Size 2) was verified. The specimens were randomly divided into four groups according to the adhesive protocol employed:

- Group 1: All-Bond 3 (AB3, Bisco Inc., Schaumburg, IL, USA), a three-step etch-and-rinse adhesive, was applied on the acid-etched post space following the manufacturer's instructions;

- Group 2: The acid-etched dentin was pre-treated with an aqueous solution of 0.3 M EDC for one minute, air-dried, and treated with AB3;

- Group 3: Prime&Bond XP (XPB, Dentsply Sirona), a two-step etch-and-rinse adhesive, was mixed with the Self-Cure Activator and applied on the acid-etched post space following the manufacturer's instructions;

- Group 4: The acid-etched dentin was pre-treated with 0.3M EDC as described in Group 2, and then treated with Prime&Bond XP.

Each fibre post was cleaned in ethanol for 30 seconds prior to the application of a silane coupling agent (Ceramic Primer; 3M ESPE). The primer-coupled fibre post was air-dried for 5 seconds. A dual-cure resin luting cement (Core-X Flow, Denstply Sirona) was used according to the manufacturer's instructions and inserted into the post-space with a suitable-sized mixing tip. After the post was inserted into the canal space for one minute, the luting cement was light-cured with a light-emitted diode curing light (Translux Power Blue, Heraeus Kulzer, Hanau, Germany). Light-curing was performed for 40 seconds each from the cervical surface of the root in the direction of the longitudinal axis, and then obliquely from the buccal and palatal surfaces (total 120 seconds). After polymerisation, the post-luted specimens were stored in distilled water at 37 °C for 24 hours.

Six 1-mm thick slices were prepared from each specimen using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) using water cooling. A mark was placed on the coronal side of each section with an indelible marker. The marked specimens were stored in artificial saliva at 37 °C [2].

Resistance of fibre post to dislodgement

A micro push-out test (N=8) was used to evaluate the ability of the fibre posts to resist dislodgement from the bonded canal walls (N = 8). Testing was conducted after 24 hours and after one year of storage in artificial saliva. Push-out was performed by applying an axial load

to the post at a crosshead speed of 0.5 mm/min, using an Instron Machine I model 10/D (Sintech, MTS, USA). The apical surface was placed facing the punch tip, ensuring that loading forces were introduced from an apical to coronal direction. Bond failure was manifested by the dislodgment of the fibre post from the root section. Push-out strength data were converted to MegaPascals (MPa) by dividing the load in Newtons by the bonded surface area (SL) in mm^2, and SL was calculated as the lateral surface area of a truncated cone using the formula: $SL= ((R+r))*((h^2 + (R-r))^2)^{0.5}$ where R is the coronal radius of the canal with the post, r the apical radius and h the thickness of the slice. The wider and the narrowest diameters were digitally measured using ImageJ software on a picture of the slice taken on a millimetre paper to set the scale, while the thickness of the slice were individually measured using a pair of digital callipers with 0.01 mm accuracy. A single observer evaluated the debonded specimens using a stereomicroscope at 40× magnification. Failure modes were classified as: adhesive failure between dentine and cement (AD), adhesive failure between the cement and post (AP), cohesive failure within the cement (CC), cohesive failure within the post (CP) and mixed failure (M). The percentage of each type of failure mode within each group was calculated.

Nanoleakage

Specimen were sectioned into 1mm-thick slices and immersed in 50 wt% ammoniacal silver nitrate solution for 24 hours. The silver ion-infiltrated specimens were subsequently immersed in photo-developing solution to reduce the silver ions into metallic silver grains. The specimens were fixed on glass slides, flattened with silicon carbide paper under running water and observed using a light microscope (Nikon E800; Nikon, Tokyo, Japan). Images of the adhesive interfaces were obtained (original magnification: 100X) and the degree of interfacial nanoleakage was scored using a four-point scale by two observers. Scoring was performed using the methodology described by Saboia *et a* [8]. Intra-examiner reliability was evaluated using the kappa (κ) test.

In-situ zymography of the hybrid layer

Six additional non-carious human premolars with two distinct roots, extracted for orthodontic reasons, were used for *in-situ* zymography. Crown removal and root canal treatment were performed in the manner described in previous sections. A standardised post-space was created in all canals and the radicular dentine was etched for 15 seconds with 36% phosphoric acid, rinsed for 60 seconds and dried with paper points. The etched canals were treated according to the aforementioned adhesive procedures. The same adhesive (AB3 or XPB) was used for each tooth; one post-space was pre-treated with 0.3 M EDC while the other post-space was bonded without EDC pre-treatment. The Core-X Flow resin cement was used for luting of the fibre posts in the manner previously described. Six 1-mm thick sections were prepared from each tooth. Each section was fixed to a glass slide using glue and polished with 4000-grit silicon carbide papers with water cooling to obtain specimens with a final thickness of ~ 50 μm.

In-situ zymography was performed with self-quenched fluorescein-conjugated gelatine as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA)[7]. Briefly, the fluorescent gelatine mixture was placed on top of each slab and covered with a glass cover-slip. Each glass slide was light-protected and incubated in a humidified chamber at 37 °C for 24 hours. Hydrolysis of quenched fluorescein-conjugated gelatine within the hybrid layer, indicative of endogenous gelatinolytic enzyme activity, was evaluated by examination of the glass slides with a multi-photon confocal laser scanning microscope (TCS SP5-AOBS 5-channel, Leica Microsystems, Buffalo Grove, IL, USA), using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Images were acquired using a HCX PL APO 40x/ 1.25 NA oil immersion objective always maintaining the same microscope setting. Optical sections (350 nm thick) were acquired from different focal planes. The stacked images were analysed, quantified, and processed with ImageJ software (National Institute of Health, Bethesda, MD, USA). The fluorescence intensity emitted by the hydrolysed fluorescein-conjugated gelatine was quantified and the amount of gelatinolytic activity assessed through the green signal within the hybrid layer was expressed in arbitrary units.

Statistical analysis

After ascertaining the normality (Shapiro-Wilk test) and homoscedastic (modified Levene test) assumptions of the data sets, the bond strength data were analysed with three-way analysis of variance to examine the effects of the adhesive system, EDC application and storage time, and the interaction of those three factors on micro push-out bond strength. Post-hoc pairwise comparisons were performed using the Holm-Šidák multiple comparison procedure. Chi-square tests were used to analyse nanoleakage scores and differences in the failure modes. Evaluation of the quantified data obtained from *in-situ* zymography was performed two-way analysis of variance to examine the effect of adhesive and EDC pre-treatment on potential gelatinolytic activities. Pot-hoc comparisons were conducted using the Holm-Šidák statistic. For all tests, statistical significance was pre-set at $\alpha = 0.05$. All statistical analyses were performed using Stata 12.0 (StataCorp, College Station, Texas, USA).

Results

Push-out strength

Bond strength data were expressed as means and standard deviations and summarised in Table 5.

Results of the three-way ANOVA showed that significant difference was observed for the factors: “EDC treatment” ($p = 0.002$) and “time” ($p = 0.000$). The factor “adhesive” had no effect on the push-out bond strength ($p > 0.05$). The interactions between the factors ‘‘EDC treatment” and “adhesive”, “adhesive” and “time”, “EDC treatment” and “time” were not significant ($p > 0.05$). Furthermore, also the interaction of the three factors “EDC treatment”, “time” and “adhesive” was not significant ($p > 0.05$).

	T=0			T=12			Bond change after storage
	CORONAL	MIDDLE	APICAL	CORONAL	MIDDLE	APICAL	
G1: AB3	7.99 (±1.61)	5.52 (±2.36)	4.50 (±2.48)	6,08 (±1.56)	4.14 (±1.16)	3.34 (±1.67)	C= - 23.9% M= - 25% A= -25.7%
G2: EDC+AB3	8.33 (±2.46)	6.14 (±3.24)	4.95 (±1.68)	8.13 (±1.50)	5.52 (±1.64)	4.47 (±1.78)	C= -2.4% M= -10.1% A= -9.7%
G3: XPB	8.01 (±2.07)	5.37 (±1.61)	4.24 (±1.17)	5.84 (±1.78)	3.30 (±1.12)	2.71 (±1.29)	C= -27.1% M= -38.5% A= -36.1%
G4: EDC+XPB	8.11 (±1.14)	5.83 (±1.66)	4.50 (±1.83)	7.05 (±1.76)	4.41 (±1.48)	4.01 (±1.50)	C= -13.1% M= -24.3% A= -10.9%

Table 5. Push-out strength (mean ± standard deviation) obtained from the four experimental groups at 24 hours and after 12 month of aging in artificial saliva. Strength reduction after aging was expressed as a percentage of the 24-hour push-out strength. Root location designation: C, coronal region; M, middle region; A, apical region.

Failure mode

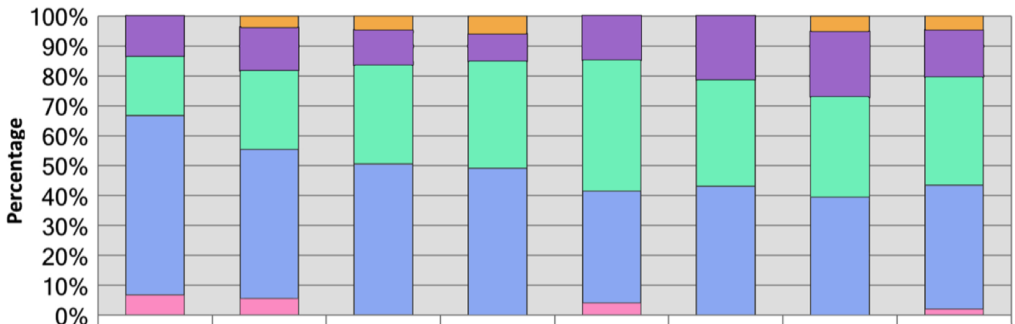
Failure modes distribution of the debonded specimens, expressed as percentages of the total number of specimens tested, are summarised in Table 6. More than 95% of the failures in each group were either adhesive failure between dentine and resin cement, or mixed failures. Other failure modes were inconspicuously identified in specimens that were tested after 24 hours. After artificial aging for 12 months, Groups 1 (AB3) and 3 (SPB) showed an increased number of adhesive failures between dentine and cement, although the increases were not statistically significant.

		AD	AP	CC	CP	M
G1	T=0	75.7	0.7	0	0.2	23.4
	T=12	86.9	0.6	0	0	12.5
G2	T=0	73.5	1.2	0	0	25.3
	T=12	77.4	1.1	0	0	21.5
G3	T=0	78.6	1.4	0	0	20
	T=12	84.1	0.9	0	0	15
G4	T=0	77.3	1.3	0	0	11,4
	T=12	78.9	1.7	0	0	19.4

Table 6. Failure mode of debonded specimens from the four experimental groups at 24 hours and after one year of aging in artificial saliva. Data were expressed as percentages of the total number of specimens tested for each group. Failure mode designation; AD, adhesive failure between dentine and cement; AP, adhesive failure between cement and post; CC. cohesive failure within the cement; CP, cohesive failure within the post; M, mixed failure.

Nanoleakage

Descriptive statistics of interfacial leakage scores are represented in Figure 1. No statistically significant differences were found among the groups in the extent of silver nitrate penetration (p = 0.052), as shown in Figure 11.



	AB3 24 hours	EDC + AB3 24 hours	AB3 One year	EDC + AB3 One year	XPB 24 hours	EDC + XPB 24 hours	XPB One year	EDC + XPB One year
>75	0.0	4.0	5.0	6.2	0.0	0.0	5.5	5.0
50≤75	13.3	14.0	11.2	8.8	14.5	21.2	21.2	15.2
25≤50	20.0	26.6	33.3	36.0	44.0	35.8	33.8	36.3
<25	60.0	50.0	50.5	49.0	37.5	43.0	39.5	41.5
0	6.7	5.4	0.0	0.0	4.0	0.0	0.0	2.0

Figure 11. Percentage of interfacial nanoleakage expression in resin-dentine interfaces created in radicular dentine with All-Bond 3 (AB3) or Prime&Bond XP (XPB), with or without carbodiimide (EDC) pre-treatment of the acid-etched dentine, at 24 hours or after one year of aging in artificial saliva.

In-situ zymography

Gelatinolytic activity, expressed as the percentage of the green fluorescence within the hybrid layer, is represented in Figure 12.

Results of the two-way ANOVA showed that significant difference was observed for the factors: “EDC treatment” ($p = 0.000$) and “adhesive” ($p = 0.000$). The interaction between the factors “EDC treatment” and “adhesive” was not statistically significant ($p > 0.05$).

Application of the two-step etch-and-rinse adhesive XPB to acid-etched dentin resulted in significantly more extensive gelatinolytic activity within the hybrid layer when compared to the application of the three-step etch-and-rinse adhesive AB3 ($p < 0.05$). For both adhesives, pre-treatment of the acid-etched dentin with 0.3 M EDC prior to adhesive application resulted in significantly reduced gelatinolytic activity within radicular hybrid layer, compared with the corresponding adhesive ($p < 0.05$). Confocal laser scanning microscopic examination of *in-situ* zymography specimens derived from unaged premolars in the four experimental groups revealed intense green fluorescence within the dentinal tubules of mineralized radicular dentine as well as within the hybrid layer (Figure 13). Because the original gelatine substrate was heavily quenched with conjugated fluorescein and did not fluoresce, regions in the root slice that exhibit strong fluorescence are indicative of hydrolysis of the extrinsic gelatine substrate, which in turn, released the conjugated fluorescein molecules.

No fluorescence was detected in negative controls prepared with non-specific inhibitors, including: 1) EDTA-treated, 2) specimens incubated with 2 mM 1,10-phenanthroline or 3) with standard nonfluorescent gelatin (data not shown).

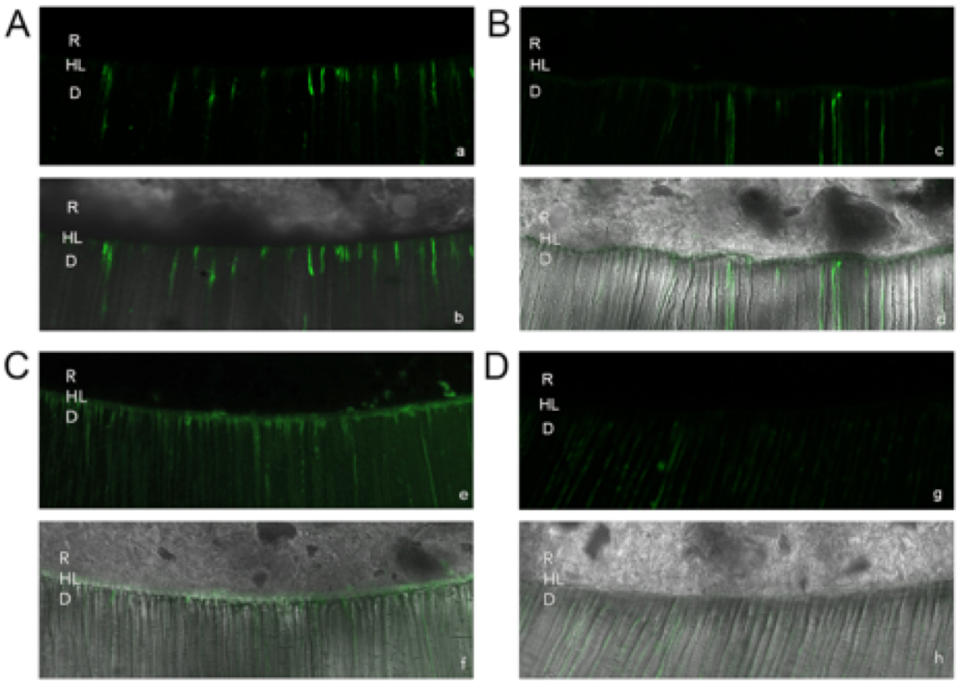


Figure 12. Representative confocal laser scanning microscopy images of resin-bonded radicular dentine interfaces that were incubated with quenched fluorescein-labelled gelatine. Abbreviations: D, dentine; HL, hybrid layer; R, resin cement. **A**. Dentine bonded with All-Bond 3 without EDC pre-treatment. **B**. Dentine bonded with All-Bond 3 with EDC pre-treatment. **C**. Dentine bonded with Prime&Bond XP without EDC pre-treatment. **D**. Dentine bonded with Prime&Bond XP with EDC pre-treatment. For each set of images, the top image was acquired in the green channel. The green fluorescence represented areas with intense endogenous gelationolytic activity within the dentinal tubules and the hybrid layer. The bottom image was produced by merging the differential interference contrast image (showing the optical density of the resin-dentine interface) and the image acquired in green channel.

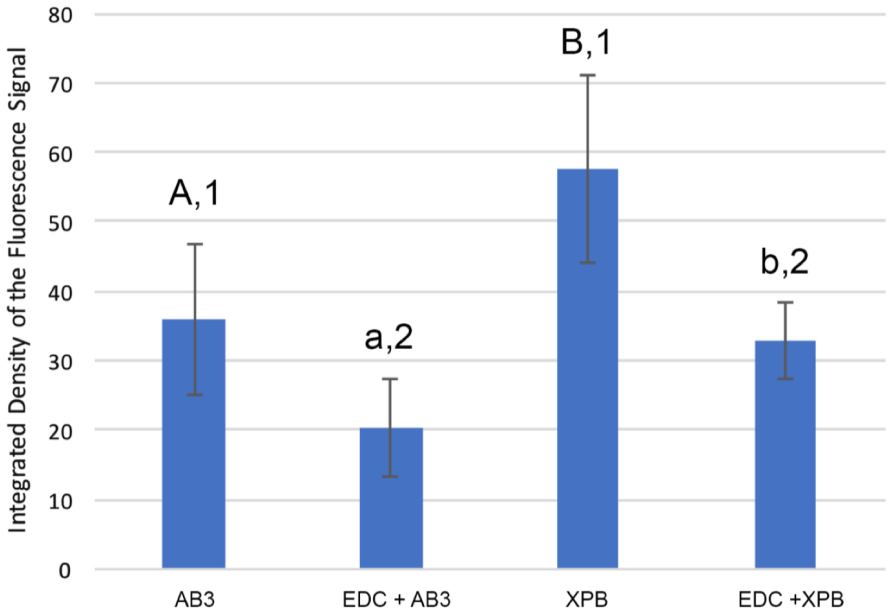


Figure 13. Gelatinolytic activity, expressed as the percentage of the green fluorescence within hybrid layers created with All-Bond 3 (AB3) or Prime&Bond XP (XPB), with or without carbodiimide (EDC) pre-treatment of the acid-etched dentine. Values are means and standard deviations. For comparison of the factor "adhesive type", columns of labelled with the same upper case letters (24 hours) or lower case letters (aging for one-year) are not significantly different ($p > 0.05$). For comparison of the factor "with or without EDC pre-treatment", columns labelled with the same numerals are not significantly different ($p > 0.05$).

4- DCC Cross-linking effect on dentin bond strength and MMPs activity

Materials and methods

Microtensile bond strength test (μTBS)

Freshly extracted sound human third molars were obtained from anonymous individuals following their signed consent under a protocol approved by the University of Bologna (Italy). Eighty tooth crowns (n=20 for each group) were selected to conduct microtensile bond strength tests, flattened using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) under water cooling, and a standardized smear layer was created with 600-grit silicon-carbide (SiC) paper on each tooth surface.

Specimens were then randomly assigned to four different groups as according to the adhesive procedure performed:

- Group 1 (G1): DCC pretreatment and Scotchbond Universal (3M ESPE) in *etch-and-rinse* mode. Then the dentin surface was etched with 37% phosphoric acid and pretreated with an ethanol solution of 0.5M DCC for 1 min, air-dried and bonded with Scotchbond Universal according to the manufacturer's instructions;

- Group 2 (G2): SBU was applied on etched dentin without DCC pre-treatment as suggested by manufacturer's instructions;

- Group 3 (G3): DCC pretreatment and Scotchbond Universal (3M ESPE) in *self-etch* mode. Then the dentin surface was pretreated with an ethanol solution of 0.5M DCC for 1 min, air-dried and bonded with SBU according to the manufacturer's instructions;

- Group 4 (G4): SBU was applied on unetched dentin without DCC pre-treatment as suggested by manufacturer's instructions;

Each bonded specimen was then light-cured for 20s using a LED curing light (Demi Light, Kerr). Four 1-mm-thick layers of microhybrid resin composite (Filtek Z250; 3M ESPE) were placed and polymerized individually for 20s. Specimens were serially sectioned to obtain approximately 1 mm-thick beams in accordance with the microtensile non-trimming technique. The dimension of each stick (ca. 0.9mm×0.9mm×6mm) was recorded using a digital caliper (±0.01mm) and the bonded area was calculated for subsequent conversion of microtensile

strength values into units of stress (MPa). Beams were stressed to failure after 24h (T0) or 1 year (T12) of storage in artificial saliva at 37°C [2] using a simplified universal testing machine (Bisco, Inc., Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. The number of prematurely debonded sticks in each test group was recorded, but these values were not included in the statistical analysis because all premature failures occurred during the cutting procedure and they did not exceed the 3% of the total number of tested specimens and were similarly distributed within the groups. A single observer evaluated the failure modes under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH) at magnifications up to 50× and classified them as adhesive, cohesive in dentin, cohesive in composite, or mixed failures.

As values were normally distributed (Kolmogorov-Smirnov test), the collected data were statistically analyzed with the three-way ANOVA test for the variable DCC pretreatment, etching mode and aging. The Chi-square test was used to analyze differences in the failure modes. For all tests, statistical significance was pre-set at $p<0.05$. Statistical analysis was performed using SPSS 21.0 software for Mac (SPSS Inc., Chicago, IL, USA).

Nanoleakage analyses

Additional thirty teeth (N=5 per group) were prepared. Slabs of mid-coronal dentine were treated with the same bonding procedures as previously described for μBST. Further, the specimens were cut vertically into 1-mm-thick slabs to expose the bonding surfaces. After storage in artificial saliva at 37◦C for 24h (T0), and 12 months (T12), specimens were immersed in 50 wt% ammoniac AgNO3 solution for 24 h in a dark environment following the protocol described by Tay et al. [9], thoroughly rinsed in distilled water, and immersed in a photo developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grain within voids along the bonded interfaces.

For the light microscopy (LM), the specimens were processed in the following manner: fixed, dehydrated, embedded in epoxy resin (LR White resin, Sigma-Aldrich, St. Louis, MI, USA), fixed on glass slides using cyanoacrylate glue, fattened on a grinding device (LS2; Remet, Bologna, Italy) under water irrigation using a series of abrasives (180-, 600-, 1200-, 2400-, and 4000-grit SiC). The precipitation of the silver tracer was analysed along the bonded

interface using LM (E800; Nikon, Tokyo, Japan), under 20X magnification, and the interfacial nanoleakage was scored based on the percentage of adhesive surface showing AgNO3 deposition by two trained investigators, following the method of Saboia et al. [8]. The scores were placed on a scale 0-4 under the following criteria: (0) no nanoleakage; (1) <25% surface with nanoleakage; (2) 25% to 50% surface with nanoleakage; (3) 50% to 75% surface with nanoleakage; and (4) >75% surface with nanoleakage. Because the data were normally distributed, statistical differences among nanoleakage group scores were analyzed with Chi^2 test and statistical significance was set for $\alpha=0.05$.

In-situ Zymographic analysis

Additional 3 teeth per group were processed for in-situ zymography analyses. The procedures were performed using the method reported by Mazzoni and Nascimento, et al. [7,10]. After aging for the designated period, each bonded slab was glued to a glass slide and polished to produce an approximately 40-μm-thick section. To produce the substrate, 1.0 mg/ mL of a stock solution containing self-quenched fluorescein-conjugated gelatin (E-12055; Molecular Probes) was prepared by adding 1.0 mL deionized water to the vial containing the lyophilized gelatin. The substrate was stored at −20°C until use. The gelatin stock solution was diluted 10 times with dilution buffer (NaCl 150 mm, CaCl2 5 mm, Tris-HCl 50 mm, pH 8.0), followed by the addition of an antifading agent (Vectashield mounting medium with 4′,6-diamidino-2-phenylindole [DAPI]; Vector Laboratories). Then, 50 μL of the fluorescent gelatin mixture was placed on top of each polished dentin section and protected with a cover slip. The glass slide assemblies were light protected and incubated in a humidified chamber at 37°C for 48 h. Detection of endogenous gelatinolytic enzyme activity within the HL was based on hydrolysis of the quenched fluorescein-conjugated gelatin substrate. The process was evaluated by examining the glass slides with a multiphoton confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss), using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Sixteen to 20 optical sections (350 nm thick) were acquired from different focal planes for each specimen. The stacked images were analyzed, quantified, and processed with ZEN 2009 software (Carl Zeiss). The fluorescence intensity emitted by the hydrolyzed fluorescein-

conjugated gelatin was isolated and quantified using Image J (ImageJ; National Institutes of Health). The amount of gelatinolytic activity was expressed as a percentage of the green fluorescence within the HL.

Negative control sections were similarly incubated, with the exception that 1) 250 mm ethylenediaminetetraacetic acid (EDTA) was dissolved in the mixture of quenched fluorescein-conjugated gelatin, or 2) 2 mm 1,10-phenanthroline, or 3) stan- dard nonfluorescent gelatin instead of fluorescent-conjugated gelatin was used. The EDTA- and 1,10-phenanthroline–containing gelatin were used as negative controls.

Results

Microtensile bond-strength

Means and standard deviations of micro-tensile bond strength (in MPa) at times T0 and T12 months are reported in Table 7.

The statistical analyses showed that SBU had higher bond strength results when employed in etch and rinse mode. At baseline, the use of the 0.5M DCC containing primer applied before the adhesive, improve the immediate bond strength of SBU only when used in self-etch mode ($p>0.05$).

However, after incubating the bonded sticks for 12 months, the etch-and-rinse groups and the self-etch control group showed significant ($p<0.05$) reduction in μTBS. The bond strength values were only preserved for the experimental group applied in self-etch mode.

A predominance of adhesive failures was detected in all groups either at T0 and T12.

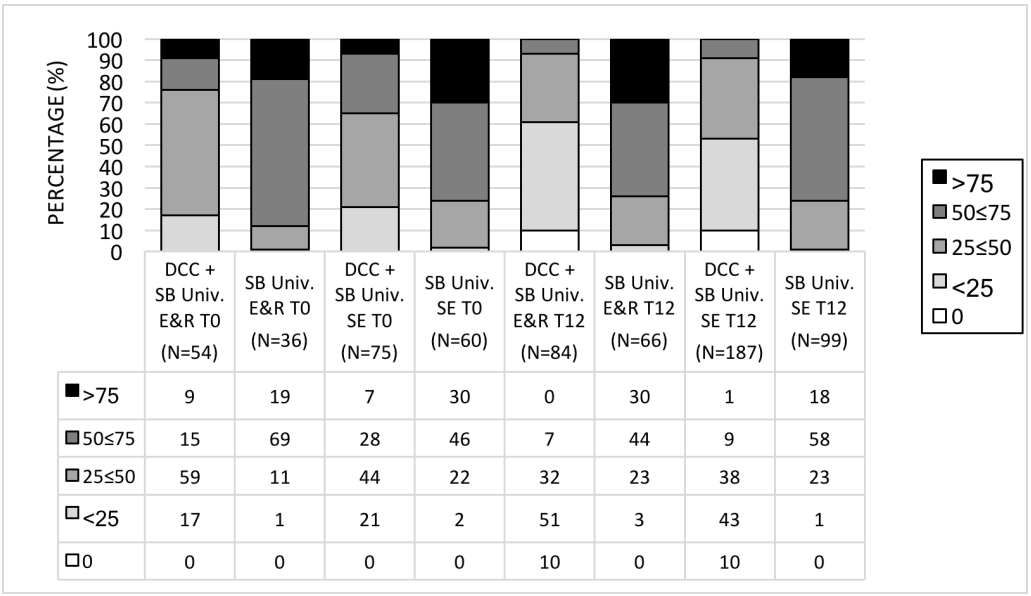
Sistema adesivo	Scotchbond Universal *Etch-and-rinse*		Scotchbond Universal *Self-etch*	
Pretrattamento	0,5 M DCC in etanolo	Controllo	0,5 M DCC in etanolo	Controllo
Test a T_0	$48,0^{bA}$ ± 17,7	$39,0^{aA}$ ± 12,6	$39,4^{a,bA}$ ± 18,4	$26,3^{cA}$ ± 17,9
Test a T_{12}	$32,3^{bB}$ ± 16,7	$29,6^{b,B}$ ± 12,7	$35,0^{bA}$ ± 15,6	$12,6^{cB}$ ± 9,2

Table 7: Means and standard deviations (MPa) of μTBS test at T0 and T12.

Nanoleakage

Figure 14 showed differences between the samples treated with and without pre-treatment with 0.5M DCC in ethanol at baseline and after one year of storage in artificial saliva. In particular, DCC treated samples expressed a lower marginal infiltration compared to control groups.

Examples of nanoleakage infiltration for each group are showed in figures 15-22.



	DCC + SB Univ. E&R T0 (N=54)	SB Univ. E&R T0 (N=36)	DCC + SB Univ. SE T0 (N=75)	SB Univ. SE T0 (N=60)	DCC + SB Univ. E&R T12 (N=84)	SB Univ. E&R T12 (N=66)	DCC + SB Univ. SE T12 (N=187)	SB Univ. SE T12 (N=99)
>75	9	19	7	30	0	30	1	18
50≤75	15	69	28	46	7	44	9	58
25≤50	59	11	44	22	32	23	38	23
<25	17	1	21	2	51	3	43	1
0	0	0	0	0	10	0	10	0

Figure 14. Percentage of interfacial nanoleakage expression in resin-dentine interfaces created in coronal dentine with SBU in etch-and-rinse and self-etch mode with or without DCC pre-treatment of dentine, at 24 hours or after one year of aging in artificial saliva.

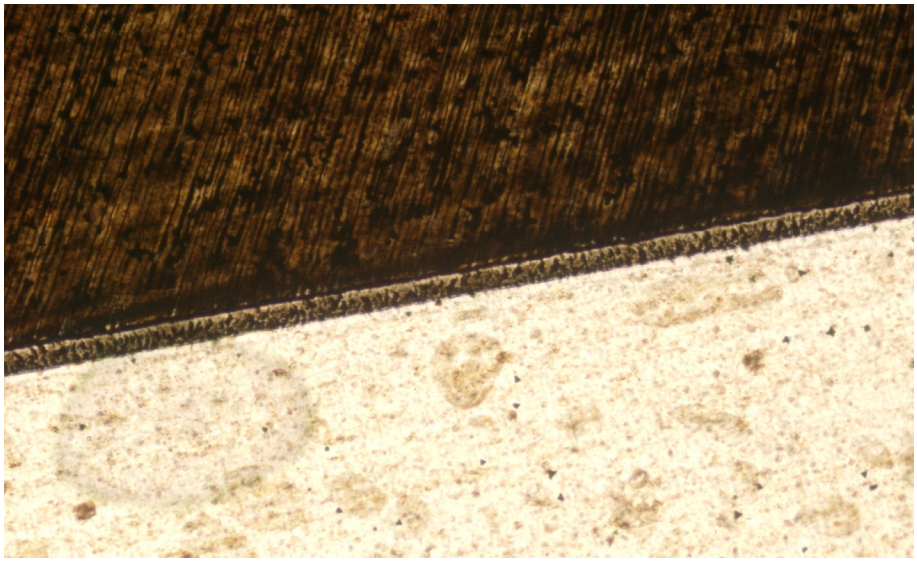


Figure 15: Sample treated with Scotchbond Universal E&R mode.

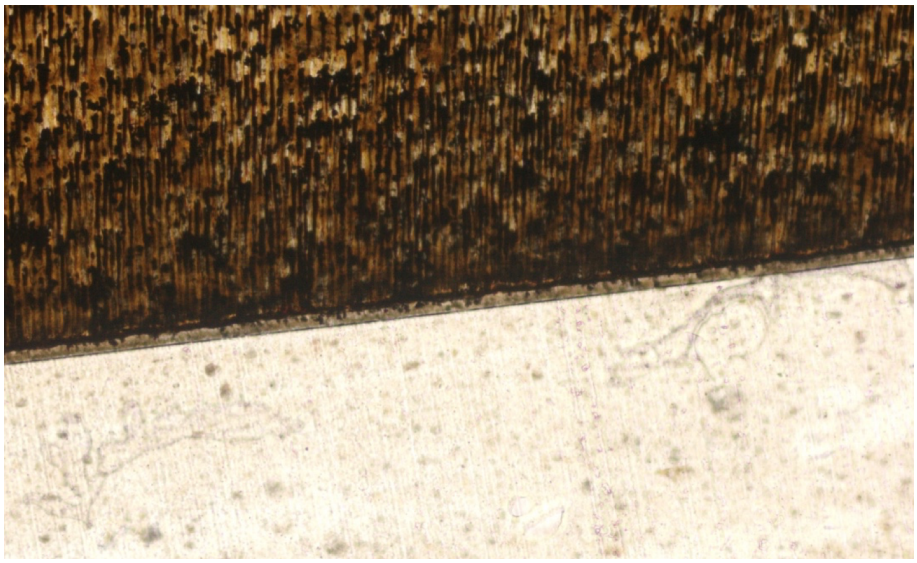


Figure 16: Sample treated with DCC and Scotchbond Universal E&R mode.

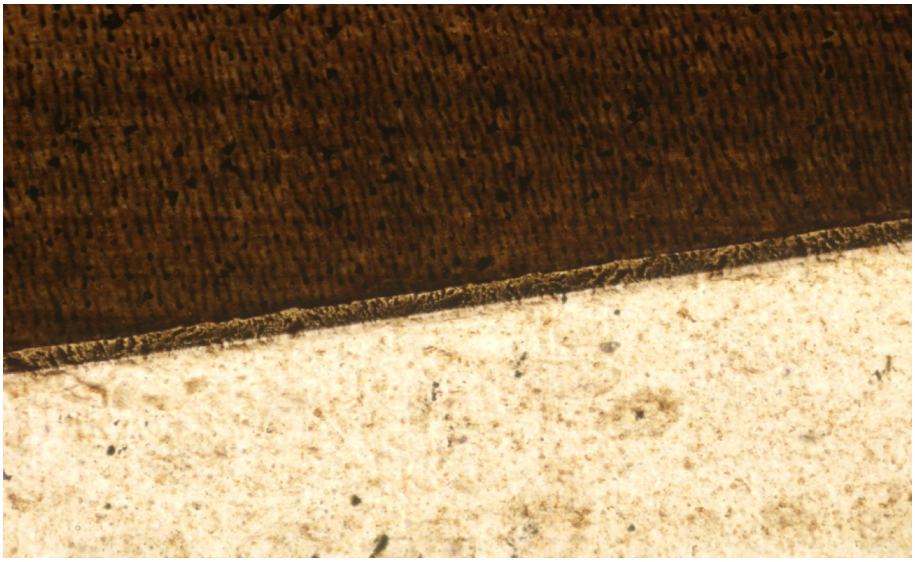


Figure 17: Sample treated with Scotchbond Universal SE mode.

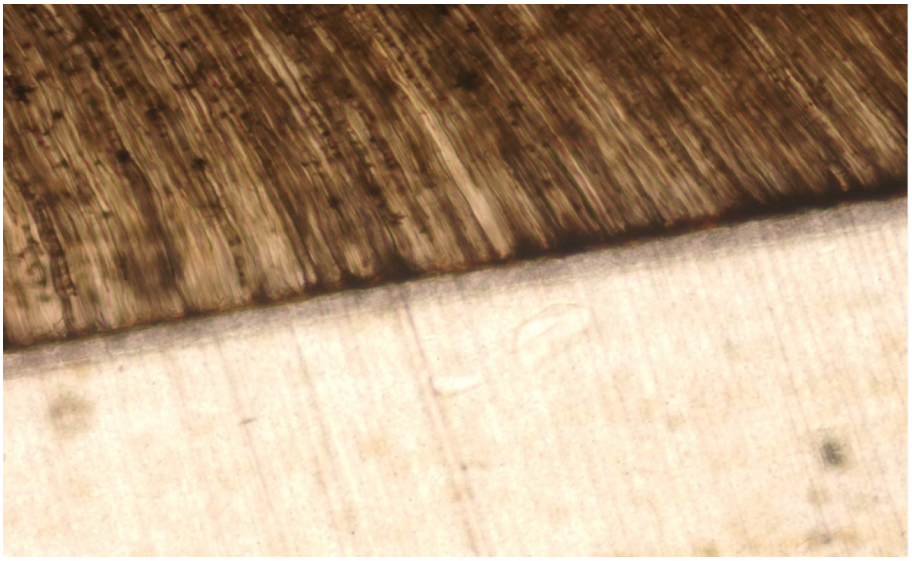


Figure 18: Sample treated with DCC and Scotchbond Universal in SE mode.

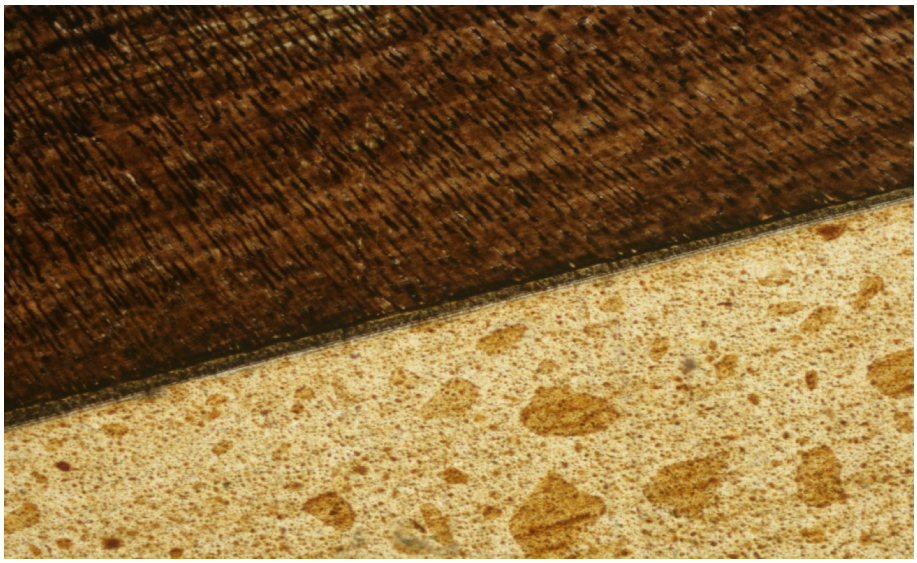


Figure 19: Sample treated with Scotchbond Universal in ER mode after 1 year of storage in artificial saliva.

Figure 20: Sample treated with DCC and Scotchbond Universal in ER mode after 1 year of storage in artificial saliva.

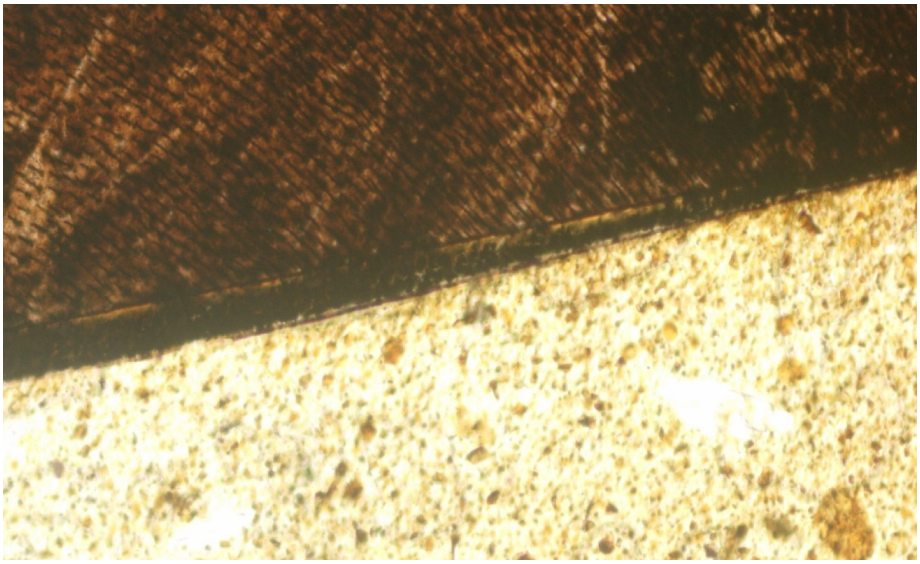


Figure 21: Sample treated with Scotchbond Universal in SE mode after 1 year of storage in artificial saliva.

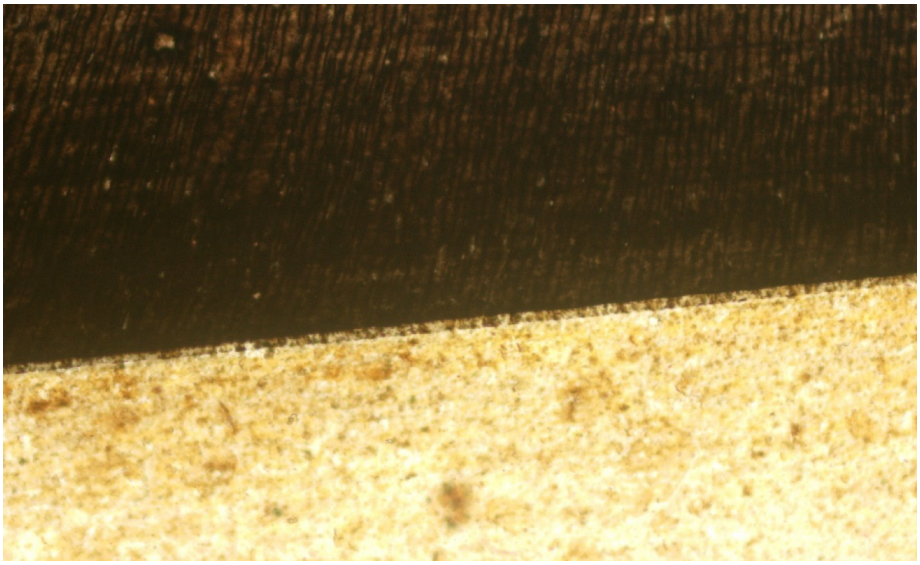


Figure 22: Sample treated with DCC and Scotchbond Universal in SE mode after 1 year of storage in artificial saliva.

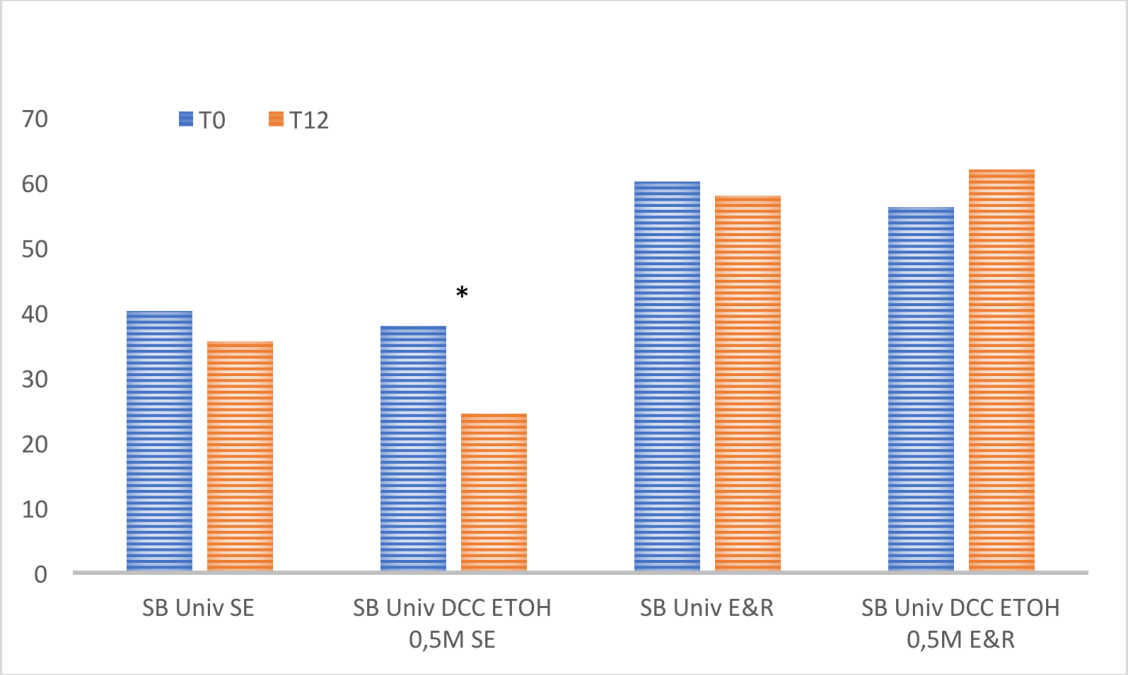
In Situ Zymography of the Hybrid Layer

Representative images of the 4 groups are reported in Figures 23 and 24, and percentages of HLs exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin in the 4 groups (T0 and T1 y aging in artificial saliva) are shown in Graph 5.

Bonded dentin slabs at T0 revealed a reduced green fluorescence within the HLs created by SBU in ER and SE mode when DCC was applied as additional primer. In control samples without DCC pretreatment, intermittent fluorescence in the HL indicated gelatinolytic activity.

At T12, both experimental and control groups showed and increased enzymatic activity apart from the experimental group with SBU in SE mode.

No fluorescence was detected in negative controls, that is, 1) EDTA-treated 2) specimens incubated with 2 mm 1,10-phenanth- roline or 3) with standard nonfluorescent gelatin (data not shown).



Graph 5. Gelatinolytic activity, expressed as the percentage of the green fluorescence within hybrid layers created with SBU or in *self-etch* or *etch-and-rinse* mode with or without DCC pre-treatment of the dentine. Values are means and standard deviations.

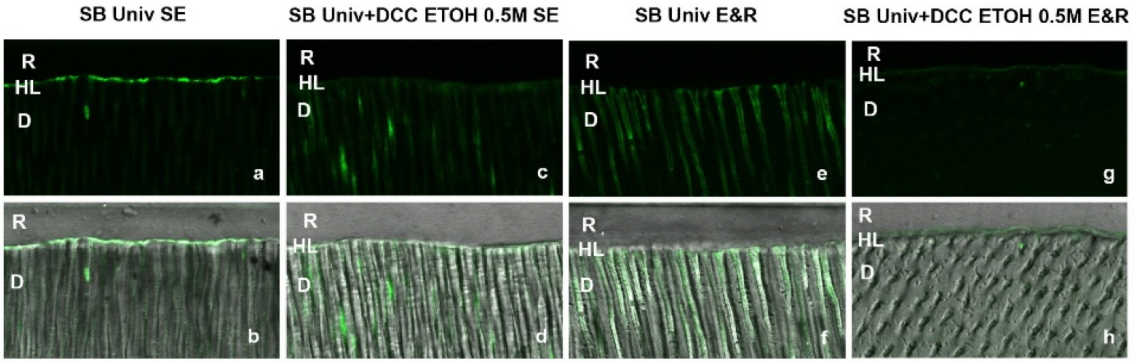


Figura 24. *In situ zymography*. Samples at T0 stored for 24 h: Resin dentine interface treated with Scotchbond Universal in *self-etch* mode (a,b); Scotchbond Universal *self-etch* mode + DCC 0,5M (c, d); Scotchbond Universal *etch-and-rinse* mode (e, f); Scotchbond Universal *etch-and-rinse* mode + DCC 0,5M (g, h). D= Dentin; HL= Hybrid Layer; R= Composite Resin.

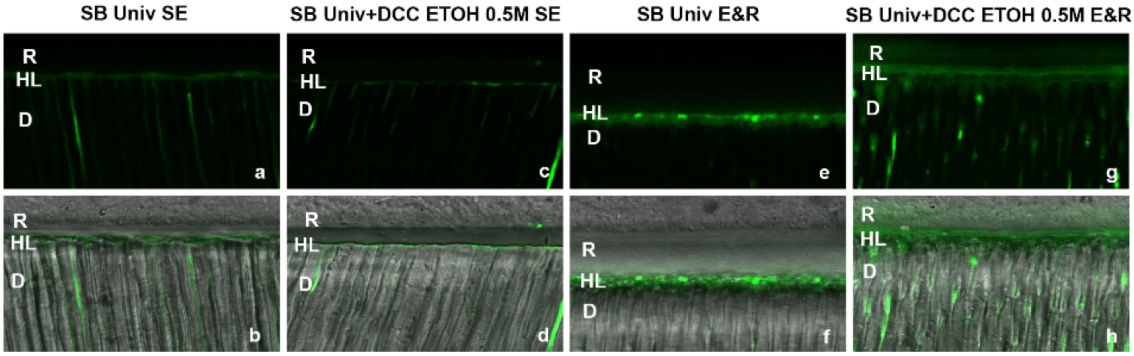


Figura 25: *In situ zymography*. Samples at T12 stored for 12 months in artificial saliva: Resin dentine interface treated with Scotchbond Universal in *self-etch* mode (a,b); Scotchbond Universal *self-etch* mode + DCC 0,5M (c, d); Scotchbond Universal *etch-and-rinse* mode (e, f); Scotchbond Universal *etch-and-rinse* mode + DCC 0,5M (g, h). D= Dentin; HL= Hybrid Layer; R= Composite Resin.

5- DCC inactivation of matrix metalloproteinases in radicular dentine

Materials and methods

Specimen preparation

36 extracted intact single-rooted teeth, single canal without decay, cervical lesions, filling, canal calcifications, open apex, and radicular reabsorption were selected. After debriding the root surfaces, Each tooth was sectioned at the CEJ perpendicular to the long axis of the tooth to visualize canal morphology using water-cooled diamond disk and stored in physiological solution at 4 ° C until use. Samples were endodontically treated following specific protocol reaching working length with 10 k-file following by Pathfiles P1-P2-P3 and Protaper Universal S1-S2 in sequence (Dentsply Maillefer, Ballaigues, Switzerland). After working length control with 10 K-file finishing Protaper Universal were used. During shaping of the canal 5% hypochlorite (Niclor 5; Ogna, Muggiò, Italy) and 10% EDTA (Tubuliclean; Ogna) were used as irrigation. the canal were dried using sterile paper points. The canal filling was obtained with endodontic sealer (Pulp Canal Sealer EWT; Kerr, Sybron, Romulus, MI, USA) and medium-sized gutta-percha points using DownPack system (Hu-Friedy, Chicago, IL, USA) and Obtura III (Analytic Technologies, Redmond, WA, USA).

The coronal sealing was made in Fuji and the samples were stored in physiological solution at 37° C for 7 days. After this time an 8 mm post space was prepared with dedicated drills(Rely-X Fiber Post, 3M ESPE, St. Paul, USA) under water-cooling . The post space was etched for 15s with 37% phosphoric acid (Conditioner36, Dentsply) with continues brushing technique and rinsed with water (endodontic needles) for 15 s.

Samples were randomized into three groups (n= 12) according to the dentinal pretreatment:

- Group 1: water wet bonding (control): post space irrigation with distilled water for 60 s -
- Group 2: Ethanol wet bonding: post space irrigation with 100% ethanol for 60 s
- Group 3: Ethanol wet bonding with DCC: post space irrigation with 0,5 M DCC in 100% ethanol for 60 s

Every group has been subjected to the same adhesive procedures:

Two applications of primer (AllBond3, Bisco, USA) with continuous brushing technique for 10 seconds and removal of excess primer by gentle blow of air for 10 seconds. One application of bonding (AllBond3, Bisco, USA) with continuous brushing technique for 10 seconds.

Meanwhile fiber posts were cleaned in ethanol for 30 seconds and air dried for 5 seconds. Then overspread with silane (Bis Silane, Porcelain Primer, Bisco Inc.) for 60 seconds and air dried again for 5 seconds.

We proceeded filling the post space with Dual-cure luting cement (Duo-Link Universal, Bisco Inc.), slowly inserting the fiber post in the post space.After 60 seconds waiting light-curing was performed with a LED polywave light (Bluephase Style, Ivoclar, Luxembourg) for 40 s each.

After polymerization, the samples were stored in distilled water at 37°C .

Push-out test

After a week every sample was cut perpendicular to the fiber post axis using a diamond saw 0,35 mm thin at low speed (Micromet; Remet, Bologna, Italy) under water-cooling . Six sections, each of 1 mm thick were obtained from each sample, representative of coronal and apical regions of post space. Each section was marked on its coronal side with an indelible marker. Samples were then divided in order to have half of them analyzed at zero time wile the other half was stored in artificial saliva at 37°C for adhesion strength evaluation at twelve months.

The push-out test of the sections was made applying an axial load to the post at a crosshead speed of 0,5 mm/ min using an Instrom Machine I (10/D model; Snitch, MTS, Canton, MA, USA). The most coronal surface of the section was always turned downward to make the test (load direction: from apical to coronal). Load peak was recorded in Newtons (N) and converted into Megapascal (MPa) dividing the value to the fiber post fragment area, that corresponds to the adhesion area according to Juloski et al. [11].

Nanoleakage

9 samples from each group were choose for nano leakage evaluation: 3 after 24 hours, 3 after 6 months and 3 after 12 months. Samples were cut as for push out test in 1mm-thick slices and the central section was selected. On top of them were applied fast nail varnish (twice) until hardening a mm from adhesive interface. Specimen were then put in silver nitrate solution in 50wt% ammoniacal silver nitrate ($AgNO_3$) 9.5 PH and stored in the dark. After 24 hours they were rinsed with deionized water for 5 minutes and immersed in photographic developing solution under fluorescent light for eight hours. Samples were fixed on glass slides then flattened with sandpaper (600 grit SiC paper) under running water and fine-grained diamond pastes (6 μm, 3 μm, 1 μm), washed in ultrasonic bath and dried. Specimens were then positioned on strains of Aluminum placed in the dryer and sputter-coated with carbon.

Samples examination was done with scanning electron microscope 15 kV voltage. This allowed us to obtain 8 images per sample (24 per group). The degree of interfacial nanoleakage was scored on a scale of 0–4 by two observers following Saboia et al. [8].

In Situ Zymography of the Hybrid Layer

Two freshly extracted non-carious human third molars were selected for in situ zymography and stored in freezer at -20° C.

The 3 canals of each molar were treated according to the dentinal pretreatment:

- Group 1: water wet bonding (control): post space irrigation with distilled water for 60 s -
- Group 2: Ethanol wet bonding: post space irrigation with 100% ethanol for 60 s
- Group 3: Ethanol wet bonding with DCC: post space irrigation with 0,5 M DCC in 100% ethanol for 60 s

Each root of the molar was treated as a single canal teeth following the same procedure illustrated before, obtaining three fiber post interfaces with radicular dentin with the three different dentinal pretreatments within the same sample.

less than One mm thick disks (70 micron) of middle deep radicular dentin were obtained from each tooth by means of a slow-speed saw (Micromet; Remet, Bologna, Italy). Each section

was marked on its coronal side with an indelible marker, glue on Plexiglas slides and ground down to obtain specimens ca. 50 micron thick. The samples surface was demineralized using freshly prepared 1% H3PO4 for 10s and rinsed for 1 min using distilled water. The excess of water was removed and the surface was bolt dried using laboratory adsorbent paper.

In situ zymography was performed with self-quenched fluorescein conjugated gelatin as the MMP substrate in accordance with Mazzoni et al.[7].

50 microliter of the fluorescent gelatin mixture was placed on top of each slabs in order to completely cover the adhesive/dentin interface and immediately covered with coverslip. This coverslip was glued with a thin layer of nail varnish just on the edge. The plexiglass slides were than put in humid chamber, light protected and incubated at 37°C for 12/16 hours. the hydrolysis of quenched fluorescein-conjugated gelatin substrate, indicative of endogenous gelatinolytic enzyme activity, was assessed by examination with a confocal laser scanning microscope

Statistical Analysis

Data were analyzed with three-way ANOVA to examine rewetting solution, time and post space region, and the interaction of these three factors on push-out strength. Post-hoc pairwise comparisons were performed using the Tukey test. The chi-square test was used to analyze differences in the failure modes. For all tests, statistical significance was pre-set at $p< 0.05$.

One way ANOVA was employed to analyze in situ zymography data at T0 for the variable treatment.

Results

Push-out test

Mean and standard deviation for the different groups are showed in table 8 and graphs 6 and 7.

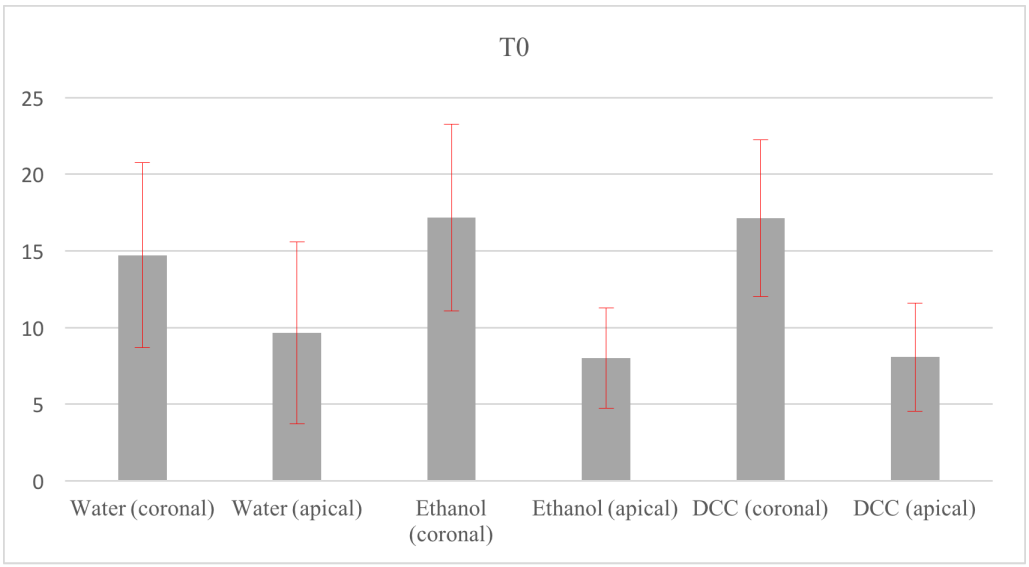
The ANOVA test showed that both the coronal and apical regions obtained overlapping results for groups 2 and 3, while for water-wet bonding the coronal region had significantly higher results ($p = 0.0001$).

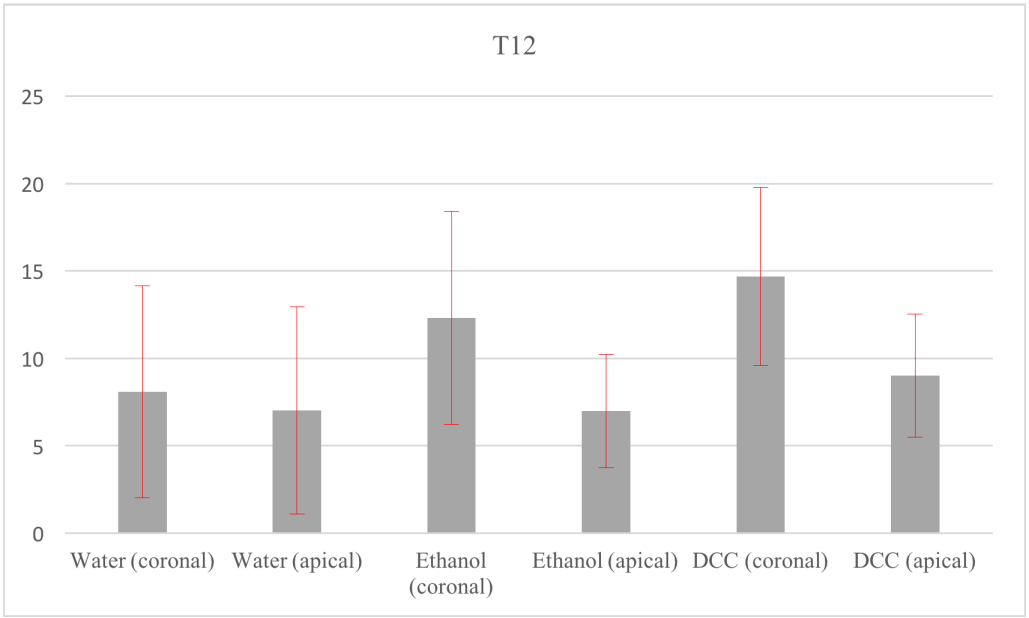
No differences were found between the three pretreatment techniques with respect to the region.

In the aged samples, a statistically significant decrease in adhesion strength compared to the baseline was observed. Furthermore, the DCC shows a significantly higher protective effect on adhesion strength than water, but not ethanol (Graph 8).

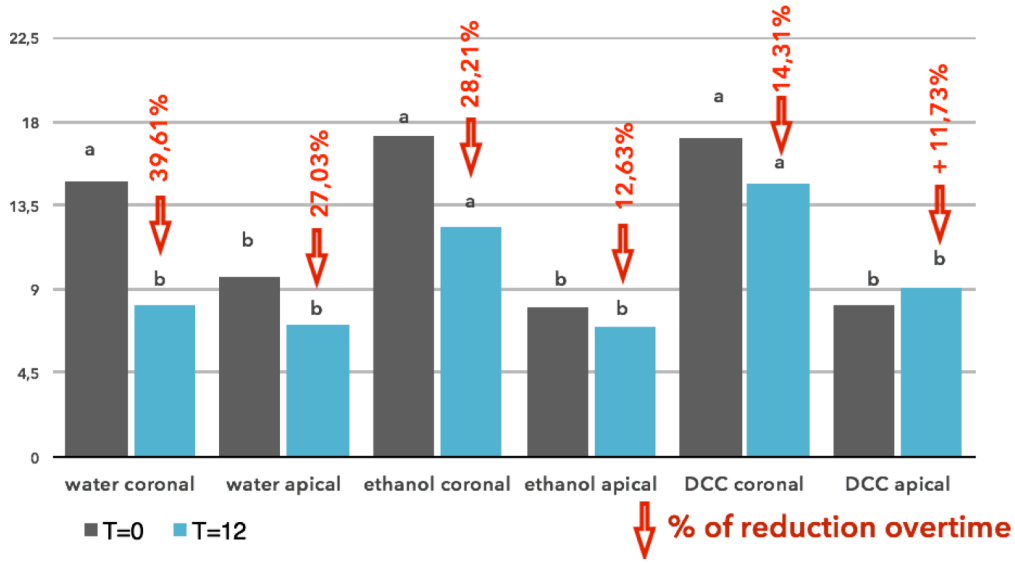
	Area	T0	T12
Water	Coronal	14,72 (±6,05)	8,09 (±3,19)
	Apical	9,66 (±5,93)	7,02 (±4,27)
Ethanol	Coronal	17,16 (±6,09)	12,32 (±6,90)
	Apical	8,00 (±3,26)	6,99 (±2,25)
DCC	Coronal	17,13 (±5,11)	14,68 (±3,89)
	Apical	8,07 (±3,53)	9,02 (±2,41)

Table 8. Push-out strength (mean ± standard deviation) obtained from the four experimental groups at 24 hours and after 12 month of aging in artificial saliva.





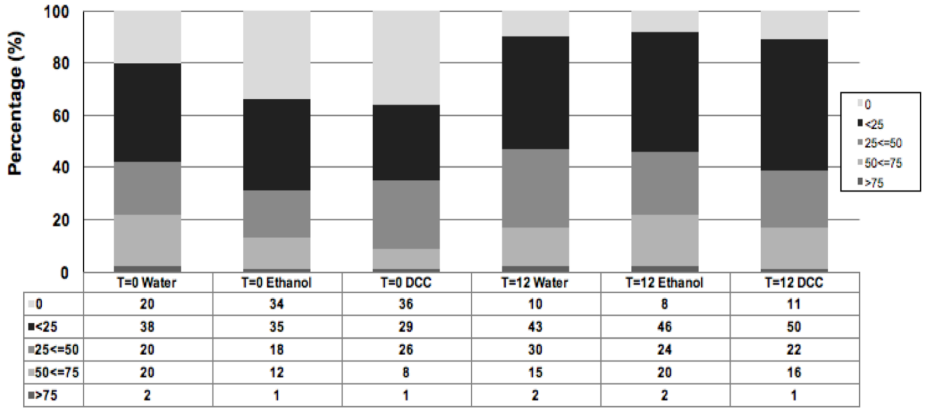
Graphs 6 and 7. Push-out strength (mean ± standard deviation) obtained from the four experimental groups at 24 hours and after 12 month of aging in artificial saliva.



Graph 8. Strength reduction after aging was expressed as a percentage of the 24-hour push-out strength

Nanoleakage

No significant differences were detected between the different treatments. Time was the only variable significantly affecting the results. A significant difference was found in between T=0 and T=12 groups of all treatment (Figure 26).



	T=0 Water	T=0 Ethanol	T=0 DCC	T=12 Water	T=12 Ethanol	T=12 DCC
0	20	34	36	10	8	11
<25	38	35	29	43	46	50
25<=50	20	18	26	30	24	22
50<=75	20	12	8	15	20	16
>75	2	1	1	2	2	1

Figure 1. Percentage of interfacial nanoleakage expression in resin-dentine interfaces created in radicular dentine in all different groups at 24 hours or after one year of aging in artificial saliva.

In-situ zymography

Gelatinolytic activity, expressed as the percentage of the green fluorescence within the hybrid layer, is represented in Figure 2.

Results of the one-way ANOVA showed that significant difference was observed for the factors: “treatment” ($p = 0.000$).

Application of water to acid-etched dentin resulted in significantly more extensive gelatinolytic activity within the hybrid layer when compared to the application of the ethanol and DCC ($p < 0.05$). However, pre-treatment of the acid-etched dentin with DCC prior to adhesive application resulted in significantly reduced gelatinolytic activity within radicular hybrid layer, ($p < 0.05$). Confocal laser scanning microscopic examination of *in-situ* zymography specimens derived from unaged premolars in the three experimental groups revealed intense green fluorescence within the dentinal tubules of mineralized radicular dentine as well as within the hybrid layer. Because the original gelatine substrate was heavily quenched

with conjugated fluorescein and did not fluoresce, regions in the root slice that exhibit strong fluorescence are indicative of hydrolysis of the extrinsic gelatine substrate, which in turn, released the conjugated fluorescein molecules.

No fluorescence was detected in negative controls prepared with non-specific inhibitors, including: 1) EDTA-treated, 2) specimens incubated with 2 mM 1,10-phenanthroline or 3) with standard nonfluorescent gelatin (data not shown).

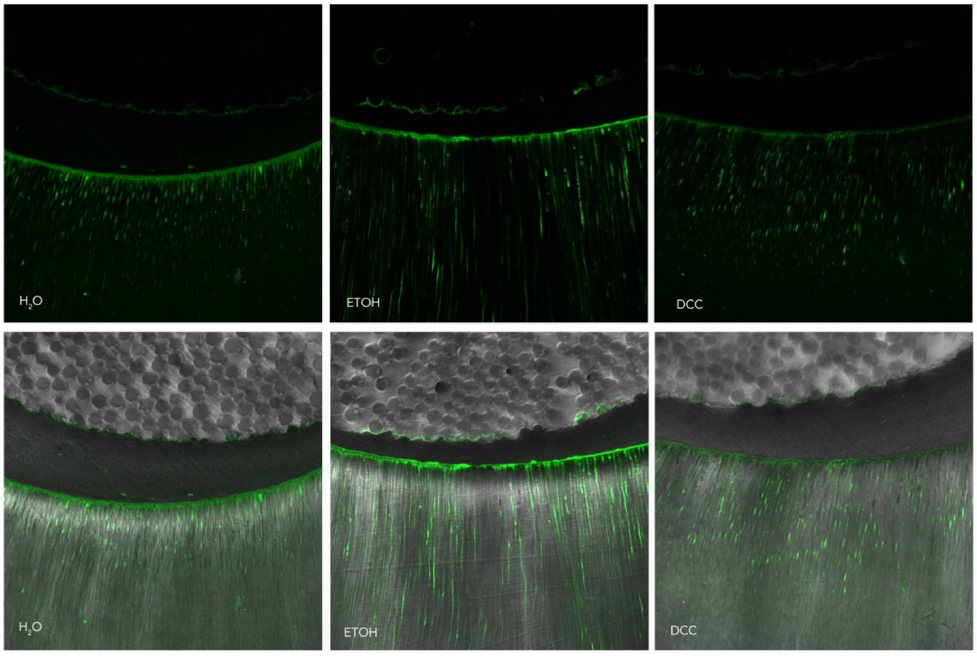


Figure 2. Representative confocal laser scanning microscopy images of resin-bonded radicular dentine interfaces that were incubated with quenched fluorescein-labelled gelatine at T=0. For each set of images, the top image was acquired in the green channel. The green fluorescence represented areas with intense endogenous gelationolytic activity within the dentinal tubules and the hybrid layer. The bottom image was produced by merging the differential interference contrast image (showing the optical density of the resin-dentine interface) and the image acquired in green channel.

References

[1] Breschi L, Mazzoni A, Nato F, Carrilho M, Visintini E, Tjäderhane L, et al. Chlorhexidine stabilizes the adhesive interface: a 2-year in vitro study. Dent Mater 2010;26:320–5. doi:10.1016/j.dental.2009.11.153.

[2] Pashley D, Tay F, Yiu C, Hashimoto M, Breschi L, Carvalho R. Collagen degradation by host-derived enzymes during aging. J Dent Res 2004;83:216–21.

[3] Mazzoni A, Scaffa P, Carrilho M, Tjäderhane L, Di Lenarda R, Polimeni A, et al. Effects of etch-and-rinse and self-etch adhesives on dentin MMP-2 and MMP-9. J Dent Res 2013;92:82–6. doi:10.1177/0022034512467034.

[4] Mazzoni A, Apolonio FM, Saboia VPA, Santi S, Angeloni V, Checchi V, et al. Carbodiimide inactivation of MMPs and effect on dentin bonding. J Dent Res 2014. doi: 10.1177/0022034513516465.

[5] Pashley D, Tay F, Imazato S. How to increase the durability of resin-dentin bonds. Compend Contin Educ Dent 2011;32:60–64.

[6] Mazzoni A, Apolonio FM, Saboia VPA, Santi S, Angeloni V, Checchi V, et al. Carbodiimide inactivation of MMPs and effect on dentin Bonding. J Dent Res 2014;93:263–8. doi:10.1177/0022034513516465.

[7] Mazzoni A, Nascimento F, Carrilho M, Tersariol I, Papa V, Tjaderhane L, et al. MMP activity in the hybrid layer detected with in situ zymography. J Dent Res 2012;91:467–72. doi: 10.1177/0022034512439210.

[8] Saboia V, Nato F, Mazzoni A, Orsini G, Putignano A, Giannini M. Adhesion of a two-step etch-and-rinse adhesive on collagen-depleted dentin. J Adhes Dent 2008;10.

[9] Tay FR, Pashley DH, Suh BI, Carvalho RM, Itthagarun A. Single-step adhesives are permeable membranes. J Dent 2002;30:371–82. doi:10.1016/S0300-5712(02)00064-7.

[10] Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, et al. Cysteine cathepsins in human carious dentin. J Dent Res 2011;90:506–11. doi: 10.1177/0022034510391906.

[11] Juloski J, Fadda GM, Radovic I, Chieffi N, Vulicevic ZR, Aragoneses JM, et al. Push-out bond strength of an experimental self-adhesive resin cement. Eur J Oral Sci 2013. doi: 10.1111/eos.12005.

CHAPTER 5
Discussion, Conclusions & Future Directions

Discussion

The present series of studies aimed to investigate the effect of various collagen crosslinkers on dentin protease activity and their effect on bond-strength to the dentinal substrate. Thus, selected collagen crosslinkers were tested to determine their effect on MPa values on coronal and radicular dentine, the mariginal infiltration at the resin dentin interfaces, the presence of endogenous enzymatic activity after treatment and the localization of the gelatinase activity on dentin. Additionally, the effect of the cross-linkers and the effect of treatment period (duration) on the degradation of collagen matrices were examined.

Over the last few years, the experimental use of collagen cross-linking agents to increase the longevity of resin-dentin bonds gained increased popularity. The use of cross-linkers can be considered as a biological tissue engineering approach where dentin tissue repair/regeneration is the development of a biomimetic strategy to enhance the tissue properties by modifying the chemistry of the tissue [1]. The biomodification of the existing tooth hard tissue structures is a novel approach to improve the biomechanical properties of the tissue for preventive and reparative/restorative purposes. The approach was thought to be determined by non- enzymatic inter- or intra-molecular collagen cross-linking [2]. However, the multiple interactions between bioactive agents with various extracellular components of the dentin matrix are likely the determinants of the tissue enhanced biomechanics and biostability. Therefore, the term biomodification is more appropriate to define the bioactivity of these highly bioactive chemical mediators.

Role of Collagen Cross-Linkers on microtensile bond-strength

The present study demonstrated that increased bond strength can be obtained by the additional use of different biochemical cross-linkers in association to different dentin bonding adhesive systems both on coronal and radicular dentin.

The ability of the tested cross-linking agent in increasing the bond strength of the adhesive interface with several bonding agents, both on coronal and radicular dentin, was assess through microtensile bond strength test and the cross-linkers application period was established in 1 min according to the recent finding published by Tezvergil-Mutluay *et al.* [3], and with the intention

to test a contact time period feasible in clinical practice. Previous studies investigated the use of different cross-linkers, such as glutaraldehyde, genepin, proanthocidin and EDC, as biomodifier agent, although the application time required to be effective (10 min to several hours [4,5]) could not be considered clinically acceptable. For this reason, more recent studies concerning the use of EDC were conducted to evaluate the capabilities to increase the mechanical properties of the etching-dentin matrix within 1 min application time, revealing that this short application time is sufficient to inactivate endogenous protease activity of dentin without significantly stiffening the collagen matrix[6]. These findings were further confirmed by a recent study conducted by Mazzoni et al. [6] demonstrating that 1 min is an adequate timing to positively influence the durability of resin-dentin bond over time.

Bond strength tests are the most frequently used tests to screen adhesives. A tensile bond strength test is defined "microtensile" when the bonded surface analyzed is 1 mm^2 or less. The microtensile bond strength test is calculated as the tensile load at failure divided by the cross-sectional area of the bonded interface [7].

In the present thesis, in order to reproduce the oral environment as realistically as possible the specimens were stored in *artificial saliva* solution at 37°C.

Many studies in literature demonstrated that bond strength values decrease even after brief period of storage [8,9][10] and Pashley *et al.* [11] demonstrated that the degradation of the hybrid layer can occur also in absence of bacteria. Furthermore, several in vitro studies have provided morphological evidence of resin elution and/or hydrolytic degradation of collagen in resin-dentin bonds and highlighted that the adhesion interface area is inversely proportional to the bond strength maintenance, in the same time-line storage [9,12]. Hence, this technique of storage could to be considered as a method of accelerating aging.

The application of acrolein before bonding to acid-etched dentine resulted in comparatively better preservation of the μTBS after one year of *in vitro* ageing. The advantage of using cross-linking instead of MMPs inhibitors to preserve the integrity of the exposed collagen matrix within the hybrid layer is that the covalent cross-links formed by cross linking agents are permanent, whereas inhibitors that are not covalently bonded with the polymerised resin network may leach out of the hybrid layer after 18-24 months[13–15]. Cross-linking agents

increase collagen stiffness by creating interfibrillar and intrafibrillar cross-links, thereby strengthening the collagen network structure[16]. This may prevent unravelling of the triple helix of collagen molecules, which is necessary for exposure of the catalytic site of the MMPs to cleave the collagen molecule. This results in the increase in resistance of the collagen matrix to MMP activities[17].

Several studies have investigated the effect of cross-linking agents, including glutaraldehyde, on the mechanical properties of the dentine organic matrix, as well as on the preservation of the hybrid layer [18–27]. Glutaraldehyde improves the mechanical properties and reduces the degradation rate of biological tissues [28,29]. Glutaraldehyde increases type I collagen covalent bonding by bridging the amino groups of lysine and hydroxylysine residues of different collagen polypeptide chains with monomeric or oligomeric cross-links. The exogenous cross-links introduced by glutaraldehyde improve the mechanical properties of the exposed dentine matrix[5,18,30–32]. Similarly to glutaraldehyde, ACR is used as a tissue fixative and is a well-known cross-linking agent [33,34]. Hence, ACR was included in the present experiments to investigate the influence of a simple unsaturated aldehyde molecule on cross-linking of demineralised dentine. The ACR cross-linking reaction starts with two molecules of ACR that react with the free amino terminal groups of lysine (or hydroxylysine), forming Nε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine). The electrophilic α,β-unsaturated carbonyl moiety is retained in FDP-lysine, allowing it to react further with sulfhydryl compounds of glutathione, creating new intra- and inter-molecular cross-links [35].

As for acrolein, the 0.3M EDC solution as a pre-treatment showed a preservation of the bond strength after one year of storage in artificial saliva of bot coronal and radicular dentin adhesive interface.

In coronal dentin, the μTBS results showed that EDC pretreatment can improve the durability and the structural integrity of the resin/dentin interfaces created either with etch-and-rinse or self-etch adhesive systems. The results of the μTBS of the EDC experimental groups showed that bond strength values, even when at baseline were comparable to the control groups, remained stable over time, or at least more stable than the control groups. Furthermore, in terms of percentage of bond strength reduction, the self-etch adhesive lost less bond strength

following EDC pretreatment, compared to etch-and-rinse adhesive system. These data further confirm previous *in vitro* findings that showed the improved stability of the 2-step self-etch system vs the two-step etch-and-rinse system due to the increased hydrophobicity [36] and curing ability [37,38]. The observed decline in bond strengths of the present study can be related to the loss of integrity of resinous components within the hybrid layer due to polymer swelling and resin leaching that occur after water/oral fluid sorption, which is recognized to be more pronounced for simplified (two-step) etch-and-rinse adhesives than unsimplified systems (three-step)[39,40]. The 2- step self-etch adhesive is considered the most durable bond [40]. This can be due to the fact that the self-etch adhesives do not completely expose the dentin collagen matrix [40]. Self-etch adhesives maintain more residual hydroxyapatite crystal in their hybrid layers which minimizes activation of dentin MMPs [41–43]. Those calcium ions that are released from the matrix during self-etching are thought to form relatively insoluble calcium salts with the functional monomers like 10-MDP (10-methacryloyloxydecyl dihydrogen phosphate) in SE Bond. The slow solubilization of the insoluble salts over 12 months may allow control SE Bond to lose more bond strength than their EDC-pretreated experimentals [41–43]. The present μTBS results revealed that bonded dentin interfaces created with EDC pretreatment improves the durability of the resin-dentin bonds. These results are similar to a recently published study in which a 3-step etch-and-rinse adhesive (Optibond FL) and a 2-step self-etch adhesive (CSE) were tested using chlorhexidine as a conditioning primer and MMP inhibitor [41–43]. The results of that study showed that the use of chlorhexidine stabilized bond strength values over time for both tested adhesives. Unlike chlorhexidine which only binds to dentin electrostatically [41–43], EDC reacts with collagen to forming covalent bonds [41–43].

As concern radicular dentin, push-out strength was improved when both adhesives tested were applied after EDC pre-treatment of the acid-etched dentine, however, the results of the present study showed that EDC application did not result in statistically significant difference in immediate bond strength. The reduced quantity of exposed collagen network, due to difficulties in secondary smear layer removal in deep post spaces [44,45], and the degradation of collagen network in endodontically-treated teeth [11] could explain the minimal effect of EDC pre-treatment on immediate fibre post push-out strength from bonded radicular dentin. Indeed, the

present findings were in agreement with Mazzoni *et al.* [46], who stated that EDC pre-treatment has no effect on immediate bond strength to coronal dentine, though reducing the endogenous enzymatic activity [47].

Whereas EDC pre-treatment did not improve immediate bonding performance, the real benefits of EDC pre-treatment can only be realised after aging.

Also, a third cross-linking molecule has been tested both on coronal and radicular dentin, such as DCC. Dicyclohexylcarbodiimide is crosslinker belonging to the same family of EDC, which activates carboxylic groups for the spontaneous formation of peptide bonds with primary amines. It differentiates from EDC because of the different solubility. Hence, EDC is soluble in water, while DCC in organic solvents. In the present study, the cross linker was tested in a 0.5M ethanol solution and the results on coronal and radicular dentin were in accordance to that obtained for EDC. No influence was detected on immediate bond-strength, while a preservation of the values was obtained in the experimental groups after one year of aging in artificial saliva.

EDC and DCC are the most favorable cross-linkers clinical practice use. DCC could be very promising since its hydrophobicity and its solubility in acetone or ethanol. Indeed, water molecules present in the HL are responsible of the ester-bonds in adhesive polymers and peptide bonds in collagen causing the failure of resin-tooth interface. For this reason, many studies focused on the concept of “ethanol-wet bonding” which provides an opportunity for coaxing hydrophobic monomers into a demineralized collagen matrix without sacrificing any additional matrix shrinkage. Infiltration of hydrophobic monomers into a collagen matrix decreases water sorption/solubility, resin plasticization, and enzyme-catalyzed hydrolytic cleavage of collagen [15,48], thereby creating more durable resin bonds. Sauro *et al* [49] demonstrated that ethanol wet-bonding is capable of increasing resin uptake and producing better sealing of the collagen matrix, even with the use of hydrophilic adhesives. The presence of ethanol probably also increases the degree of conversion of the hydrophilic adhesives.

Role of Collagen Cross-Linkers on nanoleakage analyses

Sano *et al.* [50] described the nanoleakage for the first time as submicron spaces in the hybrid layer of the order of 20-100 nm in width. After that many studies have confirmed that small ions or molecules can diffuse into the hybrid layer in the absence of detectable interfacial gap formation [50]. Nanoleakage phenomenon has been defined as the passage of a tracer such as silver nitrate through the hybrid layer. According to its original definition, nanoleakage is created by the discrepancy between dentin demineralization and adhesive infiltration that occurs in total-etch adhesive systems, in the absence of marginal gap formation along the resin– dentin interface [51]. This phenomenon is not necessarily caused by disparities between the depths of demineralization and resin infiltration. It may also represent the presence of areas in which the retention of residual water in etched dentin and/or adhesive results in regions of incomplete polymerization or increased permeability within the resin matrices of the adhesives [52].

In the present thesis, the role of different cross linker molecules in creating a uniform and durable interface was tested. Independently from the adhesive system associated with the pre-treatment, the hybrid layer of the experimental groups was less susceptible to time degradation apart from the radicular dentin evaluation where no differences were detected among the groups. This could be explained with the different nature of the radicular dentin and the reduced quantity of exposed collagen network, due to difficulties in secondary smear layer removal in deep post spaces[44,45], and the degradation of collagen network in endodontically-treated teeth [11] could explain the minimal effect of EDC pre-treatment on nanoleakage results.

Detection of MMP Inactivation Using Gelatin Zymography

Gelatin zymography is a simple and inexpensive method to measure the activity of several proteases with same substrate [53]. Its high sensitivity to detect proteolytic activity in samples with levels of pg of MMPs render it favorable in comparison to ELISA [53][54]. Mazzoni et al. [55] employed gelatin zymography for the first time, to determine the gelatinase activity of dentin MMPs using extracted molar teeth. The activation of dentin MMPs following the demineralization of dentin using acidic monomers has been known [42,56][38]. Mazzoni et al. [46] reported for the first time the active and pro-forms of MMP-2 and -9 on demineralized

dentin following the application of both self-etch and etch-and-rinse adhesives by using gelatin zymography.

To support positive mechanical data concerning the bond strength preservation and reduce interfacial nanoleakage of the dentinal substrate pre-treated with cross-linkers, the effect on endogenous enzimatic activity was detected.

Apart from the creating exogenous cross-links within the dentine collagen matrix, bond preservation in acid-etched dentine that is treated with cross-linking agents may also be related to the ability of the cross-linking agents to inactivate endogenous MMPs in the exposed dentine collagen matrix [42][41,56]. It has been hypothesised that cross-linking agents alter the three-dimensional conformation of the catalytic domain of MMPs, or change their negatively-charged ionised carboxyl groups into positively-charged amide groups, to inactivate the MMPs. The advantage of inactivating proteolytic enzymes in the dentine matrix by cross-linking is that it is a non-specific mechanism [57,58]. These cross-links involve covalent bonds that are stable over time. Because MMPs do not turn over in dentine, their inactivation by cross-linking agents should last for a long time and may be even more effective than MMP inhibitors.

The zymographic results obtained for ACR support the speculation that stabilisation of hybrid layers created after priming with acrolein is related to the inactivation of dentine gelatinolytic activities. Several authors [59,60] have hypothesised that enzyme inactivation involves modification of Cys residues critical for the catalytic site, but no definitive structure characterisation or molecular dynamics simulation have been provided. Hence, the inactivation capability of ACR may be associated with the modification of Cys residues in the MMPs, which, in turn, results in the inactivation of these proteolytic enzymes.

The results of the zymographic analysis performed for EDC study are in accordance with previous findings [41][42,43] and acrolein experiments; thus, the use of the adhesive systems tested results in increases in MMP-2 and -9 activity, but EDC pretreatment resulted in reduction or almost complete inhibition of the gelatinolytic activity.

Based on the outcomes of the project, the effectiveness of EDC in improve both the mechanical properties of collagen over time and in inhibiting the gelatinolytic activity within the HL has been successfully demonstrated. Previous studies suggested that this may be

attributed to silencing mechanism of MMPs and probably other exogenous collagen degradation enzymes via conformational changes in the enzyme 3-D structure. The use of cross-linking agents may create multiple cross-links between amino acids within their catalytic sites that irreversibly alter the 3-D conformation or flexibility of the cleft-like catalytic domain and prevent its optimal recognition and complexing with the type I collagen substrate [61]. Although there is no evidence that the catalytic domain of collagenolytic MMPs can be cross-linked to inactivate their functions, it has been hypothesized that the use of cross-linking agents may also contribute to MMPs silencing via allosteric control of non-catalytic domains [62]. For example, the catalytic domains in collagenolytic MMPs can cleave non-collagen substrates, but the hemopexin-like domain of these enzymes is crucial to initially unwind and subsequently cleave the three triple-helical fibrillar elements of the collagen molecule in succession [63]. For MMP-2, there are three fibronectin-like repeats that form a domain for binding to collagen or gelatin substrates. This collagen-binding domain binds preferentially to the α1 chain and mediates local unwinding and gross alteration of the triple helix prior to the cleavage of the β2 chain [64]. Regardless of which of the two collagen-binding mechanisms is involved, cross-linking of either the hemopexin-like or fibronectin-like domains may contribute to inactivation of the associated MMPs and reduction in their collagenolytic efficacy. Cross-linking may also affect MMP activities known to be modified by non-collagenous proteins [65]. In dentin, MMP activities and resistance to degradation may be regulated by fetuin-A[66] and the SIBLINGs Bone Sialoprotein (BSP) and Dentin Matrix Protein-1 (DMP-1) [67] all of them being present in dentin. Thus, cross-linking of these non-collagenous proteins may indirectly block MMPs via inactivation of the functional domains of these glycoproteins. Since MMPs do not turn over in peripheral dentin, their inactivation by cross-linking agents should last for a long time and may be even more effective than inhibitors such as chlorhexidine [43].

Localization of Gelatinolytic activity Using in-situ Zymography

The final part of the present thesis focused on the localization of the enzymatic activity on the hybrid layer after different cross-linkers pre-treatment both on coronal and radicular dentin.

Homogenization of tissues for gelatin zymography is mandatory, while *in situ* zymography analysis was performed to localize the MMP activity previously detected by zymography. Although gelatin zymography is a powerful technique for showing the location and presence of hydrolytic enzymes, which is based on the degradation of substrate; it does not determine potential enzymatic activities. The technique was introduced by Galis et al. [68] to localize the gelatinolytic activity in human atherosclerotic plaques and was adapted to hard tissue sections such as dentin and bone by Porto et al. and Mazzoni et al.[38], for the first time, indicated the localization of gelatinolytic activity in the hybrid layer by using in situ zymography after adhesive application.

Precise localization of gelatinase activity in sections and cells became possible with the introduction of dye-quenched (DQ)-gelatin, which is gelatin that is heavily labeled with FITC molecules so that its fluorescence is quenched [69–71][72,73]. After cleavage of DQ-gelatin by gelatinolytic activity, fluorescent peptides are produced that can be visualized against a weakly fluorescent background (EnzCheck; Molecular Probes, Eugene, OR). The use of DQ-gelatin instead of labeled or unlabeled gelatin is superior for in situ zymography because fluorescence is produced at sites of gelatinolytic activity instead of decreased staining intensity at gelatinolytic areas.

In-situ zymography of the resin-dentine interfaces confirmed that ACR is efficient in inactivating endogenous enzymes, since a decrease in MMP activity was observed in demineralised dentine treated with ACR. Nevertheless, enzymatic activity was detected in the ACR-treated dentin at around 100 kDa. This activity may be ascribed to the presence of a pro-MMP-9 complex.

Similar to glutaraldehyde, a disadvantage of ACR compared to the other cross-linking agents is the cytotoxicity that may arise from residues of unreacted or degraded cross-linking agents [26]. Although ACR possesses severe cellular toxicity that is similar to other aldehydes, ACR is ubiquitously present in cooked foods as well as in the environment [35]. Due to its intrinsic cytotoxicity, a very low concentration of ACR was employed (0.01%) in the present study. Studies on the adverse effect of chronic ACR ingestion have been performed on rats, and general recommendations for the safety level of ACR ingestion have been published by the U.S.

Environmental Protection Agency. The no-observed-adverse-effect-level (NOAEL) of ACR is 0.05 mg/kg per day. Hence, after the application of a total uncertainty factor of 100, the recommended reference dose (RfD) is 0.0005 mg/kg per day. The World Health Organisation suggests a tolerable ACR intake of 0.0075 mg/kg per day [74]. One drop of the solution that was used in the present study contains 0.05 mg of ACR. This means that the ACR primer may be safely used on adults, especially considering that the intake of ACR during dental procedures would be rare. In addition, ingestion of the primer may be completely avoided by placement of a rubber dam during the restorative procedure. Nevertheless, because there are other sources of the ACR in the environment, the toxicity of ACR should be further investigated by testing serial dilutions of ACR on dental pulp stem cells prior to its recommendation for clinical use.

Although a wide range of cross-linkers is available, their specific antienzymatic effects are still not clear. Among these cross-linkers, EDC pre-treatment of demineralized dentin matrices increases the mechanical properties of the dentin matrix and also completely inhibits the endogenous protease activity [3,32]. Zymographic studies, in fact, have shown that the pretreatment of acid-etched dentin with a conditioner containing 0.3 M EDC could completely inhibit the collagenolytic activity of endogenous enzymes [46], even after adhesive application [41,75].

In situ zymography showed a gelatinolytic activity clearly detectable within the hybrid layer and along the tubular dentin walls extending from the dentinal tubules in the control groups for both coronal and radicular dentin. The effectiveness of EDC used as conditioner primer before the bonding application was evident in the different experiments by the reduced protease activity detectable within the hybrid layer. These correlative results confirmed and validated the zymography analysis outcomes.

As concern DCC, also gelatinolitic activity was reduced in experimental groups. Thus, based on the findings of the present project the double effectiveness of cross-linkers tested in improve mechanical properties and in inhibit gelatinolytic activity within the hybrid layer has been demonstrated. The use of DCC in ethanol solution could strengthen the collagen matrix and creating an hybrid layer in absence of water. Nevertheless the application of ethanol on dentin

matrix does not completely avoid the presence of water caused by outward fluid flows without the use of adjunctive tubular occlusion agents [15,76].

Conclusions and Future Directions

Based on the studies included in this thesis, the following conclusions were drawn:

- Collagen cross-linkers tested do not affect bond strength on coronal and radicular dentin, and are effective molecules in preserving the adhesive interface over time.
- Acrolein, EDC and DCC help in preserving the degradation of dentin organic matrix by inactivating dentin endogenous enzymes
- The inhibiting effect of cross-linkers detected in the hybrid layer, even if has the tendency to increase over time, is still present after one year of storage in artificial saliva
- One minute application time of the cross-linkers on dentin is clinically acceptable and is effective in preventing degradation of the adhesive interface overtime and inhibiting endogenous enzymatic activity

While ongoing studies have searched for alternative ways to protect dentin collagen matrices from degradation clinically, the results of this thesis suggest that collagen crosslinkers can inactivate e the collagen degradation through the inactivation of dentin proteases. Thus, the use of collagen crosslinkers may help collagen matrices to resist the degradation. The incorporation of these compounds into current adhesive systems may protect demineralized collagen matrix to create a favourable tooth-biomaterial interface.

Further studies are needed to better understand the structure of the hybrid layer created using cross-linkers as conditioning primer, considering the preliminary but encouraging results obtained from this study. Additionally, *in vivo* studies will be essential to better understand the feasibility of the tested molecules as dentin conditioning primer during bonding procedures.

References

[1] Bedran-Russo AK, Pauli GF, Chen SN, McAlpine J, Castellan CS, Phansalkar RS, et al. Dentin biomodification: Strategies, renewable resources and clinical applications. Dent Mater 2014;30:62–76. doi:10.1016/j.dental.2013.10.012.

[2] Stenzel K, Miyata T, Rubin A. Collagen as a biomaterial. Cas Lek Cesk 1989. doi: 10.1146/annurev.bb.03.060174.001311.

[3] Tezvergil-Mutluay A, Mutluay MM, Agee KA, Seseogullari-Dirihan R, Hoshika T, Cadenaro M, et al. Carbodiimide cross-linking inactivates soluble and matrix-bound MMPs, in vitro. J Dent Res 2012. doi:10.1177/0022034511427705.

[4] Bedran-Russo AK, Pauli GF, Chen SN, McAlpine J, Castellan CS, Phansalkar RS, et al. Dentin biomodification: Strategies, renewable resources and clinical applications. Dent Mater 2014. doi:10.1016/j.dental.2013.10.012.

[5] Macedo G V, Yamauchi M, Bedran-Russo a K. Effects of chemical cross-linkers on caries-affected dentin bonding. J Dent Res 2009;88:1096–100. doi: 10.1177/0022034509351001.

[6] Mazzoni A, Angeloni V, Apolonio FM, Scotti N, Tjäderhane L, Tezvergil-Mutluay A, et al. Effect of carbodiimide (EDC) on the bond stability of etch-and-rinse adhesive systems. Dent Mater 2013;29:1040–7. doi:10.1016/j.dental.2013.07.010.

[7] Breschi L, Ferracane JL, Cadenaro M, Mazzoni A, Hilton TJ. Adhesion to enamel and dentin. Fundam. Oper. Dent. A Contemp. Approach, Fourth Ed., 2013.

[8] Kato G, Nakabayashi N. The durability of adhesion to phosphoric acid etched, wet dentin substrates. Dent Mater 1998. doi:10.1016/S0109-5641(99)00003-2.

[9] Shono Y, Terashita M, Shimada J, Kozono Y, Carvalho RM, Russell CM, et al. Durability of resin-dentin bonds. J Adhes Dent 1999. doi:10.1177/0022034508328910.

[10] De Munck J, Van Meerbeek B, Satoshi I, Vargas M, Yoshida Y, Armstrong S, et al. Microtensile bond strengths of one- and two-step self-etch adhesives to bur-cut enamel and dentin. Am J Dent 2003.

[11] Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, et al. Collagen degradation by host-derived enzymes during aging. J Dent Res 2004. doi: 10.1177/154405910408300306.

[12] Armstrong SR, Keller JC, Boyer DB. The influence of water storage and C-factor on the dentin-resin composite microtensile bond strength and debond pathway utilizing a filled and unfilled adhesive resin. Dent Mater 2001. doi:10.1016/S0109-5641(00)00081-6.

[13] Hashimoto M, Ohno H, Kaga M, Endo K, Sano H, Oguchi H. In vivo Degradation of Resin-Dentin Bonds in Humans Over 1 to 3 Years. J Dent Res 2000;79:1385–91. doi: 10.1177/00220345000790060601.

[14] Komori PCP, Pashley DH, Tjäderhane L, Breschi L, Mazzoni A, de Goes MF, et al. Effect of 2% Chlorhexidine Digluconate on the Bond Strength to Normal Versus Caries-affected Dentin. Oper Dent 2009;34:157–65. doi:10.2341/08-55.

[15] Sadek FT, Braga RR, Muench A, Liu Y, Pashley DH, Tay FR. Ethanol wet-bonding challenges current anti-degradation strategy. J Dent Res 2010;89:1499–504. doi: 10.1177/0022034510385240.

[16] Frassetto A, Breschi L, Turco G, Marchesi G, Di Lenarda R, Tay FR, et al. Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability - A literature review. Dent Mater 2016;32:e41–53. doi:10.1016/ j.dental.2015.11.007.

[17] Chung L, Dinakarpandian D, Yoshida N, Lauer-Fields JL, Fields GB, Visse R, et al. Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. EMBO J 2004;23:3020–30. doi:10.1038/sj.emboj.7600318.

[18] Bedran-Russo AKB, Pereira PNR, Duarte WR, Drummond JL, Yamaychi M. Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. J Biomed Mater Res - Part B Appl Biomater 2007. doi:10.1002/jbm.b.30593.

[19] Bedran-Russo AKB, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. J Biomed Mater Res - Part B Appl Biomater 2008;86:330–4. doi:10.1002/jbm.b.31022.

[20] Seseogullari-Dirihan R, Tjäderhane L, Pashley DH, Tezvergil-Mutluay A. Effect of ultraviolet A-induced crosslinking on dentin collagen matrix. Dent Mater 2015;31:1225–31. doi:10.1016/j.dental.2015.08.145.

[21] Chen C, Mao C, Sun J, Chen Y, Wang W, Pan H, et al. Glutaraldehyde-induced remineralization improves the mechanical properties and biostability of dentin collagen. Mater Sci Eng C 2016;67:657–65. doi:10.1016/j.msec.2016.05.076.

[22] Hass V, Luque-Martinez IV, Gutierrez MF, Moreira CG, Gotti VB, Feitosa VP, et al. Collagen cross-linkers on dentin bonding: Stability of the adhesive interfaces, degree of conversion of the adhesive, cytotoxicity and in situ MMP inhibition. Dent Mater 2015;32:732–41. doi:10.1016/j.dental.2016.03.008.

[23] Liu R, Fang M, Xiao Y, Li F, Yu L, Zhao S, et al. The effect of transient proanthocyanidins preconditioning on the cross-linking and mechanical properties of demineralized dentin. J Mater Sci Mater Med 2011;22:2403–11. doi:10.1007/s10856-011-4430-4.

[24] Hiraishi N, Sono R, Sofiqul I, Yiu C, Nakamura H, Otsuki M, et al. In vitro evaluation of plant-derived agents to preserve dentin collagen. Dent Mater 2013;29:1048–54. doi:10.1016/j.dental.2013.07.015.

[25] Xu C, Wang Y. Cross-linked demineralized dentin maintains its mechanical stability when challenged by bacterial collagenase. J Biomed Mater Res - Part B Appl Biomater 2011;96 B:242–8. doi:10.1002/jbm.b.31759.

[26] Han B, Jaurequi J, Tang BW, Nimni ME. Proanthocyanidin: a natural crosslinking reagent for stabilizing collagen matrices. J Biomed Mater Res A 2003;65:118–24. doi:10.1002/jbm.a.10460.

[27] Zhou J, Chiba A, Scheffel DLS, Hebling J, Agee K, Tagami J, et al. Cross-linked dry bonding: A new etch-and-rinse technique. Dent Mater 2016;32:1124–32. doi:10.1016/j.dental.2016.06.014.

[28] Nimni ME. The cross-linking and structure modification of the collagen matrix in the design of cardiovascular prosthesis. J Card Surg 1988;3:523–33. doi:10.1111/j.1540-8191.1988.tb00446.x.

[29] Sung H, Chang Y, CT C, Chen C, Liang H. Crosslinking characteristics and mechanical properties of a bovine pericardium fixed with a naturally occurring crosslinking agent. J Biomed Mater Res 1999;47:116–26.

[30] Al-Ammar A, Drummond JL, Bedran-Russo AK. The use of collagen cross-linking agents to enhance dentin bond strength. J Biomed Mater Res - Part B Appl Biomater 2009;91:419–24. doi:10.1002/jbm.b.31417.

[31] Adkins K. Acrolein fixation for light microscopy of dental tissues. Arch Oral Biol 1970;15:1255–62.

[32] Bedran-Russo AKB, Vidal CMP, Dos Santos PH, Castellan CS. Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds. J Biomed Mater Res - Part B Appl Biomater 2010;94:250–5. doi:10.1002/jbm.b.31649.

[33] Cheung D, Perelman N, Ko E, Nimni M. Mechanism of crosslinking of proteins by glutaraldehyde III: Reaction with collagen in tissues. Connect Tissue Res 1985;13:109–115.

[34] Cheung D, Tong D, Perelman N, Ertl D, Nimni M. Mechanism of crosslinking of proteins by glutaraldehyde IV: In vitro and in vivo stability of a crosslinked collagen matrix. Connect Tissue Res 1990;25:27–34.

[35] Stevens JF, Maier CS. Acrolein: Sources, metabolism, and biomolecular interactions relevant to human health and disease. Mol Nutr Food Res 2008;52:7–25.

[36] Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldeli S, et al. Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dent Mater 2013. doi:10.1016/j.dental.2012.08.004.

[37] Carrilho MRO, Carvalho RM, de Goes MF, di Hipólito V, Geraldeli S, Tay FR, et al. Chlorhexidine preserves dentin bond in vitro. J Dent Res 2007;86:90–4. doi: 10.1177/154405910708600115.

[38] Mazzoni A, Nascimento F, Carrilho M, Tersariol I, Papa V, Tjaderhane L, et al. MMP activity in the hybrid layer detected with in situ zymography. J Dent Res 2012;91:467–72. doi: 10.1177/0022034512439210.

[39] Pashley DH, Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M, et al. State of the art etch-and-rinse adhesives. Dent Mater 2017;27:1–16. doi:10.1016/j.dental. 2010.10.016.

[40] Van Meerbeek B, Yoshihara K, Yoshida Y, Mine A, De Munck J, Van Landuyt KL. State of the art of self-etch adhesives. Dent Mater 2011;27:17–28. doi:10.1016/j.dental. 2010.10.023.

[41] Mazzoni A, Apolonio FM, Saboia VPA, Santi S, Angeloni V, Checchi V, et al. Carbodiimide inactivation of MMPs and effect on dentin Bonding. J Dent Res 2014;93:263–8. doi:10.1177/0022034513516465.

[42] Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Mannello F, Tj??derhane L, et al. Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives. Biomaterials 2006;27:4470–6. doi:10.1016/j.biomaterials. 2006.01.040.

[43] Cova A, Breschi L, Nato F, Ruggeri A, Carrilho M, Tjäderhane L, et al. Effect of UVA-activated riboflavin on dentin bonding. J Dent Res 2011. doi:10.1177/0022034511423397.

[44] Serafino C, Gallina G, Cumbo E, Ferrari M. Surface debris of canal walls after post space preparation in endodontically treated teeth: a scanning electron microscopic study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:381–7. doi:10.1016/ S1079210403006061.

[45] Kim J, Arola DD, Gu L, Kim YK, Mai S, Liu Y, et al. Functional biomimetic analogs help remineralize apatite-depleted demineralized resin-infiltrated dentin via a bottom–up approach. Acta Biomater 2010;6:2740–50. doi:10.1016/j.actbio.2009.12.052.

[46] Mazzoni A, Scaffa P, Carrilho M, Tjäderhane L, Di Lenarda R, Polimeni A, et al. Effects of etch-and-rinse and self-etch adhesives on dentin MMP-2 and MMP-9. J Dent Res 2013;92:82–6. doi:10.1177/0022034512467034.

[47] Mazzoni A, Angeloni V, Sartori N, Duarte S, Maravic T, Tjäderhane L, et al. Substantivity of carbodiimide inhibition on dentinal enzyme activity over time. J Dent Res 2017;96:902–8. doi:10.1177/0022034517708312.

[48] Hosaka K, Tagami J, Nishitani Y, Yoshiyama M, Carrilho M, Tay FR, et al. Effect of wet vs. dry testing on the mechanical properties of hydrophilic self-etching primer polymers. Eur J Oral Sci 2007;115:239–45. doi:10.1111/j.1600-0722.2007.00452.x.

[49] Cadenaro M, Antoniolli F, Sauro S, Tay FR, Di Lenarda R, Prati C, et al. Degree of conversion and permeability of dental adhesives. Eur J Oral Sci 2005;113:525–30. doi:10.1111/j.1600-0722.2005.00251.x.

[50] Sano H, Yoshiyama M, Ebisu S, Burrow MF, Takatsu T, Ciucchi B, et al. Comparative SEM and TEM observations of nanoleakage within the hybrid layer. Oper Dent 1995.

[51] Suppa P, Breschi L, Ruggeri A, Mazzotti G, Prati C, Chersoni S, et al. Nanoleakage within the hybrid layer: A correlative FEISEM/TEM investigation. J Biomed Mater Res - Part B Appl Biomater 2005. doi:10.1002/jbm.b.30217.

[52] Tay FR, King NM, Chan K, Pashley DH. How can nanoleakage occur in self-etching adhesive systems that demineralize and infiltrate simultaneously? J Adhes Dent 2002;4:255–69.

[53] Kleiner DE, Stetlerstevenson WG. Quantitative zymography: Detection of picogram quantities of gelatinases. Anal Biochem 1994. doi:10.1006/abio.1994.1186.

[54] Leber TM, Balkwill FR. Zymography: A single-step staining method for quantitation of proteolytic activity on substrate gels. Anal Biochem 1997. doi:10.1006/abio.1997.2170.

[55] Mazzoni A, Mannello F, Tay FR, Tonti G a M, Papa S, Mazzotti G, et al. Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. J Dent Res 2007;86:436–40. doi:10.1177/154405910708600509.

[56] Nishitani Y, Yoshiyama M, Wadgaonkar B, Breschi L, Mannello F, Mazzoni A, et al. Activation of gelatinolytic / collagenolytic activity in dentin by self etching adhesives. Eur J Oral Sci 2006;114:160–6.

[57] Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, et al. Cysteine cathepsins in human dentin-pulp complex. J Endod 2010;36:475–81. doi:10.1016/j.joen.2009.12.034.

[58] Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, et al. Cysteine cathepsins in human carious dentin. J Dent Res 2011;90:506–11. doi:10.1177/0022034510391906.

[59] Aldini G, Orioli M, Carini M. Protein modification by acrolein: Relevance to pathological conditions and inhibition by aldehyde sequestering agents. Mol Nutr Food Res 2011;55:1301–19. doi:10.1002/mnfr.201100182.

[60] Gugliucci A, Lunceford N, Kinugasa E, Ogata H, Schulze J, Kimura S. Acrolein inactivates paraoxonase 1: changes in free acrolein levels after hemodialysis correlate with increases in paraoxonase 1 activity in chronic renal failure patients. Clin Chim Acta 2007;384:105–12. doi:10.1016/j.cca.2007.06.012.

[61] O'Farrell TJ, Guo R, Hasegawa H, Pourmotabbed T. Matrix metalloproteinase-1 takes advantage of the induced fit mechanism to cleave the triple-helical type I collagen molecule. Biochemistry 2006. doi:10.1021/bi060849d.

[62] Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, et al. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. J Dent Res 2011;90:953–68. doi:10.1177/0022034510391799.

[63] Castellan CS, Bedran-Russo AK, Karol S, Pereira PNR. Long-term stability of dentin matrix following treatment with various natural collagen cross-linkers. J Mech Behav Biomed Mater 2011. doi:10.1016/j.jmbbm.2011.05.003.

[64] Pashley D, Tay F, Imazato S. How to increase the durability of resin-dentin bonds. Compend Contin Educ Dent 2011;32:60–64.

[65] Malla N, Sjøli S, Winberg JO, Hadler-Olsen E, Uhlin-Hansen L. Biological and pathobiological functions of gelatinase dimers and complexes. Connect Tissue Res 2008. doi: 10.1080/03008200802151755.

[66] Ray S, Lukyanov P, Ochieng J. Members of the cystatin superfamily interact with MMP-9 and protect it from autolytic degradation without affecting its gelatinolytic activities. Biochim Biophys Acta - Proteins Proteomics 2003. doi:10.1016/j.bbapap.2003.08.004.

[67] Fedarko NS, Jain A, Karadag A, Fisher LW. Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. FASEB J 2004. doi:10.1096/fj.03-0966fje.

[68] Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 1994. doi:10.1172/JCI117619.

[69] Goodall S, Crowther M, Hemingway DM, Bell PR, Thompson MM. Ubiquitous elevation of matrix metalloproteinase-2 expression in the vasculature of patients with abdominal aneurysms. Circulation 2001. doi:10.1161/01.CIR.104.3.304.

[70] Lindsey M, Wedin K, Brown MD, Keller C, Evans AJ, Smolen J, et al. Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion. Circulation 2001. doi:10.1161/01.CIR.103.17.2181.

[71] Teesalu T, Hinkkanen AE, Vaheri A. Coordinated induction of extracellular proteolysis systems during experimental autoimmune encephalomyelitis in mice. Am J Pathol 2001. doi:10.1016/S0002-9440(10)63073-8.

[72] Platt CI, Krekoski CA, Ward R V., Edwards DR, Gavrilovic J. Extracellular Matrix and Matrix Metalloproteinases in Sciatic Nerve. J Neurosci Res 2003. doi:10.1002/jnr.10783.

[73] Lee S-R, Tsuji K, Lee S-R, Lo EH. Role of matrix metalloproteinases in delayed neuronal damage after transient global cerebral ischemia. J Neurosci 2004. doi:10.1523/JNEUROSCI.4243-03.2004.

[74] Gomes R, Meek ME, Eggleton M. Concise international chemical assessment document 43: Acrolein. Geneva: World Health Organization; 2002.

[75] Tezvergil-Mutluay A, Agee KA, Mazzoni A, Carvalho RM, Carrilho M, Tersariol IL, et al. Can quaternary ammonium methacrylates inhibit matrix MMPs and cathepsins? Dent Mater 2015;31:e25–32. doi:10.1016/j.dental.2014.10.006.

[76] Cadenaro M, Breschi L, Rueggeberg FA, Suchko M, Grodin E, Agee K, et al. Effects of residual ethanol on the rate and degree of conversion of five experimental resins. Dent Mater 2009;25:621–8. doi:10.1016/j.dental.2008.11.005.