Nanostructural analysis of the adhesive interface in dentistry

UNIVERSITA’ DEGLI STUDI DI TRIESTE

DOTTORATO DI RICERCA IN
NANOTECNOLOGIE

XXVI CICLO

Nanostructural analysis of the adhesive interface in dentistry
(Settore Scientifico Disciplinare MED 28)

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ANNO ACCADEMICO 2012/2013
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INTRODUCTION
Introduction

The subject of this thesis is the stability of the adhesive interface in dentistry. Success in adhesive dentistry means long lasting restorations. However, there is substantial evidence that this ideal objective is not achieved. Current research in this field aims at increasing the resin-dentin bond durability. This doctoral research examines the fundamental processes responsible for the aging mechanisms involved in the degradation of resin-bonded interfaces, as well as some potential approaches to prevent and counteract this degradation. Resin-dentin bond degradation is a complex process that is not completely understood, involving the hydrolysis of both the resin and the collagen component of the hybrid layer. The hydrophilic and acidic characteristics of current dentin adhesives have made hybrid layers highly prone to water sorption, which causes polymer degradation and results in decreased resin–dentin bond strength over time. These unstable polymers inside the hybrid layer may result in an incomplete encapsulation of collagen fibers, which become vulnerable to mechanical and hydrolytical fatigue, as well as degradation by host-derived proteases with collagenolytic activity. These enzymes, such as matrix metalloproteinases (MMPs) and cysteine cathepsins, have a crucial role in the degradation of type I collagen, the organic component of the hybrid layer. The first part of this thesis aims to review the current knowledge regarding adhesion to the tooth substrate (Chapter 1), focusing on the fundamental processes that are responsible for the degradation of the adhesive interface (Chapter 2). Since the permeability of adhesives to water is particularly evident in simplified adhesive formulations, the research activity was focused on self-etch and universal adhesive systems’ behaviour and the results are presented in Chapter 3 and 4 respectively. The last part of the thesis is focused on both the strategies to inhibit the proteolytic and collagenolytic activity of the endogenous proteases and the methods to
increase the mechanical strength of collagen network and its resistance to enzymatic degradation (Chapter 5). In particular, the ability of a cross-linker agent, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), to prevent collagen degradation was evaluated under occlusal cycle loading (Chapter 6), showing that EDC contributes to stabilize the collagen network reducing the enzymatic degradation rate. Thus, dentin collagen reinforcement and strengthening through EDC cross-linking might be of importance to improve the bond strength and structural integrity of the resin-dentin interface over time against the enzymatic and hydrolytic degradation. Unlike typical tissue engineering applications where a synthetic scaffold is designed to be resorbed and replaced by the host’s own tissues with 2-3 months, in situ tissue engineering is designed to create a resin-enveloped collagen scaffold that will remain in place for decades. This is made possible by the unique location of teeth where the crowns of teeth project into the oral cavity and are free of cells on their outer surfaces. We are entering a new era in adhesive dentistry, where resin-bonding to enamel and dentin is beginning to be understood at the nanoscopic level. The long-term success and durability of resin-dentin bonds depends upon the ability of dentists to capitalize on new discoveries being made in nanotechnology.
CHAPTER 1

DENTAL ADHESION
Dental adhesion

1.1 Introduction

The concept of adhesion to dental tissues was introduced in the field of dentistry in the middle of the past century, when the Swiss chemist Oskar Hagger developed the archetype for adhesive monomers based on glycerophosphoric acid dimethacrylate [1]. This material was first used for a dental filling by McLean and Kramer, who published the first paper on dentin-bonding agents (DBA) [2]. Poor adhesion of this restorative material to prepared teeth, however, led these authors to advocate the surgical removal of sound tissue during cavity preparation to ensure mechanical macro-retention.

The “adhesive revolution” of the 1950s was led by Buonocore, who found that acid etching could significantly enhance the bonding of restorations to enamel [3]. At that time, the “Adhesive Dentistry Age” began: traditional mechanical methods of preparing teeth for filling that had been based on Black’s concept of “extension for prevention” [4] were replaced by a more conservative approach in many branches of dentistry. Indeed, the ability of clinicians to bond resin materials to enamel and dentin has changed the concepts of cavity preparation, orthodontic treatment, caries prevention, and cementation of fixed prostheses [5].

1.2 Concept of adhesion

The word “adhesion” is derived from the Latin term adhaerere, composed of ad (to) and haerere (to stick). Adhesion is defined by the Specification D907 of the American Society for Testing and Materials as, “the state in which two surfaces are held together by interfacial forces, which may consist of valence forces or interlocking forces or both” [6].
In adhesive terminology, the adhesive material is referred to as the *adherent* and the substrate as the *adherend*. The adhesive, frequently a fluid, joins two substrates and solidifies. *Bond strength* designates the force necessary to divide the two adherends and *durability* is the time during which the bond is effective [7].

Adhesion (bonding) is the process of forming an adhesive joint that consists of two joined substrates. Most adhesive joints involve only two interfaces; bonded composite restoration (Fig. 1) is an example of a more complex adhesive joint.

![Fig. 1 A bonded interface is an example of a complex adhesive joint.](image)

Five theoretical approaches have been proposed to explain the mechanisms of adhesion [8]:

1. *Mechanical* theories state that the solidified adhesive interlocks micromechanically with the rough and irregular adherend surface.

2. *Chemical* theories describe all chemical bonds between the adhesive and the adherend. In the strongest joints, atoms of the two materials exchange (ionic bonding) or share (covalent bonding) outer electrons. Weaker bonds are formed when oxygen, nitrogen, or fluorine atoms of the two materials share a hydrogen nucleus (hydrogen bonding).
3. *Adsorption* theories propose that adhesion is the result of bonding between mobile molecules. Two molecules are attracted by a Van der Waals force, wherein each molecule has a positively or negatively charged region (pole). In the simplest bonds, such molecules have different poles with respect to average charge density; larger or more complex molecules may exhibit multiple poles of greater positive or negative charge. These poles may be a permanent molecular property (Keesom forces) or a transient effect produced by the random movement of electrons within the molecules, resulting in a temporary concentration of electrons in one region (London forces).

4. *Electrostatic* theories state that an electrical double layer is formed at the interface between a metal and a polymer, contributing to bond strength. Some conducting materials may pass electrons that alter the electrical charge at the joint, producing attractive electrostatic forces between the materials.

5. *Diffusive* theories propose that adhesion is the result of an interface through which molecules can cross to react with molecules on the other side. This mechanism is involved in sintering. The compression and heating of metal or ceramic powders causes particles to join with each other through the diffusion of atoms.

All of these interfacial phenomena require that the two materials being joined are sufficiently close and in intimate contact. Wetting, which depends on surface tension, also plays an important role in adhesion [9]. The theory of wetting claims that a solid with high surface tension is more wettable than one with low surface tension, and good adhesion is achieved when the energy of the adhesive is smaller than that of the solid. The free energy of the bonding agent should thus be as large as possible to allow penetration into surface gaps.
The wettability of a surface by a liquid is characterized by the contact angle of a droplet placed on the surface [10]. Low contact angles (< 90°; wettable) produce favorable wetting because the fluid will cover a larger surface area; high angles (≥ 90°; non-wettable) produce unfavorable wetting because the fluid will remain compact on the surface (Fig. 2). In the context of water, a wettable surface is defined as hydrophilic and a non-wettable surface is called hydrophobic. According to the theories of wetting and surface energies, adhesion to enamel can be more readily achieved than adhesion to dentin. Enamel is primarily composed of hydroxyapatite, which has a high surface energy; dentin also contains collagen and has a low surface energy.

Fig. 2 (A) The role of contact angle in wetting theory. (B) Fluids exhibiting increasing wettability (a–c).

1.3 Advantages of adhesion

Adhesive bonds have many advantages over mechanical bonds, such as greater resistance to fatigue, vibrations, and corrosion, and the ability to concurrently seal and bond.

Adhesive bonds are superior to mechanical bonds because they produce lower stress concentrations and can fully utilize the properties of adherends. However, adhesive bonds require much larger areas than mechanical bonds to bear the same load. Bonding agents are polymeric materials that can be attached without the application of forces, permitting the assembly of fragile materials (such as ceramics) that cannot be attached with mechanical
loading. A primary disadvantage of adhesives is their reliance on adhesion for load transfer through the assembly. Because adhesion is a surface phenomenon, the properties of the adhesive bond are correlated directly with surface properties. The adequacy of a surface is thus a critical factor in adhesive bonding [11].

1.4 Chemical and histological properties of enamel and dentin

1.4.1 Enamel

Enamel is the hardest and most mineralized tissue in the human body: it is composed of 96 wt% hydroxyapatite crystals. The remaining tissue is bound in an organic matrix that consists of proteins (i.e., amelogenins, enamelines) and contains trace amounts of water in the intercrystalline space and in a network of micropores opening to the external surfaces [12]. Maturing ameloblast cells develop cytoplasmic extensions through the Tomes’ process, in which the enamel protein matrix is secreted and the mineralization and orientation of enamel crystals are initiated simultaneously. Hydroxyapatite crystals are up to 1 mm long, 50 nm wide, and 25 nm thick. They extend from the dentin toward the enamel surface, perhaps reaching this surface unbroken. They are arranged in bundles of approximately 1,000 crystals, called enamel prisms (Fig. 3). Cross-sectional prism profiles range from circular to keyhole-shaped. Carbonate-apatite crystals are arranged primarily with their long axes parallel to the long axes of the prisms (Fig. 4). At the periphery of each prism, however, the crystals deviate somewhat from this orientation to produce an interface between prisms that tends to contain more intercrystalline space and forms the interrod enamel.
Fig. 3 Human enamel after etching with 35% orthophosphoric acid for 30s. (A) SEM image, 300× (B) SEM image, 1500× (C-D) SEM images, 5000×.

Fig. 4 Schematic representation of a rhomboidal crystallographic cell (bold lines).
1.4.2 Dentin

Dentin is composed of primarily small, thin apatite crystal flakes embedded in a protein matrix of cross-linked collagen fibrils. It is formed by the interaction of ectodermal and ectomesenchymal components, inducing odontoblast differentiation and dentinogenesis. As odontoblasts make dentin, they leave tracks that form dentinal tubules of differing densities and orientations in distinct tooth locations (Fig. 5). The tubules make up about 10% of the dentin volume; they are approximately 0.63 µm in diameter near the dentinoenamel junction and 2.5 µm in diameter near the pulp chamber. About 45,000/mm² tubules are present near the pulp and about 20,000/mm² are present near the enamel [13].

Unlike enamel, which is acellular and predominantly mineralized, dentin volume is composed of 45–50% inorganic apatite crystals, about 30% organic matrix, and about 25% water. Two main types of dentin occur in human teeth: intertubular dentin, the structural component of the hydroxyapatite-embedded collagen matrix that forms the bulk of dentin structure, and peritubular dentin, the lining of the tubule walls (Fig. 5d) [14]. The scaffold containing the tubules and intertubular dentin is formed by odontoblast-produced collagen fibrils; hydroxyapatite precipitates between the fibers and within fiber nanospaces during dentinogenesis.
Mineral, in the form of carbonate-rich apatite, constitutes approximately 50% of dentin volume. The precipitation of mineral substances on the collagen fibrils during dentinogenesis results in the final mineralized structure. The odontoblastic extensions are immersed in a fluid with a pressure of 25–30 mm Hg [15], and they transmit stimuli that change the intratubular pressure through contact with the pulp [16]: thus, dentin should be considered a dynamic living tissue [17].

Fig. 5 Dentin etched surface with 35% orthophosphoric acid for 15s showing dentinal tubules. (A) SEM image, 300×. (B) SEM image, 1300×. (C) SEM image, 2500×. (D) SEM images, 5000×.
1.5 Mechanism of adhesion

The primary aim of dental adhesives is to simultaneously adhere to dental hard tissue (i.e., enamel, dentin) and bond the resin composite. The mechanism of bonding to enamel and dentin is essentially an exchange process involving the replacement of minerals removed from the hard tissue by resin monomers. Upon setting, these monomers become interlocked micromechanically in the created porosities [18]. Diffusion and capillarity are the primary mechanisms of micromechanical retention. Microscopically, this process is called hybridization. Nakabayashi’s group was the first to demonstrate that resin could infiltrate into acid-etched dentin to form a new structure composed of a resin-matrix reinforced by collagen fibrils; he named this new biocomposite the hybrid layer (Fig. 6) [19].

Fig. 6. Schematic of a hybrid layer (HL) created by an etch-and-rinse adhesive. Modified from Pashley et al. (2011) The collagen fibrils in the HL are continuous with the underlying mineralized matrix. A single dentinal tubule is shown devoid of a resin tag to illustrate its presence.

1.6 Enamel adhesion

Bonding to dental tissues has posed a major challenge since the introduction of the acid-etching technique nearly 50 years ago [3]. Acid etching transforms smooth enamel into an irregular surface (Fig. 3). A resin monomer or adhesive mixture is then applied to the
enamel surface and drawn into surface microporosities by capillary action. When monomers in the fluid resin polymerize, they become mechanically interlocked with the enamel structure. The formation of resin microtags within the enamel structure has been considered the essential mechanism of resin–enamel adhesion [20,21]. Etching of dental tissues with an acidic solution is a standard clinical procedure that results in the demineralization of the superficial enamel and dentin layers. A variety of acids have been proposed for this purpose, including phosphoric (10–50%), fluoridated phosphoric, pyruvic, citric, maleic, oxalic, tannic, ethylenediaminetetraacetic, trichloracetic, and polyacrylic acids [22,23].

Recent studies have shown that the pH and pKa of the acid solution are important parameters that influence the aggressiveness of acids and their ability to demineralize the surface [24]. The physical state of the solution (gel or liquid) is another important parameter. Gel-type etching agents are easier to apply to the enamel surface than liquid forms, and have been shown to achieve wider and deeper enamel penetration [25]. During the application of an acid-etching gel, a continuous brushing technique may better define the etched pattern, thereby improving the marginal adaptation of the composite resin restoration [26].

Enamel etching produces three types of patterns [27]. The Type I etching pattern is achieved by the preferential removal of prism core material while leaving the periphery intact. The Type II pattern is defined as the preferential removal of peripheral core material while leaving the prism core relatively unaffected. The Type III pattern results in a more random etching morphology, in which adjacent areas of the tooth surface correspond to Type I and II patterns and other regions exhibit patterns unrelated to prism morphology.
1.7 Dentin adhesion

Effective adhesion to enamel has been achieved with relative ease and has proven repeatedly to be a durable and reliable clinical procedure in routine applications [28]. Conversely, adhesion to dentin is not as reliable as adhesion to enamel. The dentin adhesion strategy involves several procedures and two are the main approaches. The “etch and rinse” technique requires the use of an acid, usually phosphoric acid, followed by rinsing as a separate phase to expose the microporous network of collagen. On the other hand, on the “self-etch approach” there is no longer needs of the rinsing phase, since in this technique acidic monomers play the role of the acid agent.

Independently to the approach chosen, bond to dentin is usually critical. The dentin smear layer produced during preparation procedures should be removed by the acid-etching phase, which concurrently results in the demineralization of the dentin surface [29]. This procedure exposes a microporous network of collagen for the micromechanical interlocking of monomers [18,28,30].

1.7.1 Smear layer

The preparation of a carious tooth surface with rotary and manual instrumentation smears cutting debris over the enamel and dentin surfaces, forming the smear layer. The smear layer has been defined as, “any debris, calcific in nature, produced by reduction or instrumentation of dentin, enamel or cementum,” [31] or as a contaminant [32] that precludes interaction with the underlying pure tooth tissue. Scanning electron microscopy (SEM) has revealed that the smear layer is a 0.5–2-µm-thick layer of debris with a primarily granular substructure that completely covers the dentin surface [31-33]. The morphological features, composition, and thickness of this layer are determined largely by the type of instrument used, the method of irrigation employed, and the tooth substrate site [34]. The
smear layer obstructs dentin tubule entrances and may extend 1–10 µm into the tubules, forming *smear plugs* that are contiguous with the smear layer and decrease dentin permeability by up to 86% [35,36]. Submicron porosity of the smear layer allows the continued flow of dentinal fluid [36]. The layer acts as a physical obstacle that decreases dentin permeability, and must be removed or made permeable to allow contact and interaction between monomers and the dentin surface. Removal of the smear layer greatly increases the permeability of dentin tubules. Pashley [37] reported a primarily outward fluid flow of 20–70 cm/H$_2$O under pulpal pressure.

1.7.2 *Dentin adhesion strategies*

Due to the hydrophilic nature of dentin matrix, the combined use of hydrophilic and hydrophobic monomer groups has been suggested to improve adhesion. Hydrophilic functionality facilitates permeation of the monomer into the collagen matrix, leading to the formation of a hybridized collagen–resin layer. Hydrophobic functionality facilitates bonding to the hydrophobic resin matrix of the restoration.

Dentin adhesion procedures involve the application of a conditioner or acid etchant, followed by a primer or adhesion-promoting agent, and, finally, a bonding agent or adhesive resin. Such procedures have significantly improved bonding and sealing at the dentin–restoration interface. Hybridization is believed to result from the infiltration of primer into the open spatial network of the collagen matrix that has been exposed by dentin demineralization, and its *in situ* polymerization [38].

The smear layer that forms on the tissue surface during cavity preparation is an important consideration in the adhesion process. Two strategies have been used to overcome the low attachment strength of the smear layer: removal of this layer before bonding (etch-
and-rinse approach), and the use of bonding agents that penetrate the layer and make it permeable to subsequently applied monomers (self-etch approach).

*Etch-and-rinse adhesives:* The use of etch-and-rinse adhesives involves a separate rinsing phase. A variety of conditioning agents have been used, including maleic, citric, phosphoric, and nitric acids; 30–37% phosphoric acid is currently preferred. Dentinal etching removes the smear layer and hydroxyapatite mineral phase from the tissue surface, exposing a network of collagen fibrils that form the underlying substrate. A 15 s application of 37% phosphoric acid to dentin dissolves the smear layer and the top 1–6 µm of hydroxyapatite. The resulting layer is very permeable [37] facilitating the infiltration of adhesives into the spatial network of the fibrils (Fig. 7). The etch-and-rinse procedure characterized by the simultaneous and separate application of etching, primer and bonding is often considered to be the gold standard for predictable adhesion to teeth and is used in several bonding systems [39]. Simplified two-step etch-and-rinse adhesives combine the primer and adhesive resin into a single application.

![Fig. 7 SEM images and diagrams of etch-and-rinse and self-etch adhesion strategies.](image-url)
Self-etch adhesives: The use of self-etch adhesives is an alternative approach to the etch-and-rinse strategy; it is based on the use of rinse-free acidic monomers. These monomers simultaneously condition and prime dentin. Clinically, this approach has shown the greatest user-friendliness and reduced technique sensitivity. Indeed, self-etch techniques do not require a separate rinsing phase, which significantly reduces clinical application time, technique sensitivity, and the risk of application errors. The application of self-etch adhesives may follow a one- or two-step procedure, and involves the addition of one or more carboxylic or phosphate acid group to the monomers [40]. Self-etch adhesives may be “mild” or “strong” [28]. Strong adhesives have a very low pH (< 1) and exhibit a bonding mechanism and interfacial ultramorphology on dentin that resemble those produced by etch-and-rinse adhesives. With these adhesives, the hybrid layer reaches a thickness of 3–4 µm and exhibits the typical interfacial characteristics of a loosely organized collagen–fibril network. Individual fibrils are separated by interfibrillar spaces to produce a “shag carpet” appearance at the top of the hybrid layer. Mild self-etch adhesives (pH ~2) partially dissolve the dentin surface to a depth of ≤ 1 µm, leaving a substantial number of hydroxyapatite crystals within the hybrid layer. The hybrid layer produced with these adhesives is much thinner than those produced with strong self-etch or etch-and-rinse adhesives, but this does not impact the effectiveness of the bond [41]. Specific carboxyls (e.g., 4-methacryloxyethyl trimellitic acid [4-MET]) or phosphate groups (e.g., 2-(methacryloxy)ethyl phenyl hydrogen phosphate [phenyl-P], 10-methacryloxydecyl dihydrogen phosphate [10-MDP]) of functional monomers can then chemically interact with this residual hydroxyapatite [42].
1.8 Chemical composition of dental adhesives

1.8.1 Resin components

To ensure a good covalent bond between the adhesive and the lining composite, dental adhesives contain resin monomers that are similar to those of resin composites. The matrix is the key constituent of adhesives and, like the composite, provides structural continuity and beneficial mechanical properties. Monomers may be cross-linking or functional molecules (Figs. 8, 9). Cross-linking monomers have two polymerizable groups (vinyl groups, C=C) and form cross-linked polymers, whereas functional monomers have a single polymerizable group and form linear polymers. Most functional monomers also contain a functional chemical group that imparts monomer-specific properties. Cross-linked polymers have exhibited better mechanical strength than linear polymers, and are thus important for adhesive resin reinforcement. Some monomers (e.g., pentaerythritol [Penta], biphenyl dimethacrylate [BPDM], tetrachlorobenzene [TCB]) have a more intricate molecular structure than others and contain several polymerizable and functional groups [43].

![Fig. 8 Principal cross-linking monomers used in dental bonding agents.](image)

Fig. 8 Principal cross-linking monomers used in dental bonding agents.
Monomer structure can be divided into three distinct parts: one or more polymerizable groups, a spacer (onto which the polymerizable group(s) is grafted), and a functional group.

Acrylates, especially methacrylate monomers, are the most common polymerizable groups. Acrylic systems show greater radical-polymerization reactivity and may thus have biocompatibility and shelf-life problems [44]. This polymerizable group generally exhibits hydrophobic behavior.

Monomer spacers function solely to separate functional or polymerizable groups, but exert an important influence on the monomer [45]. Spacers are usually composed of an alkyl chain, but may also contain several other groups, such as esters, amides, or aromatic groups. Spacer group size determines monomer viscosity and, consequently, monomer wetting and penetration behavior.

In functional monomers, the functional group is hydrophilic. The hydrophilic properties of “adhesion-promoting” functional monomers enhance the bond strength of adhesives to dentin [46].

The most commonly used functional groups are phosphate, carboxyl acid, and alcohol groups. In addition to adhesion-promoting or wetting effects, these functional groups may achieve some extent of surface demineralization when applied in sufficient concentration.
The most frequently used monomers in commercial adhesives are listed below [47].

• *Methacrylic acid:* A strong irritant and corrosive due to its high acidity, methacrylic acid is present in varying amounts in the majority of adhesive resins (Fig. 10).

![Methacrylic acid](image)

Fig. 10 Methacrylic acid

• *Methyl methacrylate:* One of the oldest monomers, methyl methacrylate is sporadically added to adhesives (Fig. 11).

![Methyl methacrylate](image)

Fig. 11 Methyl methacrylate

• *Hydroxyethyl methacrylate (HEMA):* HEMA is a small monomer used widely in dentistry and other fields. Its hydrophilic properties make it an excellent adhesion-promoting monomer. High concentrations of HEMA in an adhesive may deteriorate the mechanical properties of the resulting polymer (Fig. 12).

![2-hydroxyethyl methacrylate (HEMA)](image)

Fig. 12 2-hydroxyethyl methacrylate (HEMA)
• **4-MET**: A frequently used monomer, 4-MET was originally developed as an adhesion promoter. It improves the wetting of metals such as amalgam and gold (Fig. 13).

![4-MET](image)

**Fig. 13** 4-methacryloyloxyethyl trimellitic (4-MET)

• **10-MDP**: Originally synthesized by Kuraray (Osaka, Japan), this monomer forms strong ionic bonds with calcium (Fig. 14).

![10-MDP](image)

**Fig. 14** 10-methacryloyloxydecyl dihydrogenphosphate (10-MDP)

• **Bisphenolglycidyldimethacrylate (Bis-GMA)**: Also called Bowen’s resin, Bis-GMA is the most frequently used cross-linking monomer in adhesive systems and composites. Due to its high molecular weight, Bis-GMA is highly viscous and confers superior mechanical qualities to polymers (Fig. 15).

![Bis-GMA](image)

**Fig. 15** 2,2-bis[4-(2-hydroxy-3-methacryloyloxy propoxy)]-phenyl propane (Bis-GMA)
• **Triethylene glycol dimethacrylate (TEGDMA):** This dimethacrylate is usually used with Bis-GMA or urethane dimethacrylate (UDMA). The higher flexibility of TEGDMA compensates for the rigidity of Bis-GMA and such mixtures produce resins with higher conversion rates (Fig. 16).

![Triethylene glycol dimethacrylate (TEGDMA)](image)

Fig. 16 Triethylene glycol dimethacrylate (TEGDMA)

• **UDMA:** This monomer is most commonly used in adhesive composites. Its molecular weight is comparable to that of Bis-GMA and it exhibits lower viscosity (Fig. 17).

![Urethane dimethacrylate or 1,6-di(methacryloyloxyethylcarbamoyl)-3,3,5-trimethylhexaan (UDMA)](image)

Fig. 17 Urethane dimethacrylate or 1,6-di(methacryloyloxyethylcarbamoyl)-3,3,5-trimethylhexaan (UDMA)

1.8.2 Initiator systems

To obtain a radical-polymerization reaction, bonding adhesive systems must contain small amounts of initiator. Initiators are molecules whose atomic bonds have low dissociation energy; these bonds form radicals under certain conditions. Radicals can be produced by a variety of thermal, photochemical, and oxidization-reduction (redox) methods. Redox and photo-activated initiators are used in composites and adhesives. Photo-initiators absorb electromagnetic energy (photo-curing), and redox initiators must be mixed with another component (chemical or self-curing). The main advantage of polymerization
by irradiation is the controllability of the reaction onset. Depending on initiator and system types, 0.1–1 wt% of initiator is usually added to an adhesive system.

1.8.3 Photo-initiators

Photo-initiating systems based on camphoroquinone (CQ) have been most widely and successfully used in dental restorations (Fig. 18).

![Camphoroquinone (CQ)](image)

Fig. 18 Camphoroquinone (CQ)

Although the photopolymerization process may be initiated by CQ alone, the reaction is usually facilitated by co-initiators or accelerators, such as tertiary amines. After excitation with blue light, a radical-yielding complex is formed through hydrogen abstraction. Amines are efficient hydrogen donors and have been extensively used. CQ is an excellent photo-initiator that absorbs a wide spectrum of wavelengths (360–510 nm), with peak absorbance around 468 nm (blue light). Dissolution of CQ in TEGDMA produces a bathchrome shift in absorption that peaks at 474 nm [48]. Although it is used in very small amounts (0.03–0.1%), one disadvantage of CQ is its yellowish-brown color, which can significantly influence the color of adhesives [49].

Recently, 1-phenyl-1,2-propadione (PPD) diketone has been introduced as a photo-initiator for dental resin. Its peak absorbance is around 400 nm. Photo-initiator systems may eliminate the need to use acyphosphine oxide. These systems exhibit strong absorption of
near-ultraviolet (UV) and visible light [50]. Diphenylphosphine oxide or 2,4,6-
trimethylbenzoyl (TPO) are used in commercially available products (Fig. 19) [51].

Fig. 19 Diphenylphosphine oxide or 2,4,6-trimethylbenzoyl (TPO)

The neutral color of these products provides an advantage over CQ. TPO has two
disadvantages: it is not appropriate for use with first-generation light-emitting diode (LED)
curing units and its stability is not guaranteed in the presence of water and ethanol [52].

1.8.4 Chemical initiators

The use of chemical initiators is usually restricted to cements and resin that cannot
be polymerized by light-curing. With chemically cured adhesives, the setting reaction is
initiated by mixing the initiator and co-initiator. The two components must thus be mixed
before the adhesive is applied to the tooth surface. Benzoyl peroxide (BPO) with a tertiary
amine is the most common initiator in self-curing resins (Fig. 20) [53].

Fig. 20 Benzoyl peroxide (BPO)
1.8.5 Inhibitors

Inhibitors added to dental resins are antioxidants that scavenge free radicals originating from prematurely reacted initiators. In extreme storage conditions, such as high temperature (e.g., during transport), some initiator molecules may decompose or react spontaneously. Large amounts of inhibitor, however, can result in decreased cure rates. The most frequently used inhibitors in adhesives are butylated hydroxytoluene (or butylhydroxytoluene; BHT) (Fig. 21) and monomethyl ether hydroquinone (MEHQ) (Fig. 22).

![Fig. 21 Butylhydroxytoluene (BHT)](image)

![Fig. 22 Monomethyl ether hydroquinone (MEHQ)](image)
1.8.6 Solvents

Adhesives that must bond to dentin require the addition of solvents. Due to the wet nature of dentin, good wetting can only be achieved with hydrophilic bonding. The wetting behavior of the adhesive is improved significantly by the addition of hydrophilic monomers and a solvent [54]. The low viscosity of primers and/or adhesive resins is due in part to the dissolution of the monomers in a solvent, which improves diffusion into the microretentive tooth surface. The etch-and-rinse approach uses a primer containing a solvent to promote good penetration of the monomers into the collagen network of demineralized dentin. Water, ethanol, and acetone are the most commonly used solvents in adhesives.

1.8.7 Fillers

Whereas all composite resins contain filler particles to improve their mechanical properties, adhesive resins may not. Adhesives containing fillers are said to be “filled,” in contrast to “unfilled” adhesives. Fillers may be added to adhesives for several reasons. Situated between the composite filling and the tooth, the adhesive layer is considered to be a weak link due to its low tensile strength and elastic modulus [55]. Through comparison with composites, several authors have suggested that the addition of fillers may fortify the adhesive layer [56] and modify the viscosity of adhesives. A thicker adhesive layer may also relieve contraction stresses produced by the restorative materials [57]. Depending on their chemical composition, fillers may also provide fluoride release and radio-opacity, important factors for thick adhesive layers that aid the differential diagnosis of recurrent caries. Secondly, filler particle size is a key factor enabling filled resins to penetrate dentin tubules and potentially also the collagen network. After etching, the interfibrillar spaces of demineralized collagen networks are about 20 nm in size; filler particles should thus be
smaller than 20 nm. The most frequently added fillers are thus nanometer-silized silicas (pure silicon dioxide; $\leq 7$ nm) of colloidal or pyrogenic origin. Heavy metal atoms, such as barium and strontium, are included to provide radio-opacity to the resin. Fluorine-containing reactive silicate glasses can be added to release fluoride and prevent the recurrence of caries. Acidic monomers are assumed to react with fluoro-alumino-silicate glass to produce a glass-ionomer reaction and fluoride release.

1.9 Variables related to substrate treatment

The wetness of dentin surfaces, the presence of pulpar pressure, and the thickness of dentin are extremely important variables affecting bonding procedures. These factors are especially relevant during in vitro testing of adhesive bond strength that attempts to simulate in vivo conditions.

Pashley [58] described dentin as a porous biological composite composed of apatite crystal filler particles in a collagen matrix. Marshall [59] stated that the various structural components and properties of dentin could directly affect the adhesive bond. Dentin is a dynamic substrate that is subject to continuous physiological and pathological changes in composition and microstructure.

1.9.1 Carious and tertiary dentin

Dental caries is the most common pathological challenge to dentin. Based on the pioneering work of Fusayama [60], carious dentin consists of superficial and inner layers. The outer layer is highly decalcified, physiologically unrecalcifiable, and contains decalcifiable collagen fibers. The inner layer is intermediatedely decalcified, physiologically recalcifiable, and exhibits expanded odontoblastic processes and apatite crystals bound to
sound collagen fibers. *Tertiary* or reparative dentin is produced in the pulp chamber in response to insults, such as caries, dentinal procedures, or attrition [60].

Hypermineralization, obstruction of tubules by whitelockite crystalline deposits, and apposition of reparative dentin adjacent to the pulp are well-documented processes affecting dentin [60]. The resulting sclerotic material differs from physiological sclerosis, which occurs throughout the life of the tooth; the formation of tertiary dentin is localized in the pulp chamber wall of the affected area.

Much of our understanding of dental bonding is based on research using healthy (“laboratory”) dentin. This substrate differs from that typically encountered in clinical practice, as dentists frequently bond to carious dentin during the replacement of carious tissues.

Carious dentin is softer than normal dentin. Using a nanoindentation technique, Marshall *et al.* [61] found that the mean elastic modulus (18.2 GPa) and nanohardness (0.8 GPa) of transparent intertubular dentin are slightly but significantly lower than those of unaffected intertubular dentin (20.6 GPa, 1.0 GPa). The use of micro-Raman spectroscopy in a previous study revealed structural and chemical changes in carious dentin, namely, it has less mineral phosphate and carbonate than unaltered dentin [62].

The bond strength of carious dentin is also typically 20-50% lower than that of unaffected dentin, regardless of the adhesive type used [63–65]. Hybrid layers are usually thicker (6–8 µm) and more porous in carious dentin than in healthy dentin (1 µm) [62]. Successful bonding to carious dentin may depend on adhesive composition. One study [66] compared the bond strengths of Scotchbond Multi-Purpose adhesive (3M ESPE) with and without polyalkenoic acid in the primer. The absence of polyalkenoic acid resulted in lower bond strength to carious dentin, suggesting that the residual calcium in such dentin may be
crucial for the establishment of ionic bonding with the polyalkenoate in the primer. In a study that used a two-step self-etching adhesive (Clearfil SE Bond; Kuraray Co., Ltd.), hydrostatic pressure significantly reduced the bond strength to normal dentin after 1 month of water storage, but did not affect the bond strength to carious dentin [67]. The higher mineral contents of carious dentin regions may have resulted in stronger chemical bonding to the MDP molecule in Clearfil SE, which bonds chemically to hydroxyapatite [68].
References Chapter 1


CHAPTER 2:

DEGRADATION OF THE ADHESIVE INTERFACE
Degradation of the adhesive interface

2.1 Stability of the hybrid layer

It is generally accepted that the fundamental process in dental adhesion is the creation of an adequate hybrid layer. Micromechanical retention through its formation ensures the primary source of bond strength in adhesive resins bonded to dentin [1]. Since bonding is created by the impregnation of the dentin substrate by blends of resin co-monomers, the stability of the adhesive interface relies on the creation of a compact and homogenous 3-dimensional polymer and collagen network, that would provide a continuous and stable link between resin materials and tooth substrate [2-4]. Thus, the hybrid layer is a mixture of dentin organic matrix, residual hydroxyapatite crystallites, resin monomers, and solvents [5]. Moreover, the stability of hybrid layers ultimately depends on the intrinsic resistance of their individual components to the degradation phenomena.

Despite significant improvements in adhesive systems, the adhesive interface remains the weakest area of tooth-colored restorations [6] and the contingent exposure of the dentin–adhesive interface to the oral cavity often results in marginal discoloration, poor marginal adaptation, and subsequent loss of restoration retention [7].

Although contemporary adhesives can provide good initial bonding to enamel and dentin for composite restorations, the long-term durability of bonded-interface restorations remains unclear. Several studies have reported excellent immediate and short-term bonding effectiveness of dental adhesives [8,9], but the durability and stability of some bonding systems on dentin remained unknown [8,10].

In etch-and-rinse approaches, stable bonds can be achieved only with complete adhesive infiltration of the etched substrate, rather than differential or incomplete impregnation (Fig. 1) [4]. Self-etch approaches use acids that simultaneously demineralize
and infiltrate dentin, and adhesive stability is related to the effective coupling of co-
monomers with the infiltrated substrate.

![Fig. 1 Scanning electron microscopy (SEM) images of resin-dentin interface formed by a etch-and-
rinse adhesive system. Specimens were decalcified in 6N HCl for 30s, followed by deproteinization in 2% NaOCl for 10min. (A) SEM images, 1300×. (B) SEM images, 2500×.](image)

Although the incorporation of hydrophilic and acidic resin monomers has substantialy improved the initial bonding of contemporary etch-and-rinse and self-etch adhesives to intrinsically wet dental substrates, potential problems associated with these hydrophilic formulations have been reported in several in vitro and in vivo studies [11-20].

Hashimoto et al. [21] described two degradation patterns within the hybrid layer after storage of a three-step etch-and-rinse adhesive system in water for 1 year that included disorganization of collagen fibrils, and hydrolysis of resin from interfibrillar spaces within the hybrid layer, thereby weakening the strength of resin–dentin bond.

Therefore, resin–dentin bonds created by the infiltration of hydrophilic resin monomers into the demineralized dentin matrix are imperfect [22]. Fluid movement within the dentinal tubule anastomosis complex during resin infiltration may cause incomplete permeation of resin monomers into the hybrid layer [23]. The exposed collagen fibrils
resulting from incomplete resin infiltration thus remain unprotected against denaturation challenges and are susceptible to creep [24]. These denuded collagen matrices are also filled with water, which serves as a functional medium for the hydrolysis of resin matrices (by esterases) and collagen (by endogenous and exogenous collagenolytic enzymes).

The clinical longevity of the hybrid layer appears to be affected by physical and chemical factors. Determinants such as occlusal mastication forces, repetitive expansion and contraction stresses caused by temperature changes, and acidic chemical agents (e.g., fluid, saliva, beverages, bacteria) within the oral cavity may affect interface stability [2,4,25,26].

The exact mechanism responsible for hybrid layer degradation is not completely understood yet [4,22,27]. However, it seems that the first stage of biodegradation involves extraction of the resins that had infiltrated into the dentin matrix via water-filled nanometer-sized voids within the hybrid layer and enzymatic attack of the exposed collagen fibrils, leading to their depletion [3,6].

Degradation of the hybrid layer could be divided into two major categories: hydrolytic degradation of the adhesive resin and hydrolytic degradation of the collagen matrix within the hybrid layer [27].

### 2.2 Degradation of the adhesive resin

Chronic deterioration of the hybrid layer involves hydrolysis and leaching of the adhesive that has infiltrated the demineralized dentin matrix [27,28,29]. Leaching is facilitated by water penetration into the loosely cross-linked or hydrophilic domains of the adhesive. The hydrophilic domain exhibits limited monomer/polymer conversion because of adhesive phase separation [30] and lack of compatibility between the hydrophobic photoinitiator and hydrophilic phase [31]. The poorly polymerized hydrophilic phase degrades rapidly in the aqueous environment. Resin elution continues to occur while water
movement along the length of the hybrid layer becomes more rapid as transport pathways form relatively large water-filled channels [4]. The previously resin-infiltrated collagen matrix is exposed and vulnerable to attack by proteolytic enzymes [10,32].

The structure of methacrylate adhesives, presenting carbon and oxygen or nitrogen in their backbones [33], suggests a general mechanism for their chemical and enzymatic degradation. In addition, their structure shows the presence of hydrolytically susceptible groups, such as ester and urethane, as well as hydroxyl, carboxyl, and phosphate groups [34].

On prolonged exposure of the restoration to oral fluids water begins to penetrate the resin. Water initially enters the matrix by diffusion into loosely cross-linked or hydrophilic domains or may be trapped within the matrix during photopolymerization [35,36]. Mechanical wear of the exposed adhesive may further accelerate matrix degradation by abrading the surface, increasing the surface area and allowing greater entrance of both water and enzymes. The presence of water promotes the chemical hydrolysis of ester bonds in methacrylate materials. Over years of exposure to salivary fluids, local domains of the methacrylate network may become sufficiently degraded and/or hydrophilic to permit access by esterases, which greatly accelerate ester bond hydrolysis.

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

2.2.1 Nanoleakage

As already described, the ultimate goal of dentin bonding is the complete infiltration of resin monomers into demineralized collagen fibrils exposed by acid-etching or self-etching adhesives [4].

Etch-and-rinse procedures rely on the use of phosphoric acid to demineralize the
superficial dentin layer, and on the ability of adhesive monomers to infiltrate the etched substrate without leaving porosities in the hybrid layer. Many studies have shown that small ions or molecules can diffuse into the hybrid layer in the absence of detectable interfacial gap formation. Sano et al. [25,37,38] defined nanoleakage as the presence of microporous polymerized zones beneath or within the hybrid layer that permitted tracer penetration to occur in the absence of interfacial gaps. Nanoleakage is created by the discrepancy between dentin demineralization and adhesive infiltration that occurs with total-etching adhesive systems, in the absence of marginal gap formation along the resin–dentin interface [39].

Self-etching procedures use acidic adhesive resin monomers to simultaneously demineralize and infiltrate the bonding substrate, theoretically avoiding incomplete infiltration. Tay et al. [40], however, reported variable degrees of silver uptake following the use of self-etching adhesives. Thus, nanoleakage is not necessarily caused by disparities between demineralization and resin-infiltration depths. The retention of residual water in etched dentin and/or adhesives may result in regions of incomplete polymerization or increased permeability within adhesive resin matrices [41]. Moreover, simplified one-step self-etching adhesives are highly hydrophilic and have been found to behave as permeable membranes after polymerization. This permeability results in hybrid layers that behave as semi-permeable membranes, permitting water movement throughout the bonded interface after polymerization [40,42].

Using a replica technique, Chersoni et al. [43] observed water movement from dentin through the hybrid and adhesive layers with transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Tay et al. [40] used ammoniacal silver nitrate to trace the distribution of absorbed water, and observed reticular and spotted patterns of silver tracer deposition in nanoleakage
expression (Fig. 2). In the reticular mode, silver deposits were oriented perpendicular to the hybrid layer surface. The spotted mode of nanoleakage is thought to represent microdomains in the resin matrices that contain primarily hydrophilic and/or acidic functional groups, in contrast to adjacent, relatively hydrophobic, domains [44]. The same author reported that silver uptake likely indicated areas of increased permeability within the resin matrix. Water has been incompletely removed in these zones, resulting in regions of incomplete polymerization and/or hydrogel formation of the HEMA in the adhesive.

![SEM images of resin-dentin interface after 6 months of storage in artificial saliva showing silver nitrate deposits.](image_url)

Fig. 2 Scanning electron microscopy (SEM) images (backscattered mode) of resin-dentin interface after 6 months of storage in artificial saliva showing silver nitrate deposits. The typical “water trees” aspect of silver nitrate accumulation confirm nanoleakage formation (A-B) SEM images, 2500×.

During my research stage abroad, a two-photon laser fluorescence microscopy technique was assessed to evaluate the interfacial micromorphology of the hybrid layer in bonded restorations. Micropermeability of the hybrid layer was characterized by means of simultaneously contrasting a dye-containing adhesive with a differently colored dye placed into the pulp chamber and allowed to diffuse toward the different-colored hybrid layer. A fluorescent red dye (rhodamine B) was incorporated into a commercial adhesive system that was used to perform adhesive restorations to dentin surfaces of crown segments from...
extracted human third molars. An aqueous solution of a blue dye (calcein blue) was then placed into the pulp chamber for 12 h, allowing time to diffuse toward the different-colored bonded interface. The teeth were then embedded, sectioned, and microscopically analyzed using two-photon laser microscopy at 100X magnification. Two-photon laser microscopy provided high quality, high-resolution images of the bonded interface and surrounding areas, showing an accurate analysis of the structure of the hybrid layer with the localization of the fluorescence tracer to seep wherever there were water-filled submicron channels from the pulp to the hybrid layer. Thus, the entire hybrid layer and part of the above adhesive one were fluorescent, detecting water presence (calcein blue in aqueous solution) within hybrid and adhesive layer (Fig. 3).

Fig. 3 Confocal laser scanning electron microscopy (CLSM) images of resin-dentin interface. After polymerizing the resin, the pulp was filled with calcein blue and placed under 20 cm H2O pressure to allow the fluorescent tracer to seep. (A) CLSM image with calcein blue, 100×. (B) CLSM image with rhodamine B, that was incorporated into a commercial adhesive system before bonding procedure and calcein blue as Fig. 3A, 100×. Note that there is a fluorescence continuum from dentinal tubules, around resin tags, into the hybrid layer.

An immunohistochemical approach [45] that distinguishes native collagen fibrils from resin-embedded fibrils has confirmed the interpretations of nanoleakage that implicate
hybrid layer porosity. This study found that collagen fibrils within the hybrid layer were not fully embedded by dentin adhesives. Thus, etch-and-rinse and self-etch adhesive systems both fail to fully infiltrate the collagen network, instead achieving different degrees of infiltration from the top to the bottom of the hybrid layer.

2.3 Degradation of the collagen fibrils

Degradation may also affect the collagen matrix of the hybrid layer. Complete coverage of the nanoscale irregularities on the collagen fibrils surface via passive monomer penetration may be difficult to achieve. Thus, fibrils that remain unprotected by hydrophobic resin coatings may be vulnerable to degradation and water is claimed to be a major cause of collagen degradation. Two degradation patterns have been observed within the hybrid layer: loss of resin from interfibrillar spaces and disorganization of collagen fibrils [21]. The degree of collagen fibril envelopment varies, depending on the type of bonding agent [4] that could lead to an incompletely infiltrated zones containing denuded collagen fibrils along the bottom of the hybrid layer [47].

Collagen fibrils in the hybrid layer that have been incompletely encapsulated by resin monomer can be identified immunohistochemically [45,48]. Advances in reagent purification and the production of highly specific monoclonal antibodies have permitted the establishment of reproducible and selective immune-labeling protocols for visualizing collagen or proteoglycans [49,50]. This visualization uses secondary antibodies that are conjugated with gold nanoparticles of different size. Breschi et al. [51] showed that the hybrid layer created by etch-and-rinse adhesives was characterized by different degrees of resin–collagen fibril interactions.
2.3.1 Intrinsic collagenolytic activity of mineralized dentin

Recent studies have examined the contribution of host-derived proteinases to the breakdown of collagen matrices in the pathogenesis of dentinal caries [52,53] and periodontal disease [54]. These findings had important implications for dental bonding but the first evidence of an intrinsic collagenolytic activity of host-derived matrix metalloproteinases (MMPs) in human non carious mineralized dentin came when Pashley et al. [32] demonstrated that dentin demineralized by acid etching slowly degrades in absence of bacteria. These authors speculated that such proteolytic activity could be exerted by dentinal matrix metalloproteinases (MMPs), which on that occasion had already been shown to be potentially expressed in the dentin-pulp complex [53,55,56].

Matrix metalloproteinases

MMPs are a class of zinc- and calcium-dependent endopeptidases. These endogenous enzymes are important components in many biological and pathological processes because of their ability to degrade almost all extracellular matrix components [57]. Within the oral environment, considerable interest has been devoted to the detection, distribution, and function of host-derived MMPs [58,59].

Several MMPs have been identified within the dentin-pulp complex compartments. Dentin matrix has been shown to contain at least four MMPs: stromelysin-l (MMP-3) [60,61], collagenase (MMP-8) [62], and gelatinases A and B (MMP-2 and MMP-9, respectively) [63,64].

Description of the involvement of MMPs in the degradation of demineralized dentin matrix was first mentioned in a study by Tjäderhane et al. [52]. Human MMP-2, MMP-8, and MMP-9 were identified in demineralized dentinal lesions. The experiments conducted in the mentioned study provided critical evidence that bacterial acids are required for the
removal of minerals in tooth decay and for the subsequent activation of host MMPs, but bacteria alone could not cause dentin matrix degradation. Therefore, it was assumed that after demineralization, activated host-MMPs would ultimately be responsible for destroying the dentin matrix in caries lesion progression [52]. As previously mentioned, more recently Pashley et al. [32] began discussing the likely involvement of MMPs in the degradation of poorly resin-infiltrated hybrid layers. Nowadays it is thought that the release and the subsequent activation of these endogenous enzymes during dentin bonding procedures [32,65-67] could be responsible for the almost complete disappearance of portions of hybrid layers from resin-dentin bonds that were aged in water [12].

**Cysteine cathepsins**

More recently, another group of proteases were identified in compartments of both sound and carious human dentin, and they formed part of the cysteine proteases (CPs) [68,69]. Human CPs are best known from the ubiquitously expressed lysosomal cathepsins B, H, and L, and dipeptidyl peptidase I, which until recently were thought to mediate primarily housekeeping functions in the cell [70]. Of 15 CPs genes detected, 10 were expressed in native pulp tissue and 11 in odontoblasts.84

In studies by Tersariol *et al.* [68] and Nascimento *et al.* [69] the potential correlation between the MMPs and cysteine cathepsin activities in intact or carious dentin, respectively, was also investigated. Results showed that MMPs and cysteine cathepsin activities expressed highly significant correlations between intact and carious dentin, even though the activities in carious lesions were approximately 10 times higher than in intact dentin. In conclusion, these data indicate the collagenolytic/ gelatinolytic activity of dentin may be due not only to the presence of MMPs but also to cysteine cathepsin synergic activities.
References Chapter 2


CHAPTER 3

INFLUENCE OF AGING ON SELF-ETCH ADHESIVE:
ONE-STEP VS. TWO-STEP SYSTEMS
The influence of aging on self-etch adhesives: one-step vs. two-step systems.

3.1 Introduction

In the first chapter the current knowledge regarding adhesion to enamel and dentin was described, while the second chapter was focused on the fundamental processes that are responsible for the degradation of the adhesive interface, particularly in dentin. Indeed, bonding to enamel is stable over time, but in vivo [1, 2] and in vitro [3] studies have revealed the limited durability of resin–dentin bonds.

Among contemporary dentin bonding systems, self-etch adhesives have become popular because they are easy to use and can be applied rapidly [4]. The advantage of the self-etch approach over the etch-and-rinse approach is that the clinical procedure does not require a separate rinsing step after etching, as the acidic monomer formulations simultaneously demineralise and infiltrate the substrate [5]. Thus, acidic resin blends are simply air-dried on the substrate to evaporate the solvents. For this reason, these systems are also called ‘etch-and-dry’ adhesives [1]. Non-rinsing self-etch adhesives were formulated initially as two-step systems employing an acidic etching primer followed by a separate bonding resin. Recent formulations have shifted to one-step systems, in which all components are combined into a single solution. Despite their user-friendliness and low technique sensitivity, one-step adhesives have shown lower bonding effectiveness in vitro compared with two-step systems [6, 7]. Thus, the first part of this research was focused on the evaluation of the aging mechanisms involved in the degradation of the resin-bonded interfaces produced by simplified adhesives.

The bond strength and interfacial nanoleakage expression of two-step and one-step self-etch dentin bonding systems were assayed.
Whereas data on immediate bond strength are available, to our knowledge, no data on the longevity of adhesive interfaces (i.e., stability) created by recently formulated self-etch adhesives have been reported. The null hypotheses tested in this research were that: 1) no difference in immediate bond strength or interfacial nanoleakage expression would exist between the tested two-step vs. one-step self-etch adhesives, and 2) storage for 6 months or 1 year in artificial saliva at 37°C would not affect the adhesive interface of two-step or one-step adhesives.

3.2 Material and methods

Tooth Selection and Preparation

Sixty extracted non-carious human third molars, stored for no more than 1 month in 0.5% chloramine-T solution at 4°C, were selected for the study. These teeth were collected after obtaining patients’ informed consent to their use for research purposes under a protocol approved by the institutional review board of the University of Trieste (Italy).

Flat dentin surfaces were prepared by removing the coronal tooth portion parallel to the occlusal surface with a low-speed diamond saw (Micromet; Remet, Bologna, Italy) under water irrigation. The absence of enamel and/or pulp tissue exposure on dentin surfaces was verified under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH, Jena, Germany). The exposed middle to deep dentin surfaces were polished with 320-grit wet silicon carbide (SiC) abrasive to create standardized, smear layer–covered dentin substrates [8], and specimens were then divided randomly and equally into four groups ($n = 15$ each).
Bonding Procedures

Four self-etch adhesives were selected for this study: two two-step systems (group 1: Optibond XTR; Kerr Corporation, Orange CA, USA; group 2: Clearfil SE Bond; Kuraray, Tokyo, Japan) and two one-step systems (group 3: Bond Force; Tokuyama, Tokyo, Japan; group 4: Adper Easy Bond; 3M ESPE, Seefeld, Germany). The compositions of the dentin bonding systems are listed in Table 1. The tested dentin bonding systems were applied in strict accordance with the manufacturers’ guidelines (Table 1).

After bonding, 4-mm-thick coronal build-ups were created using a resin composite (Filtek Z250; 3M ESPE). Each 2-mm-thick increment was polymerised for 20 s [9] using a quartz-halogen light unit (Curing Light 2500; 3M ESPE). The irradiance level of the light was monitored periodically with a radiometer (3M ESPE) to ensure that it remained ≥600 mW/cm².

Microtensile Bond Strength (μTBS) Testing

Dentin–resin sticks (approximately 0.9 × 0.9 × 8 mm) were created using the low-speed saw in accordance with the non-trimming technique. Sectioned specimens were assigned equally and randomly to three storage groups: time 0 (T0, stored for 24 h), time 6 months (T6m), and time 1 year (T1yr). Storage was performed in artificial saliva at 37°C.

Prior to stressing until failure, the dimension of each stick was measured with a digital calliper (±0.01 mm) and the bonded area was calculated for subsequent conversion of microtensile strength values into units of stress (MPa). Each specimen was then glued to a modified jig with cyanoacrylate adhesive (Zapit; Dental Ventures of America, Corona, CA, USA) for microtensile testing (Bisco, Schaumburg, IL, USA) and stressed until failure under tension at a crosshead speed of 1 mm/min.

The number of prematurely debonded sticks in each test group was recorded, but
these values were not included in the statistical analysis because all premature failures occurred during the cutting procedure, they did not exceed 3% of the total number of tested specimens, and they were distributed similarly within the groups.

A single observer evaluated the failure modes under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH) at magnifications up to 50× and classified them as adhesive, cohesive in dentin, cohesive in composite, or mixed failures.

<table>
<thead>
<tr>
<th>Material</th>
<th>Composition</th>
<th>Application Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optibond XTR</td>
<td>Primer: GPDM, hydrophilic co-monomers, water, ethanol, acetone</td>
<td>Scrub primer with a brushing motion for 20s on dentin.</td>
</tr>
<tr>
<td>Kerr corporation</td>
<td>Adhesive: resin monomers, HEMA, inorganic fillers, ethanol</td>
<td>Air thin.</td>
</tr>
<tr>
<td>Orange CA, USA (B4FS5F)</td>
<td></td>
<td>Application adhesive for 15s.</td>
</tr>
<tr>
<td>pH primer: 2.4*</td>
<td></td>
<td>Light-cure for 10s.</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearfil SE Bond</td>
<td>Primer: 10-MDP, HEMA, water.</td>
<td>Scrub primer for 20s on dentin.</td>
</tr>
<tr>
<td>/Kuraray, Osaka, Japan (01076A)</td>
<td>Adhesive: TEGDMA, UDMA, GPDM, HEMA, Bis-GMA, hydrophobic dimethacrylate, colloidal silica</td>
<td>Gently air blow. Application bonding.</td>
</tr>
<tr>
<td>pH primer: 2.2*</td>
<td></td>
<td>Air thin for 5s.</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td></td>
<td>Light-cure for 10s.</td>
</tr>
<tr>
<td>Adper Easy Bond/3M ESPE, Seefeld, Germany (3860005)</td>
<td>HEMA, Bis-GMA, methacrylated phosphoric esters, 1,6 hexanediol dimethacrylate, Methacrylate functionalised Polyalkenoic acid (Vitrebond™ Copolymer), finely dispersed bonded silica filler with 7 nm primary particle size, ethanol, water, initiators based on camphorquinone, stabilisers</td>
<td>Scrub adhesive for 20s on dentin. air thin for 5s. Light-cure for 10s.</td>
</tr>
<tr>
<td>pH: 2.4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond Force/Tokuyama, Tokyo, Japan (051E40)</td>
<td>HEMA, Bis-GMA, initiators based on camphorquinone</td>
<td>Scrub adhesive for 20s. Air thin for 5s. Light-cure for 10s.</td>
</tr>
<tr>
<td>pH: 2.4*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Components, compositions, and application procedure of the tested adhesives (information supplied by the manufacturer). Abbreviations: Bis-GMA, bisphenol A diglycidyl ether dimethacrylate; GPDM, glycerol phosphate dimethacrylate; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, triethylenglycol-dimethacrylate; UDMA; urethane dimethacrylate; 10-MDP, 10-methacryloyoxydecylyl. *Information as received from manufacturer.
Dentin–resin interfacial nanoleakage expression

Three additional teeth were prepared for each group, cut vertically into 1-mm-thick slabs to expose the bonded surfaces, and submitted to the three storage times (T0, T6m, T1yr) as described previously. Specimens were covered with nail varnish, leaving 1 mm exposed at the bonded interface, and processed for interfacial nanoleakage evaluation. Nanoleakage analysis was performed by light microscopy (LM) and scanning electron microscopy (SEM).

Bonded interfaces were immersed in 50 wt% ammoniacal silver nitrate (AgNO₃) solution in darkness for 24 h according to the protocol described by Tay et al. [10]. After immersion in the tracer solution, specimens were rinsed in distilled water and immersed in photo-developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grain within voids along the bonded interfaces.

For LM, the silver-impregnated specimens were fixed, dehydrated, and embedded in epoxy resin (Epon 812; Fluka, Buchs, Switzerland). The specimens were then fixed on glass slides (Menzel, Bielefeld, Germany) using cyanoacrylate glue (Super Cyanolit; Panacol-Elosol GmbH, Steinbach, Switzerland) and flattened with a series of abrasives (180-, 600-, 1200-, 2400-, and 4000-grit SiC) under water irrigation using a grinding device (LS2; Remet). Bonded interfaces were analyzed under LM (E800; Nikon, Tokyo, Japan) at 100× magnification, and the amount of silver deposit was counted and scored by two investigators using the method of Saboia et al. [11]. Interfacial nanoleakage was scored based on the percentage of adhesive surface showing AgNO₃ deposition: 0, no nanoleakage; 1, <25% surface with nanoleakage; 2, 25% to ≤50% surface with nanoleakage; 3, 50% to ≤75% surface with nanoleakage; and 4, >75% surface with nanoleakage. Intra-examiner reliability was assessed using the kappa (κ) test.

For SEM analysis, specimens were polished with 2400-grit SiC paper and 2–1-µm
diamond paste using a polishing cloth, then ultrasonically cleaned, air dried, mounted on the SEM stubs, and carbon coated. Resin–dentin interfaces were analyzed by an SEM (Quanta 250; FEI, Hillsboro, OR, USA) operating in mixed secondary/backscattered electron mode at 10 kV.

**Statistical Analyses**

Microtensile bond strength test data were evaluated using commercial software (SPSS, ver. 20.0 for Windows; SPSS Inc., Chicago, IL, USA). Because the groups did not exhibit normal data distributions (Kolmogorov–Smirnov test), a nonparametric test was used (Kruskall Wallis Analysis of Variance). Pairwise differences between group means were analyzed using the Mann–Whitney U-test (level of significance, \( p < 0.05 \)). The level of significance was adjusted according to the Bonferroni’s correction.

\( \chi^2 \) test was used to analyse the differences in the failure modes and statistical significance was set at \( p<0.05 \).

Statistical differences among nanoleakage group scores were analyzed with the Kruskall Wallis Analysis of Variance. Pairwise differences between group means were analyzed using the Mann–Whitney U-test (level of significance, \( p < 0.05 \)). The level of significance was adjusted according to the Bonferroni’s correction.

### 3.3 Results

**Microtensile Bond Strength**

The mean values and standard deviations of \( \mu \)TBS and failure modes are listed in Table 2.

The means of immediate \( \mu \)TBS (T0) for the tested adhesive systems ranked in the following order: Adper Easy Bond (43.8 ± 15.6 MPa) = Optibond XTR (40.6 ± 13.0 MPa) ≥
Clearfil SE (37.7 ± 12.4 MPa) = Bond Force (36.1 ± 12.5 MPa; p < 0.05).

Storage in artificial saliva for 6 months (T6m) significantly reduced the μTBS of Adper Easy Bond (36.6 ± 9.5 MPa) and Bond Force (23.9 ± 9.3 MPa) in comparison with T0 μTBS results (p < 0.05), whereas no significant reduction from T0 values was found for Optibond XTR (37.1 ± 12.1 MPa) or Clearfil SE Bond (34.5 ± 13.0 MPa; p > 0.05).

After 1 year of storage (T1yr), Bond Force (17.6 ± 7.8 MPa) showed a significant reduction in μTBS compared with T0 and T6m values (p < 0.05), whereas μTBS values for Optibond XTR (34.3 ± 9.5 MPa), Clearfil SE Bond (28.6 ± 9.5 MPa), and Adper Easy Bond (31.5 ± 9.3 MPa) were reduced significantly from T0 values (p < 0.05), but comparable to T6m values.

The lowest bond strengths were recorded for Bond Force at T1yr and the highest were observed for Adper Easy Bond and Optibond XTR at T0 (p < 0.05).

The failure mode distribution of the debonded specimens (Table 2) revealed that >50% of failures were cohesive in the bonding resin for Optibond XTR and Clearfil SE Bond at T0, whereas the predominant failure mode at T6m and T1yr was adhesive for all tested bonding systems. The statistical analysis showed that there was significant relationship between the type of failure and time for all tested adhesives and between type of failure and adhesive (p<0.05), except for Adper Easy Bond (p>0.05).

Dentin–Resin Interfacial Nanoleakage Expression

The kappa test confirmed intra-examiner reliability (κ = 0.84). Table 3 presents the extent of AgNO₃ deposition along the bonded interface at each aging interval. Interfacial nanoleakage analysis showed the following results at T0: Clearfil SE Bond = Adper Easy Bond = Optibond XTR > Bond Force (ρ < 0.05). Storage in artificial saliva for 6 months or 1 year significantly increased interfacial nanoleakage expression in all tested adhesive
systems ($p < 0.05$). At T6m or T1yr, interfacial nanoleakage expression was lower for Optibond XTR, Clearfil SE Bond, and Adper Easy Bond than for Bond Force ($p < 0.05$). Scanning electron microscopy images showed the accumulation of significant deposits of AgNO3 (i.e. nanoleakage) over time at all adhesive interfaces examined in this study (Figure 1).
<table>
<thead>
<tr>
<th>Adhesive System</th>
<th>Storage time</th>
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<tbody>
<tr>
<td></td>
<td>T 0</td>
</tr>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
</tr>
<tr>
<td>Optibond XTR</td>
<td>40.6 (13.0)$^g$</td>
</tr>
<tr>
<td></td>
<td>(N=83/0)</td>
</tr>
<tr>
<td></td>
<td>(%30A/54CC/16CD/0M)</td>
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<tr>
<td><strong>Group 2</strong></td>
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<tr>
<td>Clearfil SE Bond</td>
<td>37.7 (12.4)$^f$</td>
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<tr>
<td></td>
<td>(N=74/1)</td>
</tr>
<tr>
<td></td>
<td>(%40A/48CC/12CD/0M)</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td></td>
</tr>
<tr>
<td>Adper Easy Bond</td>
<td>43.8 (15.6)$^b$</td>
</tr>
<tr>
<td></td>
<td>(N=70/0)</td>
</tr>
<tr>
<td></td>
<td>(%66A/23CC/7CD/4M)</td>
</tr>
<tr>
<td><strong>Group 4</strong></td>
<td></td>
</tr>
<tr>
<td>Bond Force</td>
<td>36.1 (12.5)$^{de}$</td>
</tr>
<tr>
<td></td>
<td>(N=71/1)</td>
</tr>
<tr>
<td></td>
<td>(%47A/45CC/8CD/0M)</td>
</tr>
</tbody>
</table>

**Table 2.** Means and standard deviations (MPa) of micro-tensile bond strength (number of intact sticks tested/number of de-bonded specimens during cutting) to dentin at time zero and after storage in artificial saliva (MPa).

Mean (S.D.=standard deviation); N=total number of specimens. Means with the same superscript letters are not significantly different (p>0.05). Premature failures due to preparation procedures were not included in the statistical evaluation.
Table 3. Distribution of nanoleakage within the different treatment groups. Numbers indicate percentage of specimens with respective nanoleakage values (from 0 to >75% of adhesive joint) as observed with ×100 magnification. Aging increased nanoleakage expression for all tested adhesives; after 1 year of aging adhesives ranked in the following order: Group 1<Group2=Group 3< Group 4. Same letters indicate no significant difference (p>0.05). n = number of analyzed sections.
3.4 Discussion

In this study, the bonding effectiveness and interfacial AgNO₃ deposition within the bonded interfaces created by two-step vs. one-step self-etch adhesives were assayed. At T0, differences were found among the tested adhesives (Table 1); Adper Easy Bond and Optibond XTR showed the highest μTBS values and Bond Force the lowest (p < 0.05). Similarly, differences in interfacial nanoleakage expression were also found among systems at T0; Clearfil SE Bond, Optibond XTR and Adper Easy Bond showed lower AgNO₃ uptake than did Bond Force (p < 0.05). Thus, the first null hypothesis was partially rejected.

These results are consistent with those of previous investigations [12,13,14] showing no difference in immediate bond strength between one-step and two-step self-etch adhesives, but contrast with the results of other in vitro studies showing better bond strengths in two-step than in one-step self-etch adhesives [15,16].

The results of this study also showed that the μTBS to dentin after 6 months (T6m) and 1 year (T1yr) was material-dependent, as aging in artificial saliva reduced the bond strengths of one-step dentin bonding systems (Adper Easy Bond and Bond Force) at T6m (p < 0.05) and all materials showed μTBS reductions at T1yr (p < 0.05). Aging for 1 year also increased interfacial nanoleakage in all tested materials compared with T0 values (p < 0.05). Thus, the second null hypothesis was partially rejected. These findings are in agreement with those of previous studies showing significant bond strength reduction and extensive interfacial nanoleakage expression within the adhesive interface produced by simplified adhesives after only 6–12 months of aging under in vitro and in vivo conditions [17,18].

The adhesive capabilities of self-etch adhesives show important differences among formulations, due mainly to differences in pH. Typically, ‘strong’ self-etch systems have a pH < 1.0; this high acidity results in deep demineralisation similar to that of phosphoric acid. The application of such adhesives to dentin exposes the collagen fibrils and dissolves
nearly all hydroxyapatite. Conversely, ‘mild’ self-etch adhesives have a reduced etching potential due to their higher pH (1.5–2.5) [19].

Because the pH values of the adhesive systems tested in the present study were 2.2–2.5 (Table 1), the smear layer was not removed fully and the dentin bonding systems interacted with smear layer-covered dentin up to a few hundred nanometres. As not all hydroxyapatite was dissolved from the interaction zone, much calcium was available for additional chemical interaction with specific monomers [5,20,21].

Clearfil SE Bond, a well-documented mild two-step self-etch adhesive system, is considered the gold standard for self-etch adhesives and its bonding ability has been validated by several in vitro studies [5,22]. In particular, its improved ability to bond to dentin in vitro has been related to the presence of 10-MDP, which is able to react with residual hydroxyapatite within the hybrid layer [5,20]. Clinically, Peumans et al. [23] reported that the use of Clearfil SE Bond achieved a 98% retention rate at 8 years in non-curious cervical lesions with or without separate enamel etching. Similarly, another in vivo study showed that Clearfil SE Bond achieved better annual retention rates up to 8 years in comparison with a two-step etch-and-rinse system [24]. In the present study, Clearfil SE Bond did not yield the highest µTBS values; Adper Easy Bond performed better at T0, but no difference was found between these adhesives after aging.

Optibond XTR, a recently formulated mild two-step self-etch adhesive, yielded µTBS values similar to those of Adper Easy Bond and Clearfil SE Bond at all aging intervals (p > 0.05). Few previous studies have investigated the performance of this adhesive, and only T0 values have been reported. Walter et al. [25] reported that the mean shear bond strength of Optibond XTR to bovine dentin at 24 h was similar to that of Clearfil SE Bond and higher than that of Optibond FL (Kerr), a three-step etch-and-rinse adhesive.

Interestingly, our results did not show superior performance of two-step vs. one-step
self-etch adhesives at T0, as reported in some previous studies [13]. After aging, Bond
Force showed lower μTBS values ($p < 0.05$) and Adper Easy Bond showed μTBS values
similar to those of the two-step systems (Clearfil SE Bond and Optibond XTR; $p > 0.05$).

Bond longevity and stability are adversely affected by physical and chemical factors
over time. Thermal changes (which determine expansion and contraction forces), occlusal
loads, acidic compounds in dentinal fluids [26], salivary enzymes, and bacterial
collagenolytic factors have been claimed to affect adhesive stability.

Recently, the activation of endogenous dentin matrix metalloproteinases (MMPs)
has also been shown to contribute to the degradation of suboptimally impregnated collagen
fibrils within the hybrid layer, expediting adhesive interface degradation [27,28]. Although
this phenomenon has been related mainly to the degradation of hybrid layers created by
etch-and-rinse adhesives, the involvement of endogenous MMPs in the aging of self-etch
adhesive interfaces remains controversial.

Several in vitro aging protocols have been proposed to simulate adhesive interface
degradation. Specimens can be submitted to thermocycling or storage in artificial saliva at
body temperature to mimic the natural aging process of the bonded interface or to simulate
occlusal cyclic loading. The use of a NaOCl solution to degrade exposed collagen fibrils
and expedite in vivo degradation processes has also been proposed [29].

Among currently available systems, one-step self-etch adhesives contain the highest
percentages of hydrophilic monomers. Simplified (one-step) self-etch adhesives are
characterised by increased water sorption (vs. two-step adhesives), which promotes polymer
swelling and other water-mediated degradation phenomena [1,2,30,31]. Recent studies have
also indicated that adhesive permeability is correlated with the presence of unreacted
monomers, and one-step self-etch adhesives have shown greater permeability associated
with the lowest degrees of conversion in comparison with unsimplified (two-step) systems
Van Landuyt et al. [33] also found a 1–3-µm-thick oxygen inhibition layer on the uppermost parts of adhesive resins when using 2-hydroxyxylethyl methacrylate (HEMA)-rich one-bottle adhesives. They speculated that a hypertonic solution was present in the oxygen inhibition layer, leading to diffusion of water from the dentin through the adhesive layer.

In the present study, Bond Force showed the lowest bond strength and the highest AgNO₃ uptake at T6m and T1yr. Bond Force contains mainly bisphenol-a-glycidyl methacrylate (Bis-GMA) and HEMA, with camphoroquinone (CQ) as the initiator; the simultaneous presence of hydrophilic and hydrophobic domains within the bonding agent might affect the curing process [34]. In fact, previous studies revealed that poly(HEMA)-rich domains separate from poly(Bis-GMA)-rich areas, creating heterogeneous polymers that cannot polymerise properly with CQ [35]. To improve the degree of conversion, the use of hydrophilic photo-initiators in addition to standard CQ activation has been proposed. A recent study showed that the use of ethyl 4-dimethacrylaminobenzoate and diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide (TPO) improved the degree of conversion of hydrophilic domains in dental adhesive blends [34]. Adper Easy Bond contains TPO, and it is reasonable to speculate that its stability after 6 months and 1 year may be related to improved curing due to the inclusion of this hydrophilic initiator in its formulation [36,37]. A recent study clarified this relationship by demonstrating a significantly higher degree of cure within hybrid layers produced by Adper Easy Bond in comparison with those produced by two one-step self-etch adhesive systems lacking TPO, as assessed in situ by Raman microspectroscopy [38]. The manufacturers of the other tested adhesives disclosed no information about the photo-initiator systems used.

SEM images showed the accumulation of significant AgNO₃ deposits (i.e. nanoleakage) over time at all adhesive interfaces examined in this study. As with reduced bond strength, the increased AgNO₃ deposition within the resin–dentin interfaces of Bond
Force (vs. other tested systems) may be attributed to areas of suboptimal polymerisation within the resin matrix.

Since Optibond XTR (two-step), Clearfil SE Bond (two-step), and Adper Easy Bond (one-step) yielded similar results but Bond Force (one-step) showed reduced stability over time, it can be concluded that immediate bond strength, nanoleakage expression, and stability over time are not related to the number of steps characterising the bonding systems, but to their chemical formulations.
Figure 1. Representative backscattered scanning electron microscopic images of resin–dentin interfaces bonded with the tested self-etch adhesive systems after 24 h (T0) and 1 year (T1yr) of storage. At T0, almost no AgNO₃ was present in the hybrid layers of all tested adhesives. At T1yr, increased AgNO₃ uptake occurred, particularly in Bond Force specimens. C, composite; AL, adhesive layer; HL and arrow, hybrid layer; D, dentin.
References Chapter 3


CHAPTER 4

ADHESIVE PERFORMANCE OF A MULTI-MODE ADHESIVE SYSTEM:
1-YEAR IN VITRO STUDY

4.1 Introduction

Chapter 3 described how current adhesive technology tends to simplify bonding procedures by reducing application steps, shortening clinical application time and decreasing technique sensitivity, thus improving their standardization [1]. Thus, the most recent developments of self-etch adhesives have simplified the traditional concept of bonding [2], because these materials are easy-to-use, and have a faster application procedure when compared with multi-step etch-and-rinse adhesives. Another important clinical benefit in using self-etch adhesives is the absence (or reduced) incidence of post-operative sensitivity experienced by patients [3,4].

In relation to the application mode, self-etch adhesive systems reduce the possibility of iatrogenically-induced clinical mis-manipulation during acid conditioning, rinsing and drying, which may occur when etch-and-rinse systems are used [2]. Self-etch adhesives do not require a separate etching step, as they contain acid resin monomers that simultaneously “condition” and “prime” the dental substrates [5]. Additionally, as it can be seen in the materials and methods tested in the previous study, manipulation has been further simplified by reducing the number of steps from the initial two solutions (an acidic primer followed by the application of a relatively hydrophobic bonding resin on top of the primed surface) to a one-step system, in which all components (etchant, primer, and bonding resin) are incorporated into a single solution [6]. In terms of immediate performance these simplified one-step adhesives showed promising results [7,8]. However, as also described in the study presented in the previous chapter, the long-term performance of simplified one-step adhesives is inferior in terms of bond durability [6,9].
Even if all self-etch adhesive systems rely on the same bonding mechanism, they differ from each other in many aspects, such as acidic resin monomer composition, water content and acidity. Thus, these adhesives may be further classified according to the pH of the adhesive solution as mild (pH > 2), moderate (1< pH < 2) or strong (pH < 1). Indeed the adhesive acidity influences the ability of the system to interact with the underlying enamel and dentin [1]. Recent findings revealed that some mild self-etch systems are able to establish a chemical bond between specific carboxylic or phosphate groups of functional resin monomers (in particular 10-MDP) and residual hydroxyapatite crystals on the dentin collagen scaffold [10]. This chemical bond seems to be important in stabilising the adhesive interface on dentin over time [5].

The role of dentin endogenous matrix metalloproteinases (MMPs), explained in chapter 2, has also been involved in the stability of the hybrid layer over time. Direct evidences of increased MMP-2 and -9 activities following adhesive application were found, with higher levels of activities reported for etch-and-rinse compared to self-etch adhesives. These findings are probably correlated to the fact that the etching step of the etch-and-rinse adhesives exposes more dentin matrix than the use of self-etch adhesives [11].

Recently, a new type of one-step self-etch adhesive has been introduced. This type of self-etch adhesive is classified as “universal” or “multi-mode” as they can be applied either with the etch-and-rinse or the self-etch technique [12]. This multi-approach capability enables the clinician to apply the adhesive with the so-called selective enamel etching technique that combines the advantages of the etch-and-rinse technique on enamel, with the simplified self-etch approach on dentin with additional chemical bonding on remnant carbonated apatite crystallites in those bonding substrates.

Therefore, the purpose of the part of the research presented in this chapter was to evaluate the bond strength to human sound dentin, interfacial nanoleakage expression and
adhesive-induced endogenous MMP activities of a “multi-mode” adhesive system (used either with the self-etch or the etch-and-rinse approach) compared with a two-step etch-and-rinse adhesive (applied in accordance with manufacturer’s instructions). The null hypotheses tested were that: 1) no differences in immediate bond strength and interfacial nanoleakage expression exist between the tested groups, 2) storage does not affect the stability of the tested adhesive interfaces after 6 months or 1 year aging in artificial saliva at 37 °C, 3) activation of endogenous MMPs is not related to the adhesive system or strategy employed.

4.2 Material and methods

Teeth selection and preparation for microtensile bond strength test

Sixty extracted non-caries human third molars were selected for the study. They were stored for less than 1 month in 0.5% chloramine-T solution at 4 °C. The teeth were collected after obtaining patients’ informed consent to their use for research purposes under a protocol approved by the Institutional Review Board of the University of Trieste (Italy). Flat coronal dentin surfaces were prepared by removing the coronal tooth portion parallel to the occlusal surface with a low-speed diamond saw (Micromet; Remet, Bologna, Italy) under water irrigation. The absence of enamel and/or pulp tissue exposure on the dentin surfaces was verified under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH, Jena, Germany).

The exposed middle to deep dentin surfaces were polished with 180-grit wet silicon carbide (SiC) abrasive paper to create standardized, smear layer-covered dentin substrates. The polished specimens were randomly and equally assigned to four treatment groups (N=15 each): Group 1: Scotchbond Universal (3M ESPE, Seefeld, Germany, experimental version EXL759) was applied in the self-etch mode on smear layer-covered dentin in
accordance with the manufacturer’s instructions (i.e. the adhesive was applied for 20 s and gently air-dried for 5 s); Group 2: Scotchbond Universal was applied with the etch-and-rinse wet-bonding approach (i.e. the adhesive was applied for 20 s on 37% phosphoric acid-etched wet dentin using an etching time of 15s), in accordance with the wet-bonding technique. This was followed by gentle air drying for 5 s; Group 3: Scotchbond Universal was applied with the etch-and-rinse dry-bonding approach (i.e. the adhesive was applied for 20 s on 37% phosphoric acid-etched, dried dentin, using an etching time of 15s and an air-drying time of 10 s after water rinsing). This was followed by air thinning for 5s; Group 4: Prime&Bond NT (Dentsply DeTrey, Konstanz, Germany; Lot 1003001635) was applied in accordance with the manufacturer’s instructions, i.e. the adhesive was applied on 37% phosphoric acid-etched (etching time 15s) wet dentin for 20 s, followed by air thinning for 5 s (i.e. wet-bonding technique). The composition of the adhesive systems used and the different application modes are listed in Table I.

Adhesives were light-cured for 10 s using a halogen light-curing unit (Curing Light 2500, 3M ESPE). The irradiance level of the light was monitored periodically with a radiometer (3M ESPE) to ensure an output of at least ≥ 600 mW/cm². After adhesive application, 4-mm-thick coronal buildups were created on the bonded specimens using a resin composite (Filtek Z250, 3M ESPE) applied in 2-mm thick increments that were polymerised for 40 s per increment.

Dentin-resin sticks (approximately 0.9x0.9x8 mm) were created using a low-speed saw, under copious water irrigation, in accordance with the non-trimming technique. Sectioned specimens were equally and randomly assigned to three storage groups: baseline (T0, stored for 24 h), 6 months (T6m), and 1 year (T1yr). Storage was performed at 37 °C in artificial saliva; the latter was prepared in accordance with the protocol reported by Pashley et al. [13].
Prior to be stressed until failure, the dimensions of each stick were individually measured with a pair of digital callipers (±0.01 mm) and the bonded area was calculated for subsequent conversion of microtensile strength data into units of stress (MPa). Each specimen was then glued to a modified jig (Bisco Inc., Schaumburg, IL, USA) with cyanoacrylate adhesive (Zapit; Dental Ventures of America, Corona, CA, USA) for microtensile testing and stressed until failure under tension at a crosshead speed of 1 mm/min.

The number of prematurely debonded sticks in each test group was recorded, but these values were not included in the statistical analysis. This is because all premature failures occurred during the cutting procedure and they did not exceed 3% of the total number of tested specimens and were similarly distributed within the groups. A single observer evaluated the failure modes under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH) at magnifications up to 50× and classified them as adhesive, cohesive in dentin, cohesive in composite, or mixed failures.

Because the groups did not exhibit normal data distributions (Kolmogorov–Smirnov test), a nonparametric test was used (Kruskal-Wallis Analysis of Variance). Pair-wise differences between group means were analyzed using the Mann–Whitney U-test (level of significance, p < 0.05). The level of significance was adjusted according to the Bonferroni’s correction. The χ² test was used to analyse the differences in the failure modes and statistical significance was set at p<0.05.
<table>
<thead>
<tr>
<th>Material</th>
<th>Composition</th>
<th>Application Procedures</th>
</tr>
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<tbody>
<tr>
<td>Scotchbond Universal</td>
<td>MDP Phosphate monomer</td>
<td>Group 1 (Self-Etch): Scrub adhesive for 20s, Air-thin 5s, Light-cure for 10s.</td>
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<td>3M ESPE, Seefeld, Germany</td>
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<td>HEMA</td>
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<td></td>
<td>Methacrylate functionalised Polyalkenoic acid (Vitrebond™ Copolymer)</td>
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<td>Air-drying for 5s</td>
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<td>Scrub adhesive for 20s</td>
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<td>Air-thin 5 s</td>
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<td>Prime&amp;Bond NT, Dentsply, De Trey,</td>
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Table 1. Components, compositions, and application procedure of the tested adhesives (information supplied by the manufacturer).

Abbreviations: HEMA, 2-hydroxyethyl methacrylate; MDP, 10-methacryloyoxydecyl, UDMA, urethane dimethacrylate.
**Dentin/resin interfacial nanoleakage expression**

Three additional bonded teeth were prepared for each tested group as previously described, cut vertically into 1-mm-thick slabs to expose the bonded surfaces, and tested using the three aforementioned storage protocols (T0, T6m, T1yr). Specimens were covered with nail varnish, leaving 1 mm exposed at the bonded interface, and processed for interfacial nanoleakage evaluation. Nanoleakage analysis was performed by light microscopy (LM) and scanning electron microscopy (SEM). Bonded interfaces were immersed in 50 wt% ammoniacal AgNO3 solution in a dark environment for 24 hours according to the protocol described by Tay et al [14]. After immersion in the tracer solution, specimens were rinsed in distilled water and immersed in a photo-developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grain within voids along the bonded interfaces.

For LM, the silver-impregnated specimens were fixed, dehydrated, and embedded in epoxy resin (Epon 812; Fluka, Buchs, Switzerland). The specimens were then fixed on glass slides (Menzel, Bielefeld, Germany) using cyanoacrylate glue (Super Cyanolit; Panacol-Elosol GmbH, Steinbach, Switzerland) and flattened with a series of abrasives (180-, 600-, 1200-, 2400-, and 4000-grit SiC) under water irrigation using a grinding device (LS2; Remet). Bonded interfaces were analyzed using a light microscope (E800; Nikon, Tokyo, Japan) at 100× magnification. The amount of silver deposit was counted and scored by two investigators using the method of Saboia et al. [15]. In brief, interfacial nanoleakage was scored based on the percentage of adhesive surface showing AgNO3 deposition: 0, no nanoleakage; 1, <25% surface with nanoleakage; 2, 25% to ≤50% surface with nanoleakage; 3, 50% to ≤75% surface with nanoleakage; and 4, >75% surface with nanoleakage. Intra-examiner reliability was assessed using the kappa (κ) test.
For SEM interfacial nanoleakage analysis, silver impregnated specimens were polished with 2400-grit SiC paper and 2–1-µm diamond paste using a polishing cloth. The polished specimens were then ultrasonically cleaned, air-dried, mounted on the SEM stubs, and carbon-coated. Resin–dentin interfaces were analyzed with a SEM (Quanta 250; FEI, Hillsboro, OR, USA) operating in mixed secondary/backscattered electron mode at 10 kV.

Statistical differences among nanoleakage group scores were analyzed with the Kruskal-Wallis Analysis of Variance. Pair-wise differences between group means were analyzed using the Mann–Whitney U-test (level of significance, p < 0.05). The level of significance was adjusted according to the Bonferroni’s correction.

**Zymographic assay**

Zymographic assay of the expression of endogenous dentin MMPs was performed on dentin extracts in accordance with the protocol reported by Mazzoni et al. [11]. In brief, mineralised (to simulate the self-etch approach) and demineralised dentin powder aliquots (performed with 1 mL of 1% phosphoric acid for 10 min to simulate the etching procedure in the etch-and-rinse bonding technique) were prepared and assigned to the following treatment groups: 1) untreated mineralised dentin (control); 2) untreated demineralised dentin (control); and 3) mineralised dentin powder was mixed for 30 min with 1 mL of Scotchbond Universal (self-etch approach); 4) demineralised dentin powder was mixed for 30 min with 1 mL of Scotchbond Universal (etch-and-rinse approach); 5) demineralised dentin powder was mixed was mixed for 30 min with 1mL of Prime&Bond NT.

Adhesive-treated dentin powder lots were treated with 1 mL of acetone, centrifuged (20,800G for 20 min), re-suspended in acetone and re-centrifuged two more times to properly remove the adhesive resin, in accordance with Mazzoni et al. [16].
Specimens were re-suspended in extraction buffer (50mM Tris-HCl pH 6, containing 5mM CaCl2, 100mM NaCl, 0.1% Triton X-100, 0.1% nonionic detergent P-40, 0.1mM ZnCl2, 0.02% NaN3) for 24 hours at 4° C in accordance with the protocol reported by Breschi et al. [17], sonicated for 10 min (at ≈ 30 pulses), centrifuged for 20 min at 4°C (20,800G), supernatant was removed and re-centrifuged. The protein content was further concentrated using Vivaspin centrifugal concentrator (10,000 KDa cut off) for 30 min at 4 °C (15,000G for 3 times). Total protein concentration of dentin extracts was determined by the Bradford assay.

Extracted proteins aliquots were diluted in Laemmli sample buffer in a 4:1 ratio. Electrophoresis was performed under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/mL gelatine, which had been fluorescently labelled with 2-methoxy-2,4-diphenyl-3(2H)-furanone. 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 hour in 2% Triton X-100, incubated in activation solution (50 mmol/L Tris-HCl, 5 mmol/L CaCl2, pH 7.4) for 48 hours and photographed under ultraviolet light illumination using long wavelength ultraviolet light (Gel Doc XR System, Bio-Rad). Gelatinases (MMP-2 and -9) in the samples were analyzed in triplicate using gelatine zymography. Control zymograms were incubated with 5mM EDTA or 2mM 1,10-phenanthroline.

4.3 Results

*Microtensile Bond Strength*

The mean values and standard deviations of the microtensile bond strength and failure modes are listed in Table II. No differences were found for the mean immediate bond strengths (T0): Scotchbond Universal self-etch (Group 1; 35.5 ± 9.7 MPa) = Scotchbond
Universal etch-and-rinse wet dentin (Group 2; 34.8 ± 9.4 MPa) = Scotchbond Universal etch-and-rinse dry dentin (Group 3; 41.6 ± 10.3 MPa) = Prime&Bond NT (Group 4; 38.4 ± 11.4 MPa) (p > 0.05).

Storage in artificial saliva for 6 months (T6m) significantly reduced the bond strength of Scotchbond Universal irrespective of the application technique (Group 1, 2, 3), when compared with immediate bond strength (p<0.05). By contrast, no reduction was found for Prime&Bond NT (Group 4; p > 0.05).

After 1 year of storage (T1yr), Scotchbond Universal applied in the self-etch mode (Group 1: 26.8 ± 9.5 MPa) and Prime&Bond NT showed no significant bond strength reduction when compared to the 6 months results (p > 0.05). These one-year bond strength values were higher than the one-year values obtained for Scotchbond Universal when the adhesive was applied using the etch-and-rinse technique either on wet or dry dentin (Group 2: 21.9 ± 9.5 MPa; Group 3: 21.8 ± 9.4 MPa, respectively).

Failure mode distribution of the debonded specimens is shown in Table II. The predominant failure mode was adhesive in most of the tested groups and time intervals. When Scotchbond Universal was applied on acid-etched wet or dry dentin at time zero (Group 2 and 3 respectively) and Group 3 at T6m, more than 50% failures were cohesive within the bonding resin.

**Dentin/resin interfacial nanoleakage expression**

The kappa test confirmed a high level of intra-examiner reliability (κ=0.84). Table III presents the extent of silver deposition along the bonded interface at each aging interval. Interfacial nanoleakage analysis showed the following results at T0: Group 1 < Group 3 < Group 2 < Group 4 (p <0.05). At T0, the SEM analysis confirmed minor nanoleakage manifestation for Scotchbond Universal applied with the etch-and-rinse approach on dry
dentin (Group 3) and Prime&Bond NT (Group 4). Nanoleakage expression was predominantly observed at the peritubular level along the deepest areas of the hybrid layer (Figure 1).

Storage in artificial saliva for 6 months or 1 year significantly increased interfacial nanoleakage expression in all tested adhesive systems (p<0.05; Table III, Figure 2). Comparison of the tested adhesives showed that interfacial nanoleakage expression at T6m was in the following order: Scotchbond Universal self-etch = Scotchbond Universal etch-and-rinse dry < Scotchbond Universal etch-and-rinse wet < Prime&Bond NT. After 1 year, Scotchbond Universal self-etch showed lower nanoleakage expression than the other groups (p < 0.05; Figure 2).

**Zymographic results**

A composite of different zymograms showing the gelatinolytic activity derived from the designated groups is shown in Fig. 3. Mineralised dentin showed minor faint gelatinolytic activity (Figure 3, lane 1). After demineralisation with phosphoric acid, MMP-2 pro-form and active-form (72-kDa and 66-kDa, respectively) and pro-MMP-9 (95 kDa) were present (Figure 3, lane 2). Treatment with Scotchbond Universal produced an increased expression of MMP-2 pro- and active-form, and MMP-9 pro-form when applied either on mineralised dentin (self-etch approach, Figure 3, lane 3) or on demineralised dentin (etch-and-rinse approach, Figure 3, lane 4) with respect to mineralised (Figure 3, lane 1) or partially-demineralised dentin (Figure 3, lane 2, respectively). Prime&Bond NT produced an increase of MMP-2 pro-form and active-form (Figure 3, lane 5) compared to partially-demineralised dentin (Figure 3, lane 2). Control zymograms incubated with 5mM EDTA and 2mM 1,10-phenanthroline showed no gelatinolytic activity.
<table>
<thead>
<tr>
<th>Adhesive System</th>
<th>Storage time</th>
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<tr>
<td></td>
<td>T0</td>
<td>T6m</td>
<td>T1yr</td>
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<tr>
<td><strong>Group 1</strong> Scotchbond Universal self-etch</td>
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<td></td>
<td>35.5 (9.7)(\text{a}^a) (N=59/0) %(56A/34CC/10CD/0M)</td>
<td>27.6 (8.8)(\text{b}^b) (N=59/0) %(64A/29CC/5CD/2M)</td>
<td>26.8 (9.5)(\text{b}^b) (N=56/1) %(59A/22CC/9CD/10M)</td>
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<td><strong>Group 2</strong> Scotchbond Universal E&amp;R wet bonding technique</td>
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<td>34.8 (9.4)(\text{a}^a) (N=55/0) %(27A/60CC/13CD/0M)</td>
<td>24.3 (7.1)(\text{b}^b) (N=69/1) %(46A/32CC/16CD/6M)</td>
<td>21.9 (9.5)(\text{c}^c) (N=54/0) %(50A/32CC/9CD/9M)</td>
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<tr>
<td><strong>Group 3</strong> Scotchbond Universal E&amp;R dry bonding technique</td>
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<td></td>
<td>41.6 (10.3)(\text{a}^a) (N=63/1) %(25A/50CC/25CD/0M)</td>
<td>24.7 (7.7)(\text{b}^b) (N=65/1) %(33A/55CC/9CD/3M)</td>
<td>21.8 (9.4)(\text{c}^c) (N=57/1) %(76A/12CC/3CD/9M)</td>
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<td><strong>Group 4</strong> Prime&amp;Bond NT</td>
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<td>38.4 (11.4)(\text{a}^a) (N=64/0) %(72A/19CC/9CD/0M)</td>
<td>32.6 (10.7)(\text{b}^b) (N=58/1) %(52A/31CC/7CD/10M)</td>
<td>32.4 (11.7)(\text{c}^c) (N=53/0) %(52A/31CC/7CD/10M)</td>
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**Table 2.** Means and standard deviations (MPa) of micro-tensile bond strength (number of intactsticks tested/ number of de-bonded specimens during cutting) to dentin at time zero and after storage in artificial saliva (MPa).

Values are mean and standard deviation. N=total number of specimens. Means with the same upper case letters in columns indicate no significant difference between groups (p > 0.05). Means with the same lower case letters in rows indicate no significant difference between storage times within each group (p > 0.05). Premature failures due to preparation procedures were not included in the statistical evaluation.
Table 3. Distribution of nanoleakage within the different treatment groups. The number values indicate the percentage of specimens with the respective degree of nanoleakage (0% to > 75% of the adhesive joint) observed at x 100 magnification. The same letters indicate no significant difference (p > 0.05).
4.4 Discussion

Clinically, limiting phosphoric acid etching to enamel during the selective etching technique can be difficult, especially in small Class II preparations in which acid may readily extend onto the dentin substrate [18]. The rationale behind this study was to determine the bonding performance of a one-step universal system when applied following a “self-etch” or an “etch-and-rinse” adhesive approach.

At T0, Scotchbond Universal used with the self-etch mode (Group 1) or with the etch-and-rinse mode either on wet (Group 2) or dry dentin (Group 3) exhibited similar bond strength values, comparable to Prime&Bond NT when applied in accordance with the manufacturer’s instructions (T0; after 24 hours of water storage; p > 0.05). However differences between the systems were found in the interfacial nanoleakage expression. Scotchbond Universal applied in the self-etch mode (Group 1) showed lower silver uptake when compared to the same adhesive applied with the etch-and-rinse mode (both on wet and dry dentin, Group 2 and 3 respectively), as well as Prime&Bond NT (T0; p<0.05). Thus the first null hypothesis was partially accepted.

These results support previous findings showing that when phosphoric acid (used to etch peripheral enamel according to the selective enamel etch technique) extends to dentin, the application of a self-etch adhesive results in higher microtensile bond strength compared with its application on smear layer-covered dentin [18]. However, our results are in contrast with previous studies on 10-methacryloyloxydecyl dihydrogenphosphate (MDP)-based adhesives (such as Scotchbond Universal) reporting that phosphoric-acid etching of dentin prior to adhesive application significantly decreased the bond strength to dentin [19-21].

The results of the present study also showed that the bond strength to dentin after 6 months (T6m) was material-dependent since aging in artificial saliva reduced the bond strength of Scotchbond Universal (irrespective of the application mode) compared to
immediate values, while no significant reduction was found for Prime&Bond NT \((p < 0.05)\). Differences related to the application mode of the universal adhesive were found after 1 year (T1yr): the microtensile bond strength of Scotchbond Universal to dentin applied with the etch-and-rinse technique on wet (Group 2) or dry (Group 3) dentin was significantly lower than the same adhesives applied with the self-etch protocol (Group 1) and Prime&Bond NT (Group 4; \(p < 0.05)\). Storage in artificial saliva also caused an increase in the silver uptake pattern after 6 months (T6m) and 1 year (T1yr). Interestingly, nanoleakage expression was lower when Scotchbond Universal was applied in the self-etch mode (Group 1) compared to the etch-and-rinse applications (Group 2 and 3) and Prime&Bond NT (Group 4; \(p < 0.05)\). Thus, the second tested hypothesis was rejected.

The different values on bond strength and nanoleakage expression between the self-etch and the etch-and-rinse technique on wet and dry dentin may be explained by the fact that MDP-containing adhesives benefit from the presence of residual apatite on the collagen fibrils, which results in additional chemical bonding and this may contribute more to the stability and longevity of the bond rather than its immediate bond strength \[5,22\]. Yoshida et al., showed that chemical bonding promoted by 10-MDP is not only more effective, but also more stable in water than other functional monomers such as 4-MET (4-methacryloxyethyl trimellitic acid) and phenyl-P \[10\]. The drop in bond strength and the high nanoleakage expression observed in the present study with both the etch-and-rinse technique on dry and wet dentin may be attributed to the fact that phosphoric acid decalcifies human dentin deeper (up to 3-6 µm) than a self-etch adhesive is designed to infiltrate \[5\].

Despite several in vitro studies on one-step self-etch adhesives, most of those studies utilised only short-term periods of observation and reported bond-strength reduction and extensive interfacial nanoleakage expression produced by simplified adhesives compared to
multi-step adhesives [23-27]. In particular, the lack of a hydrophobic bonding resin in one-step self-etch adhesive formulations has been demonstrated to reduce bond stability over time, because the bonded interfaces behave as semi-permeable membranes allowing the movement of water across them and expediting hydrolytic degradation [28,29]. The adhesive permeability of one-step adhesives has also been correlated with suboptimal curing of the hydrophilic acidic resin monomers blended into their formulations, which was associated with phase separation phenomena [30,31].

Endogenous gelatinolytic/collagenolytic enzymes can degrade the collagen structure of the bonded interface over time, accelerating the degradation of the adhesive interface and different MMPs inhibitors has been successfully proposed to stabilize the bonded interface [6,11,16,17,32-40]. While this phenomenon has been mainly related to the degradation of hybrid layers created by etch-and-rinse adhesives, the involvement of endogenous MMPs in the aging of self-etch adhesive interfaces is controversial [5,11,38].

The results of the zymographic analysis showed evident changes in dentinal MMP-2 and -9 enzyme activities after the application of the tested adhesives, revealing differences in the extent of enzyme activation. These findings require rejection of the null hypothesis that the activation of endogenous MMPs is not related to the adhesive system or the strategy employed. Additionally, the present results further confirm that MMP activation is induced by dentin adhesives irrespective of the strategy employed (i.e., etch-and-rinse or self-etch) [11] as the tested MDP-containing one-step universal adhesive activates MMPs on both mineralised and demineralised dentin due to its chemical formulation. The acidic monomers reduces the pH of the environment activating pro-forms into active forms of MMPs via the cysteine-switch mechanism that exposes the catalytic domain of these enzymes that were blocked by pro-peptides [11,16].

Conversely, our results are not in agreement with previous report employing gelatin
zymography to assay the activity of MMP-2 and -9 [41,42]. These controversial findings can be justified by different laboratory protocols since in the present study protein extraction and concentration were performed after treatment with the adhesives, thus enriching the concentration of MMPs. Additionally, since the zymographic band intensity is time-related, incubation time (48 h) may have an effect on the observed results (even if no information is available on the incubation time of the previous reports) [41,42].

Based on this study, improved bonding effectiveness of the tested universal adhesive system on dentin is obtained when the adhesive is applied with the self-etch approach. Indeed, the etch-and-rinse approaches tested (both on wet and dry dentin) resulted in immediate bond strength comparable to the self-etch mode but, expedited long-term aging resulted in reduced bond strength and increased nanoleakage expression, irrespective of dentin wetness (wet and dry).

However, regardless of the approach and the material used in bonding procedures, a stable and durable bond is not achieved. Therefore, experimental strategies that aim to enhance the adhesive interface, particularly improving the durability of the resin-dentin bond strength by increasing the resistance of dentin collagen matrix to enzymatic degradation are needed. Some of these will be further described in the next chapter.
Figure 1. Representative SEM micrographs of hybrid layers created immediately after bonding (T0) by Scotchbond Universal applied with the self-etch (Group 1; Fig. 1a) or etch-and-rinse approach on wet (Group 2; Fig. 1b) or dry (Group 3; Fig. 1c) dentin and Prime&Bond NT applied in accordance with the wet-bonding technique (Group 4; Fig. 1d). Almost no interfacial nanoleakage expression was found for Scotchbond Universal applied with the self-etch approach or on acid etched wet dentin (Figure 1a, 1b). Minor silver deposits were visible along the deepest part of the hybrid layer close to demineralised peritubular dentin for Scotchbond Universal applied in accordance with the dry-bonding technique and for Prime&Bond NT. HL: hybrid layer; A: adhesive; D: dentin; Bar: 5µm.
Figure 2. Representative light microscopy micrographs of aged (T1yr: 1 year aging in artificial saliva at 37°C) adhesive interfaces created by Scotchbond Universal applied with the self-etch (Group 1; Fig. 2a; score 2) or etch-and-rinse approach on wet (Group 2; Fig. 2b; score 1) or dry (Group 3; Fig. 2c; score 3) dentin, and Prime&Bond NT applied in accordance with the wet-bonding technique (Group 4; Fig. 2d; score 3). HL: hybrid layer; A: adhesive; D: dentin; Bar: 5µm.
Figure 3. Zymographic analysis of dentin powder treated with tested adhesives. Molecular masses, expressed in kDa, are reported in the standard lane (std). Lane 1: mineralised dentin showing the presence of MMP-2 pro-form and active-form (72- and 66-kDa, respectively) and pro-MMP-9 (95 kDa); Lane 2: proteins extracted from dentin powder demineralised with 10% phosphoric acid, showing an increase in the expression of MMP-2 and MMP-9. Lane 3: Demineralised dentin powder after incubation with Scotchbond Universal applied with the self-etch approach (Group 1) produced increase in MMP-2 active-form and of MMP-9 compared with mineralised dentin; Lane 4: Demineralised dentin powder after incubation with Scotchbond Universal applied with the etch-and-rinse approach (Group 2,3) showing increased activity of both MMP-2 and -9 compared to demineralised dentin; Lane 5: Demineralised dentin powder after incubation with Prime&Bond NT showing active MMP-2 and -9.
References Chapter 4


CHAPTER 5

STRATEGIES TO STABILIZE THE ADHESIVE INTERFACE
REDUCING THE INTRINSIC COLLAGENOLYTIC ACTIVITY
OF MINERALIZED DENTIN
Strategies to stabilize the adhesive interface reducing the intrinsic collagenolytic activity of mineralized dentin

5.1 Introduction

As previously described in chapter 2, the durability of dentin bonding systems is affected by the degradation of the resin compounds occurring via hydrolysis of suboptimally polymerized hydrophilic resins and degradation of collagen matrices by matrix metalloproteinases (MMPs) and cysteine cathepsins [1].

MMPs and cathepsins have been shown to be present in dentin [2-6] and they seem to be responsible for the slow hydrolysis of the collagen fibrils in the hybrid layer that anchors resin composites to the underlying mineralized dentin [7]. To prolong the durability of the resin-dentin bond, inactivation of these proteases has been recommended through the use of synthetic MMP inhibitors such as chlorhexidine [8-11], quaternary ammonium methacrylates, or benzalkonium chloride [12,13]. Moreover, other approaches have been proposed to reduce the hybrid layer degradation, including dentin remineralization, ethanol wet-bonding, and the use of collagen-cross-linkers [10,14,15].

5.2 Protease inhibitors

5.2.1 Chlorhexidine

Most of the experiments aimed to improve the durability of dentin bonds through enzyme inhibition have been performed with the use of chlorhexidine (CHX), a potent antimicrobial agent. CHX inhibits effectively MMP-2, -8 and -9 [16], and cysteine cathepsins [17]. In 2004, Pashley et al. [18] presented convincing evidence of its efficacy in inhibiting dentin collagenolytic enzymes. Since then, several studies have demonstrated that
CHX can preserve the structural integrity of hybrid layer collagen matrix [19-27] and reduce time-dependent reduction in dentin bond strength both *in vivo* and *in vitro* [20,21,24–33].

Even though CHX binding rate to mineralized dentin is almost 80% lower than to demineralized dentin [34], low concentrations of CHX (0.05–0.2%) are sufficient to completely inhibit the collagenolytic activity of untreated dentin powder [18,35], while 0.5–2.0% concentrations are able to only partially inhibit the activity induced with acidic SE primer [35]. Since MMPs require calcium to maintain their tertiary structure and zinc ions for their catalytic activity [36] also in dentin [37], and CHX loses its MMP inhibition in the presence of calcium chloride [16], CHX-related MMP inhibition may be related to its chelating property and calcium ions released by the primer may be responsible for the loss of inhibition by CHX. This is supported by the finding that treating dentin powder with Clearfil SE Bond primer for 2 min instead of 20 s not only increased the collagenolytic activity, but also caused the loss of inhibition by 0.5% and 1.0% CHX, with only 2.0% CHX showing significant inhibition [35]. Also the recent reports of chlorhexidine binding by dentin collagen [34,38] suggest that collagen may compete with MMPs for CHX binding, requiring the use of relatively high CHX concentrations.

Incorporation of CHX at reasonably low (0.2–2.0%) concentration into methacrylate comonomers has no effect on water sorption, cause a slight decrease in conversion rate, but may even increase flexural strength and modulus of elasticity of dental adhesives [39]. Chlorhexidine release from the polymerized adhesive is concentration-dependent and retains a slow steady-state level [39]. Since the idea behind the incorporation of inhibitors into adhesive is their continuous release to prevent collagen degradation for a prolonged time, these results are promising.
5.2.2 Galardin

The inhibitory effect of galardin on dentinal MMPs has been confirmed by zymography and interfacial nanoleakage expression after 1 year. Galardin is a synthetic MMP inhibitor that exhibits strong activity against MMP-1, -2, -8, and -9. It has a collagen-like structure that binds to the active sites of MMPs [9]. Galardin (0.2 mM) can inhibit the proteolytic activity of demineralized dentin at concentrations approximately 10–100 times lower than that of CHX (2.2 mM).

5.2.3 Quaternary ammonium compounds

Other cationic compounds, such as quaternary ammonium compounds, may also inhibit dentinal MMPs and thus stabilize the adhesive interface over time. Quaternary ammonium salts are positively charged at physiological pHs and have an effective antibacterial activity. Similar to CHX, these compounds are cationic, water-soluble, but unlike CHX they may not leach out of bonded interfaces. QAMs inhibit soluble MMP-9 as or more effectively than Galardin, and almost completely inhibit the demineralized dentin collagen degradation [12].

Polymerizable quaternary ammonium methacrylates (QAMs), especially 12-methacryloyloxydodecylpyridinium bromide (MDPB) have been incorporated into self-etching primers because they possess antimicrobial properties and can copolymerize with adhesive monomers [40,41]. In vitro and clinical experiments have also indicated that QAMs (namely MDPB in Clearfil Protect Bond) may inhibit collagenolytic enzymes in the hybrid layer [42,43]. MDPB proved to be among the most effective [12]. However, other studies have reported reductions in bond strength comparable to other adhesives [44,45], so it may be too early to make any definitive conclusions of the clinical efficacy of MDPB in the preservation of hybrid layer.
Benzalkonium chloride (BAC) is a mixture of alkylbenzyltrimethylammonium chlorides of various alkyl chains. It is a cationic surface-active agent with a quaternary ammonium group used as antimicrobial agent and surfactant [13]. BAC-containing etchants can be used with etch-and-rinse adhesives without affecting immediate bond strength to enamel or dentin [46]. Tezvergil-Mutluay et al. [13] demonstrated that 0.5% BAC concentrations completely inhibited soluble MMP-2, -8 or -9, and produced significant reduction in demineralized dentin collagen degradation.

5.3 Collagen cross-linkers

Even though CHX inhibits both MMPs [16], and cysteine cathepsins [17], the potential disadvantage is that CHX may leach out of hybrid layers within 18–24 months [42,44,47-52]. Cross-linking agents are considered an interesting option for improving the stability and resistance of collagen degradation within the demineralized dentin matrix [53,54]. Covalent cross-links produced with external cross-linkers are very stable, and may inactivate the active sites of dentin proteases by reducing the molecular mobility of the active site or by changing negatively charged ionized carboxyl groups into positively charged amides. Thus, an increase in the extent of cross-linking of the collagen fibrils prior to adhesive application may result in increased bonding durability.

This approach has been recently investigated by some authors with the aim to reinforce collagen fibrils through intermolecular crosslinking [55-57]. Since lower biodegradation rates and high mechanical properties of collagen are desirable, the use of collagen cross-linkers in adhesive procedures has gained increased popularity in recent years [7,10]. The use of glutaraldehyde, riboflavin, proanthrocyanidin and carbodiimide has been proposed to enhance the mechanical and structural stability of dentin collagen, leading
to a stable dentin matrix network that, after resin infiltration, should provide a durable hybrid layer [58-60]. Moreover, collagen cross-linkers have been reported to improve the resistance of uncross-linked or mildly cross-linked collagen matrices to degradation by bacterial collagenases [61,62], potentially contributing to the stabilization of the resin-dentin interface over time.

In addition to the proven efficacy of collagen cross-linkers in chemical or physical modification of the dentin collagen substrate, the clinical applicability of these solutions is desirable. Even though the results so far have been promising, problems remain to be solved before the clinical applications of cross-linkers become available. Glutaraldehyde works well [53] but is toxic. Grape-seed extract is also effective [53] and leads to an increase of the immediate dentin bond strength in reduced application times [63], but it stains the dentin brown and the durability of long-term bond strength remains to be examined. Low-dose riboflavin has also been tested successfully, but the need for a separate curing device (UVA) or separate curing of the cross-linker with blue light irradiation for optimal outcome [60,64] make further research for a clinically acceptable technique necessary.

1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC)

Accordingly, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), a cross-linking agent with very low cytotoxicity, has shown promising results in eliminating dentin collagen degradation and preserving dentin bond strength over time with clinically acceptable application times [15,65,66].
EDC contains a functional group with the formula RN=C=NR. The carbodiimide reacts with ionized carboxyl groups in proteins to form an O-acylisourea intermediate that can react with a non-proteinated amino group and an adjacent protein chain to form a stable covalent amide bond between the two proteins, with the only by-product being urea. This category of cross-linker may inactivate the active sites of dentin proteases by reducing the molecular mobility of the active site or by changing negatively charged ionized carboxyl groups into positively charged amides. Additionally, EDC can cross-link both helical and especially telopeptide domains in collagen and may also prevent telopeptidase activity that would normally remove bulky telopeptides from the specific peptide bond of collagenases [65]. Increases in collagen stiffness may prevent MMPs from “unwinding” collagen peptides [67]. Since this “unwinding” is necessary to allow MMPs catalytic site to cut the peptide [1,7,10,68], it would also effectively inhibit MMPs functional activity.

Thus, in a recent study Mazzoni et al. [66] demonstrated that the use of 0.3 M EDC pre-treatment improves durability and structural integrity of resin-dentin interfaces after 1 year when bonding procedure were applied with two different total-etch adhesives. In addition, the effect of 0.3 M EDC on dentinal MMPs activity by means of zymographic analysis was tested, demonstrating its efficacy to completely inactivate dentinal gelatinases.

In a further study [69] the authors showed nearly complete inactivation of MMP-2 and -9 when 0.3 M EDC pre-treatment was applied prior to bonding procedures with two
different total-etch adhesives, while the application of the same two adhesives systems alone to acid-etched dentin resulted in activation of dentinal gelatinases. Moreover, *in situ* zymography technique was performed with the aim to obtain precise localization of the MMP activity within the hybrid layer created by the tested total-etch adhesives without previous extraction of enzymes from the tissue (Fig. 2). Gelatinolytic activity was clearly detectable within the hybrid layers and along the tubular wall dentin extending from the dentinal tubules. Furthermore, the location of the activity well correlates with the demineralized uninfiltrated collagen layer simplified total-etch adhesives at the bottom of the hybrid layer, an area also known for nanoleakage expression and the presence of naked collagen fibrils [1], as well as with the effectiveness of a 0.3M EDC primer applied before bonding to inactivate protease activity within the hybrid layer.

Since self-etch adhesive systems do not required dentin surface demineralization before their application, a concern is that this strategy cannot be used for this kind of bonding agents. Future studies should consider to incorporate cross-linking agents such as EDC into adhesive formulations. Thus, cross-linking agents could be applied in association with resin monomers as a controlled drug-delivery system to recharge collagen cross-linker at the interface over time and promote preservation and self-healing of the adhesive interface.
Figure 2. Three-dimensional surface-shaded reconstruction of the acquired images. Optibond FL (OFL) and Adper Scotchbond 1 XT (SB1XT) adhesives 3D reconstructions showing intense fluorescence (evidence of gelatin hydrolysis due to endogenous proteases), throughout the hybrid layer (a and b, respectively), while reduced fluorescence was recorded when OFL and SB1XT were applied to 0.3M EDC pre-treated dentin (c and d, respectively). [69]
References Chapter 5


CHAPTER 6

REDUCTION OF ENZYMATIC DEGRADATION OF DENTIN COLLAGEN
INDUCED BY A COLLAGEN CROSS-LINKING AGENT
UNDER OCCLUSAL CYCLE LOADING
Reduction of enzymatic degradation of dentin collagen induced by a collagen cross-linking agent under occlusal cycle loading.

6.1 Introduction

Research protocols investigating in vitro aging of the hybrid layer should consider all possible parameters involved in the degradation of collagen (i.e. thermal, mechanical, physical and chemical factors) to obtain clinically reliable results. In particular, simulation of mastication cycles (i.e. chewing simulation, CS) is paramount for replicating the dynamic physiological conditions of human mastication in vitro \[1,2\]. Previously it was suggested that host-derived MMPs and cathepsins act synergistically to degrade the dentin collagen network. To date, no information is available on the effect of mechanical stresses on endogenous dentin matrix enzymatic activities, and how these stresses affect the efficacy of EDC cross-linking in preventing degradation of the demineralized dentin collagen matrix.

Thus, the purpose of the part of the research presented in this chapter was to evaluate the ability of EDC pre-treatment to improve the stability of demineralized dentin collagen matrices when those matrices were subjected to mechanical cycling, by quantifying the release of telopeptide fragments over time. The null hypotheses tested were that (1) EDC pre-treatment has no effect on the release of telopeptide fragments from demineralized dentin and (2) chewing simulation (CS) has no effect on collagen degradation over time.

6.2 Material and methods

Specimen preparation

Sixteen extracted noncarious human molars were collected after obtaining patients’ informed consent for using their extracted teeth for research purposes. The teeth were stored at 4 °C in 0.5% chloramine-T solution for no more than 1 month before use. Enamel,
cementum, and pulpal soft tissues were completely removed from each tooth. A dentin slab (1.0±0.1 mm thick) was obtained from the mid-coronal portion of each tooth using a slow-speed diamond saw (Isomet 5000, Buehler Ltd., Lake Bluff, IL, USA) under continuous water-cooling.

The dentin slabs were completely demineralized in 10 wt% phosphoric acid (pH 1) at 25 °C for 24 h. Demineralized dentin slabs were thoroughly rinsed in deionized water under constant stirring at 4°C for 72 h [3]. Collagen slabs were then cut into circular disks (6.0±0.2 mm in diameter) by means of a surgical biopsy punch (Kai Europe GmbH, Solingen, Germany). The collagen disks were then randomly assigned to four treatment groups (N=4) with different storage conditions.

Group 1 (Static): each specimen was immersed in a centrifuge tube containing 0.5 mL of artificial saliva (KCl 12.92 mM, KSCN 1.95 mM, Na₂SO₄·10H₂O 2.37 mM, NH₄Cl 3.33 mM, CaCl₂·2H₂O 1.55 mM, NaHCO₃ 7.51 mM, ZnCl₂ 0.02 mM, HEPES 5 mM, pH=7.4) and stored at 37 °C for 30 days.

Group 2 (EDC + Static): specimens were pre-treated with 0.5 M EDC solution (pH 6.3) for 60 sec, rinsed with distilled water for 10 min, and then stored in the same manner as Group 1.

Group 3 (Chewing Simulation, CS): specimens were challenged with cycling loading to simulate occlusal function. For this purpose, specimens were placed at the bottom of a chewing simulator (CS-4.4, SD Mechatronik GmbH, Germany) sealed chamber (Fig. 1), covered with 0.5 mL artificial saliva and compressed for 0.50±0.01 mm with a 50 N occlusal load. The simulated masticatory load was applied for 30 sec, followed by 30 sec in which specimens were left unloaded, before the application of a new occlusal cycle. The
mastication cycle was repeated for 30 days at a temperature of 37 °C (approximately 43,200 cycles in toto).

Group 4 (EDC + CS): specimens were treated with 0.5 M EDC solution for 60 sec, rinsed with distilled water for 10 min, and then challenged with CS as in Group 3.

For all the specimens, the artificial saliva was changed every 24 h and the aging medium was collected and frozen at -20°C.

Assays for CTX and ICTP

Collagen degradation was assessed using the ICTP ELISA kit (UniQ ICTP EIA; Orion Diagnostica, Espoo, Finland) for quantification of solubilized type I collagen C-terminal cross-linked telopeptide (ICTP) fragments released during the 30-day aging period (Static or CS). This assay has a measurement range from 1-50 ng ICTP/mg dry dentin. Briefly, specimens containing the aging medium were diluted 1:10 in saline and pipetted (50 µL per well) in quadruplicate into a 96-well plate. Procedures were then performed according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a plate reader (GloMax Multi Detection System, Promega Corp., Madison, WI, USA).

Cathepsin K activity was measured by quantifying the amount of solubilized C-terminal peptide (CTX) in the aging medium over the 30-day aging period, using the Serum CrossLaps ELISA kit (Urine BETA CrossLaps ELISA, Immunodiagnostic Systems, Boldon, UK). This assay has a measurement range from 0.1-40 ng CTX/mg dry dentin. Briefly, specimens containing the aging medium or the controls were diluted 1:4 in “standard 0” provided by the kit and pipetted (50 µL per well) in quadruplicate in a 96-well plate. Procedures were then performed according to the manufacturer’s instructions. Absorbance
was measured at 450 nm using the plate reader. Reference absorbance was measured at 650 nm and then subtracted from the measurement determined at 450 nm.

**Statistical analysis**

The ICTP and CTX release rates were analyzed separately. For each telopeptide, data from all groups (in ng telopeptide/mg dry dentin) were assessed to determine if their normality (Kolmogorov-Smirnov test) and equal variance (Levine test) assumptions were violated. Since the normality and equality variance assumptions of the data were valid, they were analyzed by two-way analyses of variance (one for ICTP and the other for CTX), with dentin treatment (EDC or NO EDC) and aging conditions (Static or CS) as factors. Post-hoc multiple comparisons were performed with the Tukey test using SPSS Statistics. Statistical significance was pre-set at $\alpha = 0.05$. 
Figure 1. Schematic representation of the Chewing Simulator sealed chamber used to challenge the collagen specimens over a 30-day period. 1) Chamber, 2) Lid, 3) Piston, 4) Spring, 5) O-Ring, 6) O-Ring, 7) Pivot, 8) Screw, 9) Dentin specimen, 10) Medium.
6.3 Results

The means and standard deviations of ICTP and CTX fragments detected in the storage medium are reported respectively in Figure 2 and Figure 3.

Release of ICTP fragments from specimens aged under static conditions without EDC pre-treatment (Group 1; Figure 2) was related to aging time, with a peak at day 1 (21.9±3.1 ng ICTP/mg dry dentin; p<0.05). The amount of ICTP released decreased after day 1 and was lower than the minimum amount detectable by the assay (1 ng ICTP/mg dry dentin) by day 4. Release of ICTP from specimens pre-treated with 0.5 M EDC and stored under static conditions (Group 2; Figure 2) was lower than the minimum amount detectable by the assay throughout the entire 30-day period. Specimens that were cyclically-stressed without EDC pre-treatment (Group 3; Figure 2) released the highest amount of ICTP at day 1 (33.0±1.5 ng ICTP/mg dry dentin, p<0.05), with the released amount decreasing over time. Similarly to Group 1, ICTP release was lower than the minimum detectable amount after day 4. Specimens that were cyclically-stressed but pre-treated with EDC (Group 4; Figure 2) did not show any ICTP release (i.e. less than the minimum detectable amount) throughout the 30-day aging period.

When static and CS groups were compared, no significant difference was observed in the total amount of ICTP fragments released over the 30-day aging period by specimens from Group I (43.3±7.9 ng ICTP/mg dry dentin) and Group III (47.4±5.8 ng ICTP/mg dry dentin).

The release of CTX fragments (Figure 3) showed similar decreasing release behavior as a function of time. However, CTX fragment release was at least one order of magnitude lower than ICTP release. Specimens from Group 1 showed the highest release of CTX
fragments from day 1 to day 4. The release of CTX fragments was lower than the minimum amount detectable by the assay (0.1ng CTX/mg dry dentin) from day 5 to day 30. Specimens from Group 2 and 4 (EDC pre-treatment before storage) showed CTX telopeptide release below the minimum detectable amount throughout the entire investigation period. Group 3 specimens (CS without EDC pre-treatment) showed significantly lower CTX telopeptide release from day 1 to day 4 (p<0.05) compared with Group 1 specimens (static aging condition without EDC pre-treatment). For this group, no release was detectable after day 5.

When static and CS groups were compared, the total amount of CTX telopeptide fragments released over the 30-day period (Figure 3) was statistically higher in specimens aged under static conditions than specimens aged with CS (Group 1 = 3.4±1.3 ng CTX/mg dry dentin vs. Group 3 =1.3±0.3 ng CTX/mg dry dentin; p<0.05).

Figure 2. Means ± standard deviation of C-terminal telopeptide ICTP release from phosphoric acid-deminerlized dentin disks. Values are expressed as ng ICTP/mg dry dentin. Bars identified by asterisk are significantly different (p<0.05), while n.s. indicates no significant difference (p>0.05); n.d. indicates non-detectable amounts (lower than 1 ng ICTP/mg dry dentin).
Figure 3. Means ± standard deviation of C-terminal telopeptide CTX release from phosphoric acid-demineralized dentin disks. Values are expressed as ng CTX/mg dry dentin. Bars identified by asterisk are significantly different (p<0.05); n.s. indicates no significant difference (p>0.05); n.d. indicates non-detectable amounts (lower than 1 ng ICTP/mg dry dentin).

6.4 Discussion

The results of the present research showed that EDC pre-treatment significantly reduced the release of both ICTP and CTX fragments in demineralized dentin collagen matrices that were aged under static condition or with CS. Thus, the first null hypothesis tested that EDC pre-treatment has no effect on the release of telopeptide fragments from demineralized dentin has to be rejected. The second null hypothesis that chewing simulation has no effect on collagen degradation over time has to be rejected also. This is because throughout the 30-day aging period, the release of both ICTP and CTX fragments were statistically different for the two aging conditions (static vs. CS; p<0.05), with the only exception being ICTP release at day 2 (p>0.05; Figure 2).

The mechanisms in which type I collagen molecules are enzymatically cleaved
along their C-terminal region into distinct telopeptide fragments (i.e. ICTP and CTX) was previously highlighted and correlated to MMPs and cathepsin K, respectively [4]. Recently, this quantitative technique has been adopted for evaluating collagen degradation in acid-etched dentin [4,5,6]. Using this technique in the present study, we observed that both EDC pre-treatment and CS loading affect collagen degradation.

Cross-linking agents such as EDC, riboflavin, genipin proanthocyanidin or glutaraldehyde have been used experimentally to increase the mechanical and structural stability of dentin collagen and to create a durable and more stable hybrid layer [7-10]. The zero-length cross-linking agent EDC contains a functional group with the formula \( \text{RN}=\text{C}=\text{NR} \). It reacts with ionized carboxyl groups in proteins to form an O-acylisourea intermediate that reacts with a non-proteinated amino group and a protein chain to form a stable covalent amide bond between the 2 proteins with the only product being urea [11]. Carbodiimide is considered one of the least cytotoxic cross-linking agent, and its cross-links are very stable [12].

There is ample evidence to support dentin collagen reinforcement and strengthening through EDC cross-linking to improve the bond strength and structural integrity of the resin/dentin interface over time. Indeed, EDC can reduce enzymatic and hydrolytic degradation over time through the formation of inter- and intra- molecular crosslinks [7,13,14]. In addition, EDC may inactivate exposed MMPs bound to matrix collagen, possibly by altering the 3-D structure of their catalytic or allosteric domains [13].

To explain MMP inactivation by cross-linking agents, the mechanism that has been proposed is based on 3D conformational changes in the enzyme structure that may be achieved via irreversible changes induced within the catalytic domain or allosteric inhibition of other modular domains, which co-participate in collagen degradation [9,13,15,16]. This
cross-linking ability inactivates the active sites of dentin proteases through the reduction of the molecular mobility of the active site or by changing negatively-charged ionized carboxyl groups into positively charged amides. Furthermore, EDC can cross-link both the helical and especially telopeptide domains in collagen and may also prevent telopeptidase activity that would normally remove bulky telopeptides from the specific peptide bond of collagenases [11]. Increases in collagen stiffness may prevent MMPs from “unwinding” collagen peptides [17]. Since this “unwinding” is necessary to allow the MMPs catalytic site to cleave the peptide [12,15], it would also effectively inhibit MMPs functional activity.

Compared to mineralized collagen, demineralized collagen is more susceptible to creep under compressive loading [18]. This creep behavior also increases with increased temperature [18,19]. Thus, intensification of collagen intermolecular sliding associated with mechanically cycling in the present study likely resulted in plastic deformation of the demineralized collagen fibrils within the collagen network [20]. The plastic deformation associated with compressive creep deformation of the collagen molecules is manifested at the microscopic scale as degradation of the collagen fibrils, which, in turn is responsible for decreases in bone fatigue resistance upon cyclic stresses [21,22]. In the context of the present study, intermolecular sliding of the collagen molecules caused by compressive creep induced by mechanical cycling probably resulted in plastic deformation of the collagen triple helices and their C- and N-terminals, thereby disrupting the intramolecular and intermolecular cross-links. As discussed previously, such a phenomenon probably facilitated adaptation of the catalytic sites of collagenases to the non-helical region of the collagen molecules, resulting in augmented degradation of the collagen fibrils. Pretreatment of the demineralized collagen fibrils with EDC, on the other hand, might have increased the creep resistance of the collagen molecules and rendered them less susceptible
to plastic deformation. Such a hypothesis has to be tested in the future using a mechanistic approach.

The results of this research provide evidence that endogenous proteases such as MMPs and cathepsin K are able to degrade dentin collagen when exposed to mechanical stresses (such as in physiological conditions) and that a cross-linking agent (such as EDC) stabilizes the collagen network not only by strengthening the fibrils, but also by reducing the enzymatic degradation rate. Further studies are currently ongoing to investigate the role of EDC on dentin bonding and its contribution to hybrid layer stabilization over time.
References Chapter 6


CHAPTER 7

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS
Summary, conclusion and future directions

7.1 Summary and conclusions

Most dental adhesive systems currently used show favorable immediate results that reflect good retention and sealing of bonded interfaces [1]. Despite this immediate efficacy, dentin-bonded interfaces may not adequately withstand aging and may show long-term degradation. Clinical trials evaluating dental adhesives have found dramatically low bonding effectiveness for some materials, but greater bond stability in other materials [2]. Several studies have related bond failure over time to the degradation of resin polymers, initiated with the elution of unreacted monomers and leading to water sorption and polymer swelling [3]. The incorporation of hydrophilic and acidic resin monomers substantially improved the initial bonding of contemporary etch-and-rinse and self-etch adhesives to intrinsically wet dental substrates, providing quite favorable immediate results, regardless of the bonding approach used; but, in the long term, the bonding effectiveness of most simplified etch-and-rinse and self-etch adhesives drop dramatically. Thus, the research reported in Chapter 3 showed that the bond strength and nanoleakage expression of two-step and one-step self-etch tested bonding systems were affected by storage for 6 month and 1 year in artificial saliva. Although it is generally accepted that the permeability of adhesives to water is particularly evident in simplified adhesive formulations, the stability over time was not related to the number of steps of bonding systems, but to their chemical formulations. The performance of a new universal (or multi-mode) adhesive system through storage in artificial saliva was also investigated. The results presented in Chapter 4 found that improved bonding effectiveness of the tested universal adhesive system on dentin was obtained when the adhesive was applied with the self-etch approach. Indeed, the etch-and-rinse approaches tested (both on wet and dry dentin) resulted in immediate bond strength...
comparable to the self-etch mode but expedited long-term aging resulted in reduced bond strength and increased nanoleakage expression, irrespective of dentin wetness. Moreover, the results of the zymographic analysis showed evident changes in dentinal MMP-2 and -9 enzyme activities after the application of the tested adhesives, revealing differences in the extent of enzyme activation. These findings exhibit that the activation of endogenous MMPs is not related to the adhesive system or the strategy employed.

Thus, regardless of the approach and the material used in bonding procedures, a stable and durable bond is not achieved. Therefore, experimental strategies that aim to enhance the adhesive interface, particularly improving the durability of the resin-dentin bond strength by inhibiting intrinsic collagenolytic activity and increasing the resistance of dentin collagen matrix to enzymatic degradation are needed.

Chlorhexidine (CHX) has been used as a non-specific MMP inhibitor [4] to prevent degradation of hybrid layers [5-13]. However, CHX is water-soluble and may leach out of hybrid layers, compromising its long-term anti-MMP effectiveness [14]. An entirely different approach is to treat the acid-etched dentin containing activated matrix-bound MMPs with cross-linking agents that inactivate the catalytic site of proteases [15]. Recent experiments [16-18] have sought to increase the longevity of resin–dentin bonds with various cross-linking agents, such as glutaraldehyde, genipin, proanthrocyanidin, and carbodiimide. These in vitro studies have demonstrated that the use of cross-linking agents improved the short-term mechanical properties of dentinal collagen, and reduced the susceptibility of additionally cross-linked dentinal collagen to enzymatic degradation by collagenases. In particular, carbodiimides have been used as alternative cross-linking agents to gluteraldehyde, since they contain no potentially cytotoxic aldehyde residuals [19,20]. Previous research successfully utilized EDC to increase the durability of resin-dentin bonds by increasing the mechanical properties of the collagen matrix [21]; however, the 1 to 4 hrs
required for that procedure was clinically unacceptable. For this reason, the purpose of the last part of the research, presented in Chapter 6, was to evaluate the ability of 0.5 M EDC short-time (1 min) pre-treatment to improve the stability of demineralized dentin collagen matrices by quantifying the release of telopeptide fragments over time. In accordance with Tezvergil-Mutluay A et al. [22], the results showed that EDC application for 1 min may be a clinically relevant and effective means for stabilizing the collagen network not only by strengthening the fibrils, but also by reducing the enzymatic degradation rate.

7.2 Future directions

Adhesive technology has rapidly evolved since its introduction more than 50 years ago. The primary challenge for dental adhesives is to provide an equally effective bond to two hard tissues of different natures. Bonding to enamel has been proven to be durable. Bonding to dentin remains a major challenge in adhesive dentistry because it is far more intricate and can only be achieved with more complicated and time-consuming application procedures. Relevant concerns persist with regard to interfacial aging due to degradation of the hybrid layer, which is related to water sorption, resin hydrolysis, and collagen network disappearance.

To prevent the degradation of resin-dentin bonds, another approach that consists in the use of ethanol wet-bonding with hydrophilic etch-and-rinse systems has been proposed [23]. Dentin bonding with contemporary hydrophilic etch-and-rinse adhesives produced higher solvent concentrations that result in greater matrix upon solvent evaporation and incomplete infiltration of hydrophobic monomers. Pretreatment of water-saturated collagen matrix with 100% alcohol prevents phase separation of hydrophobic monomers (e.g., Bis-GMA) and provides an opportunity to coax hydrophobic monomers into the matrix. Hydrophobic monomers decrease water sorption/solubility and resin plasticization. Recent
studies using two-photon laser confocal microscopy and micro-Raman spectral analysis [24,25] have found that a relatively homogenous distribution of hydrophobic resin within the hybrid layer can be achieved with ethanol wet-bonding. Comparison of the hybrid layers created with commercially available etch-and-rinse adhesives using water or ethanol wet-bonding found significantly less micropermeability of the fluorescent tracer in hybrid layers created with ethanol wet-bonding [24]. Nevertheless, incomplete removal of ethanol from hydrophilic adhesives may render the polymerized matrix more susceptible to water sorption, when compared to the use of hydrophobic resins [26].

Several interesting attempts to regenerate dental tissue have been recently reported. Biomimetic mineralization is a proof-of-concept strategy that utilizes nanotechnological principles to mimic natural biomineralization [27]. This strategy replaces water from resin-sparse regions of the hybrid layer with apatite crystallites that are sufficiently small to occupy the extra- and intrafibrillar compartments of the collagen matrix, and has been adopted for the remineralization of the resin–dentin bond. Specimen slabs were immersed in a remineralizing medium containing dissolved biomimetic analogs and remineralization proceeded through a lateral-diffusion mechanism. The translation of this proof-of-concept strategy into a clinically applicable technique is currently under development.

Further improvements of existent dental materials remain necessary. New dental material types may, however, be created using nanotechnologies and other novel approaches within the fields of materials science and biomaterials. These developments could include antimicrobial properties, MMPs and cathepsins inhibition, collagen strengthening properties, and dental hard tissue regeneration.

In conclusion, while there are still many unresolved problems regarding the durability of the adhesive interface, even if it is truly remarkable to see how far adhesive bonding has come in the past 50-60 years. Techniques able to create stable resin-dentin
bonds able to resist the collagenolytic hydrolysis will be probably available in the next years, improving the quality of dental therapies.

7.3 Sommario

La tesi qui presentata riguarda la stabilità dell'interfaccia adesiva in odontoiatria. Il successo delle moderne terapie conservative è rappresentato dalla longevità dei restauri adesivi. Tuttavia, vi è una sostanziale evidenza che questo obiettivo ideale non sia raggiunto. La stabilità dell’interfaccia adesiva dipende dalla formazione di uno strato ibrido, compatto e omogeneo, durante l’impregnazione del substrato dentinale da parte dei monomeri adesivi. Poiché lo strato ibrido rappresenta un’entità complessa, in cui interagiscono componenti biologiche diverse (matrice dentinale collagenica e cristalli d’idrossiapatite residui) e non (monomeri resinosi e solventi), i fenomeni d’invecchiamento interessano in maniera sinergica sia la porzione resinosa che quella dentale. L’articolato processo che porta alla degradazione dell’interfaccia adesiva coinvolge infatti la componente resinosa, attraverso l’idrolisi della resina negli spazi interfibrillari, e quella organica, attraverso la disorganizzazione delle fibre collagene dovuta ad un incompleto incapsulamento delle stesse, nonché alla degradazione da parte di proteasi intrinseche con attività collagenolitica. È stato dimostrato come questi enzimi, le metalloproteinasi della matrice (MMP) e le catepsine, abbiano un ruolo cruciale nella degradazione del collagene di tipo I, la principale componente organica dello strato ibrido. Inoltre le caratteristiche idrofile e acide degli attuali sistemi adesivi dentinali hanno reso lo strato ibrido molto suscettibile all'assorbimento di acqua, comportando, attraverso l’idrolisi, la degradazione dello stesso e andando così a contribuire ad una diminuzione della forza di legame nel tempo. Attualmente l’interesse della comunità scientifica mira ad aumentare la durata del legame adesivo con il substrato dentinale.
Dopo un’attenta analisi delle attuali conoscenze riguardanti adesione al substrato dentale (Capitolo 1), la prima parte della tesi si propone di valutare i processi fondamentali che sono responsabili della degradazione dell’interfaccia adesiva (Capitolo 2). Poiché la permeabilità all’acqua degli adesivi è particolarmente evidente nelle formulazioni semplificate, l’attività di ricerca si è concentrata sull’analisi del comportamento dei sistemi adesivi self-etch e dei recenti sistemi adesivi universali. I risultati riportati nel Capitolo 3 ha dimostrato come la forza di legame e l’espressione del nanoleakage dei sistemi adesivi self-etch two-step e one-step testati sia negativamente influenzata dall’invecchiamento in saliva artificiale per 6 mesi e 1 anno. Sebbene sia generalmente accettato che la permeabilità degli adesivi all’acqua è particolarmente evidente in formulazioni di adesivi semplificati, la stabilità nel tempo non è stata correlata al numero di passaggi dei sistemi adesivi, bensì alle loro composizioni chimiche. Sono state in seguito analizzate anche le prestazioni di un nuovo sistema adesivo universale (o multimodale). I risultati presentati nel Capitolo 4 hanno stabilito una migliore efficienza adesiva del sistema universale, testato sul substrato dentinale, quando l’adesivo è stato applicato con l’approccio self-etch. Infatti, la tecnica etch-and-rinse, testata sia su dentina umida che secca, ha comportato una forza di adesione immediata paragonabile alla modalità self-etch, ma a tempi di invecchiamento incrementali si è evidenziata una diminuzione della forza di legame e una maggiore espressione del nanoleakage, a prescindere dalla condizione di umidità dentinale. Inoltre, i risultati dell'analisi zimografica hanno mostrato evidenti variazioni dell’attività enzimatica delle metalloproteinasi MMP-2 e -9 dopo l'applicazione degli adesivi testati. Questi risultati dimostrano come l'attivazione delle MMP endogene non sia correlata al sistema adesivo o alla strategia adottata. Ne evince che, indipendentemente dal metodo e dal materiale utilizzato nelle procedure adesive, non si è in grado di stabilire un legame affidabile e duraturo. Pertanto si avverte l’esigenza di strategie sperimentali che mirino a migliorare la
stabilità dell’interfaccia adesiva, in particolare incrementando la durata della forza di
legame in dentina inibendo l'attività collagenolitica intrinseca e aumentando la resistenza
del collagene alla degradazione enzimatica.

L'ultima parte della tesi è focalizzata quindi sulle strategie per inibire l'attività
proteolitica e collagenolitica delle proteasi endogene e sui metodi per aumentare la
resistenza meccanica del collagene alla degradazione enzimatica (Capitolo 5). Un potente
agente antibatterico, la clorexidina (CHX), è stato usato come inibitore non specifico delle
MMP al fine di impedire la degradazione dello strato ibrido. Tuttavia la CHX, essendo
solubile in acqua, può dissolversi nello strato ibrido, compromettendo la sua efficacia anti-
MMP a lungo termine. Un approccio completamente diverso è quello di trattare la dentina
mordenzata con agenti cross-linker. In particolare, simulando il carico occlusale, è stata
valutata la capacità di un agente cross-linker, l’1-etil-3-(3-dimetilammino-propil)
carbodiimmide (EDC), per prevenire la degradazione del collagene. Precedenti ricerche
hanno utilizzato con successo l'EDC con lo scopo di aumentare la durata dell’interfaccia
adesiva, aumentando le proprietà meccaniche della matrice di collagene; tuttavia, il tempo
necessario (da 1 a 4 ore) richiesto per tali procedure è clinicamente inaccettabile. Per questo
motivo, lo scopo dell’ultima parte della ricerca, presentata nel Capitolo 6, è stato quello di
valutare la capacità di 0,5 M EDC nel breve periodo di pretrattamento (1 min), andando a
quantificare il rilascio di frammenti di telopeptidi di collagene nel corso del tempo. I
risultati hanno dimostrato che l'applicazione di EDC per 1 min può essere un approccio
clinicamente rilevante ed efficace nello stabilizzare il collagene, non solo rafforzando le
fibrille, ma anche riducendo la velocità di degradazione enzimatica. Di conseguenza,
l’utilizzo di questo cross-linker può garantire una valida strategia per migliorare la forza di
legame e l'integrità strutturale dell'interfaccia adesiva nel tempo contro l’attività enzimatica
intrinseca del collagene e la degradazione idrolitica.
References Chapter 7


Acknowledgements

I am very indebted to my supervisor Prof. Milena Cadenaro for her never-ending support and encouragement throughout these years. She gave me the possibility to come in touch with the research world, and she patiently and continuously reviewed my work sharing her knowledge with generosity. I will never forget her help.

I wish to express my sincere gratitude to my tutor Prof. Lorenzo Breschi for his vast knowledge in the field of adhesive dentistry and the enthusiastic dedication to the scientific work. His invaluable contributions and optimism have been essential for my research activity.

My deepest thank to Prof. Franklin Tay for the breath of perspective, knowledge and experience that I gained from him during my research stage abroad and obviously for giving me the possibility to work in his research group at Georgia Regents University in Augusta.

I would also like to express my sincere appreciation to Prof. David Pashley, an inexhaustible source of scientific advices and continuous encouragement, and Prof. Arzu Tezvergil-Mutluay for spending time on reviewing this thesis and for their precious suggestions in several scientific meetings.

I am grateful to Prof. Roberto Di Lenarda and Prof. Elettra De Stefano Dorigo for providing me with excellent research facilities in which to carry out this research at the Dental Division of the Department of Medical Sciences in Trieste.

Let me also thank the colleagues and friends who shared this challenge with me and helped me in so many ways that they cannot even imagine, especially Dr. Giulio Marchesi, Eng. Gianluca Turco, Dr. Chiara Ottavia Navarra, Dr. Annalisa Mazzoni, Chem. Luca Fontanive, Dr. Marina Diolosà and Dr. Valeria Angeloni for their support and for all those scientific and non-scientific discussions during my graduate years.
Last but not least I would like to thank my family at large, who encouraged and supported me all the times.
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