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Review

Dental adhesion review: Aging and stability of the bonded interface

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ABSTRACT

Objective. Most of current dental adhesive systems show favorable immediate results in terms of retention and sealing of bonded interface, thereby counteracting polymerization shrinkage that affects resin-based restorative materials. Despite immediate efficacy, there are major concerns when dentin bonded interfaces are tested after aging even for short time period, i.e. 6 months.

Methods. This study critically discusses the latest peer-reviewed reports related to formation, aging and stability of resin bonding, focusing on the micro and nano-phenomena related to adhesive interface degradation.

Results. Most simplified one-step adhesives were shown to be the least durable, while three-step etch-and-rinse and two-step self-etch adhesives continue to show the highest performances, as reported in the overwhelming majority of studies. In other words, a simplification of clinical application procedures is done to the detriment of bonding efficacy. Among the different aging phenomena occurring at the dentin bonded interfaces, some are considered pivotal in degrading the hybrid layer, particularly if simplified adhesives are used. Insufficient resin impregnation of dentin, high permeability of the bonded interface, sub-optimal polymerization, phase separation and activation of endogenous collagenolytic enzymes are some of the recently reported factors that reduce the longevity of the bonded interface.

Significance. In order to overcome these problems, recent studies indicated that (1) resin impregnation techniques should be improved, particularly for two-step etch-and-rinse adhesives; (2) the use of conventional multi-step adhesives is recommended, since they involve the use of a hydrophobic coating of nonsolvated resin; (3) extended curing time should be considered to reduce permeability and allow a better polymerization of the adhesive film; (4) proteases inhibitors as additional primer should be used to increase the stability of the collagens fibrils within the hybrid layer inhibiting the intrinsic collagenolytic activity of human dentin.

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1. Introduction

Contemporary restorative techniques are based on the adhesive properties of tooth colored resin-based materials. Following the pioneer approach of Buonocore in 1955 [1], researchers and manufactures improved both sealing and bonding capabilities of dental adhesives. Despite significant improvements of adhesive systems, the bonded interface remains the weakest area of tooth-colored restorations. If the dentin/adhesive interface is exposed to the oral cavity, marginal discolorations, poor marginal adaptation and subsequent loss of retention of the restoration [2,3] are frequent clinical findings. Even though several studies revealed excellent immediate and short-term bonding effectiveness of dental adhesives [4], the durability and stability of resin-bonded interfaces on dentin created by some bonding systems remain questionable [5–9]. In fact, recent studies highlighted that immediate dentin bond strength values do not always correlate with long term bond stability [7] since degradation throughout the dentin bonded interface occurs rapidly (i.e. 6 months) [8,9].

Current adhesive systems interact with the enamel/dentin substrate using two different strategies, i.e. either removing the smear layer (etch-and-rinse technique) or maintaining it as the substrate for the bonding (self-etch technique) [10,11].

The difference between the two approaches is represented by the use of a preliminary and separate etching step for etch-and-rinse systems (usually characterized by a gel of 35–37% phosphoric acid) that is later rinsed away [10], conversely the self-etch/primer agent is only air-dried, thus remaining within the modified smear layer, i.e. the self-etch approach could be called an “etch-and-dry” approach. Despite differences in etching, the other fundamental steps for adhesion are priming and bonding that can be either separate or combined, depending on the adhesive system. The current classification of adhesives relies on the number of the steps constituting the system [11]. Etch-and-rinse adhesive systems can be either three- or two-step depending on whether primer and bonding are separated or combined in a single bottle (Fig. 1a). Similarly self-etch adhesives can be either two- or one-step systems depending on whether the etching/primer agent is separated from the adhesive or combined with it to allow a single application procedure (Fig. 1b) [11].

2. Aging of the hybrid layer

Since bonding is created by the impregnation of the dentin substrate by blends of resin monomers, the stability of the bonded interface relies on the creation of a compact and homogenous hybrid layer. In the etch-and-rinse strategy,

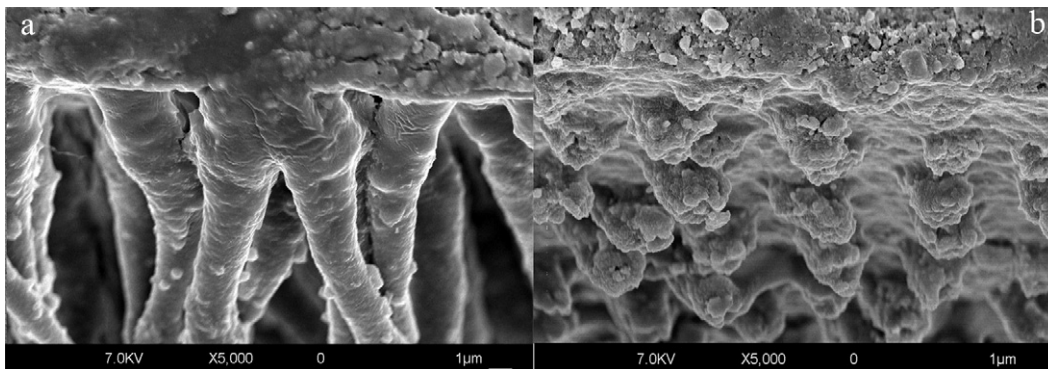


Fig. 1 – FEI-SEM micrographs of an etch-and-rinse (a) and a self-etch (b) adhesive system. Bonded interfaces were created with Scotchbond 1 (3M ESPE) and Protect Bond (Kuraray) in deep dentin tissue. Hybrid layers were then exposed with a slow speed diamond saw and dentin was dissolved by sequential rinses in hydrochloric acid and sodium hypochlorite to reveal resin penetration. Resin tags are clearly detectable in the etch-and-rinse adhesive systems (a) since they infiltrated dentin tubules funneled by the etching agent. Self-etch adhesives often infiltrate no further than the smear layer and smear plugs, revealing a more homogenous morphology that is devoid of long resin tags.

after the preliminary etching to demineralize the substrate, bonding monomers impregnate the porous etched substrate [12,13]. Thus stable bonds can be achieved if the etched substrate is fully infiltrated by the adhesive to avoid different degrees of incomplete impregnation [14–16]. Conversely, since the self-etch approach uses acidic adhesive co-monomers that simultaneously demineralize and infiltrate dentin, adhesive stability is related to the effective coupling of the co-monomers with the infiltrated substrate. Recent findings also revealed that some two-step self-etch systems (with mild acidity, i.e. showing a pH of approximately 2) may establish chemical bonds between specific carboxyl or phosphate groups of functional monomers and residual hydroxyapatite crystals still present on the dentin collagen scaffold due to the mild aggressiveness of the acidic phase [17]. This additional interaction acting synergistically with superior infiltration of adhesive monomers into the decalcified substrate is claimed to enhance bond stability over time [7].

Clinical longevity of the hybrid layer seems to involve both physical and chemical factors. Physical factors such as the occlusal chewing forces, and the repetitive expansion and contraction stresses due to temperature changes within the oral cavity [18] are supposed to affect the interface stability [7,19–21]. Acidic chemical agents in dental fluid, saliva, food and beverages and bacterial products further challenge the tooth/biomaterials interface resulting in various patterns of degradation of unprotected collagen fibrils [20,22–25], elution of resin monomers (probably due to sub-optimal polymerization) [26–28] and degradation of resin components [7,20,22,29–31].

As the hybrid layer is created by a mixture of dentin organic matrix, residual hydroxyapatite crystallites, resin monomers and solvents, aging may affect each of the individual components or may be due to synergistic combinations of degradation phenomena occurring within the hybrid layer.

3. Degradation of the resin

Hashimoto et al. [24] described two degradation patterns within the hybrid layer after storage of a three-step etch-and-rinse adhesive system (Scotchbond Multi Purpose, 3M/ESPE, St. Paul, MN, USA), 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.

Hydrolysis is a chemical process that breaks covalent bonds between the polymers by addition of water to ester bonds, resulting in loss of the resin mass: this is considered as one of the main reason for resin degradation within the hybrid layer [9,20], contributing to the reduction in bond strengths created by dentin adhesives over time [20,32–37]. Since resin degradations is related to water sorption within the hybrid layer, the degree of water sorption of recently introduced simplified adhesives was studied [34,38,39]. The latter two studies reported low water sorption by hydrophobic resin and high water sorption by hydrophilic acidic resin systems used for self-etch adhesives. Water sorption caused a significant decrease in the modulus of elasticity of the resins that is

thought to contribute to reductions in bond strength, independent of resin hydrolysis [39].

In fact since hydrolytic degradation occurs only in presence of water, adhesive hydrophilicity, water sorption and subsequent hydrolytic degradation are generally correlated [34,37–43]. In other words, irrespective of the etch-and-rinse or the self-etch strategy, by combining hydrophilic and ionic resin monomers into the bonding such as in simplified adhesives (i.e. two-step etch-and-rinse and one-step self-etch systems) the bonded interface lacks a nonsolvated hydrophobic resin coating [10]. This leads to the creation of hybrid layers that behave as semi-permeable membranes permitting water movements throughout the bonded interface even after the adhesive is polymerized (Fig. 2a–c) [44]. This water passage was revealed by studying the permeability of bonded interfaces and by using a tracer detectable by electron microscopy such as ammoniacal silver nitrate. This tracer stains pathways water-filled diffusion throughout the bonded interface that are often manifested as creating the so-called “water trees”, i.e. characteristic water channels at the surface of the hybrid layer that extends into the adhesive layer, supporting the hypothesis of complete permeation of simplified adhesive bonded interfaces to water [45]. When the tracer was previously used to stain voids, porosities (especially for etch-and-rinse systems) and water-filled regions and/or hydrophilic polymer domains (especially for self-etch systems) within hybrid layers, the silver uptake was named nanoleakage [14,37,43,46]. In etch-and-rinse adhesive systems, nanoleakage is created by the discrepancy between dentin demineralization and adhesive impregnation along the resin–dentin interface (Fig. 3a and b) [41,46–49]. Since simplified (two-step) etch-and-rinse adhesives contain higher percentages of hydrophilic monomers compared to three-step adhesives [10], they were found to exhibit high degrees of permeability after polymerization, thus facilitating silver uptake and increasing nanoleakage expression [44]. These results revealed two different modes of silver tracer deposition patterns [43,46], i.e. a reticular versus a spotted mode of nanoleakage expression. The reticular mode is the morphological characterization of water-treeing [43,45,49]; the spotted mode, visible within the adhesive layers, is thought to represent microdomains in the resin matrices containing mainly hydrophilic and/or acidic functional groups compared with the adjacent, more hydrophobic, domains [43,46,50]. After aging of resin-bonded specimens in artificial saliva, Tay et al. [37] described the transition initial nanoleakage from isolated silver grains, to water trees in the adhesive resin matrices as a series of events starting with water sorption. Water movements begin as a diffusion-type mechanism, then become more rapid as transport pathways form relatively large water-filled channels [37,45]. Similar water movements within the adhesive layer can be driven by osmotic pressure gradients due to high concentrations of dissolved inorganic ions and hydrophilic resin monomers resulting in the formation of water blisters over the adhesive layer [51,52].

A recent study that correlated the extent of polymerization and permeability of dental adhesives revealed that, irrespective of the bonding system and the number of steps required for its application, all systems exhibited variable degrees of incomplete polymerization that were correlated with their permeability to fluid movement [27]. Interestingly,

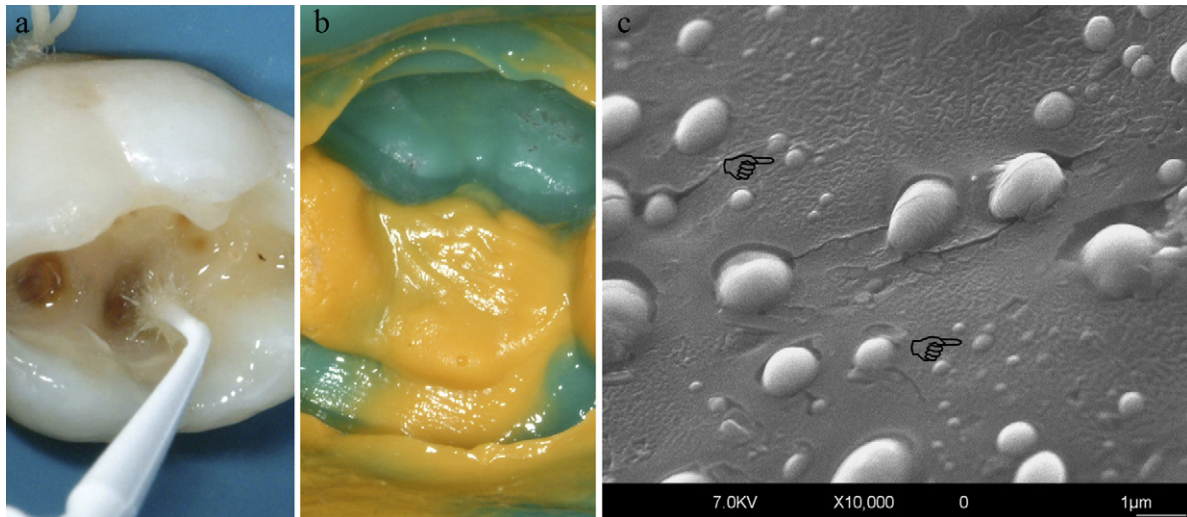


Fig. 2 – Illustrative steps of the *in vivo* analysis of the permeability of adhesives in accordance with Chersoni et al., 2004 [51,52]. A cavity was prepared and bonded (a) and an impression of the cavity floor was obtained (b). After pouring a cast with epoxy resin, specimens analyzed under FEI-SEM revealed water droplets emanating from the adhesive surface (c). These droplets are the morphological evidence of water that seeped from the adhesive layer during the setting time of the hydrophobic impression material forming major droplets as well as minor droplets (pointing finger) over the adhesive. These droplets may compromise coupling between the adhesive and the resin-based restorative material. They are thought to form at top of “water tree” as reported by Tay and Pashley [45].

incomplete polymerizations and adhesive permeability were more extensive in simplified adhesives, either two-step etch-and-rinse or one-step self-etch, probably due to the presence of higher concentrations of hydrophilic monomers. As partially cured adhesives were more permeable to fluid movement [53], they may expedite water sorption and compromise the long term integrity of the adhesive-composite bond. Conversely, dentin bonding systems that utilize the separated nonsolvated hydrophobic bonding agents showed higher extents of polymerization and were correlated with less permeability to water [27].

4. Degradation of exposed collagen fibrils

The combined degradation of resin and collagen may increase the water content of the bonded interface, leading to a further detrimental effect on the longevity of the bond; water has in fact been claimed as one of the major cause for collagen degradation. Within the hybrid layer, two degradation patterns can be observed: loss of resin from interfibrillar spaces and disorganization of the collagen fibrils [24]. Such degradation may result from the hydrolysis of resin and/or collagen,

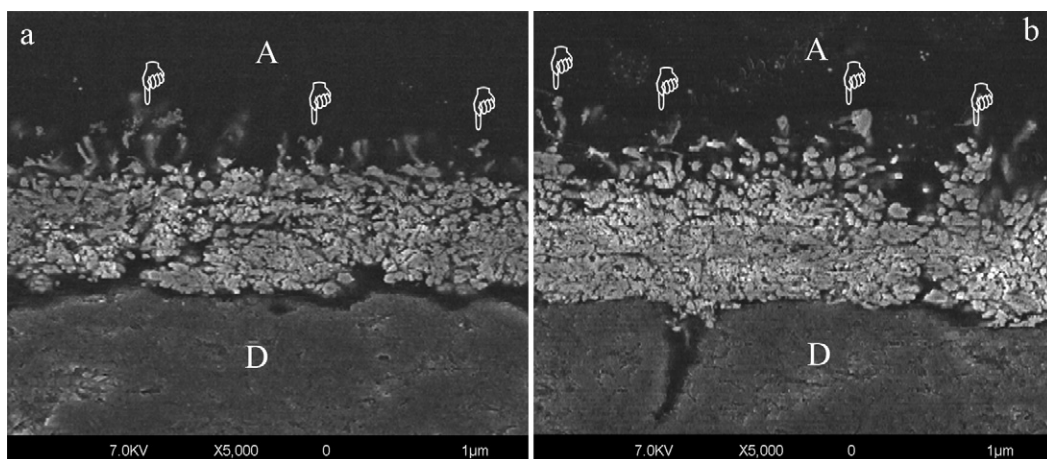


Fig. 3 – FEI-SEM micrographs illustrating the nanoleakage expression of two etch-and-rinse adhesives (a, Scotchbond 1, 3M ESPE; b, Prime&Bond NT, Dentsply). Both adhesives (A) were applied in accordance with manufactures instructions on deep dentin (D), interfaces were aged and exposed to silver nitrate in accordance with Suppa et al. [85]. Images reveal extensive nanoleakage expression characterized by an homogenous silver nitrate uptake within the hybrid layer and “water-tree like” formations protruding into the (A) adhesive layer (pointing fingers) as described by Tay and Pashley [45].

thereby weakening the physical properties of resin–dentin bond [24]. Several studies have provided morphological evidences of resin elution and/or hydrolytic degradation of collagen matrices after long-term storage [22–25,54]. In particular, the deterioration of collagen fibrils within the HL, detectable both *in vitro* and *in vivo* tests, suggests that there are many exposed collagen fibrils within the HL.

The final goal of bonding procedures is the complete infiltration, and encapsulation of the collagen fibrils by the bonding resin is recommended in order to protect them against degradation [24,55]. It is well known that the degree of envelopment of collagen fibrils is different depending on the type of bonding agents, i.e. a total-etch or a self-etch approach. For total-etch adhesives, a decreasing gradient of resin monomer diffusion within the acid-etched dentin [56] results in incompletely infiltrated zones along the bottom of the HL that contain denuded collagen fibrils [24,36,56,57] in the demineralized zone of dentin created by the discrepancy between the depth of acid etching and resin infiltration. This is thought to be due to insolubility of BisGMA in water-saturated dentin. By substituting ethanol for water, BisGMA/TEGDMA mixtures have been shown to infiltrate dentin [58] and produce high bond strengths [59]. Thus, “ethanol-wet bonding” permits the use of hydrophobic resins that absorb little water, for dentin bonding [60]. Whether this leads to more durable bonds was not yet been determined. Using self-etch adhesives, the acidic monomers dissolve the inorganic phase of dentin and simultaneously primes and infiltrates the dentin matrix, resulting in fewer exposed collagen fibrils [57].

5. Immunohistochemical analysis of the hybrid layer

Since the dentin organic matrix represents approximately 45% in volume of the sound dentin tissue (water is approximately 20% and the rest is minerals such as apatite) [61], the understanding of its three-dimensional arrangement is pivotal to clarify bonding mechanisms and how collagen fibrils interact with adhesive monomers. The main components of the dentin matrix are type I collagen fibrils (CF) and proteoglycans which are produced by the odontoblasts during tooth formation. Other minor non-collagenous protein, such as dentin sialoproteins, phosphophoryns, bone morphogenic proteins and insulin-like growth factors 1 and 2 complete the dentin organic matrix [62]. Several studies investigated the dentin organic matrix using transmission electron microscopy, field-emission scanning electron microscopy, and atomic force microscopy. These techniques revealed a complex network of fibrillar and globular structures constituting the scaffold of the dentin tissue onto which mineral is further precipitated during dentinogenesis [63–65].

Type I collagen fibrils represents the backbone of the dentin organic fibrillar network [66]. It has been demonstrated that the native collagen fibrils assembly constitutes an intricate network of fibrils (measuring approximately 70–90 nm in diameter) connected by minor branching fibrils of non-collagenous proteins (on the order of 20–40 nm in diameter) [62–65,67] giving the typical banding of 64 nm when mature

demineralized type I collagen fibrils are observed under TEM or SEM [65].

Dentin proteoglycans are claimed to have a fundamental role in stabilizing the collagen fibrillar arrangement [68,69]. Proteoglycans and phosphoproteins represent the main constituents of the non-collagenous proteins in the dentin matrix [70,69,71]. Proteoglycans are carbohydrate-rich polyanions with a high molecular weight (from 11,000 up to 220,000) constituted by a polypeptide core to which is attached one or more glycosaminoglycans, i.e. repeating disaccharide units with sulphate ester groups linked at position 4 or 6 [70]. The presence of chondroitin 4–6 sulphate is very well described on predentin, dentin and cement [70,69] and it is claimed to regulate the biophysical properties of dentin proteoglycans, which in turn may regulate the final collagen fibrils three-dimensional arrangement. In other words proteoglycans may be responsible of the three-dimensional appearance of the dentin organic matrix due to their ability to fill space, bind and organize water molecules, and repel negatively charged molecules [72–76]. Such proteoglycans may determine the water affinity of collagen in the hybrid layer by regulating water substitution which occurs during hybrid layer formation. The application of etch-and-rinse adhesives to proteoglycans-depleted dentin increased bond strengths compared to control surfaces, probably by reducing the amount of water retained within the hybrid layer (Mazzoni and Breschi, unpublished results).

Advances in the purification of the reagents and the production of highly specific monoclonal antibodies permitted the establishment of reproducible and selective immunolabeling protocols with high levels of sensitivity [77,78]. Immunohistochemical techniques provide the opportunity of identifying the nature of unknown structures observable under high-resolution microscopes, revealing the spatial relationships between the molecules of interest. These techniques applied to human dentin allowed us to visualize collagen [79] or proteoglycans [80] or both structures by means of a double immunolabeling procedure using secondary antibodies conjugated with gold nano-particles with different sizes (Fig. 4) [81]. Since apatite crystallites mask the epitopes responsible for antibody binding, preliminary demineralization of the surface is needed [82].

As etch-and-rinse adhesive systems are applied directly on the demineralized dentin collagen and proteoglycans, the maintenance of the structural integrity of these structures during and after etching should greatly improve the final stability of the hybrid layer. As the immunohistochemical positive labeling was correlated to biochemical preservation of collagen and proteoglycans in the dentin matrix, we hypothesized that 15 s application of phosphoric acid exposes the collagen fibrils without causing major structural damage, i.e. as labeling index was reasonably high when collagen fibrils maintained their integrity [79,81]. Conversely, extended application time of phosphoric acid on dentin resulted in more exposed demineralized collagen fibrils, but in lower labeling index. This was probably correlated with acid-induced structural modifications occurring when 35% phosphoric acid remains in contact with denuded collagen for more than 15 s [79,83]. Similarly, proteoglycan immunolabeling patterns were clearly related to the type of acid and to the application time

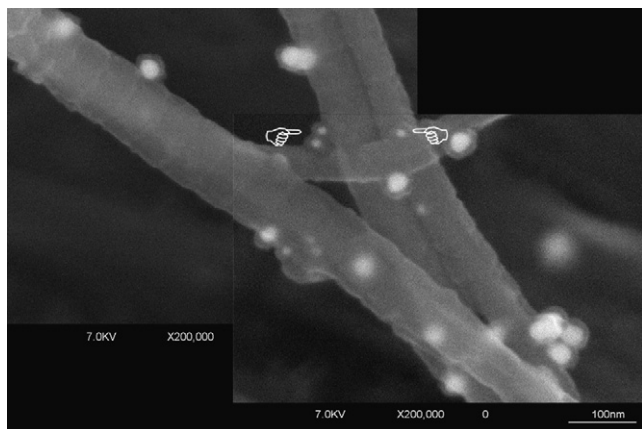


Fig. 4 – High resolution FEI-SEM micrographs obtained by mixing secondary and back-scattered electrons to image both collagen fibril morphology and the distribution of gold nano-particles used to reveal labeling. Two images at a magnification of 200,000 \times were combined to reconstruct the three-dimensional arrangement of demineralized human dentin matrix after applying a double immunohistochemical procedure. Type I collagen was labeled with secondary antibodies conjugated with gold particles of 30 nm in diameter (left side), while chondroitin 4/6 sulphate was revealed by secondary antibodies conjugated with gold particles of 15 nm (right side) in diameter. The procedure allowed imaging of major fibrils clearly characterized by the typical banding (measuring 70–90 nm in diameter), and minor branching fibrils (measuring 30–40 nm in diameter) labeled with the 15 nm gold particles, thus confirming the presence of proteoglycans (pointing fingers) on the surface of collagen fibrils.

as massive coagulation of the chondroitin sulphate occurred if phosphoric acid was applied for more than 15 s to the dentin surface [80,81]. The incorporation of either structurally altered collagen or proteoglycans into hybrid layers may represent an early stage of degradation of the hybrid layer, even before it is formed, since these molecules are destabilized prior to impregnation with the adhesive. For this reason over-etching should be avoided, not only to avoid the possibility of an impaired resin impregnation which increases nanoleakage expression [43,48], but also for maintaining dentin matrix structural integrity.

Similar to demineralized dentin matrix, collagen fibrils within the HL that are not fully encapsulated by resin monomers can be immunohistochemically identified after staining with anti-type I collagen antibodies (Fig. 5a and b) [84]. Differences were found between etch-and-rinse and self-etch adhesives in terms of immunolabeling. The hybrid layer created by the total etching systems revealed minor labeling on the top of the HL (superficial HL), indicating that adhesive resin enveloped that the collagen fibrils and prevented antibody binding. In contrast an intense labeling of collagen fibrils was seen in the deepest part of HL indicating that some collagen fibrils were not enveloped by resin [84]. This supported the hypothesis that with total etching systems, different degrees

of resin-collagen fibril interactions may occur depending on the degree of penetration of the adhesive into the demineralized dentin matrix. The collagen fibrils in the superficial HL seem to be fully impregnated (reduced labeling), while the deepest area of the HL shows a great number of exposed collagen fibrils that remain partially available for binding the antibodies. In contrast, the HL created by a two-step self-etch adhesive system did not reveal gradient in the labeling pattern for type-I collagen, but showed only a weak, uniform gold labeling [84] and minor labeling along the resin tags. Interestingly, immunolabeling of the hybrid layers correlated well with nanoleakage expression of the same adhesive systems, i.e. Scotchbond 1, a simplified etch-and-rinse adhesive, shows intense nanoleakage expression at the deepest level of hybrid layer [85], while Clearfil Protect Bond, an “unsimplified” self-etch primer adhesive, showed much less silver nitrate staining that was mainly localized only along the resin tags [85]. While silver nanoleakage studies reveal areas of incomplete infiltration of resin monomers (for etch-and-rinse adhesive systems) or areas of phase separation (for self-etch adhesive systems), immunolabeling, which may be defined as “immunoleakage”, represents sites of collagen fibrils not-encapsulated by monomers and thus available for binding of large molecular weight (i.e. 30–40 kDa) antibodies. These unprotected collagen fibrils presumably become susceptible to enzyme degradation since, as previously discussed, most of the simplified adhesives (either etch-and-rinse or self-etch systems) are permeable to water and small molecules.

6. Intrinsic collagenolytic activity of mineralized dentin

Despite the adhesive approach itself, the result of resin–dentin is often incomplete hybridization of the dentin surface, leaving collagen fibrils unprotected and vulnerable to hydrolytic degradation that also are susceptible to other degradation-promoting factors such as residual solvent of the adhesive [86] or insufficiently removed surface water. Recent studies revealed the contribution of host-derived proteinases to the breakdown of the collagen matrices in the pathogenesis of dentin caries [87–90] and periodontal disease [91], with potential and relevant implications in dentin bonding [92]. Since Ferrari and Tay [93] demonstrated that nanoleakage can occur in the absence of gaps along *in vivo* resin–dentin interfaces, this suggests that the degradation of incompletely infiltrated zones by host-derived proteinases within the dentin matrix may proceed in the absence of bacterial enzymes [94,95]. Pashley et al. [92] reported that if acid-etched dentin matrices can be slowly degraded over time by dentin-derived proteolytic enzymes, in the absence of bacteria. In this study, partially demineralized collagen matrices, obtained from human dentin, were stored in artificial saliva, while control specimens were store in artificial saliva with the addition of proteolytic enzyme inhibitors or in pure mineral oil. Demineralized collagen matrices were almost completely destroyed in the 250-day experimental specimens but not when incubated with enzyme inhibitors or mineral oil, with a significant difference in the thickness and the status of the collagen network compared to the acid-etched dentin aged in the

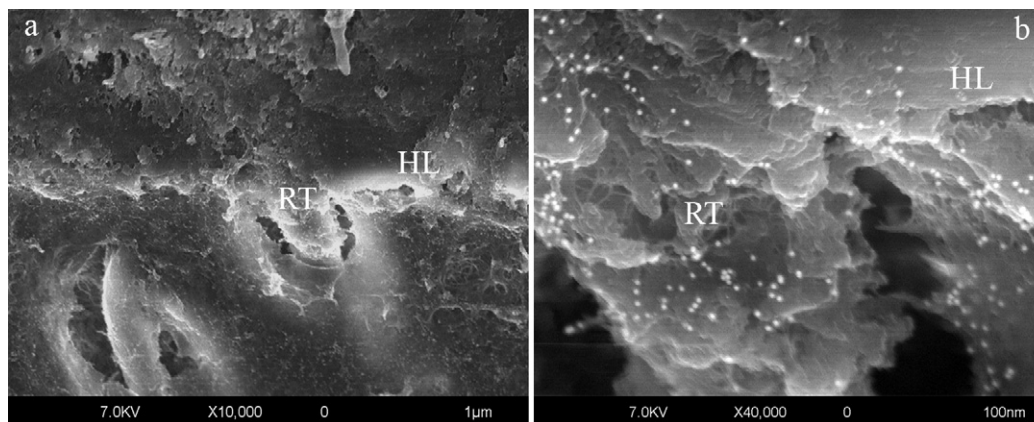


Fig. 5 – Hybrid layer created by Clearfil SE-Bond (Kuraray) and processed for immunohistochemical detection of the collagen fibrils within the hybrid layer and along the resin in accordance with Breschi et al. [84]. In contrast to the use of silver nitrate for nanoleakage analysis (areas of poor impregnation or phase separation within the adhesive), the gold labeling achieved with immunohistochemical labeling represents collagen fibril sites in the hybrid layer and resin tags that were not encapsulated by the resin and thus available for binding the antibody. These areas may be hydrolyzed by collagenolytic enzymes. (a) Low magnification image (10,000 \times) revealing the hybrid layer (HL) and a resin tag (RT) created by resin flow into an open dentinal tubule. (b) Higher magnification (40,000 \times) view of the same resin tag and peritubular area obtained by mixing back-scattered and secondary electrons as to reveal both morphology and distribution gold particles-conjugated type I collagen antibodies as white electron reflective spots. Collagen labeling was clearly located along the resin tags (RT) and in proximity of the peritubular impregnated zone, similar to the nanoleakage expression described for the same adhesive system.

control storage media [85]. Interestingly, under these conditions collagen degradation occurred in the absence of bacterial contamination as the experiment was conducted under aseptic conditions, i.e. bacterial collagenolytic activity was not responsible for the dentin collagen degradation, as is frequently advocated under *in vivo* conditions. By assaying the collagenolytic activity of mineralized dentin powder by using fluorescein-labeled type I collagen from bovine skin, Pashley et al. [92] demonstrated an intrinsic collagenolytic activity in human mineralized dentin which can be inhibited by specific protease inhibitors. Similarly, incomplete inhibition after phosphoric acid etching was found, while completely inhibition was obtained by low concentration of chlorhexidine. This pioneer study [92] on the role of host-derived enzymes for the first time supported the hypothesis that collagen degradation of human dentin occurs over time, not only due to the activity of bacteria-produced collagenases, but via host-derived enzymes that are released and activated over time.

The evidence of collagenolytic/gelatinolytic activities in partially demineralized dentin collagen matrices are indirect proofs of the existence of matrix metalloproteinases (MMPs) in human dentin [89] more recently shown to contain both MMP-2 and MMP-9 in demineralized mature dentin by gelatin zymography and Western blotting [96].

MMPs are a class of zinc- and calcium-dependent endopeptidases [97] that are trapped within the mineralized dentin matrix during tooth development [87,90]. The release and the subsequent activation of these endogenous enzymes during dentin bonding procedures [92,94,95] are thought to be responsible for the *in vitro* manifestation of thinning and disappearance of collagen fibrils from incompletely infiltrated hybrid layers in aged, bonded dentin [98–101]. The collagen

degradation that occurs at the bottom of hybrid layers has also been confirmed in *in vivo* studies [102,103]. Moreover, the application of chlorhexidine, a well-know antibacterial agent with MMP inhibiting properties [104] when applied to acid-etched human primary dentin resulted in the preservation of collagen integrity within the hybrid layers *in vivo* after the application of the etch-and-rinse bonding procedure [105,106], confirming the indirect involvement of MMPs in the collagen breakdown process. Unfortunately, a definitive cause and effect relationship between the different procedures employed in the etch-and-rinse technique and the degradation of the dentin hybrid layers has not been yet established. Presumably, phosphoric acid demineralization could have activated the MMPs, trapped within the mineralized dentin [92], resulting in the collagenolytic and gelatinolytic activities identified within the hybridized dentin. However, using fluorescein-labeled collagen enzymatic assay, it was found that treatment of mineralized dentin powder with 37% phosphoric acid gel for 15 s actually reduced the inherent collagenolytic activity of mineralized dentin, probably due to its low acidity (pH 0.7), that partially denatures the MMPs [92], leaving confusion of how dentin hybrid layers could degraded over time. In a recent study Mazzonei et al. [105], revealed the potential roles of the adhesives on dentin proteolytic activities using a modeling approach in which the relative proteolytic activities derived from dentin has been quantified before and after the sequential applications of the phosphoric acid-etchant and an etch-and-rinse adhesive. Within the limits of the study, it was concluded that simplified etch-and-rinse adhesives can activate new endogenous enzymes present in dentin that counteract the MMPs were previously inactivated by phosphoric acid-etching, providing a plausible

explanation for the *in vitro* and *in vivo* observations of the degradation of dentin hybrid layers [105].

7. How to increase bond stability

As bond strength and durability [7] seems to rely on the quality of the hybrid layer (i.e. on the proper impregnation of the dentin substrate) rather than on the thickness or morphology hybrid layer/resin tags different clinical approaches have been proposed to improve monomers infiltration, to reduce the rate of water sorption and to reduce collagen degradation.

Use of an additional layer of hydrophobic resin agent [106], multiple layer applications [107-110], enhanced solvent evaporation [111], prolonged curing time [27,28], use of MMP inhibitors [102,112,113] and use of electric current to improve monomer impregnation [114,115] are some of the modifications of standard clinical protocols which showed bonding improvements.

The use of an additional layer of hydrophobic resin agent onto the polymerized one-step adhesive agent converts a one-step in a two-step self-etch adhesive [98]. King et al. [106] reported that the use of a hydrophobic coating on three one-step adhesives (I-Bond, Xeno III and Adper Prompt L-Pop) increased bond strength and eliminated their incompatibility with auto-cured composites. For I-Bond and Xeno III an "apparent incompatibility" with auto-cured compos-

ites due to their inherent permeability was eliminated by the use of the nonsolvated more hydrophobic coating over the simplified adhesives. For Adper Prompt L-Pop, its "true incompatibility" with auto-cured composites, caused by adverse acid-base interaction and masking the inherent permeability of this adhesive, was solved by its conversion to two-step self-etch adhesive. That is, simplified adhesive was converted to a primer and further diluted by the hydrophobic monomers contained in an additional surface coating, the relative concentration of hydrophobic monomers in the adhesive layer increased thus enhancing the bonding. Moreover, the hydrophobic coating on a one-step adhesive system leads to a thicker and more uniform adhesive layer with lower concentrations of retained water and solvent, thus improving the quality of the adhesive layer [111].

The use of a multiple layer application under a continuous brushing technique has also been claimed to increase bond strength [107-109]. Hashimoto et al. [110] demonstrated that bond strengths increased with each adhesive coating up to four coats, while at the same time nanoleakage decreased with each coat, being almost absent after four or more coats. Similarly, Ito et al. [109] concluded that by simply applying more coats of adhesive, the strength and quality of dentin adhesion can be improved. Another simple approach to improve bonding efficacy and stability is correlated with enhanced solvent evaporation to avoid phase separation within the adhesive agent. The possibility of air-blowing the adhesive with full

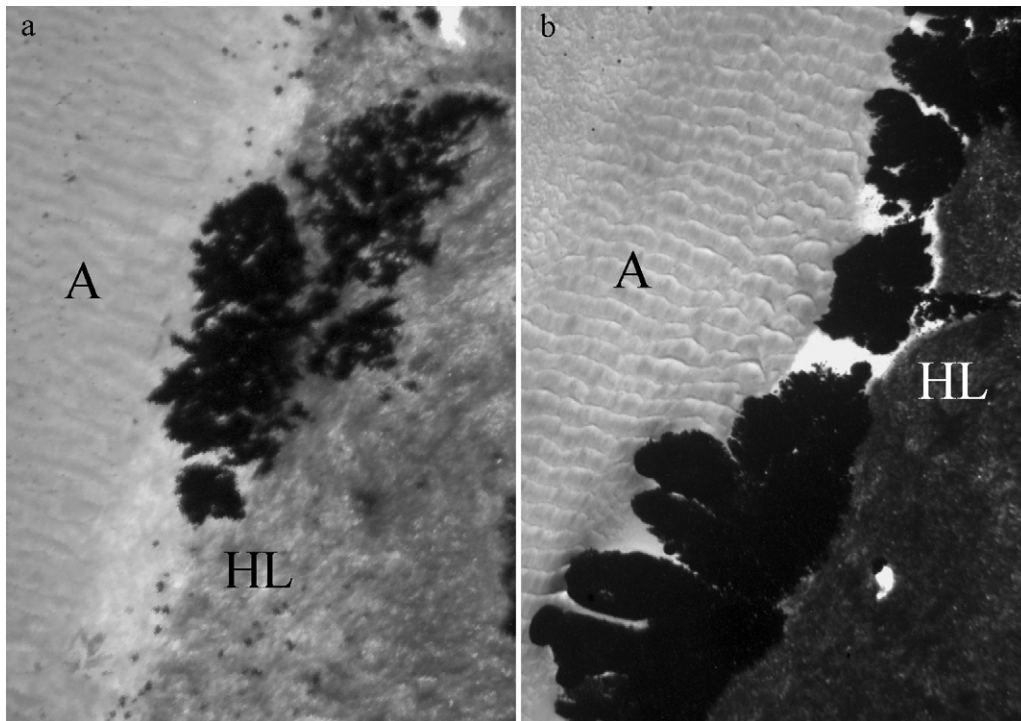


Fig. 6 – TEM micrographs of a one-step self-etch adhesive (Adper Prompt L-Pop, 3M ESPE) showing minor silver uptake (i.e. nanoleakage expression) within the hybrid layer (HL) created under the effect of an electric current generated by ElectroBond (a), or major uptake (i.e. using conventional bonding technique in accordance with the manufactures' instructions, without the use of electric current (b)). Immediate nanoleakage expression was clearly reduced if the electric current-assisted adhesive application technique was used in accordance with Breschi et al. [115]. The black hybrid layer (HL) in (b) indicates massive uniform penetration of the HL as well as accumulation of additional silver between the HL and the overlying adhesive (A) layer.

power might be a clinical technique for removing substantial interfacial water, thereby improving bonding effectiveness [111].

Since resin permeability and monomer elution are both related to suboptimally polymerized bonding systems, a recent study Cadenaro et al. [27] proposed to extend the curing beyond 20s the time period recommended by manufacturers. The study showed that extending the curing times of simplified adhesives beyond those recommend by the manufacturers resulted in improved polymerization and reduced permeability, and appeared to be a possible means for improving the performance of these adhesives.

On the other hand, the discovers that endogenous collagenolytic and gelatinolytic activities derived from acid-etched dentin result in degradation of hybrid layers, suggested the use of MMPs inhibitors in primers to slow or prevent destruction of bonded dentin matrices [112]. Hebling et al. [102] showed that hybrid layers from chlorhexidine-pre-treated teeth exhibited normal structural integrity of the collagen network compared to the progressive disintegration of the fibrillar collagen network detected in the control teeth. Similarly an *in vitro* study revealed that microtensile bond strength created with the use of chlorhexidine as additional primer in a etch-and-rinse adhesive was higher than control specimens after 6 months water storage [112].

Additionally the use of an adhesive application protocol based on the use of electric current to enhance monomer infiltration for etch-and-rinse [114] and self-etch [115] systems in dentin has recently been reported. The electric current is generated by a device (ElectroBond; Seti, Rome, Italy) consisting of a handpiece that applies an adhesive-filled disposable sponge to dentin. Release of the adhesive is triggered by the electric potential difference between the tooth surface and the adhesive. Similar to an apex locator, the second electrode (i.e. lip clip) is placed intraorally and connected via an electric circuit that creates an electrical current through a digitally controlled current modulator. The results of the studies [114,115] showed that the use of electrically assisted-adhesive application was able to improve bonding efficacy, as shown by the increased microtensile bond strength when compared with the control application technique (i.e. with a standard micro-sponge, but without the use of electric current). The bond strength data were further supplemented by FE-SEM and TEM findings that revealed reduced nanoleakage in bonded interfaces that were created by adhesive application under an assisted electrical current (Fig. 6a and b) [114,115].

8. Conclusions

Most currently marketed adhesive systems produce have immediate bond strength that allows clinician to bond to tooth structure without the use of retentive cavity preparations. Nevertheless, major concerns have been recently expressed regarding interfacial aging due to degradation of the hybrid layer, related to water sorption, hydrolysis of the resin and disruption of the collagen network. Interestingly, the new simplified adhesives exhibited not only the lowest bond strengths, but also the least predictable clinical performances when

compared with the multi-step etch-and-rinse and self-etch systems.

Various clinical procedures were proposed to optimize bonding and reduce aging:

1. Use of an hydrophobic coating: since the incorporation of hydrophilic monomer blends in simplified adhesives (two-step etch-and-rinse and one-step self-etch adhesives) dramatically reduced bond longevity, the need of an hydrophobic coating with a not-solvented bonding layer seems to be pivotal to reduce water sorption and stabilize the hybrid layer over time, i.e. etch-and-rinse three steps and self-etch two-step adhesives should be preferred to simplified ones.
2. Extended polymerization time: extending the curing times of simplified adhesives beyond those recommend by the manufacturers resulted in improved polymerization and reduced permeability, and appears to be a possible means for improving the performance of these adhesives.
3. Use of MMPs inhibitors: the use of MMPs inhibitors as additional primer has been claimed to reduce interfacial aging over time by inhibiting the activation of endogenous dentin enzymes which are responsible for the degradation of collagen fibrils in the absence of bacterial contamination.
4. Improved impregnation: various methods has been recently proposed to enhance dentin impregnation, i.e. prolonged application time, vigorous brushing technique and electric impulse assisted adhesive application. The latter technique recently revealed increased bond strength and reduced nanoleakage expression if adhesives are applied under the effects of an electric signal.

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