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Effect of bacterial collagenase on resin–dentin bonds degradation

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Abstract The objective of this study is to evaluate the effect of a bacterial collagenase on the degradation of resin–dentin bonds. Human dentin surfaces were bonded with: an etch-&-rinse self-priming adhesive (SB), a two-step self-etching primer/adhesive (SEB), and a 1-step self-etching adhesive (OUB). Composite build-ups were constructed. The bonded teeth were stored (24 h, 3 months, 1 year) in distilled water or in a buffered bacterial collagenase solution. Half of the specimens were stored as intact bonded teeth (Indirect Exposure/IE). The other half were sectioned into beams prior to storage (Direct Exposure/DE). After storage the intact teeth were sectioned into beams and all specimens were tested for microtensile bond strengths (MTBS). ANOVA and multiple comparisons tests were performed. Fractographic analysis was performed by scanning electron microscopy. The inclusion of bacterial collagenase in the storing solution did not lower the MTBS values over those seen in specimens stored in water. SB and SEB bonds strength were equal, and were superior to OUB. After 3 months of DE, SB and OUB bonded specimens showed decreases in MTBS; similar reductions required 1 year for SEB/DE. MTBS did not decrease in IE specimens except for OUB. Resin and collagen dissolution were evident in DE groups after storing.

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Introduction

Dentin bonding is usually achieved via two alternative strategies. Etch-&-rinse adhesives systems begin by removing the smear layer with phosphoric acid. This is followed by the application of a primer and an adhesive in two different steps or in a single step. With such a technique, incomplete expansion of collapsed collagen matrix after air-drying may impair resin infiltration and compromise bonding [1, 2]. In the self-etching approach, relatively high concentrations of acidic monomers and a HEMA primer are combined into one solution to form an acidic primer [3] prior to the application of a neutral adhesive. One-step self-etching adhesives have been introduced and contain all components in either a two-bottle set or in a single bottle. With the use of self-etching systems, it is generally accepted that less discrepancy occurs between the depth of demineralization and the depth of resin infiltration [3].

Resin–dentin bond strengths decrease overtime [4–6]. However the degradation mechanism is not clear. In vitro studies have demonstrated two basic mechanisms of degradation: (1) The direct hydrolysis of the decalcified and non-resin infiltrated collagen fibers at the bottom or within the hybrid layer [7–9]; (2) The extraction of resin components within the hybrid layer [10]. The degradation mechanism(s) have been shown to be different for different adhesive systems [5, 6, 11, 12].

Experimentally, it has been demonstrated that the destruction of the collagen matrix in dentin requires a combination of demineralization and activation of collagenases, whose collagenolytic activity depends on periods of oscillations in the pH [13, 14]. So, different types of acid etching may produce differences in long-term degradation [8]. Two main potential sources of proteolytic enzymes responsible for this degradation are the oral microflora and endogenous proteinases present in dentin [15].

Collagenase from *Clostridium histolyticum* has been shown to effectively cut collagen chains at several sites, thus facilitating the removal of collagen fibrils [16]. It is possible to perform in vitro aging tests for challenging resin–dentin bonds. A reduction in specimen size (dentin–resin bonded sticks of 1 mm^2 cross-sectional area) and immersion of the specimens in bacterial collagenase solution for different experimental time periods, will permit evaluation of the ability of the different resin monomers to protect the collagen matrix of dentin from bacterial or endogeneous proteolytic activity.

The aim of this study was to investigate the effect of bacterial collagenase overtime, on the resin–dentin bonds of different adhesives. The null hypothesis is that the immersion of resin-bonded specimens in a bacterial collagenase solution does not affect resin–dentin bond strength in any of the tested adhesives any more than incubating in water.

Material and methods

One hundred and eight caries-free extracted human third molars that were stored in 0.5% chloramine T (Sigma-Aldrich S.A., Madrid, Spain) at 4 °C for less than 1 month were used for the study. The specimens were sectioned below the dentin–enamel junction and ground flat (180-grit) under running water to provide smear-layer covered dentin surfaces. Tested adhesives were: Single Bond (SB) (3M ESPE Dental Products, St. Paul, MN, USA), a two-step etch-and-rinse self-priming adhesive, Clearfil SE Bond (SEB) (Kuraray Co. Ltd., Osaka, Japan), a two-step self-etching primer adhesive system and One-Up Bond F (OUB) (Tokuyama Europe GmbH, Düsseldorf, Germany), a single-step self-etching adhesive. Adhesives were applied in accordance with the manufacturers' instructions (Table 1). After the application of the adhesives to dentin, 6 mm high resin composite build-ups, were constructed incrementally (1.5 mm) with Tetric Ceram (Vivadent, Schaän, Liechtenstein). Each layer of composite was light-activated for 40 s with a Translux EC halogen light-curing unit (Kulzer GmbH, Bereich Dental, Wehrheim, Germany). The output intensity was monitored with a Demetron Curing Radiometer (Model 100) (Demetron Research Corporation, Danbury, CT, USA). A minimal output intensity of 600 mW/cm^2 was employed for the experiments.

After the preparation of resin-bonded specimens, half of the teeth, designated as ''Indirect Exposure'' (IE) were stored intact (without sectioning) at 37 °C in: (a) distilled water containing 0.02% sodium azide (Sigma-Aldrich S.A., Madrid, Spain) pH 7.0, 37 °C; or (b) 410 U/mg of collagenase from *Clostridium histolyticum* (Type II-S) (Sigma-Aldrich S.A., Madrid, Spain) in 0.05 M Tris and 0.01 M calcium acetate saline at a concentration of 0.069 mg/mL containing 0.02% sodium azide, pH 7.0, 37 °C. Both solutions were changed every 2-days, in the collagenase containing buffer solution was necessary in order to replenish the enzymes.

These teeth were aged for 24 h, 3 months and 1 year prior to bond testing. The other half of the teeth, designated as ''Direct Exposure'' (DE) were vertically sectioned into serial slabs and further into beams with cross-sectioned areas of 1 mm^2. These resin-bonded beams were aged in the same two media and for the same three periods as described above.

After each aging period, the intact teeth (Group IE) were sectioned as described above to produce beams for microtensile bond strength (MTBS) testing. The aged sectioned beams (Group DE) were retrieved from the corresponding medium and tested in the same manner. Each beam was attached to a modified Bencor Multi-T testing apparatus (Danville Engineering Co., Danville, CA, USA) with cyanoacrylate adhesive (Zapit, Dental Venture of America Inc., Corona, CA, USA) and stressed to failure in tension using a universal testing machine (Instron 4411, Instron Corporation, Canton, MA, USA) at a crosshead speed of 0.5 mm/min. The fractured beams were removed from the testing apparatus and the cross-sectional area at the site of failure was measured to the nearest 0.01 mm with digital callipers (Sylvae Ultra-Call, Li, USA). Bond strength values were expressed in MPa and analyzed with a multiple ANOVA to examine the contributions of adhesive type, storage medium, storage time and exposure method, and the interaction of these four factors on MTBS. Post-hoc multiple comparisons were conducted using Student–Newman–Keuls tests at $\alpha = 0.05$. The fractured specimens were examined with a stereomicroscope (Olympus SZ-CTV, Olympus, Tokyo, Japan) at 40× magnification to determine the mode of failure. Failure modes were classified as adhesive, mixed or cohesive failure. Representative specimens from each subgroup were dessicated and gold-coated and observed with a scanning electron microscope (SEM) (1430 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at an accelerating voltage of 20 kV to examine the morphology of the debonded interfaces.

Table 1 Bonding agents used in the experimental groups

Product	Principle ingredients (according to manufacturers)	Mode/steps of application
Single Bond		
Adhesive	2-Hydroxyethylmethacrylate, water, ethanol, Bis-GMA, dimethacrylates, amines, methacrylate-functional copolymer of polyacrylic and polyitaconic acids	Etch for 15 s Rinse with water spray for 10 s, leaving tooth moist Apply two consecutive coats of the adhesive with a fully saturated brush tip Dry gently for 2–5 s Light cure for 10 s
Clearfil SE Bond		
Primer	10-Methacryloyloxydecyl dihydrogen phosphate; 2-hydroxyethyl methacrylate; hydrophilic dimethacrylate; dl-camphorquinone; N,N-diethanol-p-touidine; water	Apply Pimer for 20 s Mild air stream Apply Bond Gentle air stream Light cure for 10 s
Bond	10-Methacryloxydecyl dihydrogen phosphate; Bis-GMA; 2-hydroxyethyl methacrylate; hydrophobic dimethacrylate; di-camphorquinone; N,N-diethanol-p-toluidine; silanated colloidal silica	
One-Up Bond F		
Bonding Agent A	Phosphoric monomer, MAC-10, multi-functional methacrylic monomers, photo-initiator	Mix Bonding Agent A and Bonding Agent B until the mixed turns homogeneously pink Apply the mixture
Bonding Agent B	Fluoroaminosilicate glass filler, water, mono-functional monomers, dye-sensitizer, borate derivate	Leave the surface undisturbed for 20 s Light cure for 10 s The pink color should turn to a pale brown after light irradiation

Bis-GMA: bis-phenol A diglycidylmethacrylate; MAC-10: Methacryloyloxyalkyl acid phosphate

Results

The adhesive type ($F = 173.0$; $p < 0.0001$), the aging period ($F = 307.7$; $p < 0.0001$) and the mode of exposure (DE/IE) ($F = 103.8$; $p < 0.0001$) significantly affected the MTBS to dentin; however the storage medium ($F = 1.10$; $p = 0.35$) did not. Interactions between factors (except for storage medium) were also significant. The mean bond strength values obtained for the different groups are shown in Table 2.

After 24 h of water storage, Single Bond and Clearfil SE Bond showed higher bond strengths than One-Up Bond F in both IE and DE groups regardless of storage media. Direct exposure of resin–dentin bonded interfaces resulted in a significant decrease in MTBS values when SB and OUB were tested after 3 months, and when CSEB was tested after 12 months, regardless of the storage solution. In indirectly exposed specimens, the only significant changes in bond strengths occurred in specimens tested after 3 months and bonded with OUB.

Table 3 summarizes the percentage failure modes of the debonded specimens according to the adhesive type, the storage medium, the aging time and their mode of exposure. Mixed fracture modes were frequently identified in all groups. No pure cohesive failures were observed in any group. Low bond strengths (i.e. OUB) were associated with higher percentages of adhesive failures. In general, the number of adhesive failures also increased with aging of directly exposed specimen beams in water.

Fractographic analysis of the debonded dentin surfaces are shown in Figs. 1–3. After prolonged immersion in collagenase, Single Bond failed at the bottom of the hybrid layer (Fig. 1a, b). Resin disappeared from dentin surfaces from the periphery to the center of the specimens. Tubule diameters became enlarged with some of them showing persistence of resin tags, and some cohesive failures of intertubular dentin (Fig. 1b). When Clearfil SE Bond was used, failures were located at the top or within the hybrid complexes (Fig. 2a, b). Some resin was left at the debonded surface (Fig. 2a), the tubules were not enlarged and intertubular dentin was not altered, especially in areas where some resin remained (Fig. 2b). When bonding with One-Up Bond F, failures were frequently at the top of the hybrid layer, as seen by polishing scratches on the surface. Very few resin tags were seen on the debonded surface, most tubules appeared empty (Fig. 3a, b).

Table 2 Mean and standard deviation MTBS (MPa) obtained for the different adhesive systems in different storage mediums and periods

Time/specimen size	Single Bond		Clearfil SE Bond		One-Up Bond F	
	Water	Collagenase	Water	Collagenase	Water	Collagenase
24 h/indirect-IE	40.85 (4.4) 1a	37.47 (3.5) 1a	42.02 (3.9) 1a	39.84 (6.2) 1a	24.11 (5.9) 1b	20.38 (3.2) 1b
24 h/direct-DE	41.24 (2.9) 1a	39.09 (4.8) 1a	41.73 (5.9) 1a	41.60 (3.9) 1a	23.57 (3.2) 1b	17.38 (3.8) 1b
3 months/indirect-IE	38.39 (5.5) 1a	34.53 (3.5) 1a	42.43 (5.7) 1a	39.61 (4.8) 1a	15.25 (5.7) 2b	14.17 (2.6) 2b
3 months/direct-DE	24.19 (4.1) 2b	20.08 (1.5) 2b	39.72 (4.9) 1a	34.23 (3.2) 1a	12.63 (3.1) 2c	10.82 (2.1) 2c
12 months/indirect-IE	42.55 (6.7) 1a	34.51 (5.9) 1a	37.12 (4.9) 1a	37.56 (6.1) 1a	11.32 (3.6) 2b	13.05 (3.7) 2b
12 months/direct-DE	26.19 (4.5) 2a	19.85 (5.8) 2a	21.29 (4.2) 2a	19.72 (5.6) 2a	9.85 (4.2) 2b	7.92 (1.6) 2b

For each horizontal row: values with identical letters indicate no statistically significant difference when maintained in the same storage media ($p > 0.05$)

For each vertical column: values with identical numbers indicate no statistically significant difference ($p > 0.05$). IE is compared to DE only when bonded with the same adhesive and within the same storage period

Table 3 Percentage distribution of failure mode: A—adhesive, M—mixed

Time	Single Bond (SB)				Clearfil SE Bond (SEB)				One-Up Bond F (OUB)			
	Water		Collagenase		Water		Collagenase		Water		Collagenase	
	A	M	A	M	A	M	A	M	A	M	A	M
24 h	23	77	26	74	24	76	29	71	48	52	58	42
3 months	44	56	65	35	26	74	27	73	76	24	80	20
12 months	75	25	69	31	45	55	82	18	85	15	77	23

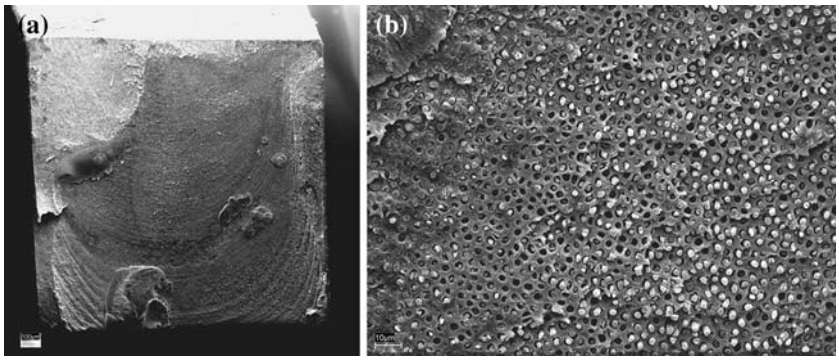

Fig. 1 SEM observations of the fractured dentin surface of a specimen bonded with Single Bond after collagenase immersion for 12 months. (**a**) A mixed failure, adhesive resin may be observed at the central part. (**b**) At a higher magnification of the periphery, main fracture is at the bottom of the hybrid layer, exposing the underlying dentin. Few rein tags remain occluding the enlarged tubules

Discussion

Microtensile bond strength values were affected by different adhesives, specimen sizes and storing periods, but as the presence or absence of bacterial collagenase did not affect MTBS, the null hypothesis must be accepted.

When the bonded interfaces were directly exposed to water (with or without collagenase) MTBS values decreased significantly ($p < 0.05$) after 3 months of immersion in the SB and OUB groups, and after 12 months when SEB was used for bonding. The time-dependency of the solubilization of collagen, may rely on locations which are completely demineralized [17], and non resin-infiltrated (Fig. 1b). It will occur after using etch-&-rinse adhesives due to restriction of the diffusion of BisGMA [18] and polyalkenoic acid copolymer [6, 19] from the base of the demineralized dentin, and in one-step self-etching adhesives because of the presence of unpolymerized acidic and aggressive monomers that continue etching the dentin [20, 21], and differential infiltration due to the phase separation of sparingly water-soluble resin components [12, 22, 23]. In the case of OUB, improper polymerization [11, 24] may accelerate resin solubilization (Fig. 3a, b).

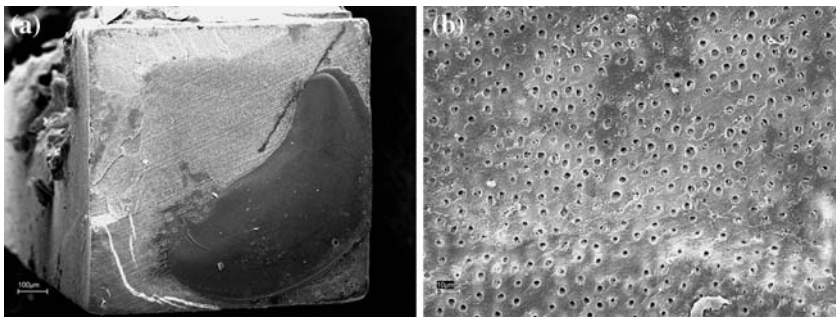

Fig. 2 SEM observations of the fractured dentin surface of a specimen bonded with Clearfil SE Bond after collagenase immersion for 12 months. (**a**) A mixed failure, the failure is mainly located at top of the hybrid complex. (**b**) At a higher magnification, an area in which the failure occurred at the bottom of the hybrid layer, there is no remaining resin but an unaltered intertubular dentin may be observed

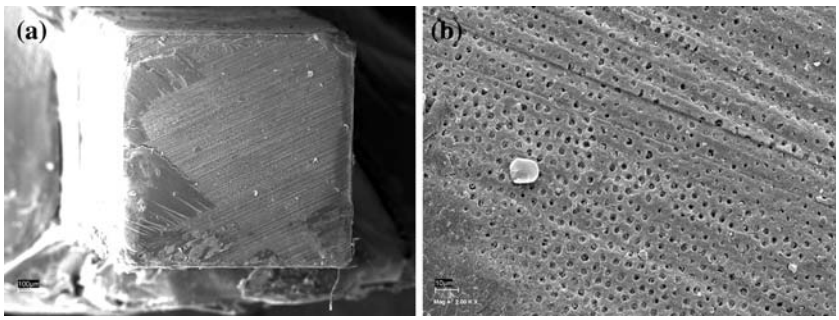

Fig. 3 SEM observations of the fractured dentin surface of a specimen bonded with One Up Bond after collagenase immersion for 12 months. (**a**) A mixed failure, with little resin remaining on the dentin surface. Main fracture is at the top of the hybrid layer, the scratches from the surface preparation may be observed. (**b**) At a higher magnification, some enlarged tubule orifices are observed

Partially demineralized collagen fibrils (i.e. SEB-treated dentin) showed a slower solubilization pattern of collagen (Fig. 2a, b) and even no decrease in MTBS at 3 months evaluation. Moreover, when SEB is applied on dentin, the monomer 10-methacryloxydecyl dihydrogen phosphate, is thought to adhered to hydroxyapatite and form a calcium salt of very low solubility rate [25], which may retard the resin solubilization process that may be responsible for decreases in MTBS [12]. Finally, the incomplete removal of water associated with hydrophilic resin monomers [9, 26], and the hydrolytic degradation of these hydrophilic resins [11] may also contribute to the hybrid layer degradation.

Pashley et al. [26] reported that hydrolytic degradation of incompletely infiltrated zones within the dentin matrix may proceed in the absence of bacterial enzymes. In our study, such endogenous collagenolytic activity of control specimens stored in water may have contributed to the decrease in MTBS over time. These results provide indirect evidence of the existence of collagenolytic activities in partially demineralized dentin collagen matrices (MMPs) [8, 26]. The additional action of added bacterial enzymes (collagenase from *Clostridium histolyticum)* did not increase or accelerate this degradation process, even though this exogenous collagenase has been shown to be effective at removing collagen from exposed dentin surfaces in vitro [16]. This collagenase is a relatively large protein (ca. 116 kDa) and it may not permeate into resin-bonded dentin, but resin dissolution at the interface (occurring over time) and the existence of a decalcified non-resin infiltrated dentin layer (SB and OUB specimens) may permit this collagenase to permeate these degraded and porous bonded interfaces.

Degradation of hybrid layers did exist in all tested systems. Phosphoric acid has been shown to partially denature the dentin MMPs due to its low pH [27, 28]. However, simplified etch-&-rinse adhesives and the less acidic versions of self-etch adhesives [29] are capable of re-activating endogenous MMPs present in etched dentin causing collagen fibrils to undergo enzymatic degradation within as little as 6 months after bonding [30], Such endogenous MMP activity may be responsible for the reported decreases in MTBS (Table 2). It has been previously been shown that pH values between 2.3 and 5 are effective in activating salivary gelatinises in a process described as ‘‘acid-activation’’ [31]. Thus, the existence of proteolytic activity after the application of SB (pH 4.3) may be reasonably explained by the increase in the quantity of

activated, MMP enzymes after acid-etching of dentin, resulting in the reduction of MTBS that was shown after 3 and 12 months of direct exposure (DE) to water. In the case of the self-etching systems, their acidity (OUB = 1.3, SEB = 1.9) decreases immediately after applying the adhesive on dentin because of the high buffer capacity of dentin [32], contributing to the activation of dentin proteolytic activity without denaturing these enzymes [27], which are trapped within the hybrid layers created by these adhesives. Thus, self-etch adhesives may activate latent MMPs and increase their activity to near-maximum levels and contribute to the degradation of resin–dentin bonds over time [29].

When bonded teeth were stored intact rather than as individual beams, their resin–dentin bond strengths did not decrease significantly for 1 year, except for OUB specimens (Table 2). This confirms a previous 4-year study [5]. While those authors speculate that resin–enamel bonds are more stable than resin–dentin bonds, another interpretation is that reducing bonded teeth to beams is a form of accelerated aging because the middle of the bond is only 0.5 mm from the outside in beams. An effective hydrophobic peripheral seal may be able to decrease the amount of water at the dentin–resin hybrid layer. Since MMPs are hydrolases, they require water to hydrolyze peptide bonds in the collagen molecules [12, 26]. When bonding with OUB, higher water sorption is expected at the resin–enamel interface and more water movement has been shown to occur within these one-step adhesive layers [33, 34]. This may provide enough water to permit rapid resin–dentin bonds degradation by MMPs. Water sorption may also plasticize the polymer networks [34], weakening resin–dentin bonds over time. Thus the peripheral resin-infiltrated enamel did not seem to provide protection against degradation of resin–dentin bonds made with the hydrophilic OUB.

Conclusions

1. At the resin–dentin bonded interfaces, hybrid layers degradation occurs over time, mainly due to resin and collagen dissolution. This process is accelerated by incomplete removal of water and water diffusion associated with the use of hydrophilic resin monomers as adhesives.
2. The additional action of added bacterial enzymes did not increase or accelerate this degradation process, as endogenous dentin MMPs are able to produce enzymatic degradation of decalcified collagen fibrils.
3. An effective hydrophobic peripheral seal (enamel-hydrophobic resin) is able to decrease the amount of water at the dentin–resin hybrid layer, retarding this degradation phenomenon.

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