

# How Stable is Dentin As a Substrate for Bonding?

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## Abstract

**Purpose of Review** This paper reviews the complexity of the composition of dentin, and the repercussions of this composition on the stability of dentin over time, particularly in relation to adhesive dental procedures.

**Recent Findings** Dentin is a complex and dynamic structure that comprises the major part of the tooth. Most adhesive procedures in dentistry involve bonding to dentin. The hybrid layer (HL) created on the very variable and dynamic organic dentin phase may fail over time, leading to the failure of dental restorations. Literature showed that the collagen fibers in the HL are prone to hydrolysis and mechanical strain, as well as endogenous proteolytic activity (collagenolytic activity of matrix metalloproteinases and cysteine cathepsins). Hydrolysis of the resin phase of the HL also occurs over time.

**Summary** Advancements in the area of dental adhesion have been huge. Silencing of collagenolytic enzymes (protease inhibitors and cross-linking agents) is one of the main strategies to decrease the degradation of the HL. In the following years, new techniques will also probably be available, and efficacy of some of the available techniques will perhaps be further clarified.

**Keywords** Dentin · Collagen · Dentin bonding systems · Hybrid layer · Metalloproteinases · Cross-linking agent · Enzyme inhibition

## Introduction

The major part of the tooth is comprised of dentin, a mineralized connective tissue that shows similarities with bone in terms of formation and composition. Dentin is a complex and dynamic structure. During the process of dentinogenesis, dentin extracellular organic matrix (ECM) is pivotal for the mineral phase formation since precipitation of the inorganic phase occurs on the formed collagen network [1]. More precisely, the intermolecular spaces between collagen fibrils are filled with tightly bound and evenly distributed water molecules [2]. With the progression of the mineralization process, minerals gradually replace the water molecules [3].

Adhesive procedures in dentistry, apart from a low percentage of cases, involve bonding to dentin. Adhesion on dentin is a type of *in situ* tissue engineering, where the collagen scaffold is enveloped in adhesive resin, creating the so-called hybrid layer (HL) [4]. The goal of adhesive procedures is to keep this bond stable for several years. However, despite the advances in dental materials, the HL created on the variable and dynamic organic dentin phase may fail over time [5, 6]. The weakening of resin-dentin bonds can lead to the failure of dental restorations [7] and, consequently, to high costs of dental restoration replacements [8].

This paper reviews the complexity of the composition of dentin and the repercussions of this composition on the

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stability of dentin over time, particularly in relation to adhesive dental procedures.

### Dentin As a Substrate: Composition and Morphology

Human dentin is composed of approximately 70 w% inorganic material, 18 w% organic material, and 12 w% water. However, the volume percentage goes in favor of organic material and water, with up to 70% being attributed to these two components (30–50% organic, 20% water) [9]. Various factors, such as the location and condition of the dentin (presence of caries, demineralization), can significantly change the composition of dentin, which has profound implications in the mechanical properties of dentin, as well as in the success rate of the adhesive procedures [10].

As 45% of the sound dentinal tissue consists of the organic matrix [11], with the main component being type I collagen fibrils and proteoglycans and the minor component being other non-collagenous proteins (dentin sialoproteins, phosphoproteins, bone morphogenic proteins, and insulin-like growth factors 1 and 2), it is of great importance to understand its three-dimensional arrangement. This understanding could in fact clarify bonding mechanisms and the interaction between collagen fibrils and adhesive resin monomers. Transmission electron microscopy, field-emission scanning electron microscopy, and atomic force microscopy studies showed that the organic matrix is a complex network of fibrillar and globular structures. Mineral is further precipitated onto these structures during dentinogenesis, using it as a scaffold [12, 13].

The dentin ECM consists of an intricate network of fibrils (approximately 70–90 nm in diameter), with type I collagen fibrils representing the pillar of the structure, connected by branching of non-collagenous proteins (20–40 nm diameter) [13–15]. The main portion of the non-collagenous proteins in dentin matrix consists of proteoglycans and phosphoproteins [16–18], with the proteoglycans being considered as fundamental in the stabilization of the collagen fibrillar alignment [16, 18]. Proteoglycans are polyanions rich in carbohydrates with a high molecular weight (11,000–220,000 kD) [18]. These proteins have the ability to fill space and bind and organize water molecules, and could play a role in the organization of the three-dimensional appearance of the dentin organic matrix [19–22]. Furthermore, proteoglycans may determine the collagen water affinity and regulate water substitution during HL formation. Owing to the advances in reagent purification and the production of highly specific monoclonal antibodies, nowadays collagen [23] and proteoglycans [24] can be analyzed using precise and sensitive selective immune-labeling protocols [25]. During this double immunolabeling procedure, secondary antibodies are conjugated with gold particles of different sizes [26], allowing

clarification of the three-dimensional relationships between the different molecules.

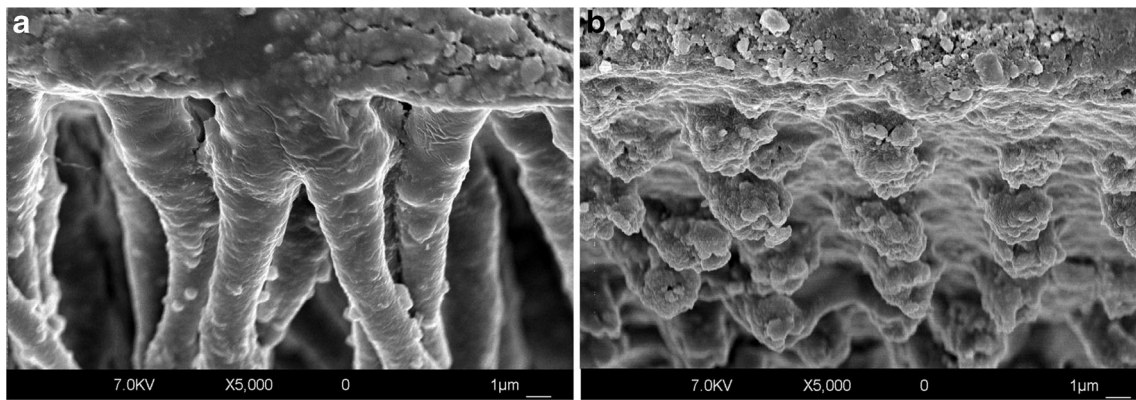
It has been shown that the application of phosphoric acid for 15 s exposes collagen fibrils without damaging their structural integrity [23, 24, 26]. On the other hand, the application of phosphoric acid to the dentin surface for more than 15 s caused weakening of the labeling index in collagen fibrils [23] and proteoglycans [19, 21]. This is probably an indication that structural changes occur within collagen or proteoglycans when exposed to prolonged acid etching. Since the introduction of structurally changed collagen or proteoglycans into the HL could jeopardize its stability, over-etching should be avoided [23].

### Dentin Bonding Agents and Adhesion Strategies

The goal of dentin bonding is to produce a homogenous and compact HL by impregnating the dentin substrate with the blends of resin monomers, which would in turn lead to durable tooth restorations. Resin monomers themselves cannot infiltrate mineralized dentin, hence the dentin surface must be previously treated with acidic agents [27]. Two different strategies can currently be employed in the bonding procedures: the etch-and-rinse technique (E&R) and the self-etch technique (SE) [Fig. 1].

In the E&R strategy, the adhesive systems remove the smear layer from the dentinal substrate and demineralize it through the process of etching. The main prerequisite for the achievement of stable bonds in this mode is full impregnation of the etched substrate by the adhesive blends. However, within this mode, the monomer diffusion within the acid-etched dentin has shown a decreasing gradient [29], with incompletely infiltrated zones noted along the bottom of the HL. Hence, this area contains denuded collagen fibrils [29–31]. This has been further confirmed by immunohistochemical labeling after staining with anti-type I collagen antibodies (Fig 2) [32]. The investigated E&R systems displayed weaker labeling on the superficial part of the HL, while intense labeling of collagen fibrils was found in the deepest parts of the HL (Fig. 2). These results indicate that the adhesive resin enveloped collagen fibrils in the superficial part of the HL, whereas denuded fibrils were present in the deeper areas due to the lack of penetration of the adhesives [32].

In the SE mode, the smear layer is maintained as the substrate for bonding. In this approach, the adhesive comonomers simultaneously demineralize and infiltrate the dentinal substrate, and the stability of the bonding depends on the effectiveness of the coupling between the substrate and the comonomers. This approach was also investigated using immunolabeling, showing, in contrast to the E&R adhesive, that the two-step SE adhesives express uniform weak labeling throughout the depth of the HL [32], allowing to hypothesize



**Fig. 1** FEISEM micrographs of an (a) E&R and (b) SE adhesive system. Bonded interfaces were created with Scotchbond 1 (3M ESPE) and Protect Bond (Kuraray) in deep dentin tissue. HLs were then exposed with a slow speed diamond saw and dentin was dissolved by sequential rinses in hydrochloric acid and sodium hypochlorite to reveal resin penetration. Resin tags are clearly detectable in the E&R adhesive

systems (a) since they infiltrated dentin tubules funneled by the etching agent. SE adhesives often infiltrate no further than the smear layer and smear plugs, revealing a more homogenous morphology that is devoid of long resin tags. Reprinted from Breschi et al. [28•], with permission. *FEISEM* field emission in-lens scanning electron microscopy, *E&R* etch and rinse technique, *SE* self-etch technique, *HL* hybrid layer

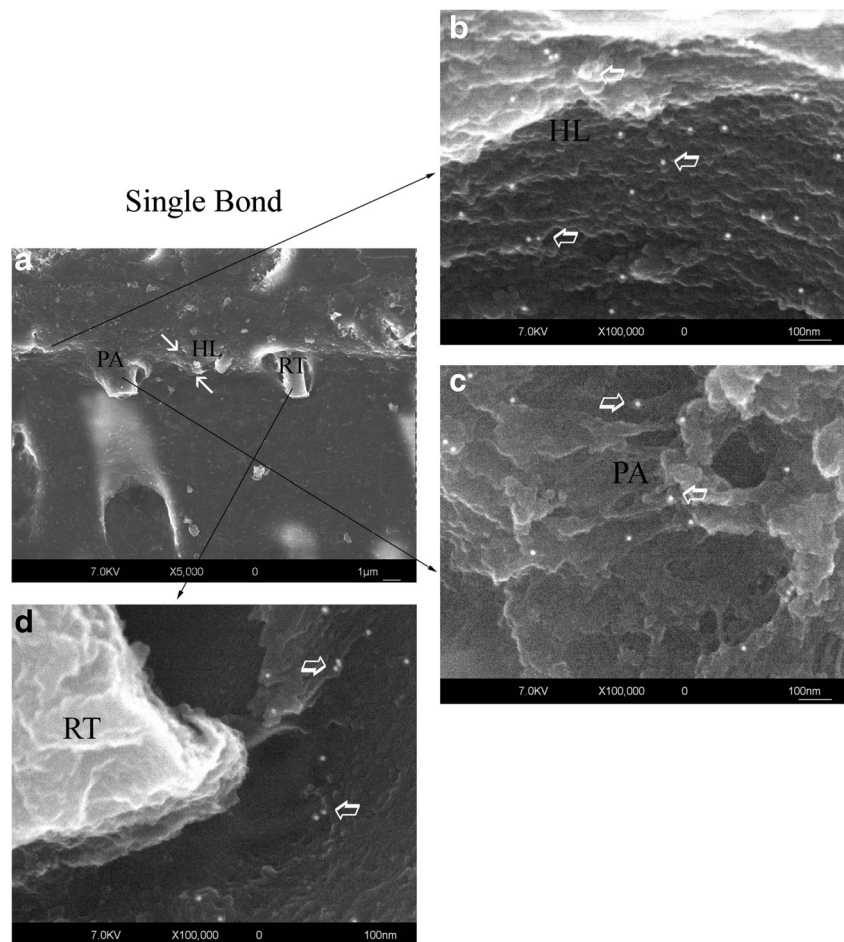
that collagen is more protected if SE adhesives are used to hybridize dentin.

It can be assumed that the collagen fibrils that are not enveloped in resin can be more prone to enzyme degradation and hydrolysis, given that free and loosely bound water from

the internal and external water compartments of collagen fibrils cannot be completely replaced either by the E&R or SE bonding approaches [33].

Factors that can influence the longevity of the HL can be physical, such as the temperature changes within the oral

**Fig. 2** FEISEM images of the dentin-SB interface after immunohistochemical labeling of collagen with a monoclonal antibody. (a) FEISEM secondary electron image after applying phosphoric acid and SB. The typical HL (between white arrows), RTs, and PA infiltration formed by total etching systems are clearly visible. (b) FEISEM mixed image of secondary and back-scattered electrons. Intense labeling is detectable on the HL. The deeper areas of the HL appear to be specially labeled (arrows) compared with the superficial HL. (c) FEISEM mixed image of secondary and back-scattered electrons. The PA infiltrated by the resin agent reveals some labeling (arrows) at the base of the resin tag, then very weak labeling present entering the lumen of the tubule. (d) FEISEM mixed image of secondary and back-scattered electrons. The RT reveals almost no gold labeling on the surface. Reprinted from Breschi et al. [32], with permission. *FEISEM* field emission in-lens scanning electron microscopy, *SB* single bond, *HL* hybrid layer, *RT* resin tag, *PA* peritubular area



cavity which produce expansion and contraction stresses, as well as the occlusal chewing forces [28•, 34–36]. Chemical factors, such as acidic agents in food and beverages, dentinal fluid, saliva, and bacterial products, can affect the stability of the interface, and can lead to elution of resin monomers [37–39] and degradation of resin components [35, 40–43], as well as degradation of the unprotected collagen fibrils [28•, 30, 35, 40, 44, 45]. Since the purpose of this review is the stability of dentin, the focus will be mainly on the degradation of the collagen fibrils, while the degradation patterns of the adhesive resin will be shortly addressed.

### Degradation of the Adhesive Resin

In order to prevent the collapse of collagen fibrils after acid etching, the water-wet bonding technique, which entails keeping dentin fully hydrated during bonding, was established in the 1990s [46]. As adhesives contain hydrophobic resin monomers, two-hydroxyethyl methacrylate (HEMA) was incorporated into many adhesive systems. Moreover, many manufacturers incorporated hydrophilic and acidic resin monomers into adhesives, aiming to improve the wetting properties of adhesives to dentin [47]. This led to a substantial improvement of immediate bonding. However, the longevity of resin-dentin bonds seems to be compromised when these systems are used [48, 49].

Degradation of the HL involves the processes of hydrolysis and leaching of the resin [50–52]. The hydrophilic domains remain poorly polymerized and, consequently, water penetrates these weak parts of the HL, which further facilitates leaching. The water movement and leaching continue along the HL, forming relatively large water channels [28•]. A larger amount of collagen matrix is exposed, leaving it susceptible to endogenous proteolytic activity [53•, 54].

### Degradation of the Collagen Fibrils

As previously reported, it is difficult to achieve complete coverage of the collagen fibrils by passive monomer infiltration, which can leave them vulnerable to hydrolytic degradation. Loss of resin from the interfibrillar spaces and disorganization of fibrils have in fact been revealed as degradation patterns within the HL [30].

Pashley et al. [53•] presented the first evidence of intrinsic collagenolytic activity in human non-cariou mineralized dentin. This pioneer study showed that degradation of the collagen matrices would occur in artificial saliva after 250 days, even in aseptic conditions. The authors hypothesized that matrix metalloproteinases (MMPs), previously found to be expressed in the dentin-pulp complex [55–57], could be responsible for this degradation. Their conclusion was based on the fact that the

groups stored in artificial saliva with the addition of proteolytic enzyme inhibitors, or in pure mineral oil, were statistically significantly more preserved after storage time.

MMPs are endogenous  $Zn^{2+}$ - and  $Ca^{2+}$ -dependent enzymes. Dayan et al. [58] were the first to report dentinal collagenolytic activity, while Tjäderhane et al. [55] showed that this activity was attributable to the MMPs. In recent years, the presence of the gelatinases MMP-2 and -9, collagenase MMP-8, stromelysin MMP-3, and MMP-20 has been reported [56, 59–66].

During the secretion of the dentin matrix, odontoblasts produce the MMPs [67], which supposedly play a role in dentin formation. The proforms of the MMPs remain inactive in the mineralized collagen matrix [68] but can be activated when re-exposed during the dentin caries process, and subsequently participate in disintegration of the dentin matrix. Hence, it can be hypothesized that these enzymes are directly involved in degradation of the HL [69–71].

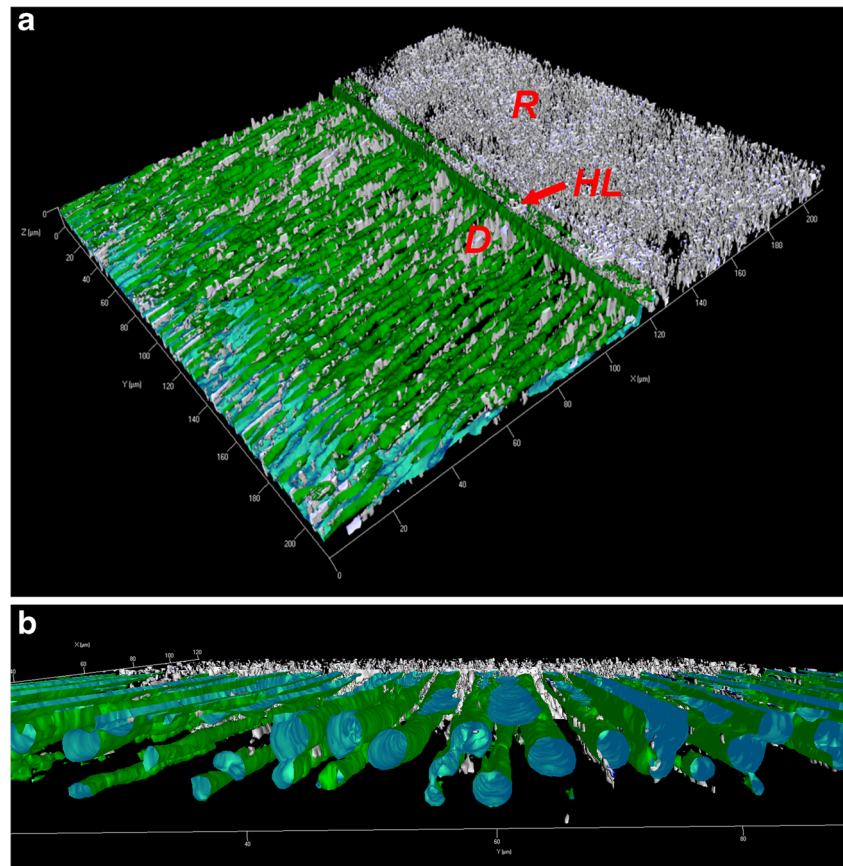
This hypothesis was supported by the studies of Mazzoni et al. [72] and Nishitani et al. [73], which showed collagenolytic and gelatinolytic activity in dentin treated with the E&R and SE modes. Further studies confirmed these initial results using specific MMP-2 and -9 assays [70, 74], and additional clarification was provided by an in situ zymographic assay technique [75•, 76]. This method confirmed the hypothesis that the collagen fibrils at the bottom of the HL are not completely infiltrated by adhesive resin since enzymatic activity was noted in this area (Fig. 3).

Cysteine cathepsins are another class of proteases more recently found in dentin [77, 78]. Cathepsins have been shown to participate in caries progression, as well as in degradation of the ECM alongside MMPs, leading to failure of adhesive restorations over time [4, 28•, 78, 79].

### How to Improve Bond Stability and Maintain the Integrity of the Dentin Substrate?

As the preservation of the HL over time can be affected by hydrolytic degradation of the suboptimally polymerized hydrophilic resins and/or degradation of the collagen matrices by MMPs and cysteine cathepsins [28•], several approaches can be used to prevent HL degradation. One of the most efficient methods could be the use of MMP inhibitors. Those investigated in the literature include chlorhexidine (CHX) [4, 80–82], quaternary ammonium methacrylates (QAMs), and benzalkonium chloride (BAC) [83, 84]. Furthermore, collagen cross-linkers were shown to inactivate MMPs and consequently improve the durability of dentin bonding [85]. Apart from the abovementioned, several other clinical approaches have been found to be useful in the preservation of the HL, including dentin remineralization and ethanol-wet bonding [4, 86].

**Fig. 3** (a, b) Three-dimensional model of the acquired image. (a) The intense fluorescence, meaning gelatin hydrolysis, throughout the entire extension of hybrid layer. (b) Higher magnification image model shows gelatinolytic activity inside dentinal tubules, shown as cylindrical tubes, in deep dentin. The high tubule density reveals this to be very deep dentin. Reprinted from Mazzone et al. [75], with permission. *R* resin composite, *HL* hybrid layer, *D* dentin



### Protease Inhibitors

The most commonly used and most investigated protease inhibitor to date is chlorhexidine, a powerful antimicrobial agent, shown to effectively inhibit MMP-2, -8, and -9 [87], as well as cysteine cathepsins [88]. Numerous *in vivo* and *in vitro* studies have demonstrated chlorhexidine-related preservation of the structural integrity of the collagen matrix within the HL, as well as a decreased reduction of bond strength over time [69, 80, 89–98]. Another MMP inhibitor is galardin, a collagen-like synthetic structure that binds to the active sites of MMP-1, -2, -8, and -9. A study by Breschi et al. showed lower MMP expression in the zymographic assay, as well as a lower level of nanoleakage after 1 year of aging [81]. Galardin has the ability to inhibit MMP activity in concentrations 10–100 times lower compared with chlorhexidine [81].

Quaternary ammonium compounds are cationic at physiological pH, are efficient antimicrobial agents, and may also inhibit dentinal MMP activity. Due to their antimicrobial properties, polymerizable QAMs, particularly 12-methacryloyloxydodecylpyridinium bromide (MDPB) have previously been incorporated into SE primers [99, 100]. *In vitro* and clinical experiments have also indicated that QAMs may inhibit collagenolytic enzymes in the HL [101, 102].

Another cationic surface-acting agent with a quaternary ammonium group is *BAC*, a mixture of alkylbenzyltrimethylammonium chlorides of various alkyl chains. It is used as an antimicrobial agent and surfactant [84]. This agent has recently come to the attention of investigators in the area of HL preservation and has shown good immediate results [84, 103] and promising long-term results [104, 105].

Furthermore, broad-spectrum antibiotics, such as the tetracyclines doxycycline and minocycline, have cationic properties and were shown to effectively inhibit MMPs [106–108]. The effect of these inhibitors on the durability of resin-dentin bonds should be further investigated. Moreover, bisphosphonates have the ability to inhibit proteases due to their chelating properties [109]. Tezvergil-Mutluay et al. showed strong inhibition of MMP-9 using polyvinylphosphonic acid (PVPA) [110]. The influence of this potent protease inhibitor group should be further investigated.

### Collagen Cross-Linking Agents

Collagen cross-linking agents could inactivate the active sites of dentinal proteases as they form stable cross-links of the collagen fibrils [111, 112]. This presents an advantage over protease inhibitors, which may leach

out of the HL within 18–24 months [40, 113–119]. Until now, several cross-linking agents have been investigated: *glutaraldehyde*, *proanthocyanidin* and *genipin* [120], *riboflavin* [121], as well as *tannic acid* [122] and *carbodiimide* [85]. All of the investigated agents seem to enhance the mechanical and structural stability of dentin collagen.

Among these, *1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)*, in particular, has been shown to reduce dentin collagen degradation and prevent degradation of the HL over time [76, 85, 123]. It has low cytotoxicity and the ability to change negatively charged ionized carboxyl groups into positively charged amides and reduce the molecular mobility of the active sites of the proteases, which could inactivate dentin proteases. Moreover, EDC can induce cross-linking of collagen [123], which in turn could prevent ‘unwinding’ of the collagen peptides by the MMPs [124] and therefore inhibit the functional activity of MMPs [4, 27, 28, 125]. This cross-linking agent was recently investigated [126] and it was shown that the application of 0.3M EDC pretreatment with the two investigated E&R adhesives can prevent degradation of the HL. Furthermore, the same authors investigated the effect of EDC pretreatment, applied with two E&R adhesives, on the inactivation of MMPs using a zymographic assay and an in situ zymographic assay [76]. The application of the adhesives without pretreatment activated dentin MMP, while EDC pretreatment lead to almost complete inactivation.

### Other Approaches

As mentioned earlier, residual water in the HL can result in matrix porosity and incomplete infiltration of hydrophobic monomers. In order to avoid this, *ethanol-wet bonding* with hydrophobic E&R systems has been proposed [127]. When a water-saturated collagen matrix is pretreated with 100% ethanol, matrix water is removed and the infiltration of hydrophobic monomers into the dentin matrix is facilitated. Using this approach, a relatively homogenous distribution of hydrophobic resin has been shown within the HL [128, 129]. Yet another concept to consider is *biomimetic remineralization*, indeed an interesting approach that uses nanotechnology in order to mimic natural biomineralization [130]. In an *in vitro* study, specimen slabs were immersed in a medium containing dissolved biomimetic analogs and remineralization occurred by means of lateral diffusion mechanism. This concept has not yet become clinically applicable.

### Conclusions

Currently available dental adhesive systems seem to be quite efficient in terms of immediate bond retention and sealing of the bonded interfaces. However, long-term maintenance of

these results was shown to be a very difficult task. The main mechanisms leading to degradation of the HL are hydrolysis and degradation by host-derived enzymes. Water sorption, promoted by the hydrophilic formulations of the new adhesives, causes slow hydrolysis of the resin. On the other hand, the endogenous MMPs and cysteine cathepsins, activated during the bonding procedures, degrade the resin uninfiltated collagen fibrils in the bottom of the HL. Hence, it seems that the key to long-term preservation of resin-dentin bonds lies in the complete replacement of water within the collagen water compartments, as well as inactivation/inhibition of the dentinal endogenous enzymes. Advancements in the area of dental adhesion have been massive. The proposed strategies to address the issue of the durability of the HL showed promising results in *in vitro* studies, while some have also been established in *in vivo* studies. In the following years, new techniques will also probably be available, and efficacy of some of the available techniques will perhaps be further clarified.

### Compliance with Ethical Standards

**Conflicts of Interest** Tatjana Maravic, Annalisa Mazzoni, Allegra Comba, Nicola Scotti, Vittorio Checchi and Lorenzo Breschi declare that they have no conflicts of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by the any of the authors.

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