Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE E NEUROMOTORIE

Ciclo 35

Settore Concorsuale: 06/F1 - MALATTIE ODONTOSTOMATOLOGICHE
Settore Scientifico Disciplinare: MED/28

Development of new collagen cross-linkers for adhesive bonding stabilization.

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Esame finale anno 2023

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ABSTRACT

Aims: This thesis aimed to investigate the influence of different collagen cross-linkers, as separate primers or contained within desensitizing agents, on the longevity of dental restorations and on the dentinal enzymatic activity immediately, or after aging in vitro. **Methods:** A series of studies was conducted using several different cross-linking molecules and several adhesive systems. Four studies investigated the longevity of the hybrid layer by means of microtensile bond strength test, and the enzymatic activity using gelatin and in situ zymography, immediately or after 1 year of aging in the artificial saliva. The first study tested samples bonded with or without a cross-linking agent, that were previously aged for 5 years. The degradation of the hybrid layer was observed using transmission electron microscopy, the enzymatic activity in the hybrid layer using in situ zymography. Raman spectroscopy was used to investigate whether the active substance was still within the hybrid layer after 5 years. **Results**: The results of the studies showed that collagen cross-linkers were efficient in preserving bond strength after aging in vitro when used as separate primers on demineralized or partially demineralized dentin. In the cases when the cross-linker was utilized on mineralized dentin, bond strength results were higher than in the control groups immediately and after aging, however, no difference in enzymatic activity was detected after aging. **Conclusions**: The tested cross-linker molecules used as separate primers in etch-and-rinse and self-etch adhesives seem to be clinically applicable, since the procedure is not overly time-consuming and seems to preserve the hybrid layer over time. As for the cross-linkers contained in the desensitizing agent, when utilized before the adhesive procedures, it has shown to increase the bond strength of self-etch adhesives, but further studies are needed to better understand its effect on the enzymatic activity and crosslinking effects on mineralized dentin.

Keywords: Dentin; collagen crosslinker; hybrid layer; enzymatic activity;

CHAPTER 1
Introduction

WHO defines health as “a complete state of physical, mental, and social well-being and not just the absence of disease” (WHO 1948). World Health Organization Constitution. Geneva, Switzerland: World Health Organization; Retrieved January 18, 2011, from http://www.who.int/governance/eb/who_constitution_en.pdf). This concept of health status embraces the biopsychosocial model of health into which symptoms, physical functioning, and emotional and social well-being are incorporated (Kleinman et al., 1988).

In this sense, oral disease impact society health severely with outcomes that are related to limitations in role performance, and in social and functional capabilities, affecting aspects of social life, including self-esteem, social interaction, school and job performance.

Moreover, oral diseases and their treatment have high social costs. Tooth decay is in fact the second reason of absence from work after respiratory diseases and affects nearly 100% of the population of developed countries. Considering that the life span is nowadays increasing, there is a large population of the elderly with a poor quality of life due to edentulousness. The estimated worldwide expenditures due to dental diseases amounts to 356.00 billion dollars, with the Western European countries accounting for 28% of this amount.

Discovering and explaining the mechanisms responsible for these diseases and the possibility of developing better preventive, diagnostics and therapeutic clinical protocols has a huge potential in terms of reducing social costs, decreasing the psychological and financial load and consequently improving patients’ life quality.

Within the last 50 years, therapy of dental disease has drastically changed allowing for previously inconceivable clinical treatment. This revolution in the approach of oral health could be summarized with the introduction of the adhesive dentistry that has allowed the dental clinicians to perform minimally invasive treatments, preserving tooth substances and allowing for a more durable and stable dental restorations, providing immediate bond strength without the need of a retentive cavity. The goal of the adhesive dentistry procedures is to form and maintain a tight adhesive-tooth interface that is stable for a number of years, providing retentive strength, marginal seal, and clinical durability (Tjäderhane, 2015).

Establishing this durable adhesion to dentin with resin monomer solutions has been an arduous task since the pioneering work of several research teams in the 1950s using the

phosphate monomer glycerol phosphoric acid dimethacrylate (GPDM), patented by Oskar Hagger in 1951 (Buonocore et al., 1956).

Dental adhesives have gone through substantial transformations in their chemistry and number of components within the last four decades as a result of the challenging pledge to create durable bonding to dentin using resin monomers. Adhesion to enamel, on the other hand, has remained consistently simple and reliable since the introduction of the acid-etch technique in 1955 by Michael Buonocore (Buonocore, 1955).

However, the adhesion of a dental restoration greatly depends on the quality of the micromechanical and, in certain adhesive systems also chemical bond between the adhesive resin and dentin. Adhesion to dentin could be considered as bioengineering and relies on the formation of a layer which contains both dentin and the adhesive resin – the hybrid layer. It is formed when a superficial layer of dentin is demineralized, either by a separate etching step, or in the case of self-etching adhesives, due to the low pH of the primer or the adhesive itself. After demineralization, dentin collagen fibrils are denuded, and ideally, the adhesive resin should penetrate the fibrils and envelope them fully, forming a stable hybrid layer. Aiming to improve the longevity of dental restorations, it is necessary to first unveil the reasons behind their failure, understand in detail all the constituents of the hybrid layer, their biomechanical and chemical properties, possible interactions between them, and the reasons why they degrade.

Since neither etch-and-rinse (E&R) nor self-etch (SE) adhesives can fully penetrate the dentin collagen network, denuded collagen fibrils surrounded by water are always present on the bottom of the hybrid layer after the bonding procedures, and this is the weak area of any resin-dentin interface. In the demineralized dentin, endogenous proteases, mainly, matrix metalloproteases (MMPs) and cysteine cathepsins are reactivated, and in the presence of water they can hydrolyze collagen fibers. MMPs are proteases dependent of zinc and calcium ions, which are required to maintain their active state. The true collagenases, MMP-1, -8, -13 and -18, are responsible for collagen degradation. However, they cannot initially bind the collagen triple helix since the binding position is protected by telopeptides. The telopeptidases (MMP-2 and MMP-9) remove the protective telopeptides which facilitates the unwinding of the triple helix and the collagenolytic activity of true collagenases. Therefore, the inhibition or inactivation of these enzymes is crucial to prolong the resin-dentin bond strength over time.

There have been numerous reports on the beneficial effect of protease inhibitors on the longevity of the hybrid layer. Primarily, agents that are able to influence the activity of endogenous dentinal enzymes can be enzyme inhibitors, via a chelating mechanism, or can be

collagen cross-linkers, reported to reinforce the collagen structure and recently also to inactivate the dentinal proteases by changing their molecular mobility.

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.

With the vast array of possibilities investigated in the literature, a confusion could be created in dental practitioners. Hence, there is a necessity for the formation of clear, clinically applicable protocols with new materials, that show predictable and favorable results even after long-term intraoral use.

CHAPTER 2
Literature Review

Adhesive Systems and Adhesion Strategies to Dentin

Adhesion to dentin could be considered as bioengineering in situ and relies on the formation of a layer which contains both dentin and the adhesive resin intertwined – the "hybrid layer" (Breschi et al., 2008; David H. Pashley et al., 2011).

Resin monomers themselves cannot infiltrate mineralized tissues, 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 (Swift et al., 1995).

These adhesive systems laid ground for contemporary dentistry and shifted the development of materials and therapeutic concepts in a new direction. Due to rapid technological development, nowadays there is a vast array of adhesive systems available on the market. The developments have aimed to resolve the most important weakness of the adhesive-dentin interface: susceptibility to hydrolytic degradation, as well as to simplify the adhesive procedure.

There are three main adhesive strategies:

- the etch-and-rinse (E&R),
- the self-etch (SE),
- and the universal adhesive systems.

They differ in terms of composition, acidity, their interaction with dentin, and the clinical protocols. They can be applied in one, two or three clinical steps (D. H. Pashley et al., 2011b; Van Meerbeek et al., 2011).

Etch-and-rinse adhesive systems

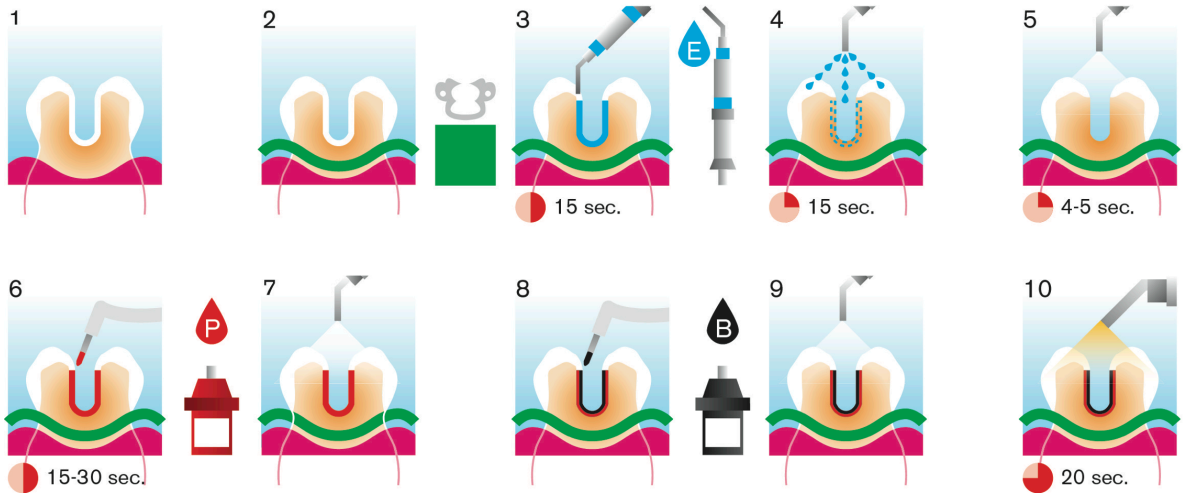


Figure 1: schematic representation of the adhesion steps required when using an etch-and-rinse 3-step adhesive.

The first requirement of this system is the demineralization of the superficial layer of dentin which removes the smear layer, exposes the organic matrix and makes it permeable for the adhesive resin. The dental substrate is etched with 35-37% phosphoric acid and thoroughly rinsed with water (Pashley et al., 1993). After dentin demineralization, collagen fibril network is exposed, surrounded by water and fragile. The specific requirement of handling demineralized dentin is not to overly dry it. In case this happens, the collagen fibrils collapse, and the adhesive resin cannot penetrate the organic network. Hence, etched dentin should be only blot dried and water-wet bonding should be applied, as suggested for the first time by Kanca et al. (Kanca, 1992). Since this layer is hydrophilic and the adhesive monomers are hydrophobic, the primers are used to increase the wettability of collagen fibrils with hydrophobic resin. The primers are amphiphilic molecules, they contain both hydrophilic and hydrophobic monomers. The hydrophilic portion is able to contact the collagen fibrils surrounded by water molecules and dislocate them. With the hydrophobic portion represented by the monomers the primers will enable the monomers of the adhesive resin to closely penetrate the fibrils granting a better diffusion of the adhesive around the collagen. In the 3-step E&R systems, the primer is in a separate bottle to facilitate the penetration of the adhesive resin (Swift et al., 1995). The 3-step E&R system is considered the gold standard in this group, since it showed the best mechanical properties and bond strength to dentin when used correctly (De Munck et al., 2003). Another advantage of the 3-step system is that therapeutic molecules

could be implemented into etchant, primer and/or adhesive, which could render the system antimicrobial or anti-enzymatic, or both (D. H. Pashley et al., 2011b). The downside of the 3-step system is that these materials are very technique-sensitive. Whenever a system contains several steps that need to be performed in a specific way, the possibility of error increases. Therefore, simpler E&R systems, 2-step, have been developed, combining the primer and adhesive into one bottle. These adhesives are applied in two layers, with the first one serving as the primer (D. H. Pashley et al., 2011b). However, the monomers in these blends are not miscible with water and are therefore dissolved in acetone or ethanol, but since demineralized collagen is comprised of approx. 70% water, the interdiffusion of these two different materials is not adequate. Hence, it was necessary to add a certain percentage of hydrophilic monomers, such as two-hydroxyethyl methacrylate (HEMA), into the adhesive blends in order to improve the interdiffusion with dentin (Swift et al., 1995; Van Meerbeek et al., 2005). However, the incorporation of the more hydrophilic monomers into these systems has rendered them more permeable and prone to hydrolytic degradation. Enamel bonds with E&R systems are more durable compared to dentin bonds (Reis and Loguercio, 2009). If in vitro bond strength test was applied to teeth that were restored and aged as intact resin-bonded crowns, and cut into sticks only before testing, the bond strength was preserved possibly due to the protective effect of peripheral enamel seal. If, on the other hand, the teeth were restored, immediately cut and aged as sticks, the bond strength deteriorated significantly (De Munck et al., 2003; Gamborgi et al., 2007). The protective properties of the peripheral enamel seal however, were shown to be less important in vivo, since clinical studies showed deterioration of the adhesive layer in class I restorations, probably due to functional and thermal stresses (M. Carrilho et al., 2007; Hebling et al., 2005).

Self-etch adhesive systems

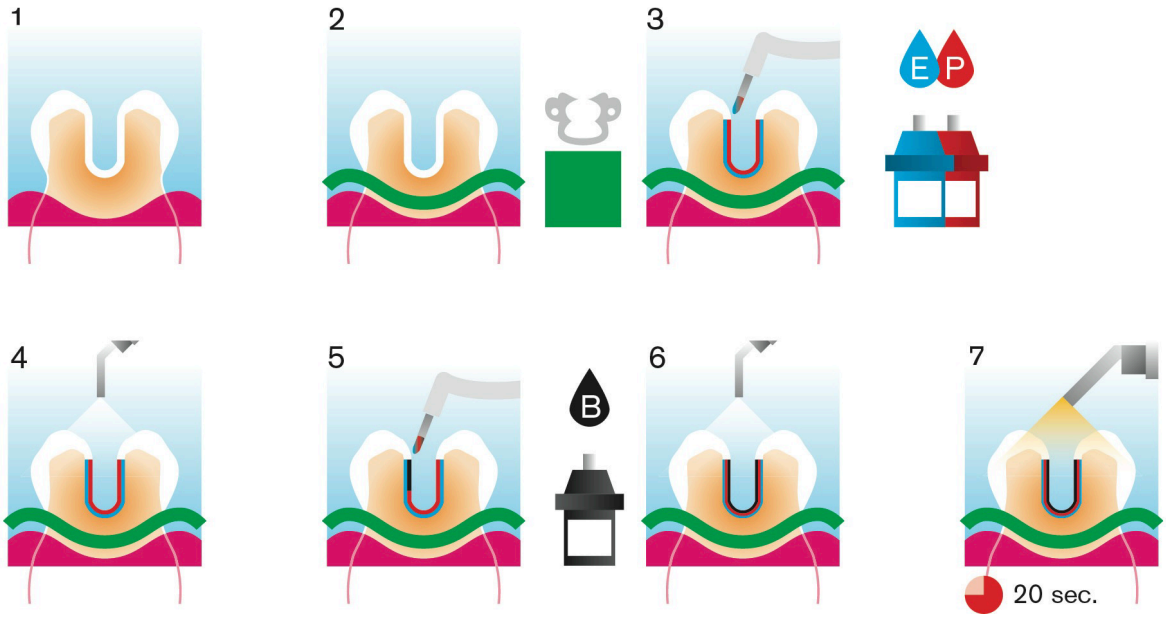


Figure 2: schematic representation of the adhesion steps required when using a self-etch 2-step adhesive.

In the SE systems on the other hand, there is no separate etching step, since the primers and/or adhesives are acidic, and they simultaneously etch dentin and penetrate the collagen network which should provide better resin infiltration compared to the E&R systems (Breschi et al., 2003; Breschi et al., 2004; Spencer et al., 2000). Further, there is significantly less post-operative sensitivity after bonding with SE compared to E&R adhesives (Franklin R. Tay et al., 2002). Depending on the aggressiveness of the system and the method of dentin preparation, SE hybrid layers contain parts of the smear layer including the smear plugs that prevent dentinal fluid from wetting the surface during bonding procedures (Koibuchi et al., 2001; Oliveira et al., 2003). With regard to the chemical composition and acidity, SE adhesives interact differently with dentin and create hybrid layer thickness of a few hundred nanometers (ultra-mild SE approach, pH > 2.5), around 1 µm (mild SE approach, pH ~2), 1-2 µm (intermediately strong SE approach, pH between 1 and 2), and several micrometers deep (strong SE approach, pH≤1) (Koshiro et al., 2006). There are 2- step systems, which contain a primer and an adhesive resin, and there are one-bottle systems. As in the case of E&R adhesives, the gold standard is the more "complex" system, since it prepares the dentinal tissue for adhesion in a more adequate way. The SE adhesives also contain functional monomers which can bind to dentin chemically, and their adhesive performance depends on the affinity of these monomers towards hydroxyapatite (Van Meerbeek et al., 2011). For instance, a

functional monomer 10-methacryloyloxydecanethylene phosphoric acid (MDP), incorporated for the first time in the Clearfil SE (Kuraray) has the ability to bind to hydroxyapatite chemically, reinforcing the adhesive bond. The Adhesion-Decalcification concept explains that in the reaction of SE adhesives with dentin, the acidic component of the adhesive initially always binds to calcium, causing the release of phosphate (PO_4^{3-}) and hydroxide (OH^-) ions from hydroxyapatite. The strength of the bond between the acid and the calcium will determine if demineralization, or chemical bonding to hydroxyapatite will occur. In mild SE adhesives with MDP, chemical bonding will take place. On the other hand, when the strong ones are used, the surface will demineralize (Inoue et al., 2003; Yoshioka et al., 2002). In general, SE bonds show adequate durability, but are still prone to loss of bond strength over time (Tjäderhane et al., 2013).

Universal adhesive systems

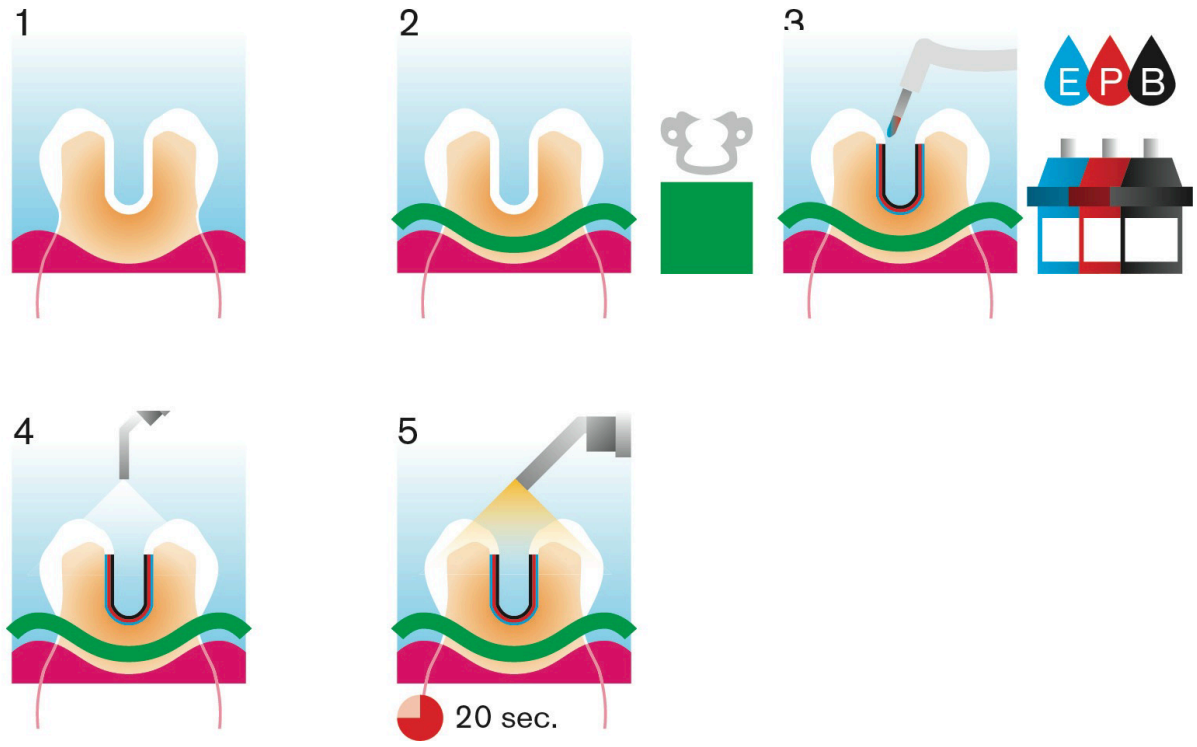


Figure 3: schematic representation of the adhesion steps required when using a universal 1-step adhesive.

The universal adhesive systems are more similar by composition to the SE systems and can be used both in the E&R and SE mode, providing more versatility to clinicians. They also contain functional monomers as the SE systems and are more acidic compared to the E&R adhesives (Hanabusa et al., 2012; Inoue et al., 2005; Toledano et al., 2001). Depending on the system, they can contain a separate primer, or be one-bottle-systems. There is still scarce evidence on bond strength of universal adhesives compared to the standard systems. So far, it seems that if used in SE mode, they present better bond strength after aging, than in the E&R mode (Marchesi et al., 2014). Further, in comparison to SE or E&R systems, the results were material-dependent. Some systems performed similarly as the controls, while others performed worse (Muñoz et al., 2013).

Different adhesive systems interact with dentin in specific ways, creating hybrid layers which are microstructurally distinct, as observed using scanning electron microscopy (SEM) or transmission electron microscopy (TEM) (Figure 4). These differences were also investigated by means of the gold immunolabeling technique (Breschi et al., 2004). Resin-infiltrated dentin specimens, both in E&R and SE mode, were incubated with a primary anti-collagen type I antibody and a secondary gold-conjugated antibody, to examine gold labelling

under SEM. The E&R adhesive showed weak gold labeling in the first half of the hybrid layers, while the bottom half showed intense gold labeling. This indicates that the deepest parts of the hybrid layer in the E&R adhesives are not fully infiltrated by the adhesive resin. On the other hand, the hybrid layer created with a SE adhesive system showed a uniform weak labeling throughout the resin-dentin interface, signaling better homogeneity. Interestingly, the distribution of the areas of interfacial nanoleakage in E&R and SE system correlated with the presented immunolabeling, demonstrating that there are more nanoporosities in the bottom of the hybrid layer, especially in the E&R adhesives, marking the initial points of hybrid layer degradation (Suppa et al., 2005)(Josic et al., 2022).

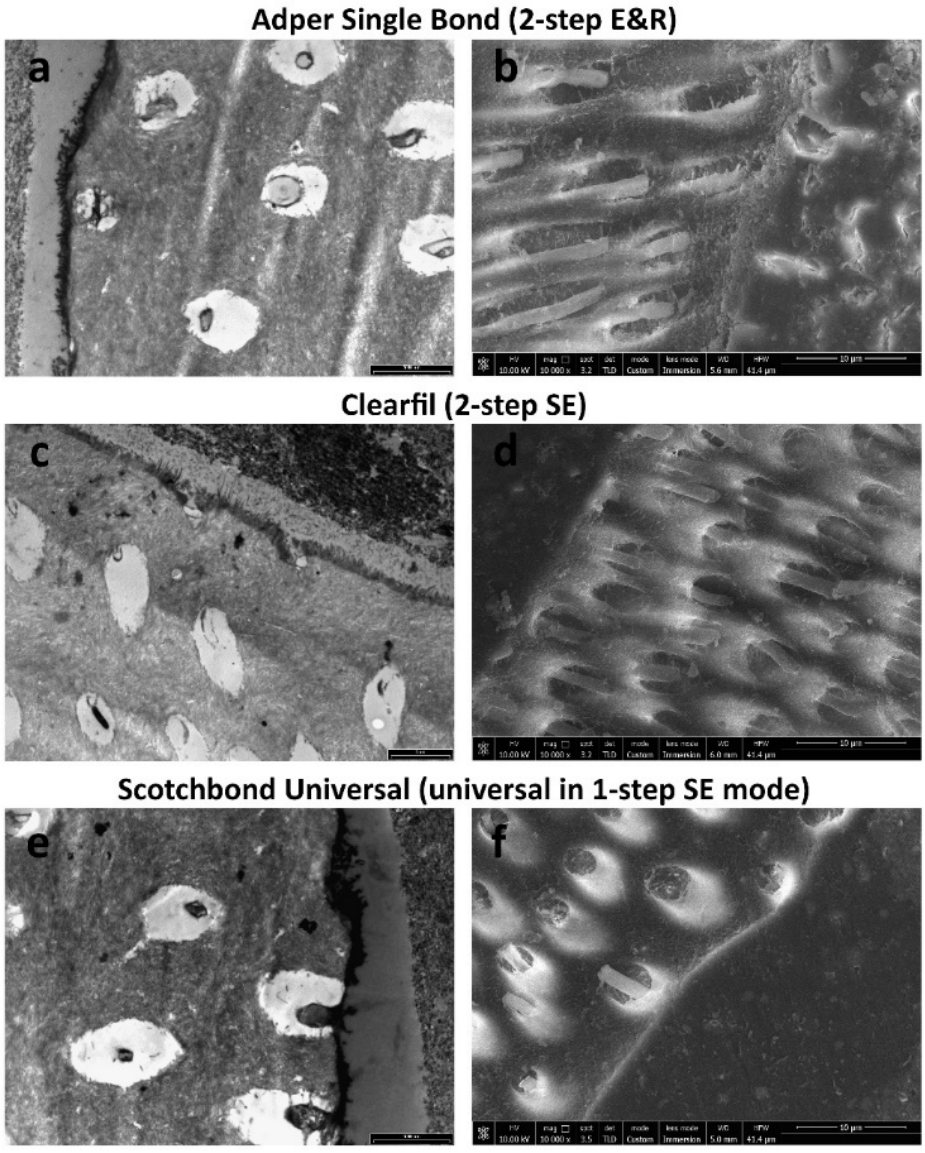


Figure 4. Hybrid layer formed with different adhesive systems as presented on TEM (a, c, e) or FEI-SEM (b, d, f): (a, b) Adper Single Bond, 3M Espe (2-step E&R); (c, d) Clearfil SE, Kuraray (2-step SE); (e, f) Scotchbond Universal (universal adhesive in 1-step SE mode).

Dentin structure in relation to bonding

The composition and structure of dentin varies in different parts of the tooth due to its tubular structure. Overall, it is composed of approximately 70 weight % or 45 volume % of mineral tissue, and 20 weight % or 33 volume % of the organic tissue, with water as the remaining fraction (A. Nanci, 2008; Tjäderhane et al., 2009).

The presence of tubules is the main characteristic of dentin. This characteristic of the dental tissue influences its mechanical behavior, permeability and bonding properties (Carvalho et al., 2012; Tjäderhane et al., 2009). Since dentinal tubules are in a radial disposition, and of a conical shape with the wider part facing the pulp, the deeper the dentin – the larger is the surface occupied by dentinal tubules. Hence, superficial dentin presents a higher surface of intertubular dentin, that is mainly organic matrix constituted of collagen fibrils, unlike peritubular dentin, which is high in mineral content. Both intertubular dentin and the tubules are equally important for the adhesion to dentin due to the interdiffusion with the adhesive resin (Gwinnett, 1993; D. H. Pashley et al., 1995; Tay and Pashley, 2004). Hence, regional variations in dentin structure could influence the quality of adhesion. Further, the surface water content is higher in deeper dentin, leading to lower bond strengths (Prati and Pashley, 1992). Different factors, such as aging, carious and reparatory processes, preparation of dentin, proximity to the pulp tissue, use of diverse chemical cleaning or whitening agents, can all influence bonding to dentin significantly (Carvalho et al., 2012).

Given that in clinical settings, the dentinal substrate available for bonding is rarely completely sound, it is important to stress out the differences in the bonding quality in these cases. Depending on the clinical situation, caries removal method, the extent of the removal of carious dentin, often governed by preferences and personal experience of the operator, the dentinal substrate available for bonding could be partially caries-infected and/or -affected (de Almeida Neves et al., 2011). The caries process causes structural changes in the dentin. The mineral content and crystallinity of the hydroxyapatite are reduced, and are followed by the changes in the structure of collagen (Wang et al., 2007). Hence, the mechanical properties of dentin are hampered, which influences adhesion to carious dentin. The hybrid layers in caries-infected and -affected dentin have been reported to be thicker and poorly infiltrated by the bonding agent (Hsu et al., 2008). Furthermore, carious dentin contains a higher percentage of

water (Ito et al., 2005), making the hybrid layer more prone to hydrolytic degradation. Polymerization of dental adhesives on caries-affected dentin has also been shown to be less efficient compared to sound dentin (Spencer et al., 2005). The majority of the studies investigating bonding to dentin *in vitro* have been performed on sound dentin, which might be less relevant form the clinical standpoint, since an operator is most often faced with cavities which include an array of different substrates, from enamel, to sound dentin, caries-infected or -affected dentin, or sclerotic dentin. The available *in vitro* research on bonding to caries-affected dentin showed a 20-50% lower bond strength of this substrate compared to sound dentin (Ceballos et al., 2003; de Almeida Neves et al., 2011; Yoshiyama et al., 2002). Further, the immediate bond strength in caries-affected dentin seems to be higher when etch-and rinse adhesives are used compared to self-etch systems (Erhardt et al., 2008; Yoshiyama et al., n.d.). However, these differences seem to disappear after short-term aging (Erhardt et al., 2008). Long-term aging studies are necessary to further investigate this matter. On the other hand, long-term clinical retrospective studies have demonstrated a high level of success of posterior composite restorations over time (Da Rosa Rodolpho et al., 2011; Opdam et al., 2010), regardless of the fact that the majority of the posterior cavities contain a portion caries-affected dentin. This disparity could be due to the fact that the cavities also contain, as previously mentioned, enamel and sound dentin, superior bonding substrates, which are usually the structures directly exposed to the oral cavity, with the caries-infected/-affected dentin being in the deeper portions of the cavity. Altogether, this could enhance the clinical durability of the restorations. Sclerotic dentin is another specific dentinal substrate to be considered in terms of adhesive bonding. It is characterized by a superficial layer of hypermineralized dentin, often containing trapped microorganisms, under which is a layer of dentin with denatured collagen fibrils. The tubules are filled with mineral crystallites (Tay and Pashley, 2004). All these features influence bonding properties of sclerotic dentin. Bond strength measured on this substrate is lower compared to sound dentin (Kwong et al., 2002), probably due to the fact that the standardized etching procedures are inadequate in this case and should be modified. It has been shown that resin tags and intertubular dentin hybridization contribute to bond strength about 20% each (Gwinnett, 1993; David H. Pashley et al., 1995; Tay and Pashley, 2004). Due to the specific structure of sclerotic dentin this hybridization is significantly reduced, which could be the cause of poor bond strength. A mere prolongation of etching time showed an improvement in some but not in all the tested adhesives (Lopes et al., 2004), indicating that this solution time might not be sufficient to resolve issues such as bacterial infiltration or denatured collagen fibrils.

Hence, it is clear that the dentin is a highly variable dynamic tissue, and it is therefore difficult to control bonding on dentin and to predict the longevity of the hybrid layer in each individual case. To better understand the mechanisms of adhesion to dentin, and its limitations, it is necessary to understand its molecular structure and organization.

Dentin Collagen Structure

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 (Perdigão et al., 1996; Van Meerbeek et al., 2000) (Figure 2A).

Collagen type I comprises the majority of the collagen structure (90%wt), that also contains traces of collagen V and III (Tjäderhane et al., 2009). 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 (Finkelman et al., 1990).

A collagen molecule consists of three α chains, two α1 and one α2 chain intertwined into a left- handed triple helix (Kramer et al., 2001; Okuyama et al., 1977; Rainey and Goh, 2009) (Figure 2B).

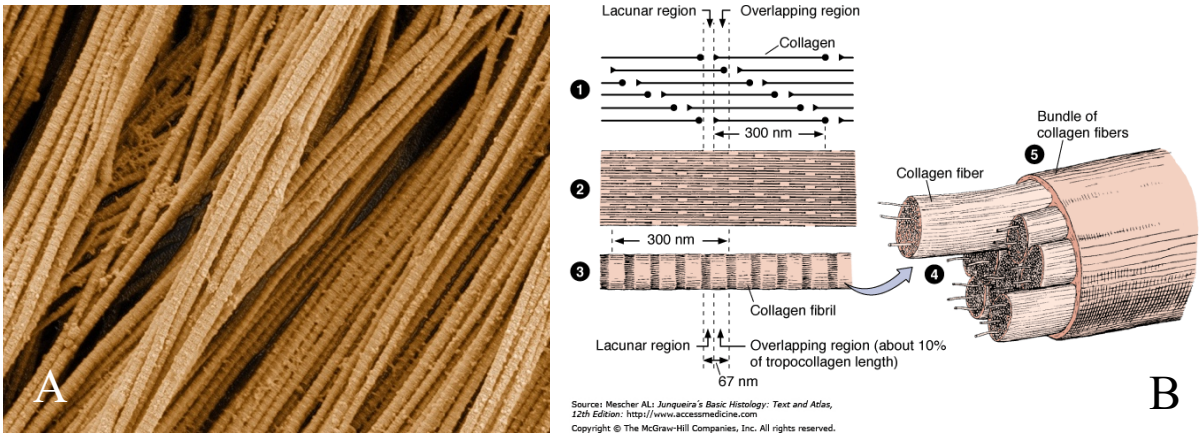


Figure 5: A: Scanning electron microscopy of collagen fibers. B: Diagram showing an aggregate of collagen molecules, fibrils, fibers, and bundles. There is a stepwise overlapping arrangement of rodlike collagen molecules, each measuring 300 nm (1). This arrangement results in the production of alternating spaces and overlapping regions (2), which cause the cross-striations characteristic of collagen fibrils and confer a 67-nm periodicity of dark and light bands when the fibril is observed in the electron microscope (3). Fibrils aggregate and are covalently cross-linked to form fibers (4), which in collagen type I aggregate further to form bundles (5) routinely called

collagen fibers when seen by light microscopy. Credit figure 5A: Tom Deerinck and Mark Ellisman, NCMIR; Credit figure 5B: Junqueira's Basic Histology Text and Atlas, 16e

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 (Yamauchi and Shiiba, 2008). 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 (Veis, 2003). During the process of dentin maturation, apatitic mineral crystallites precipitate and fill the 67 nm gaps between the collagen molecules (Perdigão et al., 1996; Van Meerbeek et al., 2000), thereby inactivating enzymes that are present in the ECM and were active during the dentinogenesis (Angélica R. Hannas et al., 2007).

Unlike insoluble collagen in other bodily systems, dentinal collagen does not metabolically turn over (Tjäderhane et al., 2009), 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 (Yamauchi and Shiiba, 2008). Because dentin collagen does not turn over, the natural cross-links accumulate over time and improve the mechanical properties of collagen fibrils (Schlueter and Veis, 1964). 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 (Veis and Schlueter, 1964).

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 (Brinckmann et al., 2005).

Type I collagen fibrils represent the pillar of the collagen structure, perpendicularly connected by non-collagenous proteins (Breschi et al., 1999; Lin et al., 1993). 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 (Goldberg and Takagi, 1993). 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 (Cheng et al., 1999; Goldberg and Takagi, 1993). 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 (Hedbom and Heinegård, 1993; Scott, 1988; Vogel et al., 1984). Sensitive selective immunolabeling protocols can reveal with great precision the three-dimensional relationships between the different structural elements of the ECM (Breschi et al., 2002; L Breschi et al., 2003; Lorenzo Breschi et al., 2003; Oyarzún et al., 2000).

Degradation of the hybrid layer

Since the hybrid layer consists of different components, deterioration of each of these components can lead to the degradation of the whole system. Primarily, the hybrid layer degrades due to hydrolysis and disorganization of the collagen fibrils and of the adhesive resin (Breschi et al., 2018).

Degradation of the adhesive resin

Hydrolytic degradation is a chemical reaction during which the covalent bonds between polymers are disrupted in the presence of water, leading to loss of resin mass (Breschi et al., 2018; Tay and Pashley, 2004).

The well-known discrepancy between dentin and the adhesive resins in the affinity towards water, along with the tendency to simplify dental procedures, caused changes in the composition of the simplified adhesives. The hydrophilic monomers that are added into simplified adhesive blends are essential for the infiltration of the resin into the collagen network (Swift et al., 1995; Van Meerbeek et al., 2005), but render the adhesive more prone to water sorption and more permeable (F.R. Tay et al., 2002; Franklin R. Tay et al., 2002). Moreover, the amphiphilic resin blends are susceptible to nano-phase separation phenomenon, especially in the more simplified systems. Hence, heterogenous resin layers are created, with the separation of the more hydrophilic and the more hydrophobic phase (Wang et al., 2006; Ye et al., 2008, 2007). The restorations are intraorally constantly exposed to occlusal forces and temperature changes, which eventually hampers the interfacial stability allowing oral fluids and water to penetrate the resin, especially in the more hydrophilic adhesives, or the hydrophilic phase of the heterogenous blends (Gale and Darvell, 1999). The degradation starts with water diffusion through the more hydrophilic part of the adhesive layer, leading to the creation of water-filled channels that degrade the hydrophilic phase of the adhesive (Jacobsen and Söderholm, 1995; Mazzoni et al., 2011a). As the solubilized resin leaches out, the underlying collagen fibrils become denuded and are more exposed to hydrolytic degradation

by endogenous proteases (Cadenaro et al., 2005). One of the key factors in the stability of the resin-dentin interface is the quality of the polymerization of the adhesive. All the adhesive systems, irrespective of the number of the components in the system, display a certain degree of incomplete polymerization, which could facilitate hydrolytic degradation (Breschi et al., 2008b; Cadenaro et al., 2005). Hence, any factor that can further adversely affect degree of conversion can facilitate the degradation of the hybrid layer. With regard to this, it is important to mention that the adhesive resins contain up to 50% of solvents, that can be water, ethanol or acetone, depending on the system. These solvents need to be evaporated by air stream before polymerization or they may remain trapped within the matrix during photopolymerization (Reis et al., 2010; Yoshida et al., 2007). Further, the standard photo-initiators, such as camphorquinone, that were adequate for the hydrophobic monomers, might not yield the optimal degree of cure of hydrophilic resins (Wang et al., 2006). The addition of alternative hydrophilic photo-initiators such as TPO (ethyl 4-dimethylaminobenzoate and diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide), in addition to conventional camphorquinone, has been proposed to improve the DC of hydrophilic adhesive systems (Ilie and Hickel, 2008; Wang et al., 2006).

Degradation of the collagen fibrils

Since neither E&R nor SE adhesives can fully penetrate the dentin collagen network, denuded collagen fibrils surrounded by water are always present on the bottom of the hybrid layer after the bonding procedures, and this is the weak area of any resin-dentin interface (Frankenberger et al., 2005; Sano et al., 1995; Wang and Spencer, 2002). In the demineralized dentin, endogenous proteases, mainly, matrix metalloproteases (MMPs) and cysteine cathepsins are reactivated (Angélica R Hannas et al., 2007; Y. Liu et al., 2011; Nascimento et al., 2011; Vidal et al., 2014), and in the presence of water they can hydrolyze collagen fibers (Pashley et al., 2004).

MMPs are a group of Zn^{2+} and Ca^{2+} dependent endogenous proteases, with implications in various physiological and pathological processes in dentin (Mazzoni et al., 2009a). The MMPs contain several prodomains, two of which are in common for all: propeptide domain which contains the key cysteine residue and the catalytic domain, which contains the Zn-binding site. These two domains form the “cysteine switch” which maintains the enzyme in an inactive form. MMPs also contain other domains which determine substrate specificity and other interactions (Angélica R Hannas et al., 2007). The MMPs are secreted in pro-form and due to several different factors, they can turn to the active form. In the pro-form, the cysteine

residue from the propeptide domain is bonded to the zinc atom on the active site. Irrespective of the activation mode, the underlying mechanism is always the same – the cysteine is dissociated from the zinc atom in the active site, and exchanged by water molecules, which transforms the pro- into active form of MMP (Van Wart and Birkedal-Hansen, 1990). So far, several MMPs have been identified in dentin: gelatinases MMP-2 and MMP-9 (Mazzoni et al., 2009b, 2007), collagenase MMP-8 (Shimada et al., 2009; Sulkala et al., 2007), stromelysin MMP-3 (Mazzoni et al., 2011b), enamelysin MMP-20 (Shimada et al., 2009; Sulkala et al., 2007) and most recently MMP-7 (Mazzoni et al., 2018). They are thought to play part in the mineralization of collagen matrix during tooth development, after which they remain trapped and inactive in the mineralized dentin. After the demineralization of dentin, these protases are reactivated. The MMPs are believed to be involved in a vast range of dental hard tissue pathologies, including dental caries (Mazzoni et al., 2015; Tjäderhane et al., 2015), degradation of the hybrid layer (Mazzoni et al., 2014) and dentin erosion (Buzalaf et al., 2015). In particular, MMP-2 and MMP-9 are important in caries progression (Tjäderhane et al., 1998; Vidal et al., 2014) and degradation of the hybrid layer (Apolonio et al., 2017; A. Mazzoni et al., 2013; Mazzoni et al., 2015, 2012).

The MMPs can degrade practically all components of the extracellular matrix (ECM). However, only true collagenases, MMP-1, -8, -13 and -18, are responsible for collagen degradation (Visse and Nagase, 2003). The cleavage occurs on specific sites, creating ¼ and ¾ fragments of the collagen molecule. These fragments are thermally unstable and can easily be degraded by other members of the MMP family, or a wide range of proteases (Perumal et al., 2008). Nevertheless, they cannot initially cleave the collagen triple helix since the binding position is sterically blocked by C-telopeptides, making collagen triple helix positioned in such a way that the large collagenase molecule cannot fit (Perumal et al., 2008). Hence, removing the protective telopeptides is the key factor which facilitates the unwinding of the triple helix and the collagenolytic collagen cleavage by true collagenases (Mirigian et al., 2013). The telopeptidases which enable this process are MMP-2 and -9 (Perumal et al., 2008). The activity of these gelatinases, the most abundant in dentin, and pivotal in the collagen degradation process, has been extensively investigated by Mazzoni et al. (Mazzoni et al., 2009b, 2007). Apart from demonstrating MMP-2and -9 as the intrinsic constituents of dentin organic matrix using immunolabeling technics (Mazzoni et al., 2009b), Mazzoni et al. also investigated the activity of these MMPs with precise identification of the pro- and active forms of both MMP-2 and -9 using gelatine zymography in dentin powder (Mazzoni et al., 2007). The same research team further demonstrated that the MMPs can be activated by acid etching, E&R as well as SE

adhesive systems (A. Mazzoni et al., 2013; Mazzoni et al., 2012, 2006). Therefore, inhibition/inactivation of the MMPs is considered a very important strategy for the preservation of the hybrid layer (Breschi et al., 2018; Tjäderhane et al., 2013).

CTs are another important intrinsic constituent of dentinal organic matrix with implications in physiological and pathological processes in dentin. They can contribute to the degradation of the ECM and foreign proteins (Mazzoni et al., 2009a). All human cathepsins have the same active site, formed by cysteine and histidine residues, with three well-defined substrate binding subsites (Turk et al., 1998). The presence and distribution of cathepsins K and B in dentin have recently been described using immunolabeling technique (Scaffa et al., 2017). Cathepsins K and B may play an important role in bone remodeling or collagen degradation during orthodontic tooth movement (Mazzoni et al., 2009a). Furthermore, there is high increase in the activity of cysteine CTs in active and chronic carious lesions (Nascimento et al., 2011). The activity of cathepsins is more pronounced with the increased depth of the cavity. Hence, it is reasonable to believe that the odontoblast-secreted CTs have implications in the active caries lesions. The increase of the activity of cysteine CTs in the chronic caries lesions could indicate their role in the activation of growth factors and consequently in the stimulation of defensive pulpal reactions (Nascimento et al., 2011; Tersariol et al., 2010).

Interestingly, almost all collagenolytic activity of the CTs can be attributed to CTK which is specific in several ways. Cathepsin K, a papain-like cysteine protease, is predominantly expressed in osteoclasts and held responsible for the degradation of bone collagen (Bromme and Okamoto, 1995). It can cleave collagen in multiple positions, in the helical as well as non-helical parts (Garnero et al., 1999), while other CTs can cleave only the non-helical parts of the collagen molecule (Brömme and Wilson, 2011). It is capable of disintegrating compact collagen fibers into fragments and further solubilizing them into soluble peptides (Panwar et al., 2013). “Productive” GAGs, such as chondroitin 4-sulfate, or chondroitin 6-sulfate are necessary for the activation of CTK. Since the active site of the CTK is too small to accommodate the whole collagen molecule, it is hypothesized that the CTK-GAG complex conformationally changes the active site of the proteases, enlarges it, enabling the cleavage of collagen (Li et al., 2002). While some GAGs activate CTK, others, such as heparin, act in its inhibition. Hence, the activity of CTK is fully regulated by GAGs (Li et al., 2004).

The mechanisms in which the MMPs and CTs degrade collagen is different, but they can also work synergistically, and create a cascade of events leading to the degradation of the hybrid layer (Nascimento et al., 2011; Tersariol et al., 2010).

In summary, there are two main pathways of collagen degradation. One involves the MMPs, where in the joint activity of telopeptidases and true collagenases, the collagen molecule is fragmented into ¼ and ¾ fragments which are thermally unstable and can further be degraded by a wide range of proteases and non-collagenolytic MMPs. The other pathway is mediated by CTK-GAG complexes which can degrade collagen without the cooperation with other proteases (Li et al., 2004).

Strategies to reduce hybrid layer 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 (Gendron et al., 1999) and cysteine cathepsins (Scaffa et al., 2012)(Gobbi et al., 2021). As low as 0.2% solutions of CHX have been shown to increase the longevity of the HL (Breschi et al., 2010b; Campos et al., 2009; M. Carrilho et al., 2007; M. R. O. Carrilho et al., 2007; Leitune et al., 2011; Loguercio et al., 2016; Mobarak, 2011; Scaffa et al., 2012; Zheng et al., 2014). 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 (Carrilho et al., 2010; da Silva et al., 2015; J. Kim et al., 2010; Stanislawczuk et al., 2014; Yiu et al., 2012).

Some studies have shown that CHX binding is a reversible mechanism, and probably due to leaching, the resin-dentin interfaces treated with CHX were shown to be unstable after

18 months (Zhou et al., 2009). Sodium chloride have been demonstrated to be able to displace CHX from both mineralized and demineralized dentin, indicating that CHX binding to dentin is only electrostatic (Imazato et al., 2003; J. Kim et al., 2010). However, there is also ample *in vitro* and *in vivo* research data that shows at most part a beneficial effect of CHX on HL longevity and bond strength (Breschi et al., 2010b; M. Carrilho et al., 2007; Hebling et al., 2005; Loguercio et al., 2016). A recent study carried on by our research group has demonstrated the anti-proteolytic effect and protection of the hybrid layer from degradation of chlorhexidine also after 10 years of accelerated aging (Breschi et al., 2020).

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) (Cadenaro et al., 2023; J. Kim et al., 2010).

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 (Carvalho et al., 2000; Thompson et al., 2012). 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 alkyl-benzyl-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 (Comba et al., 2019a; Sabatini et al., 2015; Sabatini and Patel, 2013; Arzu Tezvergil-Mutluay et al., 2011), with promising results in bond strength preservation over time (Sabatini et al., 2015). 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 (Imazato et al., 2007, 2003), showing a good ability to inhibit the MMPs activity (D. Pashley et al., 2011; Tezvergil-Mutluay et al., 2015). Following the good results obtained with MDPB, other QAMs were investigated as MMPs inhibitors with encouraging results, comparable to CHX (A Tezvergil-Mutluay et al., 2011). Researchers are still focused on the development of new monomers with quaternary

ammonium functionalities (Antonucci et al., 2012), 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, polyvinyl-phosphonic acid, has shown good immediate results, but with questionable longevity (Heikkilä et al., 2002; Tezvergil-Mutluay et al., 2010). Further, tetracycline and its analogs (doxycycline and minocycline) have shown collagenases- and gelatinases-inhibitory properties (Lauhio et al., 1995; Sulkala et al., 2001). 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 (Breschi et al., 2010a). Similarly, SB-3CT, a specific MMP-2 and -9 inhibitor showed a positive influence on HL preservation (De Munck et al., 2010). 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 (Turk et al., 2012).

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. Introducing exogenous crosslinks to dentin matrix is a stable approach to enhance the biostability of collagen through improving its mechanical properties and resistance to collagenase degradation. Khor et al. have pointed out the significance of the intramolecular and intermolecular chemical bonds of collagen molecules to the stability of collagen fibrils (Khor, 1997). The chemical crosslinking agents, which increase the number of intramolecular and intermolecular chemical bonds, can effectively increase the stability of collagen structure. Meanwhile, crosslinking may inactivate endogenous enzymes by lowering the molecular mobility of the catalytic sites, which are critical for their protease activities (A. Tezvergil-Mutluay et al., 2012). Biomodification of the collagen scaffold enhance the biomechanical properties of dentin, and makes it less prone to proteolytic attack (Bedran-Russo et al., 2008; Xu and Wang, 2011).

Aldehydes, such as glutaraldehyde (GD), are used as tissue fixatives, but have been proposed as potential cross-linking agents for the use in dentistry, since they form covalent bonds between the amino groups of proteins and the two aldehyde groups of glutaraldehyde. In particular, GD is a monomer containing five carbon atoms of aliphatic molecules, and two aldehyde groups at both ends of the hydrocarbon chain. GD is able to bind 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 (Bedran-Russo et al., 2008, 2007; Chen et al., 2016; Hass et al., 2016; Hiraishi et al., 2013; R. Liu et al., 2011; Xu and Wang, 2011). 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 (Maravic et al., 2017; Nimni, 1988; H. Sung et al., 1999). 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-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), which was shown to achieve a similar bond strength preservation to that of GD (Bedran-Russo et al., 2010; Annalisa Mazzoni et al., 2013; Mazzoni et al., 2018, 2017, 2009b; A Tezvergil-Mutluay et al., 2012) 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 (A Tezvergil-Mutluay et al., 2012). 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 (Annalisa Mazzoni et al., 2013) and in-situ zymographic three-dimensional images obtained by confocal microscopy (Mazzoni et al., 2014), 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 (Bedran-Russo et al., 2007; Hiraishi et al., 2013; Seseogullari-Dirihan et al., 2016).

Apart from chemical compounds, the cross-linking effect can also be achieved through physical and photochemical treatment, such as ultraviolet A (UVA) (Seseogullari-Dirihan et al., 2015) or gamma irradiation, heating and drying (Hayashi et al., 2010).

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 (A. Mazzoni et al., 2013; Mazzoni et al., 2006). 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 (Sabatini et al., 2014), EDC (Mazzoni et al., 2012; A Tezvergil-Mutluay et al., 2012) and plant-derived cross-linking agents (Khaddam et al., 2014; Zhang et al., 2012) 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 (Spencer and Wang, 2002).

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. (Pashley et al., 2007) 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. (Tay et al., 2007) 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 (Ayar, 2016). In the absence of water, matrix poroteases cannot cleave collagen.

Using ethanol wet-bonding permits infiltration of more hydrophobic resins (Hiraishi et al., 2005; Mazzitelli et al., 2022a; Shin et al., 2009) 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 (Agee et al., 2015). To determine whether ethanol can replace bound water from collagen matrices, Jee et al. (Jee et al., 2016) 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% (Hiraishi et al., 2005), unlike the 70 vol% (D. H. Pashley et al., 2011b) 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 (Ayar, 2015; El-Deeb et al., 2015; Makishi et al., 2015).

Alternative demineralizing agents for structural stabilization of dentin

A viable option to preserve the longevity of the hybrid layer is to maintain a thin layer of apatite mineral surrounding the collagen fibrils in the dentin.

Dental adhesion on dentin requires the removal of the smear-layer and the demineralization of the collagen substrate to correctly infiltrate the fibrils and to obtain a hybrid

layer. However, when demineralized dentin collagen is challenged by collagenase, collagenases approach to collagen molecules and unwind triple helical conformation in such a way that the catalytic active can attack the specific glycine-isoleucine peptide bonds and cleave individual chains in succession, turning collagen molecules into small peptide fragments (Chung et al., 2004). In this sense, different materials have been proposed as an alternative to traditional orthophosphoric acid dentin demineralization in order to protect the collagen fibrils from complete demineralization or to cross-link them during the demineralization process in order to strengthening them.

Nordihydroguaiaretic acid (NDGA), isolated from the creosote bush, is a natural di-catechol antioxidant, with well-known anti-inflammatory, anti-cancer and anti-cardiovascular disease properties. The structure of NDGA molecule is simple, and contains two o-catechol groups at the ends of its short alkyl chain. Recent studies have also identified its highly efficient protein crosslinking effect (Koob and Hernandez, 2003, 2002). NDGA could stabilize collagen by forming bridge-type hydrogen bonds between its phenolic hydroxyl and amide carbonyl groups of collagen, hydrophobic interaction (i.e., π-stacking) between aromatic rings, and covalent-like interactions (Fathima et al., 2010). Using an EDX Gongx et al. demonstrated that dentin etched by bio-modified 1% NDGA etchant displayed the presence of remnant calcium. It is well known that phenolic hydroxyl groups from NDGA can interact with metal ions (i.e., calcium) via metal-coordination chemistry (Gong et al., 2018; Sever et al., 2004). Free calcium ions generated during the demineralization process of dentin might have participated in the crosslinking reaction of dentin collagen. This would be an ulterior benefit for the subsequent dentin bonding, as the calcium persisting in the collagen matrix may promote remineralization of denuded collagen fibrils that were not penetrated by adhesive resin (Dhand et al., 2016). Bio-modified etchant with low concentration (0.5% or 1%) of NDGA showed significantly improved enzymatic degradation resistance and stability of the mechanical properties of dentin collagen matrix (Gong et al., 2018).

Phytic acid, known as inositol hexakisphosphate (IP6), is a saturated cyclic acid and the phosphate ester of inositol. In 2013, IP6 was evaluated as a dentine etching agent with results showing that upon etching with IP6, the bond strength of resin to dentine was significantly increased compared with phosphoric acid (Nassar et al., 2013). The speculated mechanism of action behind the increased resin-dentine bonding was attributed to two possible events. The first mechanism was due to IP6's ability to form insoluble complexes with calcium at a pH above 4 (Grynspan and Cheryan, 1983). As a result of the high dentine buffering capacity (Camps and Pashley, 2000), the pH of IP6 increases upon neutralization with dentine,

and thus facilitated the formation of the insoluble complex, which might provide a certain level of stability for the exposed collagen. The second speculated mechanism was through the collagen crosslinking action of IP6 (Lee et al., 2011; Ravichandran et al., 2013). At a pH above the isoelectric point, because both IP6 and the protein have a net negative, IP6 can interact with collagen fibrils, forming a soluble ternary protein-cation-IP6 complex, where cations such as calcium, bridges the IP6 to protein (Camps and Pashley, 2000). This newly formed protein complexes result in decreased protein solubility and might be recalcitrant to enzymatic hydrolysis or require higher quantities of the enzymes to be degraded (Ravindran et al., 1995; Selle et al., 2012). Moreover, in addition to IP6's excellent ability to chelate with calcium, it is also a potent chelator of zinc. The zinc-IP6 complex is stable and insoluble and these properties along with an ability to bind to collagen might make IP6 a potential inhibitor of dentinal matrix metalloproteinases. Despite limited evidence, it seems that controlled concentrations of IP6 are not aggressive to dentine and create a stable collagen network which might be clinically translated to better longevity of resin-based restorations (Nassar et al., 2021).

Recently, a new alternative etchant have been introduced and were tested on their enamel-bonding effectiveness (Yao et al., 2019). This experimental metal salt-based $ZrO(NO_3)_2$ conditioner, produced by Ivoclar (Ivoclar Vivadent, Schaan, Liechtenstein) represents a new category of dentin conditioners with Ca-chelating ability. Despite having a higher pH than a classic phosphoric acid etchant, the etching efficacy of this experimental material on enamel enabled durable bonding, and it could be considered as a promising phosphoric-acid alternative etchant. This experimental etchant is a Lewis acidic metal salt that is readily soluble in water. Water is necessary for the dissolution of the zirconium salt to create an acidic condition (pH = 0.56) that is conducive for demineralizing the inorganic components of dentin. During demineralization, $Ca2+$ and $PO24-$ ions are released from dentin and these ions bind to the $Zr4+$ ions as to form a solid complex that determin a self litimting demineralization factor for the etching of the material (Solovkin and Tsvetkova, 1962). The experimental etchant interfacial interaction with a universal adhesive was characterized by a twofold bonding mechanism, combining collagen-fibril envelopment by resin at the hybrid-layer top, rendering micro-mechanical interlocking as typically achieved by an E&R bonding mode, with additional chemical binding of the functional monomer 10-MDP at the HAp-richer hybrid-layer bottom as typically achieved by a mild SE bonding mode. The latter chemical interaction is potentially complemented by the methacrylated polyalkenoic acid co-polymer included in universal adhesives. In addition, the more HAp-protected collagen at the deepest

hybrid-layer area, where potentially resin failed to fully infiltrate, may reduce/retard hydrolytic degradation with time but ulterior research are needed on the matter.

Another alternative to completely demineralize the collagen fibrils would be to selectively remove the interfibrillar mineral 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 (Chiba et al., 2016; Takahashi et al., 2013; Toroian et al., 2007). 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, or nitric acid, or iron containing solution (Sebold et al., 2019), 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 (Mai et al., 2017). 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 (Mai et al., 2017; Sebold et al., 2019).

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

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 (Tay and Pashley, 2008) 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 (Gu et al., 2011; Y. K. Kim et al., 2010) 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 (Abuna et al., 2016; Sauro et al., 2015). 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 (Sauro et al., 2015) and SE (Abuna et al., 2016) 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.

The future of dentin adhesion?

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.

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 (Reiser et al., 1992).

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 non-collagenous component of dentin as well as collagen scaffold (Bedran-Russo et al., 2014).

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 (Bedran-Russo et al., 2008, 2007; Cheung et al., 1985; Hayashi et al., 2010). Collagen can be modified by using methods such as chemical, physical and photochemical crosslinking (Cova et al., 2011; A. Tezvergil-Mutluay et al., 2012).

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

Glutaraldehyde

Among the many available protein crosslinking agents, glutaraldehyde has undoubtedly found the widest application in various fields (Nimni et al., 1987; Richards and Knowles, 1968; SABATINI et al., 1963).

Glutaraldehyde, a linear, 5-carbon dialdehyde, is a clear, colorless to pale straw-colored, pungent oily liquid that is soluble in all proportions in water and alcohol, as well as in organic solvents. It is mainly available as acidic aqueous solutions (pH 3.0–4.0), ranging in concentration from less than 2% to 70% (w/v). The structure of glutaraldehyde in aqueous solution has been the subject of more debate than any of the other crosslinking reagents. In fact, glutaraldehyde structure in aqueous solution is not limited to the monomeric form (Figure 6, structure I). Figure 1 gives an overview of the possible molecular forms of glutaraldehyde in aqueous solution based on reports covering the past 40 years (Aso and Aito, 1962; Kawahara et al., 1992; Rembaum et al., 1978; Richards and Knowles, 1968).

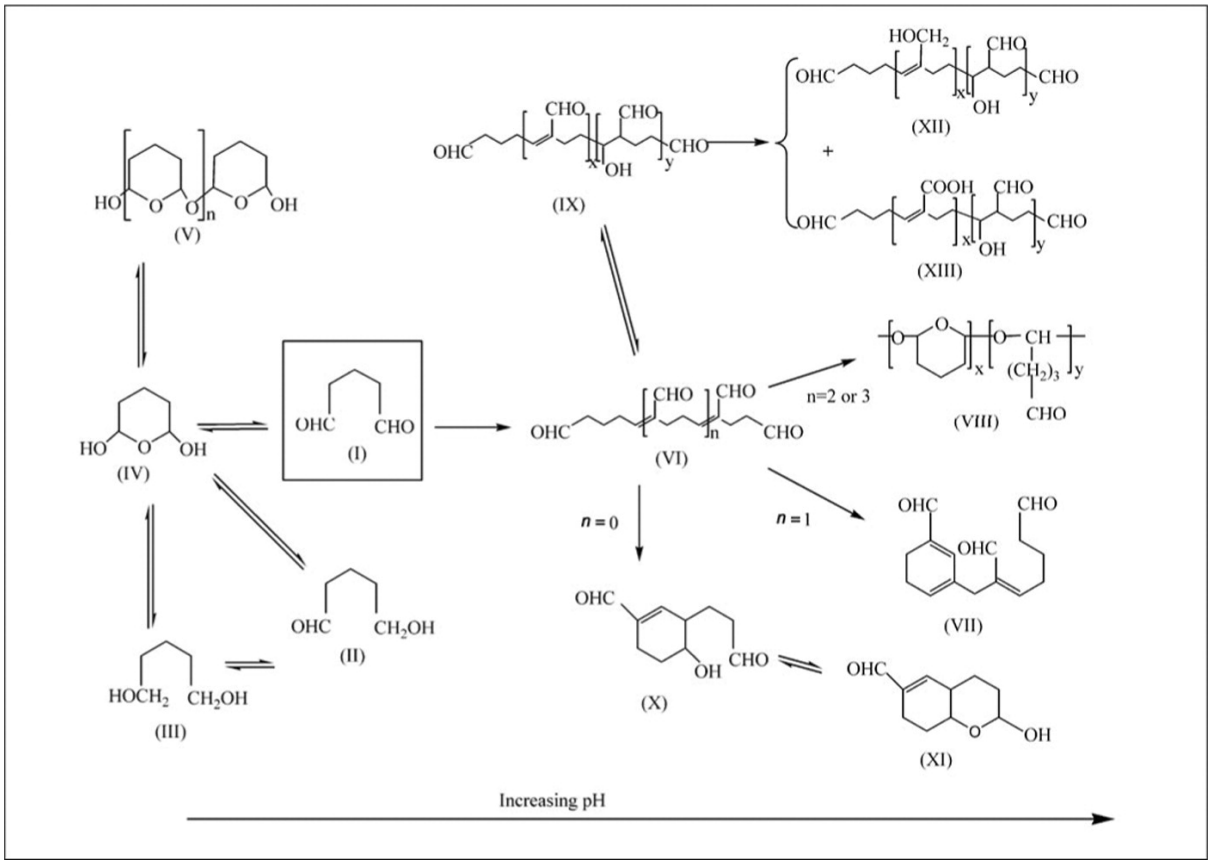


Figure 6. Summary of the possible forms of glutaraldehyde in aqueous solution.

Glutaraldehyde has had great success because of its commercial availability and low cost in addition to its high reactivity. It reacts rapidly with amine groups at around neutral pH (Okuda et al., 1991) and is more efficient than other aldehydes in generating thermally and chemically stable crosslinks (Nimni et al., 1987).

The mechanism seems to be dependent on the formation of covalent chemical bonds between the aldehyde group of GA and the amino groups of lysine and hydroxylysine residues in collagen (Ritter et al., 2001; H. W. Sung et al., 1999), which increases the resistance of collagen to enzymatic degradation (H. W. Sung et al., 1999). However, the chemical nature of the reaction of glutaraldehyde with proteins is not clearly understood, and the mechanisms of protein crosslinking reactions remain open to speculation. It seems that no single mechanism is responsible for glutaraldehyde reaction with proteins. In fact, because glutaraldehyde is present in different forms even for specific and controlled reaction conditions, several possible reaction mechanisms could proceed simultaneously (Migneault et al., 2004).

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 (Sheehan et al., 1961). 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 (Bedran-Russo et al., 2010), carbodiimides are less toxic than aldehydes (Huang et al., 1990).

Carbodiimide hydrochloride (EDC)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Figure 7) is a water soluble carbodiimide usually obtained as the hydrochloride. It is typically employed in the 4.0-6.0 pH range (López-Alonso et al., 2009). 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 (López-Alonso et al., 2009). In medical filed EDC was recently studied, with promising results, as reinforced for collagen scaffold in tissue engineering (Li et al., 2013).

The most studied carbodiimide in dentistry is 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), which was shown to achieve a similar bond strength preservation to that of GD (Bedran-Russo et al., 2010; Mazzoni et al., 2013) 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 (A Tezvergil-Mutluay et al., 2012). Its ability to increase the stiffness of demineralized dentin matrix (Bedran-Russo et al., 2010; Ryou et al., 2016) and the HL (Zhang et al., 2017) has been well documented. Recent zymographic assays on dentin powder (Mazzoni et al., 2013) and in situ zymographic three-dimensional images obtained by confocal microscopy (Mazzoni et al., 2017, 2014), clearly demonstrated that when EDC is used as additional therapeutic primer on acid-etched dentin, it also has the ability to silence dentinal MMPs, even after a 1-year storage in artificial saliva (Mazzoni et al., 2017).

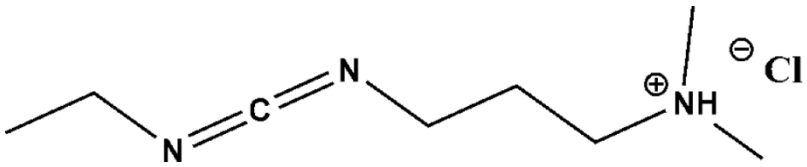


Figure 7. EDC molecular structure

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 8). 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 (Olde Damink et al., 1996; Staros et al., 1986). 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.

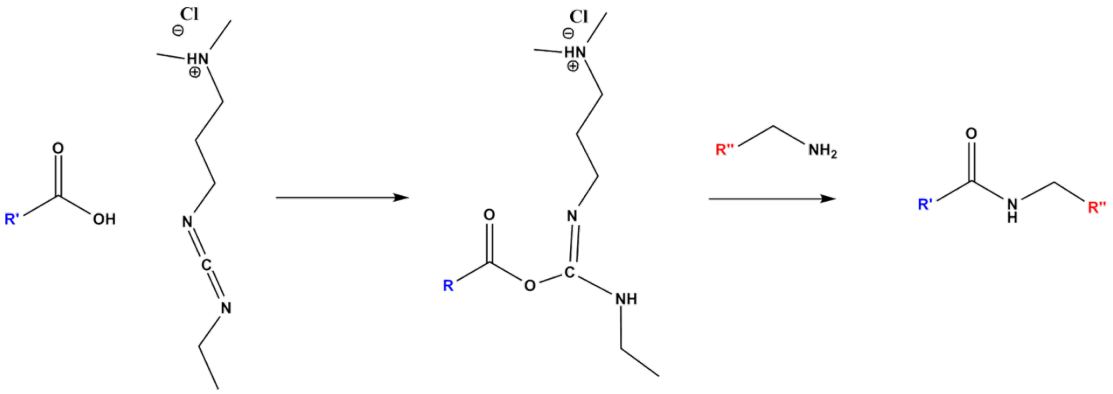


Figure 8. EDC cross-linking reaction: EDC reacts with carboxylic group of, after the elimination of O-acylisourea intermediate, the glutamic or aspartic acid bond the N residue of lysine or hydroxylysine.

Dicyclohexylcarbodiimide (DCC)

N,N'-Dicyclohexylcarbodiimide (DCC) is an organic compound with the chemical formula $C^{13}H^{22}N^2$ (Figure 9) 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 (Chen et al., 2014). 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 (Sebald et al., 1980).

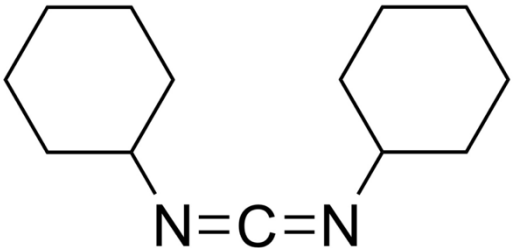


Figure 9. DCC molecular structure

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 (Sebald et al., 1980) (Figure 10).

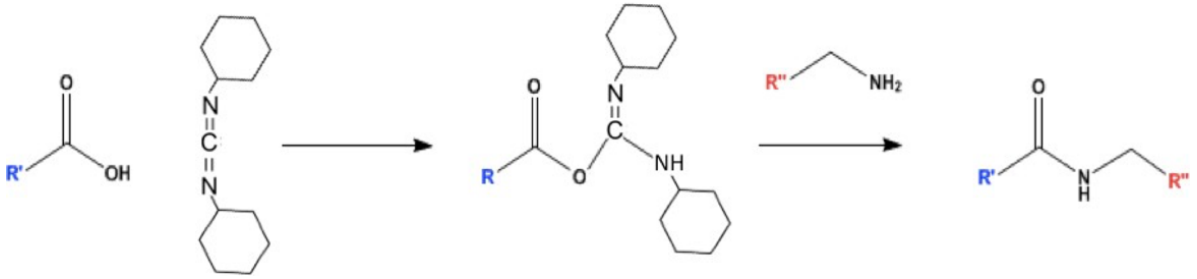


Figure 10. DCC cross-linking reaction: likewise EDC, the DCC reacts with carboxylic group of glutamic and aspartic acids, after the elimination of O-acylisourea intermediate, the glutamic or aspartic acid bond the N residue of lysine or hydroxylysine.

Chitosan

Chitosan is a macromolecule formed by the repetition of D-glucosamine, which is derived from the deacetylation of chitin, obtained from the shells of marine crustaceans (in particular from crabs and prawns). It is a fiber, chemically similar to cellulose, and it is indigestible. Chitosan is a natural nontoxic, biocompatible, biodegradable polysaccharide with tested antibacterial properties (Qasim et al., 2018). The major properties of chitosan include biocompatibility, safety, biodegradability, antimicrobial activity, and the ability to form film and gel (Fiorillo, 2019; Husain et al., 2017; M, 2017). Different formulations of chitosan are presented on the market characterized by different pH and different materials. There are gels based on chitosan containing lactic acid, some containing distilled water, and others containing

chlorhexidine. The antibacterial activity of chitosan is strongly influenced by its formulation. In conservative dentistry it has been used for the prevention of caries, as well as in to the field of conservative dentistry surgery (Ortiz and Boyce, 2008). Chitosan has been widely used as a component of toothpaste for its antimicrobial activity, and it is a biocompatible and safe characteristic. It favors the hard tissues of the tooth, such as enamel and dentin, and it can be widely used without reported side effects (Cicciù et al., 2019). In some studies, it has been hypothesized that chitosan, modified with methacrylic groups, is able to covalently bind to the resin of dental restorations and, because of the presence of residual positive charges on the polysaccharide, to interact electrostatically with the demineralized dentin. Therefore, by introducing chitosan methacrylate in the primer of a three-step "etch and rinse" adhesive system we can achieve good adhesion values and good stability of the hybrid layer when subjected to mechanical simulation of chewing and thermal stress. It would also seem to improve the characteristics of mucus-adhesion to the enamel, producing a better remineralization (Cervino et al., 2017). Chitosan biopolymer has also been highlighted among substances that can decrease degradation of the collagen matrix caused by metalloproteinases. It can form cross-links with dentin collagen, strengthening the fibrils against degradation, besides decreasing the action of MMPs (Baena et al., 2020). The structure of chitosan has free amino and hydroxyl groups, besides positive charges that form a cross-linkage with dentin collagen through ionic complexes, producing a mechanically strong fibril chain and raising the mechanical performance of restorations (Kishen et al., 2016).

Aims and hypotheses

Five separate studies were performed as a part of the present thesis, and each study was executed in accordance with their own specific aims. However, all of them share the same general objectives:

1) To investigate whether different cross-linkers influence the preservation of bond strength, immediately or after aging in artificial saliva at 37°C.

2) To investigate whether different cross-linkers influence enzymatic activity within dentin powder or the hybrid layer, immediately or after aging in artificial saliva at 37°C.

The null hypotheses were as follows:

1) The cross-linkers do not influence bond strength, immediately or after aging in artificial saliva at 37°C.

2) The cross-linkers do not influence the enzymatic activity within dentin powder or the hybrid layer, immediately or after aging in artificial saliva at 37°C.

CHAPTER 4
Experimental Studies

STUDY 1

Dentin Cross-linking Effect of Carbodiimide After 5 Years

The objective of the present study was to evaluate the ability of an EDC-containing aqueous primer applied during etch-and-rinse and self-etch adhesive procedures to preserve bond strength and collagen structure and inactivate MMPs after 5 y of accelerated aging in artificial saliva at 37 °C.

The null hypotheses tested were that dentin preconditioning with an EDC-containing primer prior to the adhesive resin application does not:

1) preserve bond strength
2) inactivate endogenous enzymes
3) influence the chemical profile of the HL at baseline (T0) and after 5 y of accelerated aging (T5).

Materials and methods

Microtensile Bond Strength

Human noncarious molars (n = 32, sample size determined with G*Power 3.1.9.7 for Windows; Faul et al. 2007) were used within 1 month after extraction. Each tooth was cut into a 4-mm-thick slab with a slow-speed diamond saw (Micromet; Remet) under water cooling.

Following exposure of the medium/deep dentin, a standardized smear layer was created on the dentin surface with wet silicon-carbide paper (600 grit) for 30 s. The tooth slabs were randomly allocated to one of the following groups and restored by an experienced practitioner:

Group 1: dentin etched for 15 s with 35% phosphoric acid (3M ESPE), rinsed abundantly with water, pretreated with 0.3M EDC-containing aqueous primer for 1 min, blot dried, and bonded with XP Bond (XPB; Dentsply Sirona) according to the manufacturer's instructions.

Group 2 (control): dentin prepared as in group 1 but with- out EDC pretreatment.

Group 3*:* dentin treated with Clearfil SE (CSE; Kuraray Noritake Dental Co. Ltd.) bond primer, air-dried, treated with the 0.3M EDC primer for 1 min, and bonded with the CSE bond according to the manufacturer's instructions.

Group 4 (control): dentin prepared as in group 3 but with- out EDC pretreatment.

All the adhesives were light cured with a light-emitting diode curing unit (output, 800 mW/cm^2) for 20 s. A 4-mm– thick core buildup was created with a hybrid resin composite (Filtek Z250, 3M ESPE) in each of the bonded teeth in 1-mm– thick increments. Each increment was light cured for 20 s. Bonded teeth were sectioned into 0.9 × 0.9 × 8–mm sticks (± 0.01 mm). Half of the randomly chosen sticks from each tooth were stored in artificial saliva for 24 h at 37 °C (designated as T0). The artificial saliva (pH 7.4) comprised the following (mmol/L): CaCl (0.7), MgCl ·6H2O (0.2), KH PO (4.0), KCl (30), NaN_3 (0.3), and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid buffer (20; Pashley et al. 2004). The other half of the sticks from each tooth were aged for 5 y in artificial saliva at 37 °C, with the saliva replaced every 2 wk (designated as T5). After aging, the bonded area of each specimen was calculated and was stressed in tension in a testing machine (Bisco Inc.) at a stretching speed of 1 mm/min until failure. Since the prematurely debonded specimens (1.2%) were evenly distributed among groups, they were not included in the statistical analysis. Fracture modes (A, adhesive failure; CC, cohesive in composite resin; CD, cohesive in dentin; M, mixed failure) were analyzed with a stereomicroscope at 30× magnification.

Three debonded sticks from each group with bond strength that approximated the mean of that group were fixed with 2.5% glutaraldehyde-cacodylate buffer (3 h, pH 7.4), dehydrated

in ascending concentrations of ethanol (50% to 100%), and dried with hexamethyldisilazane. Specimens were mounted on aluminum stubs, coated with gold-palladium, and examined with a field emission scanning electron microscope (FE-SEM; Nova NanoSEM 450 [FEI Co.]).

In Situ Zymography

Additional non-carious human molars (n = 5, sample size deter- mined per G*Power) were used to investigate gelatinolytic activity within the HL. The crown of each tooth was sectioned transversally in two 1-mm–thick slices that contained medium/ deep dentin. These slices were divided into 2 pieces (4 pieces in toto) and randomly assigned to 4 groups. This experimental design enabled all groups to be examined with the same dentinal substrate. The dentin pieces were bonded in the same manner as previously described. A 1-mm–thick composite buildup was made with the Filtek Z250 resin composite. The bonded and restored dentin pieces were sectioned to expose the HL. Half of the sectioned specimens from each group were tested after 24 h of storage in artificial saliva at 37 °C (T0), while the other half was aged for 5 y in artificial saliva (T5), as previously described.

After aging, in situ zymography was performed per the method reported by Mazzoni et al. (2012). Briefly, each specimen was glued to a microscope slide (2 specimens from each tooth per group) and progressively polished with wet silicon carbide papers with increasingly fine grit size (600, 1,200, 4,000) until ~50 μm thick and coated with fluorescein-conjugated gelatin solution (E-12055; Molecular Probes). The specimens were placed in a humid chamber and kept overnight in the dark at 37 °C. They were then examined with a confocal microscope (Leica SP8; Leica Microsystems GmbH; excitation/emission wavelength, 488/530 nm). Three z-stack images (1-μm interlayer distance) were made for each specimen (in the middle and toward both ends) by a researcher who was unaware of the designated groups. Quantification of the integrated density of the fluorescence signal was performed at the same level for each image via image analysis software (ImageJ; National Institutes of Health). A standardized rectangular selected area was used for all images. Differences in the level of fluorescence among the tested groups were statistically analyzed.

Micro–Raman Spectroscopy

The chemical profile of resin-dentin interfaces (n = 3 teeth per group, 2 bonded sticks per tooth) was examined with a dispersive Raman spectrometer/microscope (Horiba Scientific

Xplora). Specimens were polished with 1,500-grit silicon car- bide paper for 30 s and analyzed with a 638-nm diode laser (1-μm spot diameter) at 100 mW, an X100/0.90 NA air objective, and at 600 lines per millimeter of grating. The bonded interface was scanned from 400 to 2,000 cm^{-1}, with 30-s accumulation time with 5 coadditions. Spectra were acquired at 3 random sites from the top of the HL to the underlying dentin at 1-μm–thick intervals. Eleven spectra were acquired for each site via a computer-controlled x-y-z stage. The acquired spectra were analyzed by Labspec 6 software (Horiba). The band ratio between the intensity of the pyridinium ring and phenyl vibrations (1,032 cm^{-1}/1,003 cm^{-1}) was used to obtain information on the cross-linking effect of EDC.

Statistical Analyses

For the microtensile bond strength (primary outcome) and in situ zymography (secondary outcome), data were homogeneous (modified Levene test) and normally distributed (Kolmogorov-Smirnov test). As such, 3-way analysis of variance (ANOVA) and post hoc Tukey tests were performed to identify the effects of 3 variables—EDC pretreatment (with/ without), aging (T0/T5), and adhesive system (XPB/CSE) — and their interactions on bond strength and gelatinolytic activity. A random effect for the tooth variable was included to account for the split-tooth design. One-way ANOVA and post hoc Bonferroni tests were conducted to evaluate differences among the groups. Statistical significance was preset at $\alpha = 0.05$ (Stata 12.0; StataCorp).

Results

Results of the microtensile bond strength test are presented in the Table 1. Bond strength was significantly affected by aging and EDC pretreatment, with higher bond strength at T0 ($P = 0.0002$) as well as in groups pretreated with EDC ($P = 0.0223$), while adhesive system (the main variable) and all the possible interactions did not exert any effect on bond strength ($P < 0.05$). When the groups were individually considered, the increase in bond strength in the EDC-treated XPB groups was statistically significant ($P < 0.05$; 27% at T0 and 29% at T5). No significant difference was observed in the EDC-treated CSE groups (9% at T0 and 13% at T5).

The majority of the failure modes in the XPB groups were A failure, regardless of aging or EDC-treatment. In the CSE groups, A and CC failures were the most prevalent (Table 1).

Table 1. Results of Microtensile Test at T0 and T5 With and Without EDC (Carbodiimide).

		XP Bond		Clearfil SE Bond	
		No EDC (Control)	EDC	No EDC (Control)	EDC
T0		34.6 ± 13.6^{Ab}	44.1 ± 16.7 ^{Ab}	38.2 ± 9.9 ^{Aab}	41.6 ± 19.2 ^{Aa}
	A	82	65	39	57
	C	9	4	35	9
	CD	8	27	17	25
	M	0	4	9	9
T5		28.8 ± 12.3^{Ab}	37.0 ± 13.4 ^{Aa}	29.2 ± 9.6 ^{Bab}	33.1 ± 13.4 ^{Ba}
	A	67	60	24	46
	C	34	27	50	27
	CD	5	5	15	20
	M	5	7	11	7

n = 8 teeth per group. Data are expressed in MPa (mean ± SD) and percentage of failure.

T0, data obtained after 24 h of storage at 37 °C; T5, data obtained after 5 y of aging in artificial saliva at 37 °C.

A, adhesive failure; CC, cohesive failure in composite; CD, cohesive failure in dentin; M, mixed failure.

^{AB}Different superscript uppercase letters indicate differences ($P < 0.05$) within the columns.

^{ab}Different superscript lowercase letters indicate differences ($P < 0.05$) within the rows.

FE-SEM demonstrated differences in the dentin structure between the adhesives. The XPB groups had M failures that displayed demineralized collagen fibrils and open dentinal tubules (Fig. 11).

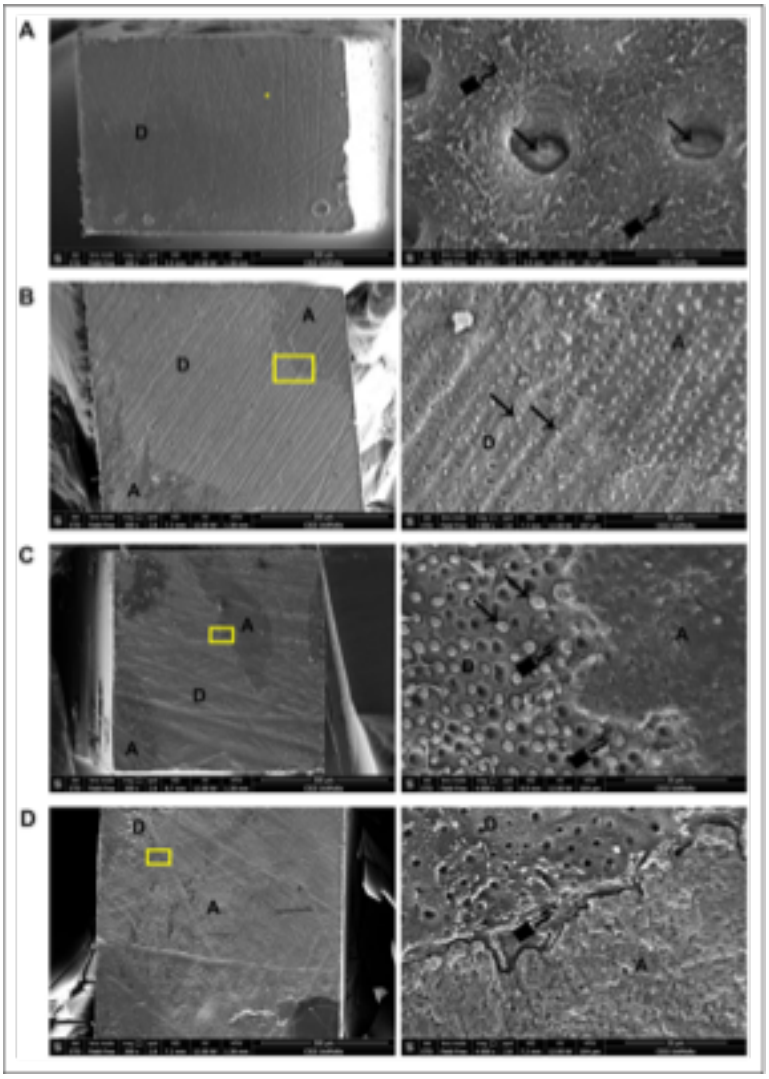


Figure 11. Field emission scanning electron microscope micrographs of the adhesive interfaces of fractured microtensile bond strength sticks (the dentin side) for the XPB groups: left, view of the whole adhesive surface; right, enlarged view of the area marked with the yellow selection. (A) XPB T0: dentin surface after adhesive failure. Denuded collagen fibrils and a small number of resin tags can be identified within the dentin tubules (bars: 500 μm, left; 5 μm, right). (B) EDC XPB T0: mixed failure with a fine layer of adhesive resin adhering to the dentin surface (bars: 500 μm, left; 50 μm, right). (C) XPB T5: mixed failure with visible dentin tubules containing resin tags and sparse denuded collagen fibrils (bars: 500 μm, left; 30 μm, right). (D) EDC XPB T5: mixed fracture with the majority of the adhesive interface covered by adhesive resin. The dentin portion is free of resin tags, with sparse denuded collagen fibrils (bars: 500 μm, left; 30 μm, right). Black arrow, resin tag in the dentinal tubule. Pointer, denuded collagen fibrils. A, adhesive resin; D, dentin; EDC, carbodiimide-pretreated group; T0, 24-h aging; T5, 5-y aging; XPB, XP Bond.

In contrast, CSE groups showed M failures with occluded dentin tubules covered with smear layers (Fig. 12).

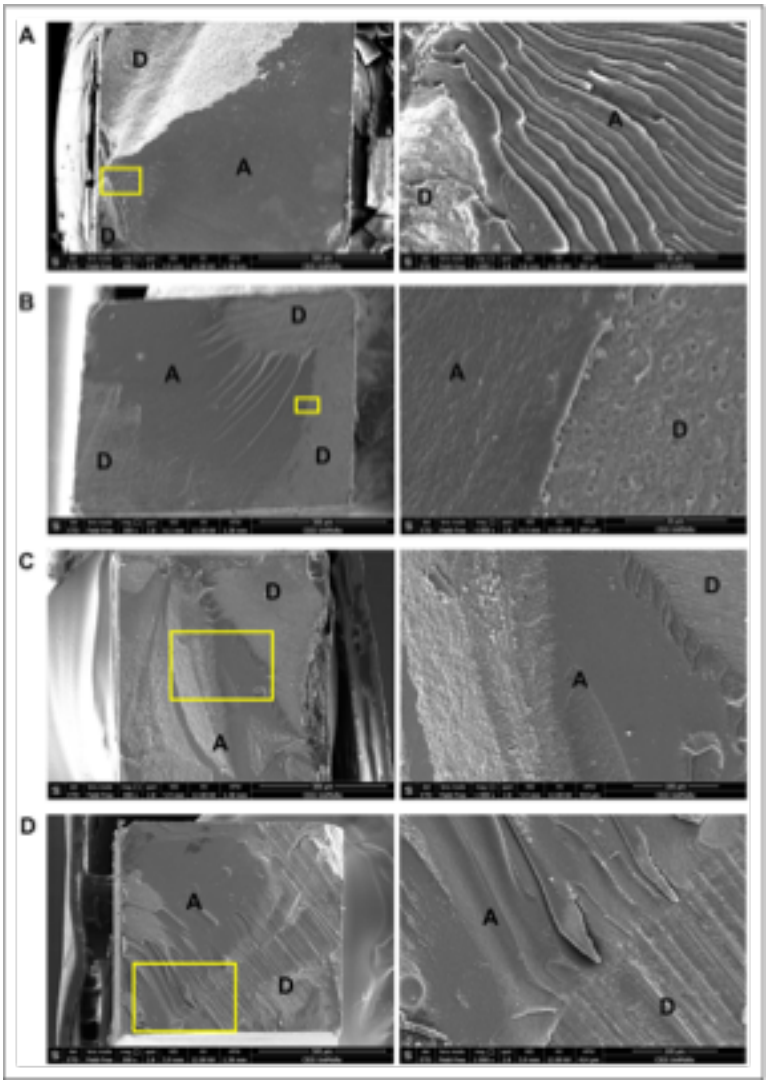


Figure 12. Field emission scanning electron microscope micrographs of the adhesive interfaces of fractured microtensile bond strength sticks (the dentin side) for the CSE groups: left, view of the whole adhesive surface; right, enlarged view of the area marked with the yellow selection. (A) CSE T0 group: mixed failure. Flat resin surface with chevron marks (river lines) of the fractured adhesive resin created during crack propagation. Side chipping of the dentin is evident. Dentin tubules are occluded by a smear layer (bars: 500 μm, left; 50 μm, right). (B) EDC CSE T0: mixed failure with a thicker layer of adhesive resin adhering to the dentin surface. Dentin tubules are occluded by a smear layer (bars: 500 μm, left; 30 μm, right). (C) CSE T5: mixed failure with a layer of adhesive resin adhering to the dentin surface. Dentin tubules occluded by a smear layer (bars: 500 μm, left; 100 μm, right). (D) EDC CSE T5: mixed failure. Dentin tubules occluded by a smear layer (bars: 500 μm, left; 100 μm, right). A, adhesive resin; CSE, Clearfil SE bond; D, dentin; EDC, carbodiimide-pretreated group; T0, 24-h aging; T5, 5-y aging.

For in situ zymography, EDC pretreatment (P = 0.0001), aging (P = 0.0001), and adhesive (P = 0.0003), as well as the interaction pretreatment × adhesive (P = 0.0341), significantly influenced dentin gelatinolytic activity. In all the tested groups, fluorescence density level was higher after aging. The EDC silenced enzymatic activity more efficiently in the XPB groups. One-way ANOVA revealed a significant decrease in the gelatinolytic activity in all the EDC-pretreated groups except for CSE at T5. Fluorescence was detected in the HL as well as in the underlying dentinal tubules (Fig. 13).

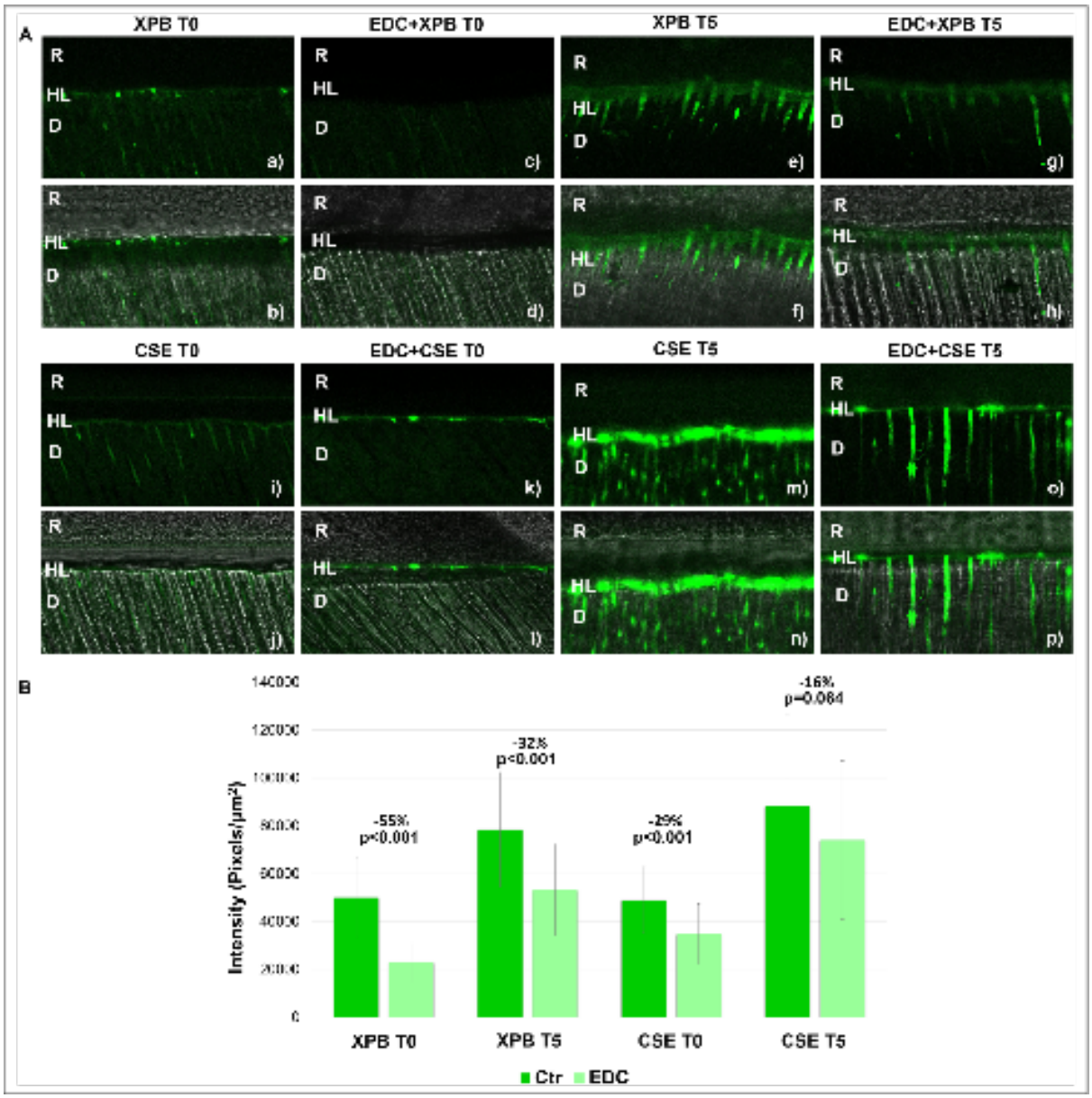


Figure 13. In situ zymography results. (A) Resin-dentin interfaces incubated with quenched fluorescein-labeled gelatin. (a, c, e, g, i, k, m, o) Images acquired in the green channel show fluorescence in dentinal tubules and within the HL of the tested groups. Fluorescence is lower in the groups treated with EDC primer and at T0 as compared with the aged specimens. (b, d, f, h, j, l, n, p) Image obtained by merging the differential interference

contrast image, showing the optical density of the resin-dentin interface, and the image acquired in the green channel. (B) Quantification of the gelatinolytic activity within the resin-dentin interfaces of the tested groups. Mean values of the fluorescence signal intensity and SDs are presented on the graph. CSE, Clearfil SE bond; Ctr, control; D, dentin; EDC, carbodiimide; HL, hybrid layer; R, resin composite; T0, 24-h aging; T5, 5-y aging; XPB, XP Bond.

Representative Raman spectra of the experimental groups are shown in Figure 14. Spectra recorded in the range of 400 to 2,000 cm^{-1} covered the fingerprint region associated with the HL and dentin collagen bands. Peaks assigned to adhesives were documented (C─O─C at 1,113 cm^{-1}, C═C at 1,610 cm^{-1}, C═O at 1,720 cm^{-1}). The relative decrease of intensity of these peaks represented the transition through the dentin. Dentin collagen bands also became evident: amide I at 1,667 cm^{-1}, amide III at 1,243 cm^{-1} and 1,273 cm^{-1}; the phenyl group at 1,003 cm^{-1} of the aromatic ring of the phenylalanine residues of collagen; and the pyridinium ring at 1,032 cm^{-1}, which has a tri- valent amino acid cross-linking residue. In addition, functional groups of carbonated apatite (phosphate at 962 cm^{-1} and carbonate 1,072 cm^{-1}) were detected within the mineralized den- tin. More aggressive demineralization was detected in the XPB groups, as shown by the abrupt reduction in the intensity of carbonate and phosphate peaks within the transition region, while mineral-associated peaks were more pronounced in the CSE groups. After aging, the intensity of the mineral-associated peaks dropped for the XPB groups (especially control), while they remained stable over time for the CSE groups.

Collagen changes were identified in the amide III peaks (1,243 to 1,248 cm^{-1}) and from 1,273 to 1,278 cm^{-1} in all EDC-treated groups, regardless of aging. In addition, the second peak of the doublet at 1,273 cm^{-1} was less well defined in the groups without EDC at T5; for XPB at T5, this peak almost disappeared. Furthermore, a drop of adhesive peaks was noted for all the T5 groups, except the EDC XPB, where well-pronounced adhesive bands were still detected.

Pretreatment with EDC did not alter the pyridinium/phenyl band ratio at T0, regardless of the adhesive system used. This band ratio was not reduced at T5 for the EDC groups in comparison with their respective adhesive sub- groups at T0 (cross-linking pyridinium/phenyl: XPB T0, 1.02; XPB T5, 0.98; EDC + XPB T0, 1.03; EDC + XPB T5, 1.16; CSE T0, 1.18; CSE T5, 1.02; EDC + CSE T0, 1.19; EDC + CSE T5, 1.19). Additional peaks assigned to EDC could not be identified.

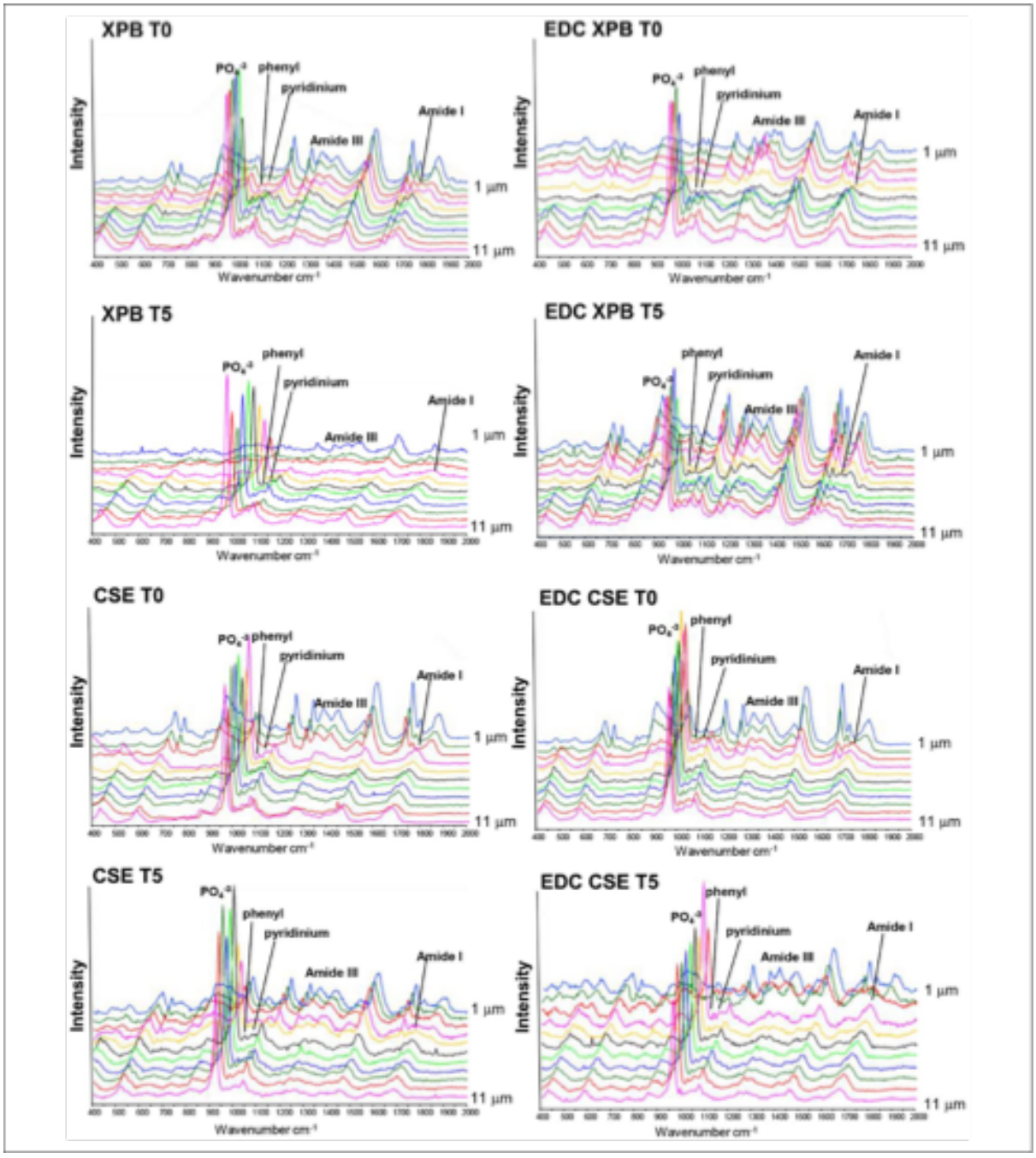


Figure 14. Micro-Raman line spectra acquired at the adhesive-dentin interface created by XPB and CSE with the application of the EDC primer and without at T0 and T5. Each spectrum was acquired from the middle of the hybrid layer (top spectra) to the underlaying dentin (bottom spectra). The relative peaks associated with methacrylate monomers, mineralized and demineralized dentin in the hybrid layer, were observed in both groups. Changes were identified at amide III peaks (1,243 to 1,248 cm−1 and 1,273 to 1,278 cm−1) for all EDC-treated groups. The band ratios between intensity of pyridinium ring and phenyl vibrations (1,032 cm−1/1,003 cm−1) were used for evaluation of the cross-linking effect of EDC. No difference was detected for EDC treatment of each adhesive subgroup at T0. This ratio was not reduced at T5 for the EDC groups when compared with the ratio at T0 for both adhesives. CSE, Clearfil SE bond; EDC, carbodiimide; T0, 24-h aging; T5, 5-y aging; XPB, XP Bond.

STUDY 2

Effect of an ethanol cross-linker on universal adhesive

The aim of the study was to evaluate the effects of N,N'-dicyclohexylcarbodiimide (DCC), an ethanol-based dentin cross-linker, on the immediate and long-term microtensile bond strength (μTBS) and nanoleakage expression of a universal adhesive employed in self-etch mode (SE) or etch- and-rinse mode (ER). The effect of DCC on the dentinal MMP activity was also investigated by means of in-situ zymography.

The null hypotheses tested were that preconditioning of dentin with DCC prior to adhesive application:

1) Does not benefit the immediate bonding performance of a universal adhesive to dentin;
2) Does not prevent interfacial degradation over time;
3) Does not inhibit endogenous dentin MMP activity.

Materials and methods

Freshly-extracted sound human third molars (N = 120) were obtained from anonymous individuals following their signed consent under the protocol ASL BO N◦ 0013852, approved on 02/01/2019 by the Ethics Committee.

Microtensile bond strength test (μTBS)

Eighty teeth were selected to conduct μTBS testing. The occlusal surface of each tooth was cut transversely to the long axis to expose mid-coronal dentin using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) with copious water cooling. A standardized smear layer was created with 600-grit silicon-carbide paper on each tooth surface. The polished teeth were randomly assigned to one of the following groups according to the dentin surface treatment and adhesive approach performed (N = 20) (Table 2):

Group 1 (G1): DCC pre-treatment and Scotchbond Universal adhesive (SBU; 3M ESPE, St. Paul, MN, USA used in the etch- and-rinse mode (ER). The dentin surface was etched with 32% H3PO4 (Scotchbond Universal Etchant, 3M ESPE) for 15 s, pre-treated with an ethanol solution of 0.5M DCC for 1 min, air-dried and bonded with SBU according to the manufacturer's instructions (Table 2).

Group 2 (G2): SBU in ER mode. No DCC was applied on dentin; SBU application was the same as G1.

Group 3 (G3): DCC pre-treatment and SBU in self-etch mode (SE). The dentin surface was pre-treated as in G1; instead of etching with 32% H3PO4, SBU was applied directly to the smear layer-covered dentin and agitated for 20 s. The adhesive was air-dried. Without light-curing, DCC was applied for 1 min. The second layer of universal adhesive was subsequently applied, air-dried and light-cured (Table 2).

Group 4 (G4): SBU in SE mode. No DCC was applied on dentin. SBU application was the same as G3.

Table 2 – Adhesive system, composite composition and application mode.

Material	Composition	ER mode	DCC + ER mode	SE mode	DCC + SE mode
Scotchbond Universal (SBU; 3M ESPE)	1. Etchant: 32% phosphoric acid, water, synthetic amorphous silica, polyethylene glycol, aluminum oxide (Scotchbond Universal Etchant) 2. Adhesive: methacryloyloxydecyl dihydrogen phosphate (MDP) phosphate monomer, dimethacrylate resins, 2-hydroxyethyl methacrylate (HEMA), methacrylate-modified polyalkenoic acid copolymer, filler, ethanol, water, initiators, and silane.	1. Apply etchant for 15 s 2. Rinse for10s3.Airdry 5s4.Applythe adhesive to the entire preparation with a microbrush and rub it in for 20s5.Directa gentle stream of air over the liquid for 5 s until it no longer moves and the solvent is evaporated completely 6. Repeat steps 4 and 5 7. Light-cure for 20 s.	1. Apply 0.5M DCC ethanol-based primer and brush it for 1 min 2. Direct a gentle stream of air over the liquid for 5 s 3. Apply adhesive as for the ER mode.	1. Apply adhesive to the entire preparation with a microbrush and rub it in for 20 s 2. Direct a gentle stream of air over the liquid for 5 s until it no longer moves and the solvent is evaporated completely 3. Repeat steps 2 and34. Light-cure for 20 s.	1. Apply first coat of adhesive 2. Apply 0.5M DCC ethanol-based primer and brush it for 1 min 3. Direct a gentle stream of air over the liquid for 5 s 3. Apply adhesive as for the SE mode.
Filtek Z250 (3M ESPE)	Triethyleneglycol dimetacrylate (TEGDMA) < 1–5%; Bisphenol-A-glycidylmethacrylate (Bis-GMA) < 1–5%; Bisphenol-A polyethylenglycol dietherdimethacrylate (Bis-EMA) 5–10%; Urethane dimethacrylate (UDMA) 5–10% Fillers: Zirconia/silica; 60 vol% inorganic fillers (particle size 0.01–3.5 ⍰m)				

Each bonded specimen was light-cured for 20 s using a light-emitting diode curing light (DemiTM Plus, Kerr Corp., Brea, CA, USA) after solvent evaporation. Four 1-mm thick layers of a micro-hybrid resin composite (Filtek Z250; 3M ESPE) were incrementally placed over the bonded dentin and individually polymerized for 20 s each to obtain a 4-mm thick composite build up for μTBS testing. Each specimen was serially-sectioned to obtain approximately 1-mm thick sticks, each containing resin composite and dentin and with the adhesive interface in between, in accordance with the non- trimming technique of the μTBS test. The dimension of each stick (0.9 mm × 0.9 mm ± 0.01 mm) was recorded using a pair of digital calipers. The bonded area was calculated for subsequent conversion of microtensile strength values into units of stress (MPa). Sticks from each tooth were randomly assigned to two storage groups: 24 h (T0) or 1 year (T12) of stor- age in artificial saliva at 37 ◦C. The artificial saliva consisted of CaCl2 (0.7 mmoles/L), MgCl2 6H2O (0.2 mmoles/L), KH2PO4 (4.0 mmoles/L), KCl (30 mmoles/L), NaN3(0.3 mmoles/L) in HEPES buffer (Sabatini et al., 2015).

Each stick was stressed to failure under tension 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 group was recorded, but those null values were not included in the statistical analysis. This is because all premature failures occurred during the cutting procedure and those failures 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 GmbH, Jena, Germany) at 30× magnification. Failure modes were classifieds adhesive failure (A), cohesive failure in dentin (CD), cohesive failure in composite (CC) or mixed failure (M).

Statistical 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 were evaluated for compliance with the normality assumption using Shapiro-Wilk test, and the homoscedasticity assumption using the modified Levene test prior to the use of parametric analytical methods. A three-way analysis of variance (ANOVA) was performed to identify the effects of three variables, DCC pre-treatment (with/without), adhesive application mode (ER/SE) and aging (T0 /T12) and their interactions on bond strength. Post-hoc comparisons were conducted using Tukey test. Additionally, one-way ANOVA was conducted to evaluate differences within each variable. For all tests, statistical significance was pre-set at $\alpha = 0.05$. Statisti- cal analyses were performed using Stata 12.0 software for Mac (StataCorp, College Station, TX, USA).

Nanoleakage expression

Twenty teeth (N = 5) were used for examination of nanoleakage within the resin-dentin interface. Mid-coronal dentin was bonded in the same manner described for μTBS testing. Each specimen was cut vertically into 1-mm-thick slabs to expose the resin-dentin interface. After storage in artificial saliva at 37 ◦C for 24 h (T0) or 12 months (T12), the specimens were immersed in 50 wt.% ammoniacal AgNO3 solution for 24 h in the dark, following the protocol described by Tay et al. (Franklin R. Tay et al., 2002). The specimens were then 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 grains within voids along the bonded interfaces.

For light microscopy, the specimens were fixed, dehydrated, embedded in epoxy resin (LR White resin, Millipore- Sigma, Burlington, MA, USA), fixed on glass slides using cyanoacrylate glue, flattened on a grinding device (LS2; Remet, Bologna, Italy) under water irrigation and polished with a graded series of silicon carbide abrasive papers of increasing fineness (180-, 600-, 1200-, 2400-, and 4000-grit). The presence of the silver tracer was examined along the bonded interface using light microscopy (E800; Nikon, Tokyo, Japan), at 20× magnification. Interfacial nanoleakage expression was scored by two trained investigators based on the percentage of adhesive surface showing AgNO3 deposition, following the method of Saboia et al. (Saboia et al., 2008). A scale 0–4 was used for evaluation: (0) no nanoleakage; (1) <25% surface with nanoleakage; (2) 25–50% surface with nanoleakage; (3) 50–75% surface with nanoleakage; and (4) >75% surface with nanoleakage. Intra-examiner reliability was evaluated using the Cohen's kappa (κ) statistic. Statistical differences among nanoleakage scores were analysed with the chi-square statistic. Statistical significance was pre-set at α = 0.05.

In-situ zymography

Twenty freshly-extracted human third molars (N = 5) were used for in-situ zymography. One mm-thick slabs of middle/deep dentin were prepared. Each slab was further divided into four parts to test the 4 control land experimental groups on the same substrate (Figure 15). Silicon carbide paper (600-grit). was used to create a standardized smear layer on each dentin surface. One surface of each quarter of a slab was treated with the adhesive systems as described for μTBS testing. was used to create a standardized smear

layer on each dentin surface. One surface of each quarter of a slab was treated with the adhesive systems as described for μTBS testing.

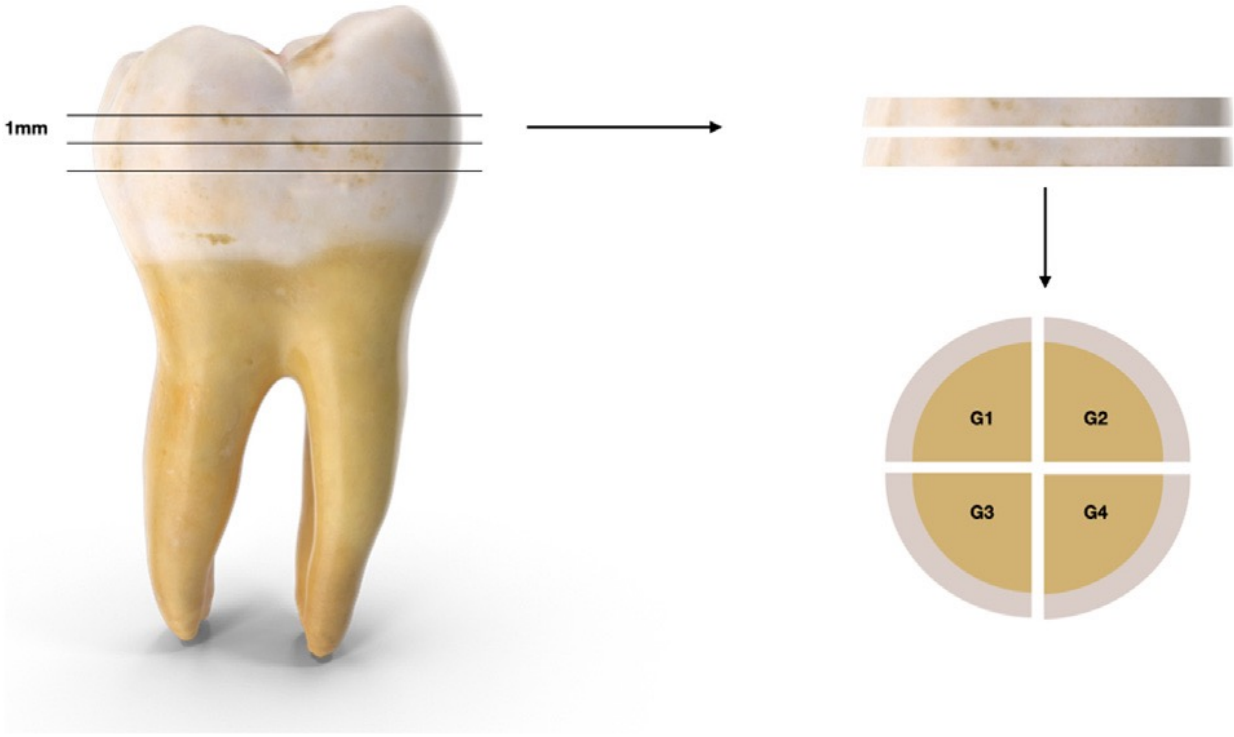


Figure 15. Schematic of tooth preparation for in-situ zymography. A dentin disk (1-mm thick) was divided into four quadrants, enabling bonding procedures of the four control and experimental groups to be performed on the same dentin substrate.

The procedure was performed using the method previously reported by Mazzoni et al. (Mazzoni et al., 2014, 2012). After aging for the designated period (24 h or 1 year), 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, Eugene, OR, USA) 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 anti-fading agent (Vectashield mounting medium with 4′,6- diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA). 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 hybrid layer was based on hydrolysis of the quenched fluorescein-conjugated gelatin substrate. The process was evaluated by examining the glass slides with a multi-photon confocal laser scanning microscope (LSM 5 Pa; Carl Zeiss), using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Samples were imaged using a HCX PL APO 40×/1.25 NA oil immersion objective. Series of x- y-z images (0.145*0.145*1 μm3 voxel size) were collected. Laser power and detector gain were set at the beginning of the experiment and kept the same for all specimens in order to have the possibility to compare different groups. Sixteen to 20 optical sections were acquired 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 Institute of Health, Bethesda, MD, USA). The amount of gelatinolytic activity was expressed as a percentage of the green fluorescence within the hybrid layer.

Negative control sections were similarly incubated, with the exception that 250 mL ethylenediaminetetraacetic acid (EDTA) or 2 mM 1,10-phenanthroline was dissolved in the mixture of quenched fluorescein-conjugated gelatin. The EDTA- and 1,10-phenanthroline-containing gelatin were used as negative controls. In addition, standard non-fluorescent gelatin was used as the third negative control.

Because the in-situ zymography data complied with normality and homoscedasticity assumptions after non-linear transformation, a three-way ANOVA was used to identify the effects of the three variables, DCC pre-treatment, adhesive application mode and aging, on the density of fluorescence signals. Additional one-way ANOVA was conducted to evaluate differences within each variable. Statistical significance was pre-set at $\alpha = 0.05$.

Results

Microtensile bond strength

Microtensile bonds strengths of the four groups tested at T0 and T12 are summarized in Table 3. Three-factor ANOVA revealed significant difference for the variables DCC pre-treatment, adhesive application mode and aging ($p < 0.05$), as well as for the interaction between DCC pre-treatment and adhesive application mode ($p < 0.05$). Post-hoc com- parisons showed that the use of a 0.5 M DCC-containing ethanol solution before adhesive application improved bond strength of SBU (G1 and G3) vs control groups (G2 and G4) ($p < 0.05$),

irrespective of the adhesive application mode and aging. Additionally, SBU generated higher bond strength when employed in the ER mode vs the SE mode ($p < 0.05$). Aging significantly reduced μTBS among all the adhesive application mode/dentin pre-treatment combinations, except for G3 ($p < 0.05$). One-way ANOVA indicated that at T0, DCC pretreatment significantly improved μTBS in both experimental groups compared to the control groups (G1 46.0 ± 15.3; G2 37.1 ± 12.5; G3 39.4 ± 11.1; G4 26.3 ± 11.4). After aging, DCC pretreatment showed a preservation of the bond strength when SBU was applied in the SE mode (T0 = 39.4 ± 11.1 and T12 = 35.3 ± 13.9) thus, shoving a final MPa value after aging comparable to that of the SBU ER groups. For all groups at T0 or T12 the predominant failure modes were the adhesive failure and the mixed failure. At baseline, for all groups, except for G2 the adhesive failures were around 70% of the failure. After aging, the number of mixed failures increased between 40% and 55% in the different groups (Table 4).

Table 3: Results of μTBS test at T0 and T12.

Application mode	**Application SBU (ER)[a]**		**SBU (SE)a**	
Pre-treatment	G1	G2	G3	G4
T_0	$46.0 \pm 15.3^{A,a}$	$37.1 \pm 12.5^{A,b}$	$39.4 \pm 11.1^{A,a,b}$	$26.,3 \pm 11.4^{A,c}$
T_{12}	$33.5^{B} \pm 13.9^{B,a}$	$31.0 \pm 11.0^{B,a}$	$35.3 \pm 13.9^{A,a}$	$13.4 \pm 9.1^{B,b}$
Different superscript upper-case letters indicate differences ($p < 0.05$) within the columns. Different superscript lower case letters indicate differences ($p < 0.05$) within the rows. a Values are means ± standard deviations (in MPa).				

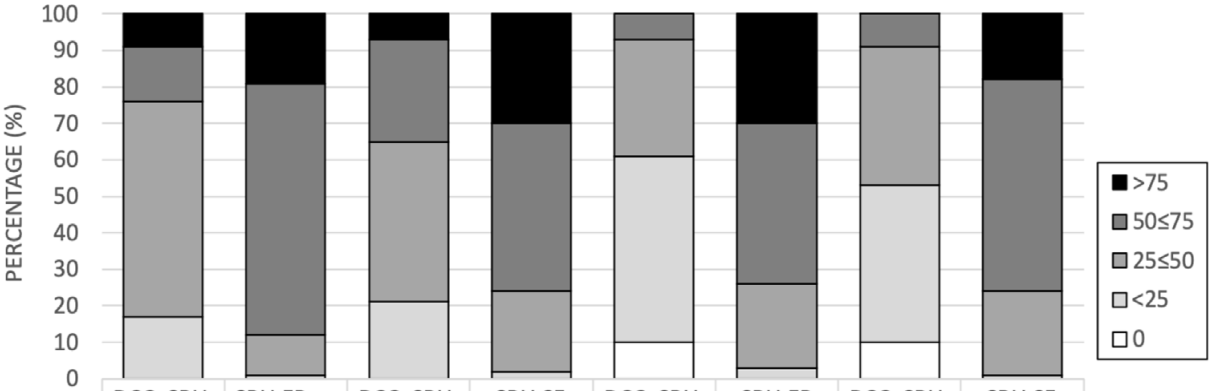
SBU (ER) Scotchbond Universal used in the etch-and-rinse mode; SBU (SE): Scotchbond Universal used in the self-etch mode. G1: 0.5M DCC SBU (ER); G2: SBU (ER), control; G3: 0.5M DCC + SBU (SE); G4: SBU (SE), control. T0: Data obtained after 24 h of storage at 37 ◦C. T12: Data obtained after 1 year of aging in artificial saliva at 37 ◦C.

Table 4: Percentages of failures mode among the different groups.

Application mode	**Application SBU (ER)[a]**		**SBU (SE)a**	
Pre-treatment	G1	G2	G3	G4
T_0	68% A 32% M	35% A 8% CD 57%	81% A 1% CD 18%	74% A 36% M
T_{12}	45% A 55% M	M 58% A 42% M	M 53% A 47% M	60% A 40% M
Different superscript upper-case letters indicate differences ($p < 0.05$) within the columns. Different superscript lower case letters indicate differences ($p < 0.05$) within the rows. a Values are means ± standard deviations (in MPa).				

Nanoleakage expression

Descriptive statistics of interfacial leakage scores are represented in Figure 16. Statistically significant differences were found among the four groups in the extent of silver nitrate penetration along the adhesive interfaces ($p < 0.05$). Specimens that were pretreated with DCC-containing solution prior to adhesive application showed lower nanoleakage expression in both the ER and SE modes, compared to control groups, at both T0 and T12 ($p < 0.05$). At baseline SBU ER and SBU SE showed a higher percentage of marginal infiltration compared to the experimental groups. However, SBU SE performed worse than SBU ER. After aging in artificial saliva, DCC pretreated groups shoved a significantly lower marginal leakage than the control groups. In addition, DCC SBU groups showed comparable results among them.



	DCC+SBU ER T0	SBU ER T0	DCC+SBU SE T0	SBU SE T0	DCC+SBU ER T12	SBU ER T12	DCC+SBU SE T12	SBU SE T12
>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 16: Distribution of interfacial nanoleakage (in %) in the resin-dentin interfaces created with the Scotchbond Universal (SBU) adhesive in the etch-and-rinse mode (ER) or the self-etch mode, with or without DCC pre-treatment of dentin. Testing was performed after 24 h (T0) or after one year of aging in artificial saliva (T12).

In-situ zymography

Representative micrographic images of the different groups are shown in Figure 17 for time T0 and Figure 8 for time T12. The percentages of hybrid layers exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin at T0 and T12 are shown in Figure 19. For all specimens, the highest enzymatic activity appeared to be concentrated in the hybrid layer and the dentinal tubules underneath the hybrid layer.

Statistical analysis of the in-situ zymography identified significant differences for the variables DCC pre-treatment, adhesive application mode and aging ($p < 0.05$), and for the interaction between application mode and aging ($p < 0.05$). For the variable "DCC pre-treatment", post-hoc comparisons showed that DCC pre-treatment significantly reduced fluorescence at the level of the hybrid layer, compared to non-treated groups ($p < 0.05$), irrespective of the adhesive application mode and the aging period. For the variable "adhesive application mode", ER groups resulted in a significantly higher enzymatic activity compared to the SE groups ($p < 0.05$), irrespective of DCC application and aging period. For the variable "aging period", especially when associated with ER mode, significant increases in fluorescence at the level of the hybrid layer was identified with the ER mode at both T0 and T12 ($p < 0.05$).

One-way ANOVA calculated across all groups indicated that at T0, there was reduced fluorescence within the hybrid layers, irrespective of the adhesive application mode, when 0.5M DCC solution was applied prior to adhesive application (G1 and G3). However, the decrease was not statistically significant for the SE mode (Figure 17). At T12, both experimental (G3 and G4) and control groups (G1 and G2) showed comparable endogenous enzymatic activity for the ER group (Figure 8). When SBU was applied in the SE mode, 0.5M DDC solution significantly reduced the activity within the hybrid layer (Figure 18).

No fluorescence was detected in two negative control groups prepared with non-specific inhibitors (EDTA or 1,10-phenanthroline) or when non-fluorescent gelatin was employed (data not shown).

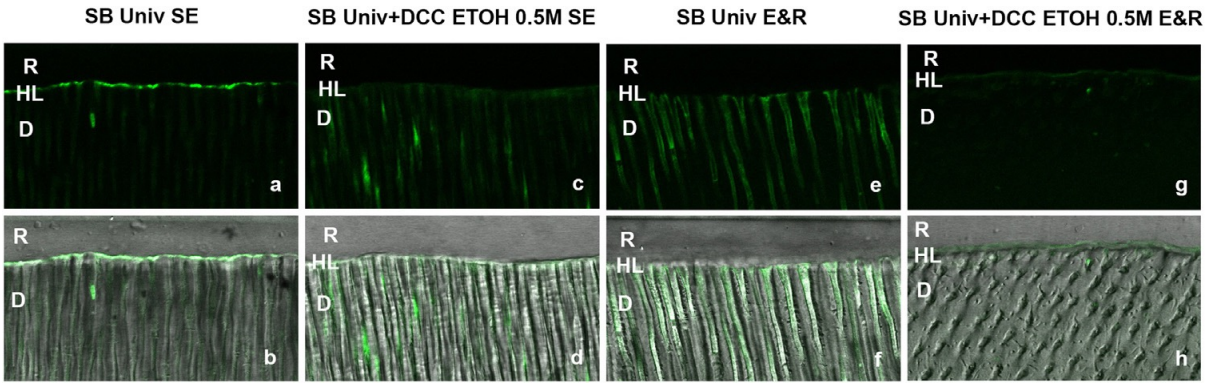


Figure 17: Representative examples of in-situ zymography of the resin-dentin interfaces at T0. Dentin treated with SBU adhesive in the SE mode (a, b); SBU (SE) + DCC 0.5M (c, d); SBU in the ER mode (e, f); SBU (ER) + DCC 0.5 M (g, h). D: dentin; HL: hybrid Layer; R: resin composite.

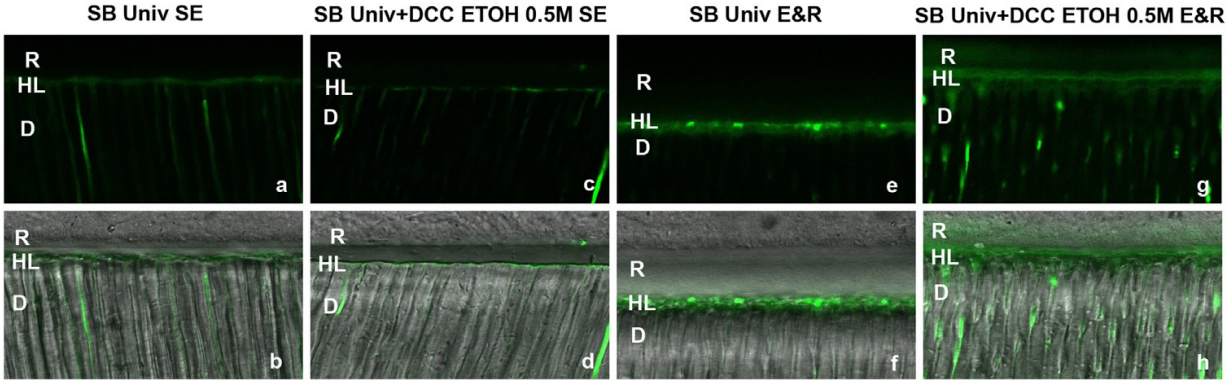


Figure 18: Representative examples of in-situ zymography of the resin-dentin interfaces at T12 (aging for 12 months in artificial saliva). Dentin treated with SBU adhesive in the SE mode (a, b); SBU (SE) + DCC 0.5M (c, d); SBU in the ER mode (e, f); SBU (ER) + DCC 0.5M (g, h). D: dentin; HL: hybrid Layer; R: resin composite.

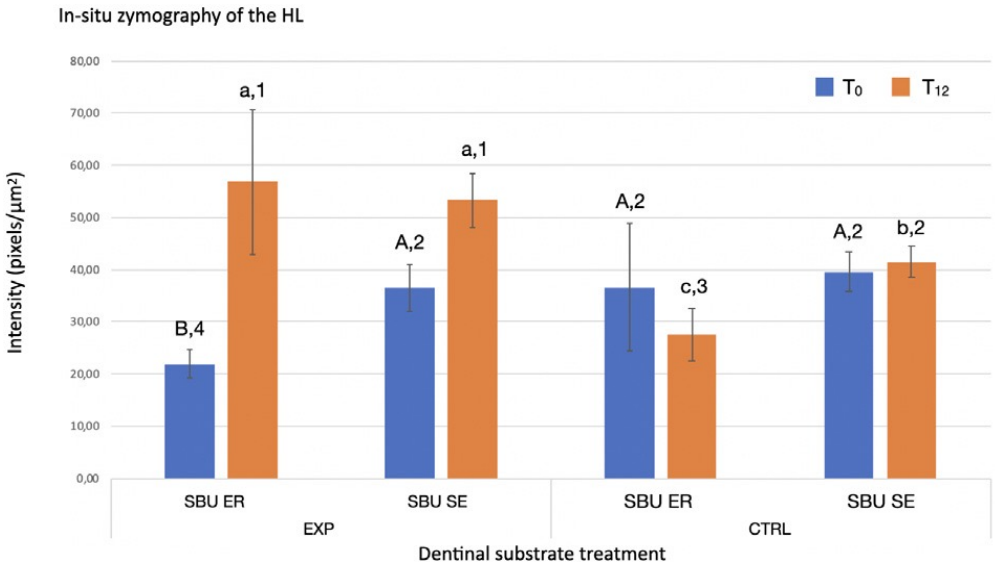


Figure 19: Gelatinolytic activity, expressed as the intensity of green fluorescence (pixels/µm2) within the hybrid layers (HL) created with SBU in ER mode or SE mode for the experimental (DCC pre-treatment) and control (no DCC pre-treatment) groups at T0 and T12. Values are means and standard deviations. For comparison of the factor "adhesive application mode", columns labelled with the same upper case letters (T0) or lower case letters (T12) are not significantly different ($p > 0.05$). For comparison of the factor "DCC pre-treatment", columns labelled with the same numerals are not significantly different ($p > 0.05$) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

STUDY 3

Endogenous Enzymatic Activity in Dentin Treated with a Chitosan Primer

The aim of the present study was to evaluate, by means of gelatin and in situ zymography, the effect of different concentrations of lyophilized chitosan polymer as a separate aqueous primer on the enzymatic activity of unrestored, as well as adhesively restored, dentin.

The null hypotheses to be tested include the following:

1) chitosan applied on acid-etched dentin does not influence MMPs activity regardless of the concentration used;
2) the application of the adhesive resin does not influence the interaction of chitosan with the endogenous dentinal enzymes.

Materials and methods

Preparation of Solutions

Three aqueous solutions of 1, 0.5 and 0.1 wt% chitosan (Chitoscience Chitosan, Heppe Medical Chitosan GmbH, Halle, Germany; average molecular weight ~50 kDa) were obtained by dissolving the lyophilized polymer in distilled water without the need for pH adjustment.

Gelatin Zymography

Ten sound human third molars were extracted after obtaining informed consent. The teeth were ground free of enamel, and pulpal tissue was completely removed. Dentin was frozen in liquid nitrogen and triturated to obtain dentin powder by using a Retsch miller (Model MM400, Retsch GmbH, Haan, Germany).

Mineralized (MD) dentin was divided into 5 experimental groups (two 100 mg aliquots per group) to be treated with 100 μL of previously prepared solutions:

Group 1 (MIN): MD left untreated;

Group 2 (DEM): MD was treated with 10 wt% phosphoric acid for 10 min at 4 ◦C, then the acid was neutralized with 4N NaOH and centrifuged;

Group 3 (1% CH): MD was etched as in Group 3 and then treated with 1% chitosan water solution for 30 min;

Group 4 (0.5% CH): MD was etched as in Group 3 and then treated with 0.5% chitosan water solution for 30 min;

Group 5 (0.1% CH): MD was etched as in Group 3 and then mixed with 0.1% chitosan water solution for 30 min.

After the treatment, dentin aliquots were rinsed twice with distilled water and centrifuged to remove the supernatant. Dentin powder aliquots were stirred with the extraction buffer (50 mM Tris–HCl pH 6 containing 5 mM CaCl2, 100 mM NaCl, 0.1% Triton X-100, 0.1% nonionic detergent P-40 and 0.1 mM ZnCl2, 0.02% NaN3) for 24h at 4 ◦C. Thereafter, the aliquots were sonicated for 10 min and then centrifuged twice in order to separate the supernatant (20 min/4 ◦C/12,000 rpm twice). The protein content was concentrated from the supernatant by using the Vivaspin centrifugal concentrator (10,000 kDa cut off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) (30 min/4 ◦C/10,000 rpm for 3 times). The total protein concentration in the dentin extracts was determined by Bradford assay.

Dentin proteins aliquots from each experimental group (60 μg) were diluted in a Laemmli sample buffer in a 4:1 ratio and electrophoresed under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) containing 1 mg/mL fluorescein-labeled gelatin. Pre-stained low-range molecular weight SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular-weight markers. After electrophoresis, the gels were washed twice for 30 min in 2% Triton X-100 and incubated in zymography activation buffer (50 mmol/L Tris–HCl, 5 mmol/L CaCl2, pH 7.4) for 48 h at 37 ◦C. Gelatinase zymograms were registered under long-wave UV light scanner (ChemiDoc Universal Hood, Bio-Rad, Hercules, CA, USA). Zymograms were quantified by taking a mineralized group as baseline and using the Image J software (NIH, Bethesda, MD, USA).

In Situ Zymography

Sound extracted third molars (N = 5, sample size determined using G*Power 3.1.9.7 for Windows, Düsseldorf, Germany) were used either within 24 h from the extraction or frozen immediately (−20 ◦C) until use. The enamel and superficial dentin were removed from all the teeth using a high-speed diamond saw with water cooling (Micromet, Remet, Bologna, Italy). When middle dentin was reached, two transversal cuts were made to obtain 1 mm thick slices of middle/deep dentin. These slices were further cut into 4 pieces, adding up to 8 dentin pieces per tooth. In order to test all the groups on the same dentin substrate, one dentin slice from each tooth was assigned to the 8 following groups:

Group 1: Dentin etched with 37% phosphoric acid for 15 s (Vococid, Voco, Cuxhaven, Germany), rinsed thoroughly, blot dried and then primed with the 1 wt% chitosan water solution for 1 min, after which it was gently air-dried for 5 s;

Group 2: Dentin etched and rinsed as in Group 1 and then primed with the 0.5 wt% chitosan water solution for 1 min, after which it was gently air-dried for 5 s;

Group 3: Dentin etched and rinsed as in Group 1 and then primed with the 0.1 wt% chitosan water solution for 1 min, after which it was gently air-dried for 5 s;

Group 4 (control): Dentin etched and rinsed as in Group 1 and gently air dried for 5 s;

Group 5: Dentin etched and pretreated as in Group 1, followed by the application of a universal adhesive system (Futurabond M, Voco, Cuxhaven, Germany) according to the manufacturer's instructions and polymerized for 10 s using a LED curing unit (Demi, Kerr, Germany) after which a 1 mm thick layer of flowable composite was applied to the bonded surface (Grandio Flow, Voco, Cuxhaven, Germany) and polymerized for 20 s;

Group 6: Dentin etched and pretreated as in Group 2, followed by the adhesive and restorative procedures as in Group 5;

Group 7: Dentin etched and pretreated as in Group 3, followed by the adhesive and restorative procedures as in Group 5;

Group 8 (control): Dentin etched as in Group 4, followed by the adhesive and restorative procedures as in Group 5.

After 24 h storage in the artificial saliva on 37 ◦C, the dentin specimens were cut into 1 mm thick sticks, glued to glass slides (2 sticks per tooth per group), ground down to approximately 50 μm thickness and subjected to the in situ zymography protocol by Mazzoni et al. (Mazzoni et al., 2012). Briefly, the samples were covered in diluted fluorescein-quenched gelatin, protected with a glass coverslip and kept in a dark humid chamber overnight at 37 ◦C, after which the specimens were observed by using a confocal microscope (Leica SP8, Leica Microsystems GmbH, Wetzlar, Germany; excitation/emission wavelength: 488/530 nm). Three images (z-stack, one image after every 1 μm into the depth of the sample) were made for each stick on randomly chosen sites by a blinded operator. Depending on the group, either the pretreated dentin surfaces or the hybrid layers were captured on the images. The integrated density of the fluorescent signal was further measured on all the images using the ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the data were statistically analyzed using SigmaPlot 14.0 (Systat Software Inc., Berkshire, UK). As the data passed the assumptions of normal distribution and homogeneity, two- way ANOVA test was used for the analysis (factors “pretreatment” and “adhesive system application”). The significance level was set at $p < 0.05$.

Results

Gelatin Zymography

Results of the gelatin zymography are shown in Figure 20. Proteins extracted from mineralized dentin powder (Lane 1) showed the expression of pro-MMP-9 with the corresponding molecular weight of 92 kDa and active form of MMP-9 (at 86 kDa). Moreover, the presence of pro-form and active forms of MMP-2 is evident (72 and 66 kDa, respectively). On the other hand, the demineralization of dentin powder with phosphoric acid (Lane 2) demonstrates an increase in the expression of MMPs, but particularly MMP-2 in pro-form and active forms. The bands are wide and less delineated.

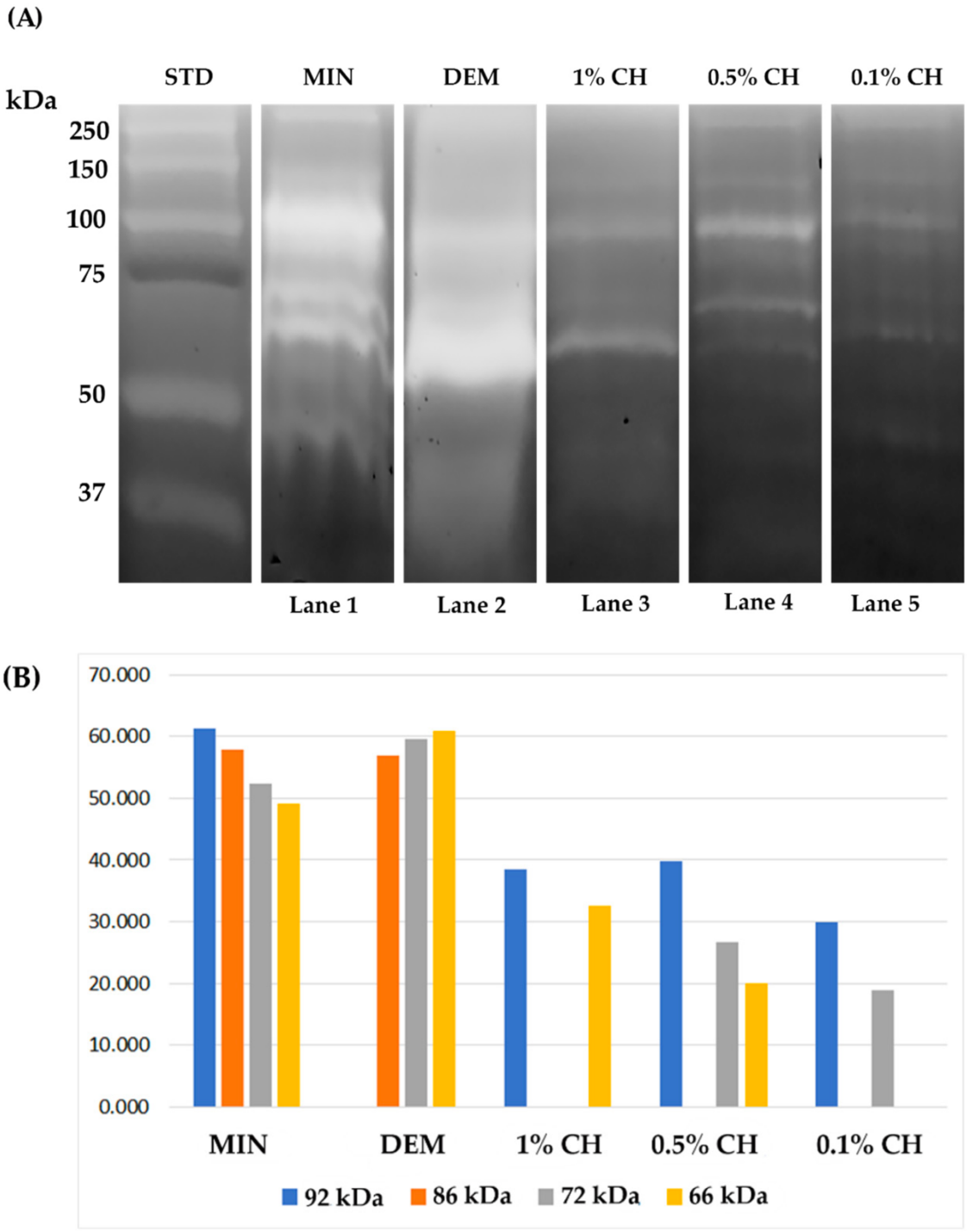


Figure 20. Zymographic analysis of proteins extracted from dentin powder. (A) Figure showing the differences in the enzymatic expression and activity between the investigated groups presented as light bands in the area of the molecular weights of MMP-2 and MMP-9. (B) Densitometric evaluation of the bands in the different treatment groups.

A lower expression of pro-MMP-9 compared to the control groups was detectable at 1% (lane 3) and 0.5% (lane 4) chitosan groups, and the faintest band was noted at 0.1% CH group (Lane 5). Furthermore, the expression of the 72 kDa MMP-2 pro-form decreased or completely disappeared after treating demineralized dentin with any of the chitosan concentrations tested (1%, 0.5% or 0.1%). In the groups treated with chitosan 0.5% and 1%, there is a faint band at the molecular weight of MMP-2 active form.

In Situ Zymography

The qualitative and quantitative results of the in situ zymography are presented on Figure 21. The gelatinolytic activity in unrestored dentin (Figure 21a–h) is concentrated mostly in the first several microns of the dentin surface (higher activity), as well as in the dentinal tubules (lower activity). The activity within the hybrid layer (Figure 21i–p) is visibly higher in all the groups compared to unrestored dentin, except for the 0.1% chitosan group. The high fluorescence level in the hybrid layers reaches deeper into the dentin tubules (~10 μm). The two-way ANOVA analysis of the quantitative data confirmed the qualitative observations and demonstrated that both investigated factors ("pretreatment" and "adhesive system application"), as well as their interaction, have a statistically significant influence on the gelatinolytic activity within dentin and the hybrid layer ($p < 0.05$). The intensity of the fluorescent signal significantly differed between all the pretreatments in the following order: 1% chitosan > control > 0.5% chitosan > 0.1% chitosan ($p < 0.05$). The application of the adhesive in all the pretreatment groups except for the 0.1% chitosan ($p > 0.05$) increased the density of the fluorescent signal ($p < 0.05$). Within the groups of unrestored dentin, significant differences were noted between all the groups except for the control and 1% chitosan, as well as 0.5 and 0.1% chitosan (control = 1% chitosan > 0.5% chitosan = 0.1% chitosan), while in the groups bonded with the adhesive and restored with composite resin, only the control group and the 0.5% groups were similar to one another (1% chitosan > control = 0.5% chitosan > 0.1% chitosan).

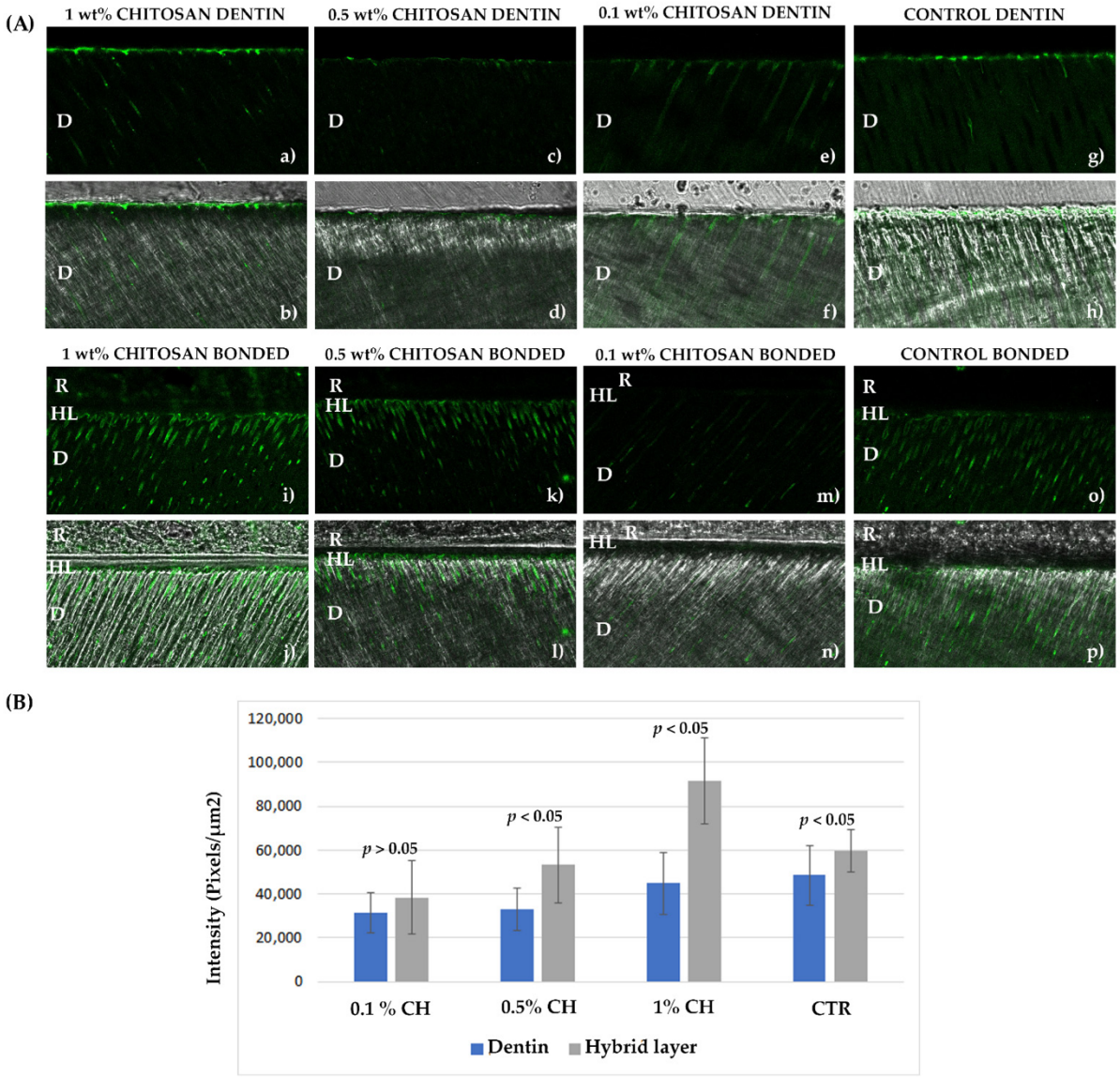


Figure 21. In situ zymography. (A) Images acquired in the green channel showing fluorescence within the dentin surface (a,c,e,g) and within the HL (i,k,m,o) of the tested groups. Images obtained by merging the differential interference contrast (DIC) image (showing optical density of the resin-dentin interface) and the image acquired in the green channel (b,d,f,h,j,l,n,p). (B) Quantification of the gelatinolytic activity within the dentin and the HL of the tested groups. D—dentin; HL—hybrid layer; R—resin composite.

STUDY 4

Effect of two glutaraldehyde-based desensitizer on dentin in combination with universal adhesives

The aim of this in vitro study was to evaluate the effects of two glutaraldehyde-based solutions (GLUMA Desensitizer and GLUMA Desensitizer PowerGel) Figure 22 on the microtensile bond strength (μTBS) and endogenous enzymatic activity (MMPs) of simplified universal adhesives (UAs) used in the self-etch mode to dentin.

The null hypotheses were that 5.0% Glutaraldehyde and 35% HEMA in the two GLUMA formulations (gel and liquid):

1) does not benefit the bonding performances of two universal adhesives to dentin immediately and after 1 year of laboratory aging.
2) does not influence endogenous dentin MMPs activity immediately or over time.

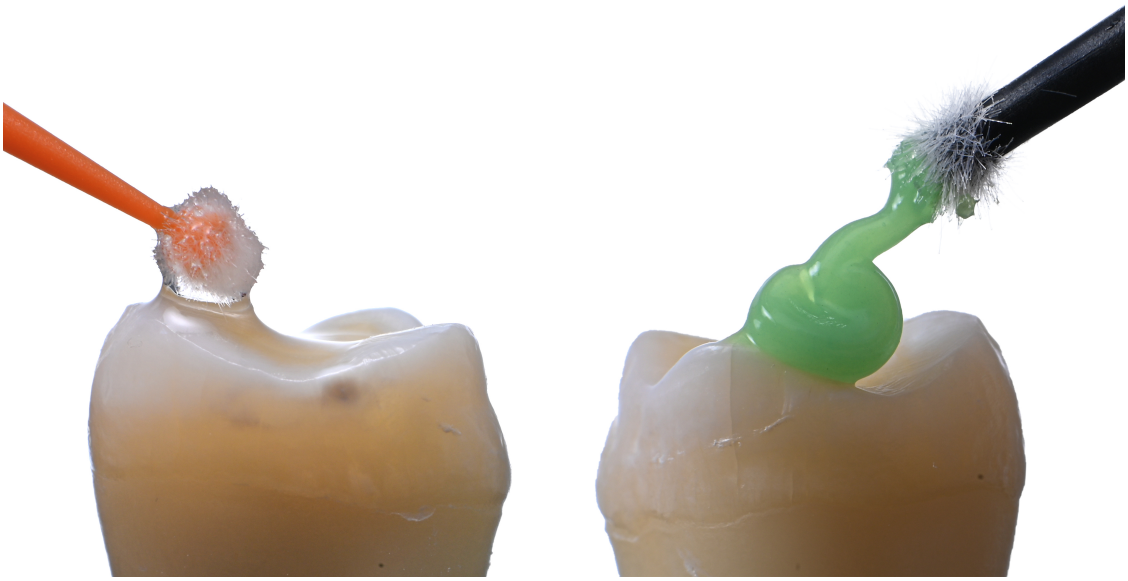


Figure 22

Application on dentin of the two desensitizer, GLUMA Desensitizer (left) and GLUMA Desensitizer Powergel (right).

Materials and Methods

Microtensile bond strength test (μTBS)

Forty-eight freshly extracted non-carious human molars were obtained from anonymous individuals following their informed consent under a protocol approved by the Ethical Committee of the University of the University of Bologna, Bologna, Italy (protocol N°: 71/2019/OSS/AUSLBO). Tooth crowns were removed with a low-speed diamond saw under water cooling (Microremet, Remet, Casalecchio di Reno, Italy) to expose enamel-free middle/deep coronal dentin. Cut dentin surfaces were examined with a stereoscopical microscope to ensure that they were devoid of defects and enamel remnants. A standardized smear layer was created on each dentin surface using #320- grit wet silicon carbide paper and water lubrication.

In the experimental groups, prior to adhesive application, the dentin surfaces were pre-treated for 30 s with one of the following desensitizers available in 2 consistencies (Kulzer GmgH, Hanau, Germany): the liquid GLUMA Desensitizer (L) and the gel GLUMA Desensitizer Powergel (G). After application, the specimens were thoroughly water-rinsed as indicated by the manufacturer. In the control groups, no dentin pretreatment was performed. Then, two universal adhesives were employed for the bonding procedures in the SE mode: IBU - iBond universal (Kulzer), or AU - Adhese Universal (Ivoclar Vivadent, Schaan, Liechtenstein). In the end, the following groups were formed according to the desensitizer/adhesive combination (n=8): 1) L/IBU; 2) G/IBU; 3) IBU (CTRL); 4) L/AU; 5) G/AU; 6 AU (CTRL). Complete details and instructions of the materials used in the study are presented in Table 5.

After polymerization of the respective adhesive, a build-up was created (two 2 mm-thick layers) with a nanohybrid resin composite (Venus Pearl, Kulzer). Light curing of the adhesive resin and each layer of composite resin was performed for 20 s with a light-emitting diode (LED) curing light (ELIPAR™ DeepCure-S, 3M, St Paul, MN, USA; light output > 1000 mW/cm² and wavelength 430-480 nm).

The bonded specimens were serially sectioned to obtain sticks with ~0.9 mm × ~0.9 mm cross-sectional area, following the non-trimming technique of the microtensile bond strength test (μTBS). The exact dimension of the sticks was measured using a pair of digital calipers. Bond testing was performed after the sticks were aged in artificial saliva at 37°C for 24 h (T0) or 12 months (T12). Each beam was stressed under tension to failure using a simplified bond testing machine (Shear Bond Tester; Bisco, Schaumburg, IL, USA) at a crosshead speed of 1 mm/min.

The number of prematurely debonded specimens in each experimental group was recorded. Null bond strength values were not included in the statistical analysis because the number of prematurely debonded sticks did not exceed 3% of the total number of tested specimens and were similarly distributed within the groups. A single observer evaluated each side of the fractured sticks with a stereomicroscope at 50× magnification to determine the mode of failure. Failure was classified as adhesive at the dentin interface (A), cohesive in dentin (CD), cohesive in composite (CC) or mixed failure (M; adhesive and cohesive fractures occurred simultaneously).

Table 5. Chemical composition of the materials used in the study and bonding procedures.

Material	Composition	Ph	Mode of use
Adhese Universal Ivoclar Vivadent, Schaan, Liechtenstein	MDP, MCAP, HEMA, Bis-GMA, D3MA, Water, Ethanol Highly dispersed silicon dioxide Initiators and Stabilizers	2.5- 3	1. The adhesive is scrub on dentin for 20 s; 2. Air-spray with oil- and moisture-free compressed air until a glossy, immobile film layer results; 3. Light-cure using a LED light-curing unit for 20 s.
iBOND Universal Kulzer GmgH, Hanau, Germany	Phosphonic acid acrylate, HEMA, D3MA Highly dispersed silica Ethanol Catalysts, stabilizers, fluoride	1.6-1.8	1. The adhesive is scrub on dentin for 20 s; 2. Disperse the adhesive with an oil- and moisture-free compressed air until a glossy, immobile film layer result; 3. Light-cure using a LED light-curing unit for 10 s.
GLUMA Desensitizer Kulzer GmgH, Hanau, Germany	Purified water, (HEMA), glutardialdehyde, pyrogenic silicic	3.66	1. Apply the desensitizer for 30 - 60 s; 2. Thoroughly rinse off the desensitizer with water spray and dry with oil-free air; 3. Continue with the bonding procedures.
GLUMA Desensitizer Powergel Kulzer GmgH, Hanau, Germany	Purified water, (HEMA), glutardialdehyde, pyrogenic silicic	3.66	1. Apply the desensitizer for 30 - 60 s; 2. Thoroughly rinse off the desensitizer with water spray and dry with oil-free air; 3. Continue with the bonding procedures.
VENUS Pearl Kulzer GmgH, Hanau, Germany	Amorphous silica, triethylen glycol dimethacrylate, trimethoxysilylpropyl methacrylate, Phenyl-1,2-propandion, methyl methacrylate, acetic acid	Not defined	

Scanning Electron Microscope (SEM) Examination

After μTBS, two representative debonded sticks per group were selected (with a bond strength value close to the mean value of the group) and prepared for scanning electron microscopy (SEM) evaluation. Each specimen was fixated in a 2.5% glutaraldehyde 0.1 M cacodylate buffer (pH 7.4), dehydrated in ascending ethanol solutions (50%, 70%, 80%, 90%, 95% and 100%), and dried using hexamethyldisilazane. Then, they were mounted on aluminum

specimen stubs, coated with 8÷10 nm gold particles and observed under a field-emission gun scanning electron microscope (FEG-SEM; Nova NanoSEM 450; FEI, Eindohoven, NL).

In situ zymography of resin-dentin interfaces

One-millimeter-thick slabs of middle/deep coronal dentin were obtained from four extracted human third molars using a low-speed saw (Micromet) under water-cooling. Two dentin slabs were obtained from each tooth. Each slab was further divided into 4 pieces so that testing of the six experimental groups was performed using the same dentin substrate. A standardized smear layer was created on each dentin surface using #600-grit silicon carbide paper under water cooling. Identical bonding procedures were performed as previously described for the μTBS forming the same 6 groups (n = 4). Resin-dentin interfaces were exposed by cutting the bonded specimens vertically into 1 mm-thick sticks using the slow-speed saw under water cooling. The sticks were fixed to glass slides with cyanoacrylate glue and polished to obtain ~50 μm thick slabs using a series of wet silicon carbide papers. Self-quenched fluorescein-conjugated gelatin was used as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA) for *in situ* zymography at T_0 and T_{12} (A. Mazzoni et al., 2013). The fluorescent gelatin mixture was placed on top of each slab and covered with a glass coverslip. The slides were incubated in a humidified chamber at 37° C overnight. 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 specimen, a series of images were made to visualize the hydrolysis of the quenched fluorescein-conjugated gelatin substrate as an indicator of endogenous gelatinolytic activity. Enzymatic activity was quantified as the integrated density

of the fluorescence signals using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data sets obtained from bond strength testing and in situ zymography were first validated individually for their normality (Shapiro-Wilk test) and equality of variance (Brown-Forsythe test). For the μTBS test, as data were normally and equally distributed, the three-way analysis of variance (ANOVA) was performed to identify the effects of the 3 independent variables involved in the testing: adhesive systems, dentin desensitizer and aging time. Post-hoc pairwise comparisons were conducted using the Tukey test.

Regarding the in situ zymography results, since data were not normally distributed (Shapiro-Wilk test, $P<0.05$), they were analyzed using the Kruskal-Wallis test and a Pairwise Multiple Comparison Procedures (Dunn's Method). All analyses were performed using a statistical software (Sigmaplot v.14.0.; StataCorp LLC, College Station, TX, USA) and the statistical significance was preset at $\alpha = 0.05$.

Results

Microtensile bond strength test (μTBS)

Mean microtensile bond strength results and standard deviations of the experimental groups tested at baseline and after 1 year of artificial storage are presented in Table 6. Statistical analysis revealed that dentin pre-treatment, the type of adhesive and aging significantly influenced the results ($P<0.05$). The interaction between the adhesive and aging was also significant ($P=0.048$). AU showed higher bond strength compared with IBU ($P<0.001$). At baseline, specimens pre-treated with L resulted in higher bond strength compared to G ($P<0.001$) and control group ($P<0.001$). No statistically significant differences were found between G and the control groups ($P=0.556$). Laboratory aging statistically decreased bonding values, irrespective of the experimental group ($P <0.001$). After 1 year of storage in artificial saliva no differences in bond strength were observed between L and G regardless of the adhesive ($P=0.001$). However, pre-treating dentin with the two desensitizers resulted in higher

bond strength when compared to the controls, independent of the adhesive product used for bonding procedures (P<0.001). Table 7 summarizes the percentage distribution of failure modes identified after μTBS test at T_0 and T_{12}. A predominance of A failures first, followed by the CC ones was observed in all groups, irrespective of the adhesive and dentin pre-treatment. Notwithstanding, after storage, the type of debondings changed trend with a reduced percentage of A failures and higher CC for pre-treated groups (irrespective of whether it was L or G), except for the control groups that did not show any different tendency (independent of the type of adhesive). A certain percentage of CD and M fractures were observed among groups, with an equal distribution independent of the adhesive, dentin pre-treatment and aging.

Table 6: Summary of microtensile bond strength results obtained from the four experimental groups immediately (T0) and after 12 months of laboratory aging (T12).

Densensitizer/Adhesive	**T_0 (MPa)** †	**T_{12} (MPa)** †
L/IBU	24.03 ± 7.43 ^{c,A}	24.37 ± 10.70 ^{a,b,A}
G/IBU	23.05 ± 14.26 ^{c,A}	18.57 ± 10.67 ^{b,A}
IBUCTRL	20.59 ± 6.86 ^{c,A}	16.59 ± 7.76 ^{b,A}
L/AU	43.16 ± 10.51 ^{a,A}	30.41 ± 9.55 ^{a,B}
G/AU	32.86 ± 1.63 ^{b,A}	27.54 ± 14.95 ^{a,A}
AUCTRL	31.50 ± 11.27 ^{b,A}	24.51 ± 11.72 ^{a,b,A}

Abbreviations: L – liquid desensitizing agent GLUMA Desensitizer; G – desensitizing agent GLUMA Desensitizer Powergel; IBU –iBond Universal; AU - Adhese Universal; CTRL – Control: no dentin pretreatment.

Different lower-case letters indicate significant differences within the same column ($p < 0.05$).

Different upper-case letters indicate significant differences within the same row ($p < 0.05$).

† Values are means ± standard deviations, in megaPascals (MPa).

Table 7. Failure mode distributions and their percentages identified among the experimental groups after bond strength testing at baseline (T_0) and 1 yr of laboratory storage in artificial saliva (T_{12}).

	Failure mode (%)							
	T_0				T_{12}			
	A	**CC**	**CD**	**M**	**A**	**CC**	**CD**	**M**
L/IBU	44%	49%	5%	2%	32%	61%	2%	5%
G/IBU	38%	51%	8%	3%	32%	57%	0%	11%
IBUCTRL	21%	64%	8%	8%	22%	63%	9%	6%
L/AU	34%	32%	19%	15%	18%	42%	22%	18%
G/AU	28%	51%	6%	15%	26%	60%	9%	5%
AUCTRL	25%	68%	3%	5%	19%	67%	10%	5%

Failure modes: A – adhesive at the dentin interface; CC - cohesive in composite; CD - cohesive in dentin; M – mixed.

Scanning Electron Microscope (SEM) Examinations

Representative SEM images at T_0 and T_{12} are shown in Fig. 23 and 24 respectively (magnifications 200x, 2000x and 5000x). SEM analysis showed the presence of smear layer when no dentin pre-treatment was performed (control groups in Figs 2C and G and Figs 3C and G), especially when compared to the experimental groups where the tubules orifices were mostly visible and wide (Figs 2A,B,D,E and Figs 3A,B,D,E). These situations were observed both at baseline (Fig. 2) and after laboratory storage (Fig.3). Remnants of resin composite bulk were observed in all the experimental groups at baseline showing, however, rough and untight surfaces cautiously indicating mixed failures (Fig.2). Higher concentration of dentinal tubules with larger amount of impregnated resin tags were observed at T_{12} for AU when compared to IBU, irrespective of the desensitizer used (Fig 3D and E). At the same timepoint, IBU revealed a softer and plastic appearance with sparsely distributed bubble formations when it was used after application of the liquid desensitizer or alone (CTRL group) (Fig. 23A and C,

respectively). This conformation was only occasionally present when IBU was used after the application of G (Fig. 24B).

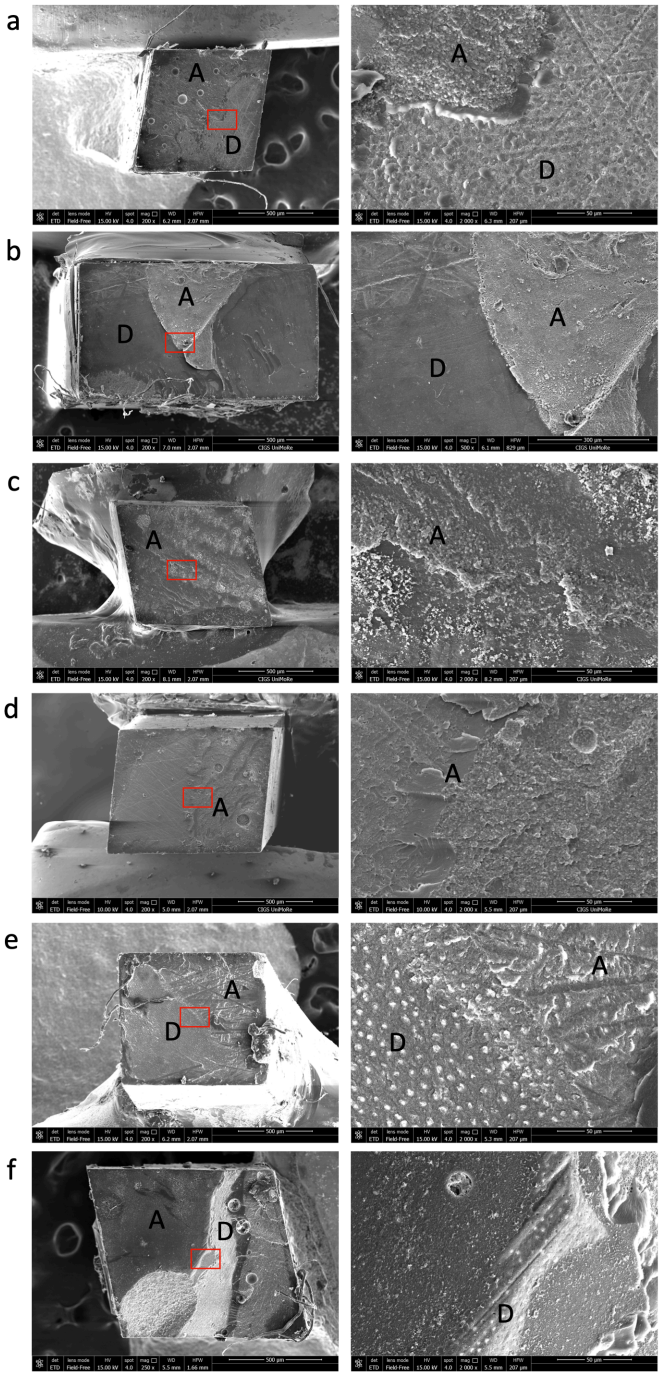


Figure 23

Representative field emission scanning electron microscope micrographs of the adhesive interfaces of fractured microtensile bond strength sticks (the dentin side) at baseline (T_0): left, view of the whole adhesive surface; right, enlarged view of the area marked with the red selection. Magnification: 200 x, left; 2000 x, right. (a) L/IBU: mixed failure with the presence of remnants of resin composite. (b) G/IBU: mixed debondings with the present of resin composite still attached on the dentin surface. (c) IBUCTRL: mixed failure, the presence of soft-appearance material was noted over the dentin surface. (d)L/AU: this group showed the highest percentage of mixed failures among groups at baseline. (e) G/AU: mixed failure, with the presence of sparsely distributed resin tags occluding dentinal tubules. (f) AUCTRL: mixed failures.

A, adhesive resin; D, dentin;

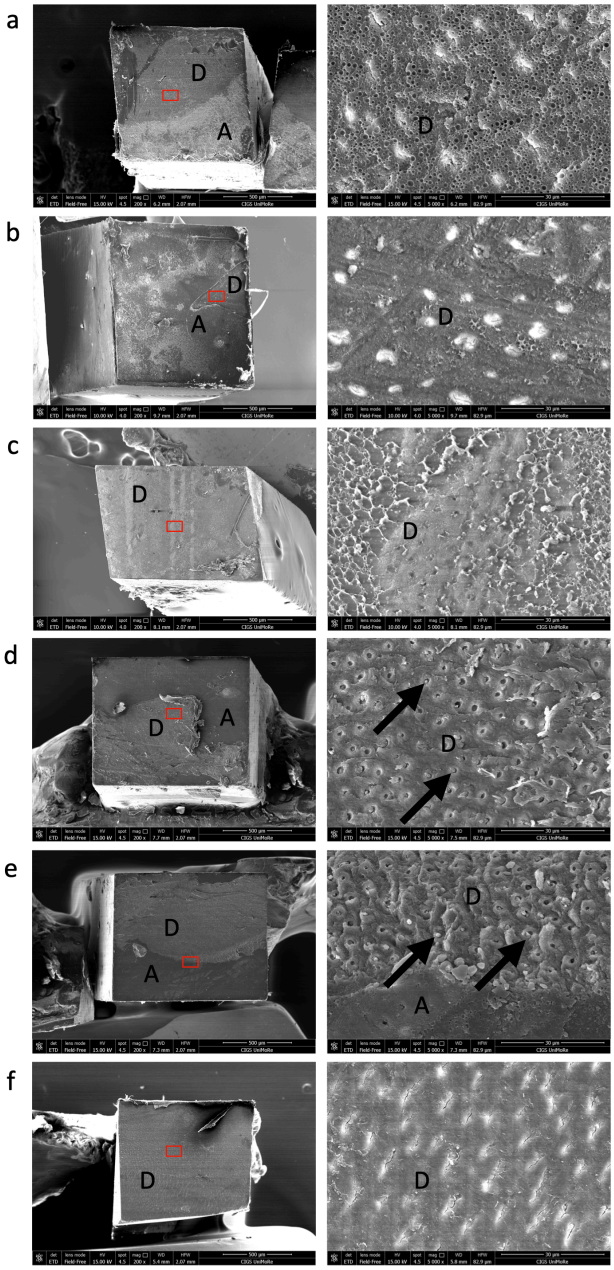


Figure 24

Representative field emission scanning electron microscope micrographs of the adhesive interfaces of aged (T_{12}) fractured microtensile bond strength sticks (the dentin side): left, view of the whole adhesive surface; right, enlarged view of the area marked with the red selection. Magnification: 200 x, left; 5000 x, right. (a) L/IBU: mixed fracture, with the presence of a porous with internmittent bubbles cross overing the resin. (b) G/IBU: mixed failure with some resin tags and sparsely distributed soft resin remnants. (c) IBUCTRL: mixed fracture with some opened dentinal tubules with no evident resin penetration. The surface was also characterized by the presence of plastic filaments, rendering the surface rough and irregular. (d) L/AU: mixed failure showing exposed dentinal tubules with thin extruding resin tags formations. (e) G/AU: mixed failure with the presence of opened dentinal tubules intermittently occluded by thin and sparse resin impregnation. (f) AUCTRL: mixed failure with the presence of smear layer on the dentin surface occluding the underneath tubule orifices.

A, adhesive resin; D, dentin; Black arrow, resin tag in the dentinal tubule.

In situ zymography

Representative confocal images of the tested groups are shown in Figures 25 and 26 for time T_0 and T_{12}, respectively. The percentages of HLs exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin at T_0 and T_{12} are shown in Figure 27. The green fluorescence signals at T_0 identified from the AU control group were statistically lower compared to those exhibited by the L/AU group (P<0,001), whilst G/IBU showed statistically higher levels of fluorescence than IBU (CTRL) group (P=0,022). In all the tested groups, the fluorescence density level was lower after aging. At T_{12} all the groups tested showed comparable endogenous enzymatic activity irrespective of the adhesive and the desensitizing agent used (P>0.05).

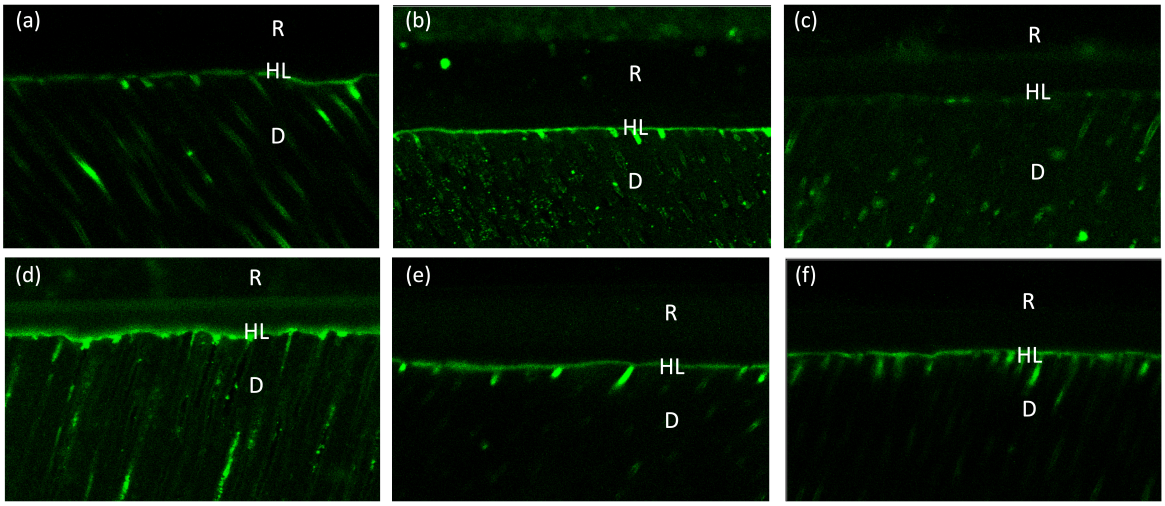


Figure 25

Resin-bonded mid-coronal dentin interfaces prepared with L/IBU (a) G/IBU (b), IBUCTRL (c), L/AU (d), G/AU (e) and AUCTRL (f) at T_0, incubated with quenched fluorescein-labeled gelatin. All the images were acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL.

D = Dentin; HL = Hybrid Layer; R = Resin Composite.

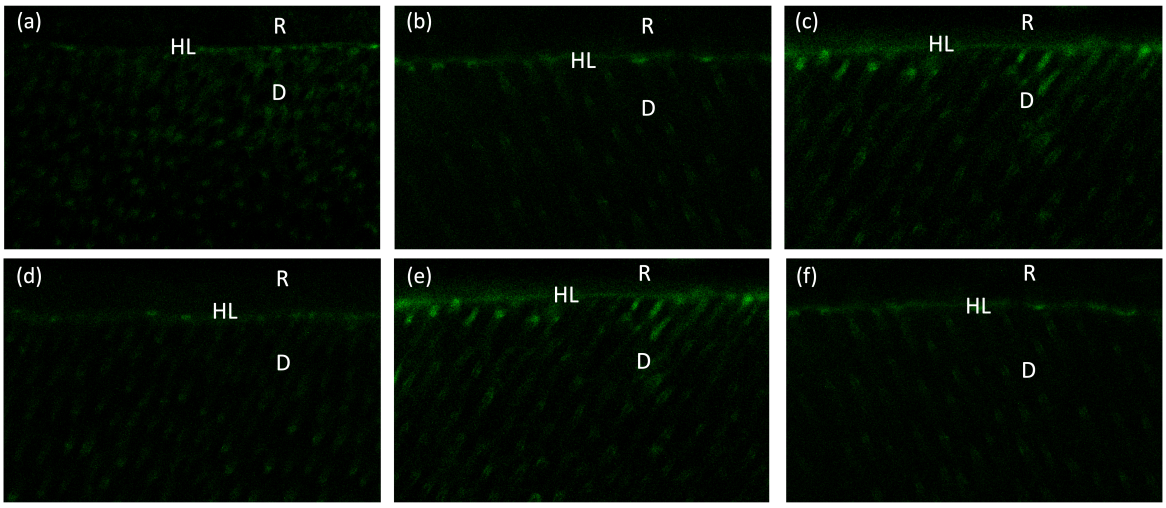


Figure 26

Resin-bonded mid-coronal dentin interfaces prepared with L/IBU (a) G/IBU (b), IBUCTRL (c), L/AU (d), G/AU (e) and AUCTRL (f) at T12, incubated with quenched fluorescein-labeled gelatin. All the images are acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL.

D = Dentin; HL = Hybrid Layer; R = Resin Composite.

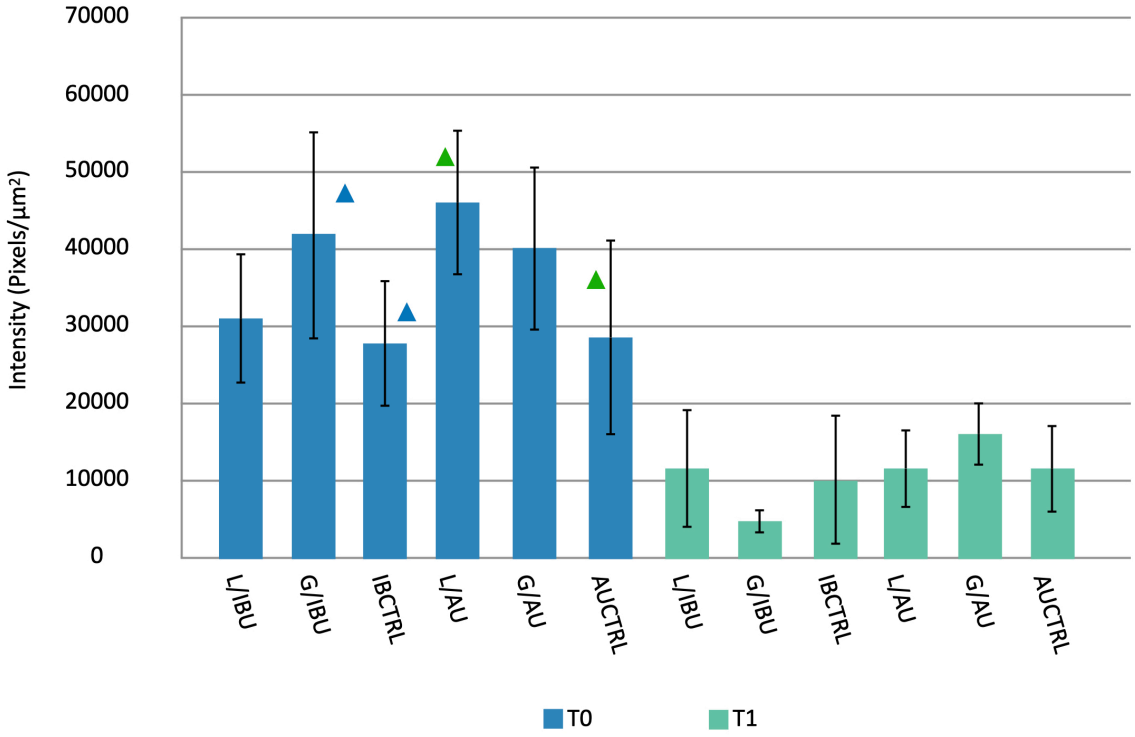


Figure 27

Quantification (Pixels/um2) of the fluorescence obtained in the different groups at T0 and T12 by means of in situ zymography showing reduced enzymatic activity in all T12 groups irrespective from the adhesive and desensitizing agent used.

STUDY 5

Bonding to dentin using an experimental zirconium oxynitrate etchant

An experimental zirconium oxynitrate conditioner [ZrO (NO3)2] has recently been introduced to adhesive dentistry. Zirconium oxynitrate has only been used previously in applied chemistry or as a radiopacifying material in endodontic cements (Camilleri et al., 2011). Promising results have recently been reported when ZrO(NO3)2 was used as an enamel etchant, in combination with different adhesive systems (Yao et al., 2019). Introduction of an etching product that can dissolve the inorganic component of dentin, reduce nanoleakage and inhibit MMP-mediated proteolytic activity at the resin-dentin interface is highly desirable to maintain bond stability over time (Pashley et al., 2004).

Capitalizing on the aforementioned considerations, the objectives of the present *in vitro* study were to evaluate the immediate (T0) *vs* 1 year (T12) microtensile bond strength to dentin, interfacial nanoleakage expression and the effects of endogenous enzymatic activity on hybrid layers created by two simplified adhesive systems (one single-component etch-and-rinse adhesive and one universal adhesive). These adhesives were applied to dentin after the latter was conditioned with a H3PO4 acid etchant or an experimental ZrO(NO3)2 etchant.

The null hypothesis tested were that the experimental ZrO(NO3)2 etchant:

1) has no effect on the immediate bonding performance of the tested ad- hesives to dentin;
2) has no effect on the maintenance of bonding per- formance after laboratory aging; and
3) has no effect on inhibiting endogenous dentin MMPs activity immediately or over time.

Material and methods

Microtensile bond strength test (μTBS)

Sixty-four freshly extracted non-carious human third molars were obtained from anonymous individuals following their informed consent. The project protocol was approved by the Ethical Committee (protocol N◦: 71/2019/OSS/AUSLBO, approved on 23/01/2019). Tooth crowns were removed with a low-speed diamond saw under water cooling (Microremet, Remet, Bologna, Italy) to expose deep cor onal dentin. Cut dentin surfaces were examined with a stereoscopical microscope to ensure that they were devoid of enamel remnants. A standardized smear layer was created on each dentin surface using 600- grit wet silicon carbide paper and water lubrication. Dentin conditioning was performed with 37 % H3PO4 – TE (Total Etch; Ivoclar Vivadent, Schaan, Liechtenstein) or an experimental zirconium oxynitrate gel (ZON) (Ivoclar Vivadent). After dentin etching, a universal adhesi – AU (Adhese Universal; Ivoclar Vivadent) was used in the etch-and-rinse mode. Alternatively, a single-component etch-and-rinse adhesive – EF (Excite F; Ivoclar Vivadent) was used in the etch-and-rinse mode. This resulted in the establishment of 4 experimental groups (n = 16): 1) ZON + AU; 2) TE + AU; 3) ZON + EF; 4) TE + EF. The chemical composition of the adhesives and etchants and their instructions for use are shown in Table 8.

Table 8: Chemical composition of the materials used in the study and bonding procedures.

Material	Composition	Ph	Mode of use
Adhese Universal Ivoclar Vivadent, Schaan, Liechtenstein	MDP, MCAP, HEMA, Bis-GMA, D3MA, Water, Ethanol Highly dispersed silicon dioxide Initiators and Stabilizers	2.5 - 3	1. The adhesive is scrub on dentin for 20 s; 2. Air-spray with oil- and moisture-free compressed air until a glossy, immobile film layer results; 3. Light-cure using a LED light-curing unit for 20 s.
Excite F Ivoclar Vivadent	Phosphonic acid acrylate, HEMA, D3MA, ethanol, highly dispersed silica, catalysts, initiator and stabilizers, fluoride	2.5	1. After acid etching, apply adhesive on dentin and agitate for at least 10 s; 2. Thin the adhesive with an oil- and moisture-free compressed air until a glossy, immobile film is obtained; 3. Light-cure using a LED light-curing unit for 20 s.
Experimental etchant (ZON) Ivoclar Vivadent	ZrO(NO3)2, water, glycerol, fumed silica, polyethylene oxide	0.56	1. Apply ZON and allow it to interact with the tooth surface without agitation for 30 s; 2. Thoroughly rinse off the etchant with water spray and dry with oil-free air; 3. Continue with bonding procedures.
Total Etch Ivoclar Vivadent	Phosphoric acid (37 wt% in water), thickening agent and color pigments	0.1-0.4	1. Apply the etchant and allow it to interact with the tooth surface without agitation for 15 s; 2. Thoroughly rinse off the etchant with water spray and dry with oil-free air. 3. Continue with bonding procedures.

Abbreviations: Bis-GMA - bisphenol A-glycidyl methacrylate; D3MA - dec- andiol dimethacrylate; HEMA – 2-hydroxyethyl methacrylate; LED – light emitting diode; MCAP - methacrylated carboxylic acid polymer; MDP - meth- acryloyloxydecyl dihydrogen phosphate.

After polymerization of the respective adhesive, a 4 mm-thick layer of composite build-up was performed with a nanohybrid resin composite (Tetric Evo Ceram Bulk Fill, Ivoclar Vivadent). Light curing was per- formed for 20 s with a light-emitting diode curing light (DemiPlus; Kerr Corp., Orange, CA, USA) with an output of 500 mW/cm2.

The bonded specimens were serially sectioned to obtain beams with ~0.9 mm × ~0.9 mm cross-sectional area, in accordance with the non- trimming technique of the microtensile test. Bond testing was performed after the beams were aged in artificial saliva at 37 ◦C for 24 h (T0) or 1 year (T12). The exact dimension of each beam was measured using a pair of digital calipers. Each beam was stressed under tension to failure using a simplified bond testing machine (Shear Bond Tester; Bisco, Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. The number of prematurely debonded beams in each experimental group was recorded. Null bond strength values were not included in the statistical analysis because the number of prematurely debonded beams did not exceed 3% of the total number of tested specimens and were similarly distributed within the groups. A single observer evaluated each side of the fractured sticks with a stereomicroscope at 50× magnification to determine the mode of failure. Failure was classified as adhesive (A), cohesive in dentin (CD), cohesive in composite (CC) or mixed failure (M).

Nanoleakage expression

Four additional teeth per group were sectioned into 1 mm-thick slices of mid-coronal dentin and bonded in the manner described for the μTBS test. After bonding, 1 mm-thick resin composite build-ups were made using TetricEvo Flow (Ivoclar Vivadent). The specimens were sectioned vertically into 1-mm thick beams to expose the bonding sur- faces. Half of the beams were stored in the artificial saliva at 37 ◦ C for 24 h (T0). The remaining half was stored the artificial saliva for 12 months (T12). After aging, the specimens were immersed in 50 wt% ammoniacal AgNO3 solution for 24 h, following the protocol described by Tay et al. (F.R. Tay et al., 2002). The silver tracer infiltrated specimens were thoroughly rinsed with distilled water and immersed in a photo-developing solution for 8 h under a fluorescent light to reduce silver ions that infiltrated voids along the bonded interfaces into metallic silver grains.

Each specimen was fixed to a glass slab and polished with a polishing device (LS2; Remet) under water irrigation. Polishing was performed using a series of silicon carbide papers with increasing fineness (180-, 600-, 1200-, 2400-, and 4000-grit). Observations were made using a light microscope (E800; Nikon, Tokyo, Japan). Images of the resin- dentin interfaces were obtained at 20x magnification. The severity of interfacial nanoleakage was quantified using a four-point scale by one experienced investigator. Scoring was performed using the method described by Saboia et al. (Saboia et al., 2008) with the scores representing the percentage distribution of silver deposits within the resin-dentin interfaces.

Gelatin zymography

Zymography was performed using the method reported by Mazzoni et al. (A. Mazzoni et al., 2013). Briefly, mineralized dentin powder was obtained from eight extracted human third molars. Each tooth was ground free of enamel and cementum; pulpal soft tissue was removed with excavators and hand files. Dentin powder was obtained by freezing dentin chips in liquid nitrogen and triturating them in a ball mill (Retsch Mill; Reimiller, Reggio Emilia, Italy). The mineralized dentin powder was pooled, sieved, dried and kept frozen until use. Aliquots of mineralized dentin powder were divided into 3 groups:

Group 1: Mineralized dentin control (C);

Group 2: Dentin powder demineralized with ZON for 10 min (ZON);

Group 3: Dentin powder demineralized with TE for 10 min (TE).

In the groups treated with a liquid etchant, the acid was neutralized after 10 min and centrifuged. The supernatant was removed and the powder was rinsed 2 more times with distilled water and re-centrifuged for 20 min at 4 ◦C. For protein extraction, dentin powder aliquots were re-suspended in extraction buffer (50 mM Tris–HCl, pH 6, containing 5 mM CaCl2, 100 mM NaCl, 0.1 % Triton X-100, 0.1 % nonionic detergent P-40, 0.1 mM ZnCl2 and 0.02 % NaN3) for 24h at 4 ◦C. The dentin powder-containing buffers were intermittently sonicated for 10 min and centrifuged for 20 min at 4 ◦C. The supernatants were discarded and recentrifuged. The extracted proteins were concentrated using Vivaspin centrifugal concentrator (10 kDa molecular weight cut-off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 25 ◦C (15 G × 3 times). Total protein concentration in the dentin extracts was determined using the Bradford assay (Bio-Rad Bradford Protein Assay; Bio-Rad, Hercules, CA, USA). Dentin proteins aliquots (60 μg each) were diluted in Laemmli sample buffer in 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-range molecular weight SDS-PAGE standards (Bio-Rad) were used as molecular-weight 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 CaCl2, pH 7.4) for 48 h. Proteolytic activity was evaluated using an ultraviolet light scanner (ChemiDoc Universal Hood, Bio-Rad). Gelatinase activities in the

extracts were analyzed in duplicate. Images were qualitatively examined and gelatinase activities were quantified using an image processing software (ImageJ; National Insti- tute of Health, Bethesda, MD, USA).

In situ zymography of resin-dentin interfaces

One-millimeter-thick slabs of middle/deep coronal dentin were obtained from three extracted human third molars using a low-speed saw (Micromet) with water-cooling. Two dentin slabs were obtained from each tooth. Each slab was further divided into 2 pieces, so that testing of the four experimental groups was performed using the same dentin substrate. A standardized smear layer was created on each dentin surface using 600-grit silicon carbide paper under water cooling. The specimens were then randomly assigned to the same 4 groups (n = 3) described for μTBS. Identical bonding procedures were performed as previously described. Each bonded specimen was light-cured for 20 s using a light emission diode light-curing unit (Demi Plus). Resin-dentin interfaces were exposed by cutting the bonded specimens vertically into 1 mm-thick sticks using the slow-speed saw under water cooling. The sticks were affixed to glass slides with cyanoacrylate glue, and polished to obtain ~50 μm-thick slabs using a series of wet silicon carbide papers. Self-quenched fluorescein-conjugated gelatin was used as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA) for in situ zymography at the baseline (T0) and after 12 months (T12) of storage in artificial saliva at 37 ◦C, as previously described (Comba et al., 2019b). The fluorescent gelatin mixture was placed on top of each slab and covered with a glass coverslip. The slides were incubated in a humidified chamber at 37 ◦C overnight. 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 specimen, a series of images were made to visualize hydrolysis of the quenched fluorescein-conjugated gelatin substrate as an indicator of endogenous gelatinolytic activity. Enzymatic activity was quantified as the integrated density of the fluorescence signals using ImageJ software.

Statistical analyses

Data sets obtained from bond strength testing, nanoleakage evaluation and in situ zymography were first validated individually for their normality (Shapiro-Wilk test) and homoscedasticity assumptions (modified Levene test) prior to the use of parametric statistical

methods for analysis. Three independent variables were involved in μTBS testing: adhesive systems, dentin conditioners and aging time. Accordingly, a three-factor analysis of variance (ANOVA) was performed to identify the effects of the three independent variables on bond strength. Post-hoc pairwise comparisons were conducted using the Tukey test. The nano-leakage results were analyzed using Chi-squared test. Data derived from in situ zymography were analyzed with one-factor ANOVA and post-hoc Tukey test. All analyses were performed using a statistical software (Stata v.12.0.; StataCorp LLC, College Station, TX, USA). For all analyses, statistical significance was preset at $\alpha = 0.05$.

Results

Microtensile bond strength test (μTBS)

Bond strength values at T0 and T12 are depicted in Table 9. Three- way ANOVA test showed that all the investigated factors (adhesive systems, dentin conditioners and aging), significantly influenced bond strength results ($p < 0.05$). In addition, significant interactions were identified between adhesive systems and dentin conditioners ($p < 0.05$) and between adhesive systems and aging ($p < 0.05$). At T0, ZON/AU had the highest mean bond strength among the tested groups ($p < 0.05$). No differences were observed among TE/AU, ZON/EF and TE/EF. Significant difference in bond strength was observed after laboratory aging ($p < 0.05$). Pairwise comparisons indicated that bond strength values after aging were in the order: ZON/AU = TE/AU > TE/AU = ZON/EF > ZON/EF = TE/EF ($p < 0.05$). Compared with the immediate bond strength results, bond strength significantly declined for all groups ($p < 0.05$) after laboratory aging except for TE/AU.

Table 9: Summary of microtensile bond strength results obtained from the four experi- mental groups immediately (T0) and after 12 months of laboratory aging (T12).

Adhesive/Densensitizer	T_0 (MPa) †	T_{12} (MPa) †
ZON/AU	54.3 ± 15.0 a,A	43.9 ± 14.2 a,B
TE/AU	39.1 ± 14.2 b,A	36.0 ± 14.1 a,b,A
ZON/EF	42.1 ± 17.9 b,A	26.9 ± 14.3 b,c,B
TE/EF	37.9 ± 16.2 b,A	20.8 ± 14.8 c,B

Abbreviations: ZON - experimental zirconia oxynitrate [ZrO(NO3)2] etchant; TE - Total Etch conventional 37 % phosphoric acid (H3PO4) etchant; AU - universal adhesive Adhese Universal; EF - 2-step self-etch adhesive Excite F.

Different lower-case letters indicate significant differences within the same column ($p < 0.05$).

Different upper-case letters indicate significant differences within the same row ($p < 0.05$).

† Values are means ± standard deviations, in megaPascals (MPa).

Table 9 summarizes the percentage distribution of different failure modes identified after μTBS test at T0 and T12. Whereas at T0 for all groups but TE/AU the majority of the failures were adhesive, followed by mixed failures and cohesive in resin composite, at T12, there were less mixed fractures and, especially in the AU groups, more cohesive failure in resin composite, irrespective of the dentin conditioner employed. After aging in the ZON/EF group there an increment in the adhesive failures.

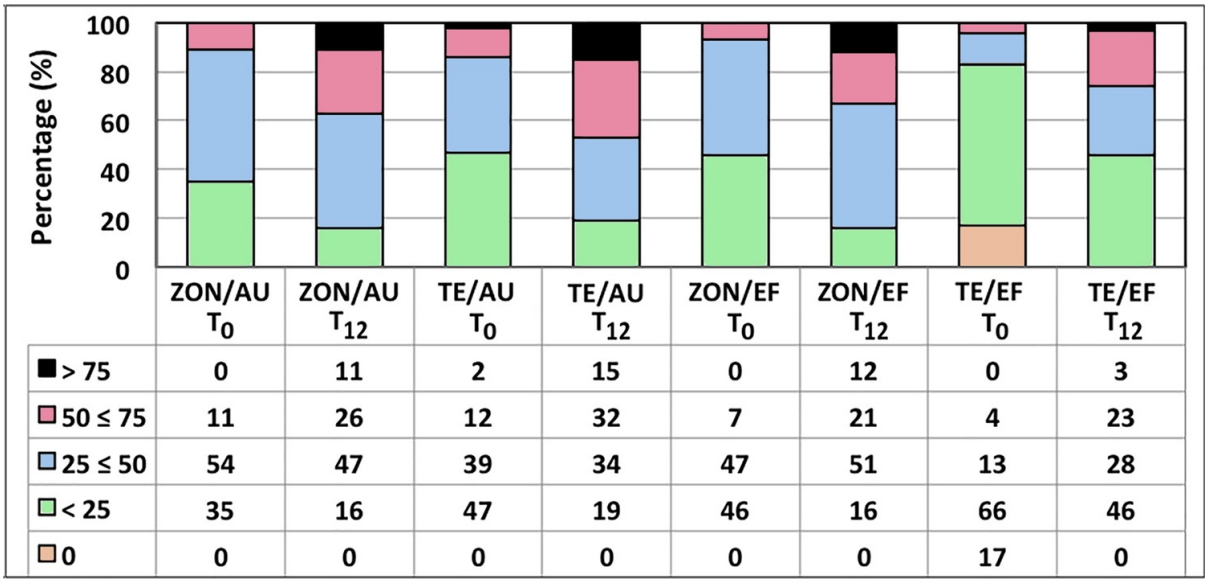
Table 9: Failure mode distributions and their percentages identified from the four experimental groups after bond strength testing at T0 and T12.

	Failure mode (%)							
	T_0				T12			
	A	CC	CD	M	A	CC	CD	M
ZON/AU	43%	34%	0%	23%	19%	73%	6%	2%
TE/AU	28%	43%	6%	24%	24%	61%	11%	4%
ZON/EF	57%	22%	2%	20%	81%	11%	3%	5%
TE/EF	47%	21%	8%	25%	52%	32%	0%	16%

Failure modes: A - adhesive; CC - cohesive in composite; CD - cohesive in dentin; M – mixed.
Abbreviations: ZON - experimental ZrO(NO3)2 etchant; TE - Total Etch 37 % H3PO3; AU - Adhese Universal adhesive; EF - Excite F adhesive.

Nanoleakage expression

Interfacial nanoleakage scores are summarized in Figure 28. Representative light microscopy images of nanoleakage expression for the four experimental groups tested at T0 or T12 are shown in Figures 29 and 30, respectively. The Chi-squared test indicated differences among the experimental groups in the percentage of silver deposition along the resin-dentin interfaces. At T0, there was no difference in nanoleakage expression among the experimental groups. There were more extensive silver deposits along the resin-dentin interfaces after the specimens were aged for 12 months (T12), compared with T0 ($p < 0.05$) and this manifestation was independent of the tested group. When the etchants were compared, the two groups that were etched with TE had more extensive interfacial silver deposits compared to the ZON groups ($p < 0.05$).



	ZON/AU T_0	ZON/AU T_{12}	TE/AU T_0	TE/AU T_{12}	ZON/EF T_0	ZON/EF T_{12}	TE/EF T_0	TE/EF T_{12}
> 75	0	11	2	15	0	12	0	3
50 ≤ 75	11	26	12	32	7	21	4	23
25 ≤ 50	54	47	39	34	47	51	13	28
< 25	35	16	47	19	46	16	66	46
0	0	0	0	0	0	0	17	0

Figure 28: Percentage of interfacial nanoleakage expression in resin-dentin interfaces created among the different groups, at T0 and T12.

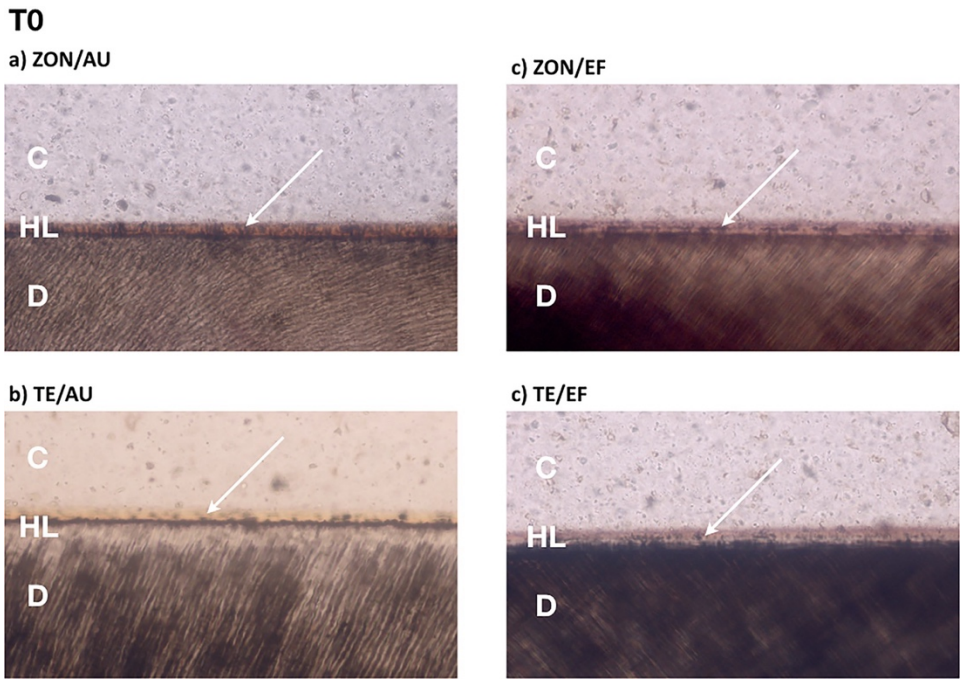


Figure 29. Representative light microscopy images (20x magnification) of the tested materials in the experimental conditions and submitted to nanoleakage with silver nitrate after 24 h of storage in artificial saliva (T0). D = Dentin; HL = Hybrid Layer; C = Composite. Arrows indicate areas of silver nitrate particles deposition.

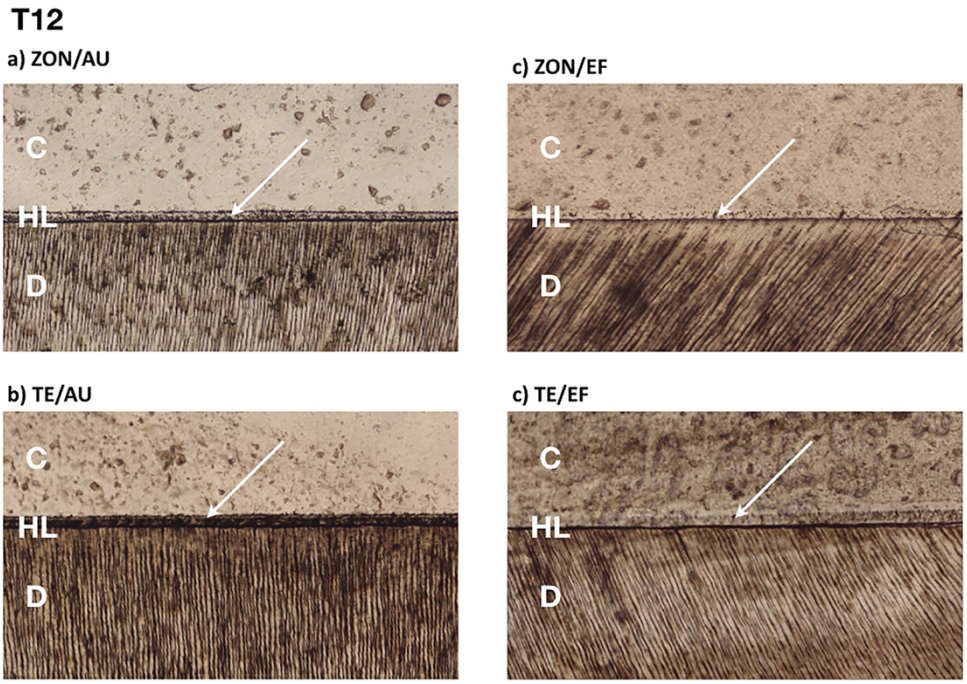


Figure 30. Representative light microscopy images (20x magnification) of the tested materials in the experimental conditions and submitted to nanoleakage with silver nitrate after 12 months of storage in artificial saliva (T12). D = Dentin; HL = Hybrid Layer; C = Composite. Arrows indicate areas of silver nitrate particles deposition.

Gelatin zymography

Zymography results are shown in Figure 31a and b. Qualitative and quantitative zymography assays identified the proform and active form of MMP-9 (≈92 kDa and 86 kDa, respectively) in mineralized dentin. Expression of MMP-9 was more pronounced in dentin demineralized with TE. In addition, an extra band that corresponded to the molecular weight of active MMP-2 was observed at ≈ 66 kDa. Dentin demineralized with ZON showed bands that corresponded to the molecular weight of pro-MMP-9, the intensity of which was slightly lower that what was presented in the TE-demineralized dentin. There was complete inhibition of MMP-2 and MMP-9 active forms.

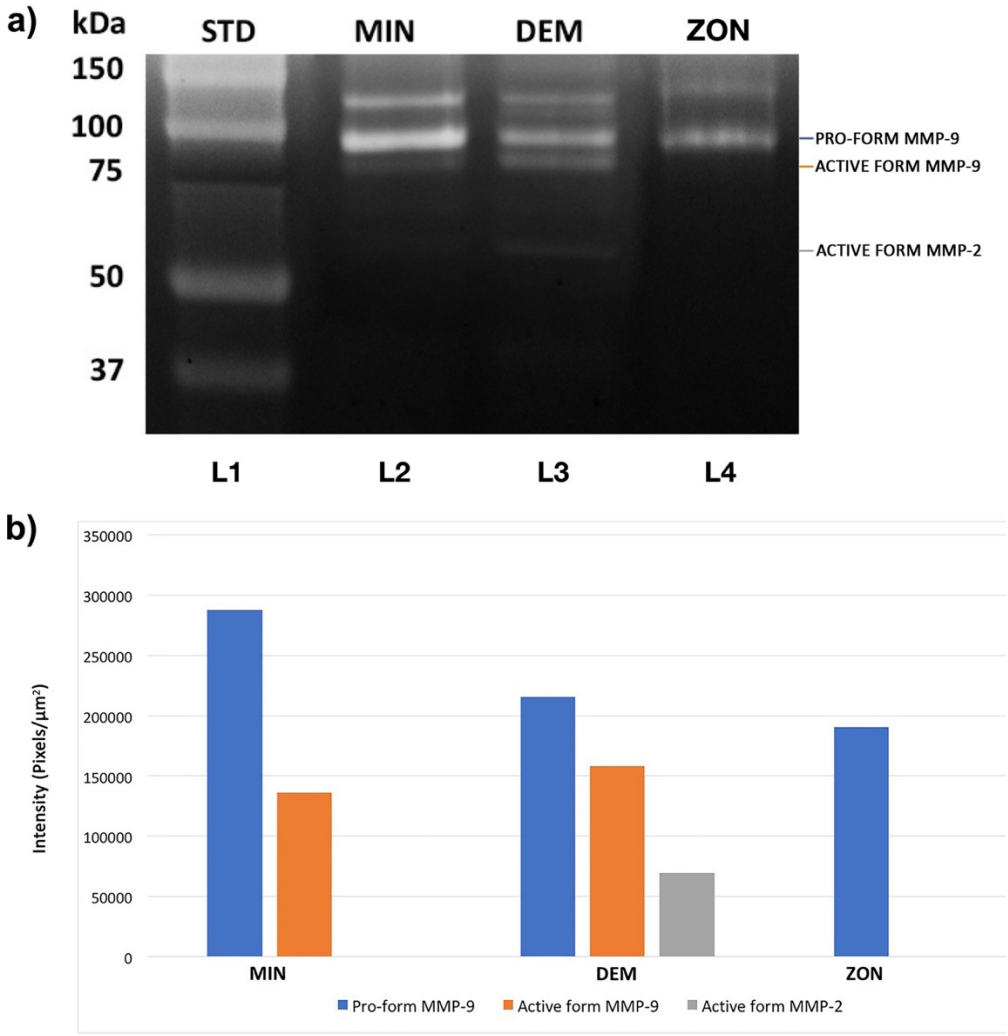


Figure 31. a) Zymographic analysis of proteins extracted from dentin powder. L1: Standards (STD); L2: mineralized dentin (MIN) showing the presence of activity of pro-form of MMP-9 (92 kDa) and active form of MMP- 2 (66 kDa); L3: demineralized dentin powder (DDP) showing an increase of MMP-9 (86 kDa) and the higher activity of the active form of MMP-2 (66 kDa); L4: Dentin powder demineralized with ZON showing inhibition of MMP-9 and MMP-2 activity. b) Graph illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder.

In situ zymography of resin-dentin interfaces

Qualitative and quantitative in situ zymography results are shown in Figures 32–34. The green fluorescence signals identified from the AU groups at T0 and T12 were lower compared to those exhibited by the EF groups. All groups demonstrated the general tendency of augmented protease activity after aging. Statistical analysis indicated that experimental groups treated with ZON had significantly lower levels of fluorescence at T0, compared to TE, irrespective of the bonding system employed ($p < 0.05$). After aging, there was also less fluorescence for ZON-etched dentin for the AU adhesive ($p < 0.05$). However, fluorescence in the ZON-etched dentin and TE-etched dentin that were bonded with the EF self-etch adhesive were very high and there was no difference between these two groups at T12 (Fig. 21, 3rd and 4th green columns; $p > 0.05$).

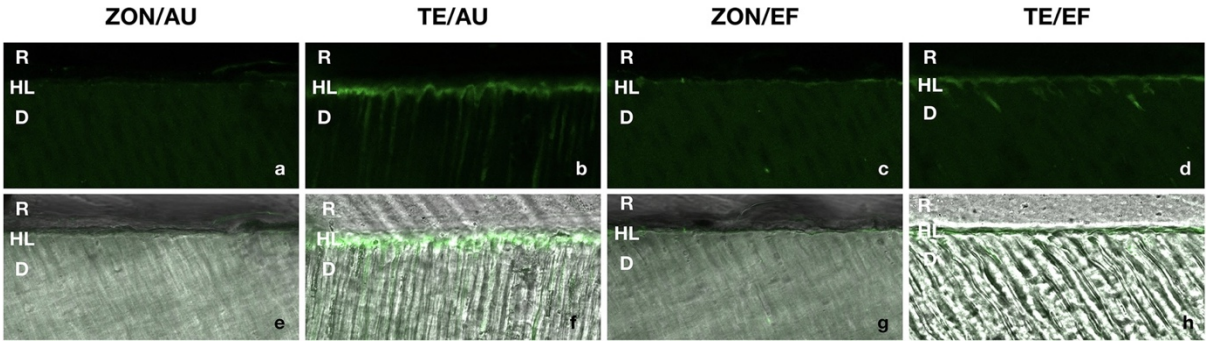


Figure 32. Resin-bonded mid-coronal dentin interfaces prepared with ZON/AU (a and e), TE/AU (b and f), ZON/EF (c and g) and TE/EF (d and h) at T0, incubated with quenched fluorescein-labeled gelatin. (a) Image acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created with ZON/AU; (b) Image acquired in green channel, showing fluorescence in dentinal tubules and within the HL created with TE/ AU; (c) Image acquired in green channel, showing fluorescence in dentinal tubules and within the HL created with ZON/EF; (d) Image acquired in green channel, showing fluorescence in dentinal tubules and within the HL created with TE/EF; (e) Image of the HL created by the application of ZON/AU obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel; (f) Image of the HL created by the application of TE/AU obtained by merging differential interference contrast image and image acquired in green channel; (g) Image of the HL created by the application of ZON/EF obtained by merging differential interference contrast image and image acquired in green channel; (h) Image of the HL created by the application of TE/EF obtained by merging differential interference contrast image and image acquired in green channel. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

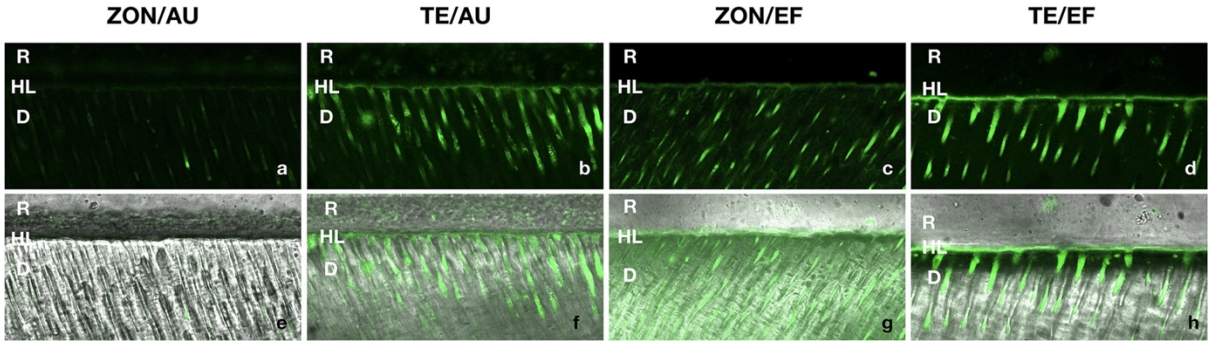


Figure 33. Resin-bonded mid-coronal dentin interfaces prepared with ZON/AU (a,e), TE/AU (b,f), ZON/EF (c,g) and TE/EF (d,h) at T12, incubated with quenched fluorescein-labeled gelatin. (a) Image acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created with ZON/AU; (b) Image acquired in green channel, showing fluorescence in dentinal tubules and within the HL created with TE/AU; (c) Image acquired in green channel, showing fluorescence in dentinal tubules and within the HL created with ZON/EF; (d) Image acquired in green channel, showing fluorescence in dentinal tubules and within the HL created with TE/EF; (e) Image of the HL created by the application of ZON/AU obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel; (f) Image of the HL created by the application of TE/AU obtained by merging differential interference contrast image and image acquired in green channel; (g) Image of the HL created by the application of ZON/EF obtained by merging differential interference contrast image and image acquired in green channel; (h) Image of the HL created by the application of TE/EF obtained by merging differential interference contrast image and image acquired in green channel. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

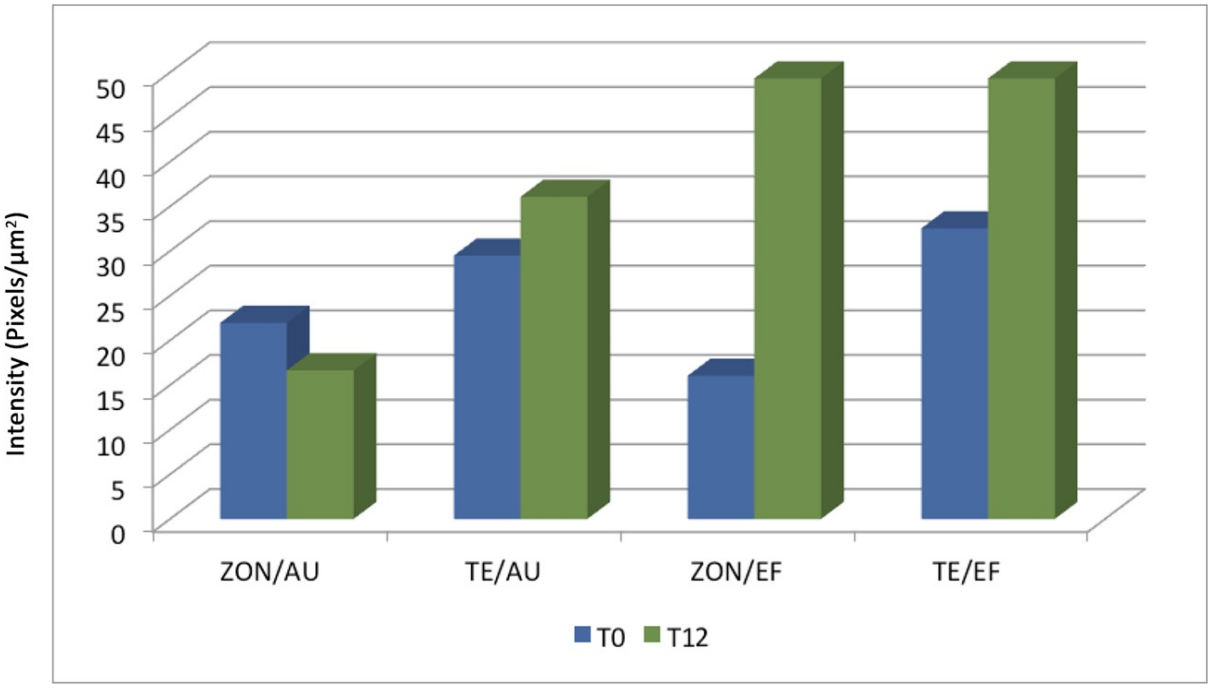


Figure 34. In situ zymography quantification at T0 and T12 showing reduced enzymatic activity in ZON groups when used in association with both AU and EF adhesive systems.

CHAPTER 5
Discussion, Conclusions & Future Directions

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 marginal infiltration at the resin dentin interfaces, the presence of endogenous enzymatic activity after treatment and the localization of the gelatinase activity in dentin. Additionally, the effect of the cross-linkers and the effect of treatment period (duration) on the degradation of collagen matrices were examined.

The presented thesis demonstrated that the cross-linkers employed were efficient in preserving bond strength after 12 months or 5 years of aging and hybrid layer integrity after aging in vitro in artificial saliva at 37°C when used as a separate primer on demineralized dentin. Hence, the first hypothesis can be rejected. The enzymatic activity was lower in all experimental groups when associated at demineralized dentin, at baseline, as well as after aging (for 1 year and 5 years) with a slight influence of the bonding mode. In the cases when the cross-linking agent was utilized on mineralized dentin before the adhesive procedures, the bond preservation was similar to the other groups. Hence, the second hypothesis of the thesis can be partially rejected.

Discussion

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 (Breschi et al., 2010b). 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 (Pashley et al., 2004). 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.

Influence 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 agents in increasing the bond strength of the adhesive interface with several bonding agents, both on coronal and radicular dentin, was assessed through microtensile bond strength test and the cross-linkers application period was established in 1 min according to the finding published by Tezvergil-Mutluay et al. (A. Mazzoni et al., 2013), 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, chitosan, proanthocidin and EDC, as biomodifying agents, although the application time required to be effective (10 min to several hours) (Mazzoni et al., 2014; D. Pashley et al., 2011) 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 (Mazzoni et al., 2014). These findings were further confirmed by a recent study conducted by Mazzoni et al. (Mazzoni et al., 2014) demonstrating that 1 min or even 30s 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 mm2 or less. The microtensile bond strength test is calculated as the tensile load at failure divided by the crosssectional area of the bonded interface (Mazzoni et al., 2012).

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 (Nascimento et al., 2011; Saboia et al., 2008; Franklin R. Tay et al., 2002) and Pashley et al. (D. Pashley et al., 2011) 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 (Armstrong et al., 2001; Hosaka et al., 2009). Hence, this technique of storage could be considered as a method of accelerating aging.

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 (Hashimoto et al., 2000; Komori et al., 2009; Sadek et al., 2010). Cross-linking agents increase collagen stiffness by creating interfibrillar and intrafibrillar cross-links, thereby strengthening the collagen network structure (Frassetto et al., 2016). 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 (Chung et al., 2004).

The present thesis demonstrated for the first time that EDC pretreatment retained significantly higher bond strength values, even after 5 y of accelerated aging in artificial saliva. In the conducted study, EDC was more efficient in bond strength preservation when applied with an etch-and-rinse adhesive, probably due to differences in the application mode and properties of the adhesive. In etch-and-rinse adhesive systems, dentin is etched, rinsed, and then pretreated with the EDC aqueous solution, enabling the cross-linker to interact freely with collagen molecules, with an abundance of collagen substrate to cross-link and exposed MMPs to inactivate (Mazzoni et al., 2006). We can speculate that during the course of aging, EDC may still exert its cross-linking potential in the resin-sparse, water-rich layer of collagen fibrils

at the bottom of the hybrid layer. As compared with an etch-and-rinse adhesive, separate EDC pretreatment associated with the use of a self-etch adhesive is conducted after placement of the self-etching primer on dentin. EDC may not be as efficient in diffusing through a bed of partially demineralized collagen fibrils that has been infiltrated with a mixture of water and resin. Moreover, CSE bond contains 10-methacryloyloxydecyl dihydrogen phosphate, an acidic phosphate monomer with the purported ability to bind chemically to dentin (Van Meerbeek et al., 2011). The HLs created with this system are thinner since the penetration of the acidic monomer is shallower, with better dentin hybridization than an etch-and-rinse adhesive (Breschi et al., 2004). In addition, there is likely to be less exposed MMPs for the EDC to inactivate and less denuded collagen fibrils to cross-link, as demonstrated in the SEM failure analysis, with dentin tubules occluded by smear layer, as compared with the etch-and-rinse groups, which presented open dentin tubules and exposed sparse denuded collagen fibrils (Figures 1, 2). The present EDC-priming protocol appears to be clinically feasible, considering that EDC is a compound with low trans- dentinal cytotoxicity (Scheffel et al., 2015) and it prolongs the adhesive procedure for merely 1 min. Clinical trials are necessary to confirm the effectiveness of EDC in the preservation of resin-dentin bonds over time. Incorporation of EDC as a component of dentin adhesive systems may result in simplification of the clinical application procedure, making it more appealing to dental practitioners.

Also, a second cross-linking molecule has been tested in this research work both on coronal and radicular dentin, the dicyclohexylcarbodiimide (DCC). DCC is a 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 with 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 have demonstrated to be primers exploitable for the 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 (Hosaka et al., 2007; Sadek et al., 2010), thereby creating more durable resin bonds. Sauro et al. (Sauro et al., 2009) 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.

Several studies have also investigated the effect of glutaraldehyde, on the mechanical properties of the dentine organic matrix, as well as on the preservation of the hybrid layer (Bedran-Russo et al., 2008, 2007; Chen et al., 2016; Hass et al., 2016; Hiraishi et al., 2013; R. Liu et al., 2011; Seseogullari-Dirihan et al., 2015) Glutaraldehyde improves the mechanical properties and reduces the degradation rate of biological tissues (Nimni, 1988; H. Sung et al., 1999). 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 (Bedran-Russo et al., 2010, 2007; Macedo et al., 2009).

A desensitizing agent called GLUMA desensitizer (Heraeus Kulzer GmgH, Hanau, Germany) or in its Gel composition GLUMA desensitizer power gel (Heraeus Kulzer GmgH), containing purified water, hydroxyethyl methacrylate (HEMA) 35%, glutardialdehyde 5% - 10% and pyrogenic silicic (Chu and Lo, 2010; Wang et al., 2010) have been proposed on the market as dentin desensitizer to reduce postoperative sensitivity. This material has been proven effective as a desensitizer applied on exposed and sensitive dentine (Sayed et al., 2021; Schüpbach et al., 1997). Its desensitizing effects are to be ascribed mainly to its components, HEMA and Glutaraldehyde. HEMA is a resin that penetrates within the dentinal tubules and physically seals them. Because HEMA is soluble in water, it fosters permeation of the glutaraldehyde in the tubules and enables in-depth action (Rajnekar et al., 2022). The desensitizing effects of glutaraldehyde are not the sole positive effects ascribed to GLUMA. Indeed, it contains glutaraldehyde, with its already described cross-linking properties on the demineralized collagen matrix if utilized before adhesive application. GLUMA desensitizer has also already shown to perform well in terms of bond strength when in association with etch and rinse adhesives and self-adhesive cements (Aranha et al., 2006; Sailer et al., 2012). However, little is known about the effect of glutaraldehyde used as dentin pretreatment on the bond strength and enzymatic interaction of self-etch adhesives. For this reason, the study presented, analyzed the effects of the application of this desensitizing agent in two formulations

on the bond strength on dentin before adhesion procedures with two universal adhesives in the self-etch mode.

According to our results, better stability of the hybrid layer was observed when the desensitizers were applied compared to the control group, irrespective of the type of adhesive.

As already stated, HEMA is a water-soluble monomer present in most current hydrophilic dental adhesives to facilitate resin infiltration in the moist dentin substrate and to facilitate the reaction with dentin collagen due to its ester and hydroxyl groups (Xu et al., 1997). Indeed, HEMA was found to decrease surface tension of water molecules and subsequently increase monomer penetration into dentin facilitating the diffusion of the hydrophilic resin monomer into the tubules, and improving the efficacy of the resin-dentin bonds (Nakabayashi et al., 1992). Previously, products containing glutaraldehyde and HEMA in their formulations have demonstrated the formation of a collagen-glutaraldehyde layer at the dentin/desensitizer interfaces ready to chemically interact with HEMA molecules (Munksgaard and Asmussen, 1984). Eventually, copolymerization occurs between the adhesive and HEMA complexes increasing the resin-dentin bonding performances (Munksgaard and Asmussen, 1984; Qin et al., 2006), and this may explain the enhanced experimental groups' bond strength in the present study.

Regarding the desensitizer used in this study, GLUMA, in both its consistencies, has a pH of ≈ 3. When it comes to the evaluation of the effect of acidity on smear layer modification, we can cautiously hypothesize that this desensitizer acted similarly to a mild SE adhesive which only superficially/partially demineralized the dentin surface, leaving the hydroxyapatite remnants still available for chemical bonding (Giannini et al., 2015). In partial support of this statement, the SEM images showed a greater dissolution of the smear layer in the groups treated with the desensitizers, irrespective of the consistency of the product (Fig. 3). It would seem that the application of an acidic universal adhesive resin after GLUMA pretreatment has provided higher smear layer dissolution, dentin interaction and possibly chemical interaction

with the underlying dentin substrates. Due to the complex mechanism of bonding to dentin, the latter information can only be assumed, as this was not taken into consideration in our methodology, thus requiring further evaluations and future studies should be performed to compare this potential etching efficacy with other dentin pre-treatment methods, such as phosphoric acid or EDTA (Mazzitelli et al., 2010). Apart from the possible chemical interaction with the dental substrate, in the presence of open dentinal tubules filled with HEMA, observed after GLUMA application, resin penetration into the dentin could be improved, leading to higher bond strength compared to the control groups, independent of the testing times (Tab. 2), and this was in accordance with previous studies [56,104]. It could be suggested that GLUMA could work as an intermediate between an acid etching and demineralized agent, thus providing more than partial smear layer dissolution and aperture of dentinal tubules ready to interact with the subsequent adhesive resin. Thus, the smear layer removal and the resin penetration might have been improved with the subsequent application of the universal adhesives after GLUMA, and this might be another reason explaining the higher results in the bonding strength of the experimental groups [56,104].

When the liquid version of GLUMA was used before adhesive application, statistically higher bonding values were recorded when compared to gel consistency (Tab. 6). These results are in accordance with the study of Lee and Sabatini where the two formulations of GLUMA were tested with an etch-and-rinse adhesive (Lee and Sabatini, 2017). It is worth mentioning that the two forms of the desensitizer, liquid and gel, possess identical formulations in terms of glutaraldehyde and HEMA content, as well as presented the same application modality (in terms of application time and the removal method). Therefore, it could be speculated that the flowability of the liquid version has enhanced its ability to diffuse among the dentin collagen fibrils and the smear layer compared to the more viscous gel. It could also be possible that the gel formulation is more difficult to rinse off from the dentin surface, leaving remnants of the

material on the surface, possibly hindering the subsequent bonding mechanisms of resin penetration and polymerization.

As the two adhesives employed in the study have shown bond strength differences but exhibited no differences in terms of enzymatic activity, the third null hypothesis must also be partially rejected. AU performed statistically better than IBU immediately and after aging in terms of bonding performance, irrespective of the dentin pretreatment with the desensitizer. The two adhesives differ in terms of their acidity and composition. IBU has a pH of ≈ 1.4-1.8 and it is classified as a strong universal adhesive while AU is reported to have a pH of around 2.6 and it is classified as a mild universal adhesive. Dentin bond strength of intermediately strong universal adhesives has been indicated as less stable over time (Cuevas-Suárez et al., 2019). These lower values could be explained by the presence of unpolymerized monomers remaining after light activation, which continue to demineralize the dentin due to their high level of acidity, thus promoting dentin-adhesive interfaces with low hydrolytic stability and low-stability chemical interactions with the collagen (Van Meerbeek et al., 2011). Furthermore, these two systems have different solvents. While AU contains ethanol, IBU contains acetone and water. Acetone-based adhesives are more prone to hydrolytic degradation (Tjäderhane et al., 2013). In fact, IBU previously demonstrated higher hydrophilicity, resulting in swallowing phenomena and plasticization after polymerization (Mazzitelli et al., 2022b, 2020), as also observed in the present SEM images.

The tested material GLUMA is indicated and has proven its efficacy, prior to adhesive restorations, in those cases where, due to extended cavity preparation, or proximity with the pulp, postoperative sensibility may develop. Contrarily to other crosslinkers, that require an additional clinical step solely to achieve improved bonding and stability of the hybrid layer, GLUMA could combine in a single step beneficial desensitizing effect and act as a bonding enhancer and stabilizer.

Despite attempts to improve adhesive performance and to promote more stable resin-dentin interfaces through the use of protease inhibitors, degradation of these interfaces have been reported with all types of bonding approaches (Abu-Hanna et al., 2004; Hashimoto et al., 2002; Van Meerbeek et al., 2011) due to the fact that the depth of etching dentin with phosphoric acid is always higher than the depth of penetration of the adhesive resin into the denuded collagen fibrils. Therefore, alternative etchants have been developed. An experimental zirconium oxynitrate conditioner [ZrO (NO3)2] has recently been introduced to adhesive dentistry. This material was investigated in this thesis for its peculiar characteristics of dissoluting prevalentely the inorganic component of dentin, thus reducing nanoleakage and inhibit MMP-mediated proteolytic activity at the resin-dentin interface maintaining bond stability over time (Pashley et al., 2004).

In the study conducted the experimental ZON etchant not only did not impair immediate bond strength, but increased that of the universal adhesive Adhese Universal (D. H. Pashley et al., 2011a). The ZON etchant is a Lewis acidic metal salt that is readily soluble in water. Water is necessary for the dissolution of the zirconium salt to create an acidic condition (pH = 0.56) that is conducive for demineralizing the inorganic components of dentin. During demineralization, Ca^{2+} and PO^{24-} ions are released from dentin and these ions bind to the Zr^{4+} ions as to form a solid complex (Solovkin and Tsvetkova, 1962). A preliminary scanning electron microscopy and energy-dispersive X-ray spectroscopy study performed by this research group confirmed the precipitation of Ca^{2+} and P^{3-} ions on the surface of dentin treated with ZON for 30 s, with tubules more closed compared to dentin etched with TE for 15 s (unpublished data).

The ZON etchant increased the immediate bond strength of AU to dentin, resulting in acceptable immediate adhesion in all the experimental groups. It has been speculated cautiously that the chemical interaction between dentin and MDP does not have any influence on the

immediate bond strength to dentin, but helps to improve bond stability (Erhardt et al., 2011). Such a speculation was validated in the present study, wherein the bond strength of AU did not decrease with the use of conventional etching after 12 months of laboratory aging.

Significant decrease in bond strength was observed for ZON/AU at T12, however, it is possible that the bond strength values in this group were underestimated, since the adhesive bond strength between the tooth and the composite exceeded the cohesive bond strength within the material itself (73 % of CC failure mode). The 10-MDP and the methacrylated carboxylic acid polymer incorporated within AU possibly enabled the adhesive to produce durable bonds to dentin (Mancuso et al., 2022; Sauro et al., 2009; Visse and Nagase, 2003) Nurrohman et al. (Nurrohman et al., 2012) investigated the interaction of MDP and the carboxylic-based functional co-polymer on the formation of crystallites on the bottom of the hybrid layer and preservation of the underlying dentin structure after the exposure to acid attack. Interestingly, the study revealed that both functional monomers/polymers were more efficient in creating a crystallite layer and protecting the underlying dentin structure in the self-etch compared to etch-and-rinse systems, since the etching with phosphoric acid always penetrates deeper into the dentin structure than the adhesive resin itself (D. H. Pashley et al., 2011b). Although in the control MDP-containing etch-and-rinse samples there was apatite formation on the bottom of the hybrid layer, after the acid attack, the destruction of the underlying dentin structure was prevented by the MDP only partially. AU in general performed better in the present study in terms of bond strength. AU contains both the 10-MDP and methacrylated carboxylic acid polymer, contributing to a possibly stronger interaction with the apatite crystals on the bottom of the etched dentin surface, leading to higher bond strength compared to EF, even after aging in the artificial saliva. Furthermore, the precipitation of Ca^{2+} and PO^{42-} ions on the bottom of the hybrid layer formed after etching with ZON might have enhanced the MDP interaction with the apatite crystals. The experimental zirconium oxynitrate etchant has the ability to improve

bond strength, particularly when used before a universal adhesive containing functional monomers.

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 (Tay FR, King NM, Chan K, 2002). Its high sensitivity to detect proteolytic activity in samples with levels of pg of MMPs render it favorable in comparison to ELISA (Leber and Balkwill, 1997; Tay FR, King NM, Chan K, 2002). Mazzoni et al. (Mazzoni et al., 2007) 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 (Mazzoni et al., 2012, 2006; Nishitani et al., 2006). Mazzoni et al. (A. Mazzoni et al., 2013) 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 enzymatic 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 (Mazzoni et al., 2014, 2006). It has been hypothesized that cross-linking agents alter the three-dimensional conformation of the catalytic domain of MMPs, or change their negatively-charged ionized 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 (Nascimento et al., 2011; Tersariol et al., 2010). 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.

As for the molecule tested, chitosan, the data available on the cross-linking effect are scarce but are in accordance with the data available on other cross-linkers. Previously, our research group demonstrated by means of gelatin zymography that the 0.1% chitosan primer was able to inhibit the expression of the MMPs when used with a universal adhesive in the SE mode. However, it activated the MMPs when used before a 3-step etch-and-rinse adhesive (Baena et al., 2020). The results of the present study are in accordance with the previous findings as chitosan dissolved in water at low concentrations was able to decrease the activity and expression of dentinal endogenous proteases when used on unrestored dentin as well as before a universal adhesive placed in the etch-and-rinse mode. This indicates that the anti-enzymatic activity of chitosan is also adhesive system dependent. It seems that chitosan (0.1% water solution) is more effective when used with universal adhesives. Another interesting point to discuss is the influence of the application of the adhesive resin over a cross-linking primer. In the present study, the adhesive resins significantly increased the enzymatic activity in the dentin underlying the HL. It has been previously shown that both the etch-and-rinse and self-etch adhesives activate the dentinal MMPs (A. Mazzoni et al., 2013; Mazzoni et al., 2011a; Nishitani et al., 2006), which is in accordance with the results of the present study (for all the groups except the 0.1% chitosan). It was observed, in the study depicted, that the chitosan concentration had an influence on the MPs expression. In this case, the lower the chitosan concentration, the lower the enzymatic activity. Chitosan 1% and 0.5% revealed gelatinolytic

bands corresponding to active MMP-2, while there were no active forms of MMPs expressed in the 0.1% CH group, and the pro-form MMP-9 and MMP-2 bands were faint.

The experimental etchant was also investigated in this thesis for its enzymatic inhibition abilities. The zymography results in the work revealed inhibition of MMP activities when the ZON etchant was used to demineralize the dentin surface, a feature that was not identified with the use of the TE etchant. In situ zymography identified more potent inhibition of enzymatic activity by ZON, when compared with TE, at T0 and T12 (Figure 18). These results could be explained by the ability of ZON to bind to Ca2+ ions via chelation. Because of this mechanism, the ZON etchant removes Ca2+ ions from the catalytic site of MMP-2 and MMP-9 that are crucial for the optimal function of these proteases (Visse and Nagase, 2003). Such a phenomenon is analogous to the chelation effect of EDTA in MMPs inhibition (Sauro et al., 2009). Further studies should be performed to validate the depth of demineralization created by the ZON etchant on dentin.

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 on 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. (Galis et al., 1994) 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.

(Mazzoni et al., 2012), 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 (Goodall et al., 2001; Lindsey et al., 2001; Teesalu et al., 2001). 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.

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 (Bedran-Russo et al., 2010; A. Tezvergil-Mutluay et al., 2012) 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 (A. Mazzoni et al., 2013), even after adhesive application (Mazzoni et al., 2014; Tezvergil-Mutluay et al., 2015).

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 a 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 (Sadek et al., 2010)(Cadenaro et al., 2009).

Considering the glutaraldehyde and HEMA compounds, in our study, the application of the desensitizing agents influenced dentin MMPs activity only at T_0, whilst after 1 year of aging no statistical significance was found among the groups.

Glutaraldehyde, in addition to its well-known disinfectant and desensitizing properties, is also widely known as a potent protein cross-linker reacting with the amino groups of proteins within minutes (Kiernan, 2000). As previously observed, GLUMA was able to inactivate matrix-bound dentin proteinases in demineralized dentin matrices almost entirely in a short time application (within 30 s of exposure) (Sabatini et al., 2014). The inactivation of MMPs induced by a collagen cross-linker is a non-specific mechanism involving covalent bonds that are claimed to be very stable over time (Tersariol et al., 2010). Studies have shown a significant decrease in MMPs activation after the treatment of demineralized dentin with glutaraldehyde solutions (Chen et al., 2016; Hass et al., 2016).

In the presented study, however, the application of GLUMA for 30s prior to the SE adhesive procedures resulted in a statistically significant increase in the MMPs expression in the HL. Differently from the cited studies where the dentin was etched and then only primed with GLUMA, in this research, GLUMA was applied for 30 s on mineralized dentin prior to the universal adhesive application in the SE mode. Thus, the application of GLUMA was not

performed on demineralized dentin but on a mineralized substrate. This should be considered, as Qin et al. (Qin et al., 2006) stated that the glutaraldehyde present in GLUMA could not form cross-links with mineralized dentin. Indeed, the enzymatic inhibition of glutaraldehyde requires contact with collagen fibrils. Its application before the demineralization procedures could result in the presence of a smear layer preventing contact with the fibrils and prone to nullify the cross-linking ability of GLUMA. Another recent study (Mazzoni et al., 2018) demonstrated that a cross-linker used before a SE adhesive was not able to inactivate the MMPs, or improve the longevity of the hybrid layer as efficiently as in the case of the etch-and-rinse adhesives.

Moreover, the mild acidity expressed by GLUMA can activate MMPs (Davis et al., 1992; Tjäderhane et al., 1998) by inducing the cysteine switch (Chaussain-Miller et al., 2006). Acidic resin monomers may activate latent forms of MMPs (pro-MMPs) via the cysteine-switch mechanism that exposes the catalytic domains of these enzymes that were blocked by pro-peptides, and also provide excellent conditions for the activation of the protease's cysteine cathepsins (A. Mazzoni et al., 2013; Tallant et al., 2010; Tersariol et al., 2010). This might explain the further activation of MMPs and the consequent activation of the enzymes at T_0.

Aging negatively affected bonding performances and determined a reduction of enzymatic activity in the tested groups. As no significant differences in terms of endogenous enzymatic activity were found irrespective of the adhesive and the desensitizing agent used, this reduction seems to be independent of the influence of the MMPs, and prevalently due to the hydrolytic degradation of the adhesive resin. Both adhesives employed in the study, IBU and AU, contain functional monomers. It has been postulated that the Ca salts created by these functional monomers on the dentin surface may inhibit MMP activity reducing the enzymatic activity over time (Shen et al., 2020; Yuan et al., 2023), explaining the decrease of the activity of the samples in all groups after 1 y storage in artificial saliva.

In situ zymography performed on samples treated with the experimental etching, identified more potent inhibition of enzymatic activity by ZON, when compared with TE, at T0 and T12 (Figures 19, 20). These results could be explained by the ability of ZON to bind to Ca2+ ions via chelation. Because of this mechanism, the ZON etchant removes Ca2+ ions from the catalytic site of MMP-2 and MMP-9 that are crucial for the optimal function of these proteases (Visse and Nagase, 2003). Such a phenomenon is analogous to the chelation effect of EDTA in MMPs inhibition (Sauro et al., 2009). Further studies should be performed to validate the depth of demineralization created by the ZON etchant on dentin.

Conclusions

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

- Collagen cross-linkers tested do not alter immediate bond strength to dentin, and are effective molecules in preserving the adhesive interface over time.
- EDC, DCC and Chitosan help in preserving the degradation of dentin organic matrix by inactivating dentin endogenous enzymes.
- The inhibiting effect of cross-linkers on the enzymatic activity 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 and for 5 years when EDC was used.
- Although the crosslinkers tested have shown to been able to reduce the enzymatic activity of demineralized and partially demineralized dentin, this inhibition might be reduced and not necessary when the crosslinkers are used in mineralized dentin.
- Short applications time of the cross-linkers (1 min or 30s) on dentin is clinically acceptable and is effective in preventing degradation of the adhesive interface over time and inhibiting endogenous enzymatic activity
- New approaches for the demineralization of the dentin substrate may promote the stabilization of the dentin hybrid layer, improving bond strength over time.

Future Directions

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 slow down/inhibit 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 or desensitizing agents 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 encouraging results obtained from this thesis. Additionally, *in vivo* studies will be essential to better understand the feasibility of the tested molecules as dentin conditioning primer during bonding procedures.

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