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ABSTRACT

Carbodiimide (EDC)-based dentin primers preserve hybrid layer (HL) integrity. However, aging longer than 1 year has not been investigated. The present study examined whether the cross-linking effect of EDC was reflected in dentin bond strength, endogenous enzymatic activity and the chemical profile of the HL after 5-year aging in artificial saliva. Non-carious human third molars (N=42) were cut to expose middle/deep coronal dentin and treated as follows: Group-1: dentin etched with 35% H_3PO_4, pretreated with a 0.3 M aqueous EDC primer for 1 min and restored with XP Bond (Dentsply Sirona); Group-2: as in Group-1 but without EDC pretreatment; Group-3: Clearfil SE Bond (CSE; Kuraray-Noritake) primer applied to dentin surface followed by EDC pretreatment as in Group-1 and application of CSE bond; Group-4: as in Group-3 without EDC pretreatment. After composite build-up, the specimens were cut into sticks or slabs, depending on the experiment. All tests were performed at baseline (T0) and after 5 years of aging (T5) in artificial saliva at 37°C. Microtensile bond strength (μTBS) was tested at a crosshead speed of 1 mm/min until failure. Endogenous enzymatic activity was investigated using *in-situ* zymography. The chemical profile of HL was determined using Raman spectroscopy. Three-way ANOVA and post-hoc Tukey test were used to analyze μTBS and *in-situ* zymography data (α=0.05). EDC pretreatment and aging significantly influenced μTBS and *in-situ* zymography results ($p<0.05$). Higher bond strength and lower gelatinolytic activity were identified in the EDC-treated groups at T5 ($p<0.05$), especially in the etch-and-rinse groups. Raman spectra revealed less defined amide III peaks in control specimens at T5. The EDC cross-linking effect persisted in the HL for 5 years in terms of bond strength, collagen structure preservation and dentinal enzymes silencing.

INTRODUCTION

Long-term bonding effectiveness remains the biggest challenge to be addressed in adhesive dentistry. The longevity of the hybrid layer (HL) depends on the stability and integrity of the resin-embedded demineralized collagen fibrils. If not well-infiltrated by resin, these fibrils are prone to degradation by host-derived enzymes such as matrix metalloproteinases (MMPs) and cysteine cathepsins (Maravic *et al*. 2017b; Breschi *et al*. 2018). These enzymes are trapped within the mineralized dentin matrix during tooth development (Mazzoni *et al*. 2015) and are exposed and reactivated after dentin demineralization (Mazzoni *et al*. 2006).

Considerable efforts have been invested in developing strategies to minimize HL degradation over time, with recent emphasis on bio-modification of tooth tissues (Bedran-Russo *et al.* 2014). Cross-linkers covalently bond collagen molecules, increasing the resistance of the resin-sparse, water-rich collagen matrix within the HL against enzymatic degradation as well as inactivate dentinal MMPs (Bedran-Russo *et al*. 2008, 2010, 2014; Cova *et al*. 2011; Mazzoni *et al*. 2013; Tjäderhane *et al*. 2013a; Maravic *et al*. 2017a; Breschi *et al*. 2018; Comba e*t al*. 2020).

The commercially-available synthetic cross-linking reagent, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), preserves bond strength and inhibits MMPs for up to 12 months (longest reported aging time so far) (Tezvergil-Mutluay *et al*. 2012; Mazzoni *et al*. 2014, 2017; Comba *et al*. 2019) as well as cysteine cathepsins (Turco et al. 2016). Although cross-linking of collagen fibrils and MMP inactivation are irreversible processes, further studies with longer aging times are required to confirm this hypothesis.

Accordingly, the objective of the present study was to evaluate the ability of an EDC-containing aqueous primer applied during etch-and-rinse and self-etch adhesive procedures to preserve bond strength and collagen structure and inactivate MMPs after 5 years of accelerated aging in artificial saliva at 37˚C. The null hypotheses tested were that dentin pre-conditioning

with an EDC-containing primer prior to the adhesive resin application does not: 1) preserve bond strength; 2) inactivate endogenous enzymes; and 3) influence the chemical profile of the HL, at baseline (T0) and after 5 years of accelerated aging (T5).

METHODS

Microtensile bond strength (μTBS)

Human non-carious molars (n=32, sample size determined using G*Power 3.1.9.7 for Windows; Faul *et al*. 2007) were used within one month after extraction. Each tooth was cut into a 4 mm-thick slab using a slow-speed diamond saw (Micromet, Remet, Bologna, Italy) under water cooling. Following exposure of the medium/deep dentin, a standardized smear layer was created on the dentin surface using wet silicon-carbide paper (600-grit) for 30 s. The tooth slabs were randomly allocated to one of the following groups and restored by an experienced practitioner:

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

Group-2 (control): dentin prepared as in Group-1 but without EDC pretreatment.

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

Group-4 (control): dentin prepared as in Group-3 but without EDC pretreatment.

All the adhesives were light-cured with a light-emitting diode curing unit (output 800 mW/cm^2) for 20 s. A 4 mm-thick core build-up was created using a hybrid resin composite (Filtek Z250, 3M ESPE) in each of the bonded teeth, in 1 mm-thick increments. Each increment was light-cured for 20 s. Bonded teeth were sectioned into 0.9x0.9x8 mm (±0.01 mm) sticks. Half of the randomly chosen sticks from each tooth were stored in artificial saliva for 24 h at 37°C (designated as T0). The artificial saliva (pH 7.4) comprised (in mmoles/L): $CaCl_2$(0.7), $MgCl_2 \cdot 6H2O$ (0.2), KH_2PO_4 (4.0), KCl (30), NaN_3(0.3) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (20) (Pashley *et al*. 2004). The other half of the sticks from each tooth were aged for 5 years in artificial saliva at 37˚C, with the saliva replaced every two weeks (designated as T5). After aging, the bonded area of each specimen was calculated and it was stressed in tension in a testing machine (Bisco Inc., Schaumburg, IL, USA) at a stretching speed of 1 mm/min until failure. Since there were only 1.2% of prematurely debonded specimens, evenly distributed among groups, they were not included in the statistical analysis. Fracture modes (A–adhesive failure, CC–cohesive in composite resin; CD–cohesive in dentin; M–mixed failure) were analyzed using a stereomicroscope at a 30× magnification.

Three debonded sticks from each group with bond strength that approximated the mean bond strength of that group were fixed with 2.5% glutaraldehyde-cacodylate buffer (3 h, pH 7.4), dehydrated in ascending concentrations of ethanol (50-100%) and dried using hexamethyldisilazane. Specimens were mounted on aluminum stubs, coated with gold-palladium and examined using a field-emission scanning electron microscope (FE-SEM; Nova NanoSEM 450; FEI Co., Hilsboro, OR).

In-situ zymography

Additional non-carious human molars (n=5, sample size determined using G*Power) were used to investigate gelatinolytic activity within the HL. The crown of each tooth was sectioned transversally in two 1-mm-thick slices that contained medium/deep dentin. These slices were

further divided in 2 pieces (4 pieces *in toto*) and were randomly assigned to 4 groups. This experimental design enabled all groups to be examined using the same dentinal substrate. The dentin pieces were bonded in the same manner as previously described. A 1 mm-thick composite build-up was made using the Filtek Z250 resin composite. The bonded and restored dentin pieces were sectioned to expose the HL. Half of the sectioned specimens from each group were tested after 24 h of storage in artificial saliva at 37 °C (T0), while the other half was aged for 5 years in artificial saliva (T5), as previously described.

After aging, *in-situ* zymography was performed using the method reported by Mazzoni *et al*. (2012). Briefly, each specimen was glued to a microscope slide (2 specimens from each tooth per group), and progressively polished with wet silicon carbide papers with increasingly-fine grit size (600-grit, 1200-grit, 4000-grit) until ~50 µm-thick and coated with fluorescein-conjugated gelatin solution (E-12055; Molecular Probes, Eugene, OR). The specimens were placed in a humid chamber and kept overnight in the dark at 37 °C. They were then examined with a confocal microscope (Leica SP8, Leica Microsystems GmbH, Wetzlar, Germany; excitation/emission wavelength: 488/530 nm). Three z-stack images (1 µm interlayer distance) were made for each specimen (in the middle and toward both ends) by a researcher who was unaware of the designated groups. Quantification of the integrated density of the fluorescence signal was performed at the same level for each image, using the ImageJ image analysis software (National Institutes of Health, Bethesda, MD). A standardized rectangular selected area was used for all images. Differences in the level of fluorescence among the tested groups were statistically analyzed.

Micro-Raman spectroscopy

The chemical profile of resin-dentin interfaces (n=3 teeth per group, 2 bonded sticks per tooth) was examined using a dispersive Raman spectrometer/microscope (Horiba ScientificXplora, Villeneuve d'Ascq, France). Specimens were polished with 1500-grit silicon

carbide paper for 30 s and analyzed using a 638-nm diode laser (1 μm spot diameter) at 100 mW, an X100/0.90 NA air objective and a 600 lines/mm grating. The bonded interface was scanned from 400 to 2000 cm–1, using 30 s accumulation time with 5 co-additions. Spectra were acquired at 3 random sites from the top of the HL to the underlying dentin at 1 μm-thick intervals. Eleven spectra were acquired for each site using a computer-controlled x–y–z stage. The acquired spectra were analyzed using the Labspec 6 software (Horiba). The band ratio between the intensity of pyridinium ring and phenyl vibrations (1032 cm^{-1}/1003 cm^{-1}) was used to obtain further information on the cross-linking effect of EDC.

Statistical analyses

As the μTBS (primary outcome) and *in-situ* zymography data (secondary outcome) were homogeneous (modified Levene's test) and normally-distributed (Kolmogorov–Smirnov test), a three-way analyses of variance (ANOVA), with the random effect for the variable "tooth" to account for the split-tooth design, and post-hoc Tukey tests were performed to identify the effects of three variables, "EDC pretreatment" (with/without), "aging" (T0/T5) and "adhesive system" (XPB/CSE) and their interactions on bond strength and gelatinolytic activity. In addition, one-way ANOVA and post-hoc Bonferroni tests were conducted to evaluate differences between each of the groups. Statistical significance was pre-set at $\alpha=0.05$ (Stata 12.0 software, StataCorp, College Station, TX).

RESULTS

Results of the μTBS test are presented in Table 1. Bond strength was significantly affected by "aging" and "EDC pretreatment", with higher bond strength at T0, as well as in groups pretreated with EDC ($p=0.0002$, $p=0.0223$, respectively), while the main variable "adhesive system" and all the possible interactions did not exert any effect on bond strength ($p<0.05$). When the groups were individually considered, the increase in bond strength in the EDC-

treated XPB groups was statistically significant ($p<0.05$; 27 % at T0 and 29 % at T5). No significant difference was observed in the EDC-treated CSE groups (9% at T0 and 13% at T5).

The majority of the failure modes in the XPB groups were A failure, regardless of aging or EDC-treatment. In the CSE groups, A and CC failures were the most prevalent (Table 1). FE-SEM demonstrated differences in the dentin structure between the two different adhesives. The XPB groups had M failures that displayed demineralized collagen fibrils and open dentinal tubules (Figure 1). In contrast, CSE groups showed M failures with occluded dentin tubules covered with smear layers (Figure 2).

For *in-situ* zymography, the three variables "EDC pretreatment", "aging" and "adhesive", as well as the interaction "pretreatment/adhesive" significantly influenced dentin gelatinolytic activity ($p=0.0001$, $p=0.0001$, $p=0.0003$, $p=0.0341$, respectively). In all the tested groups, fluorescence density level was higher after aging. The EDC silenced enzymatic activity more efficiently in the XPB groups. One-way ANOVA revealed a significant decrease in the gelatinolytic activity in all the EDC-pretreated groups except for CSE at T5. Fluorescence was detected in the HL as well as in the underlying dentinal tubules (Figure 3).

Representative Raman spectra of the experimental groups are shown in Figure 4. Spectra recorded in the range of 400-2000 cm^{-1} covered the fingerprint region associated with the HL and dentin collagen bands. Peaks assigned to adhesives were documented (C-O-C at 1113 cm^{-1}, C=C at 1610 cm^{-1}, C=O at 1720 cm^{-1}). The relative decrease of intensity of these peaks represented the transition through the dentin. Dentin collagen bands also became evident (amide I at 1667 cm^{-1}, amide III at 1243 cm^{-1} and 1273 cm^{-1}, phenyl group at 1003 cm^{-1} of aromatic ring of phenylalanine residues of collagen, pyridinium ring at 1032 cm^{-1} which has a trivalent amino acid cross-linking residue). In addition, functional groups of carbonated apatite (phosphate at 962 cm^{-1} and carbonate 1072 cm^{-1}) were detected within the mineralized dentin. More aggressive demineralization was detected in the XPB groups, as shown by the abrupt

reduction in the intensity of carbonate and phosphate peaks within the transition region, while mineral-associated peaks were more pronounced in the CSE groups. After aging, the intensity of the mineral-associated peaks dropped for the XPB groups (especially control), while they remained stable over time for the CSE groups.

Collagen changes were identified in the amide III peaks (1243-1248 cm^{-1}) and from 1273-1278 cm^{-1} in all EDC-treated groups, regardless of aging. In addition, the second peak of the doublet at 1273 cm^{-1} was less well-defined in the groups without EDC at T5; for XPB at T5, this peak almost disappeared. Furthermore, drop of adhesive peaks was noted for all the T5 groups, except the EDC XPB, where well-pronounced adhesive bands were still detected.

Pretreatment with EDC did not alter the pyridinium/phenyl band ratio at T0, regardless of the adhesive system used. This band ratio was not reduced at T5 for the EDC groups in comparison with their respective adhesive subgroups at T0 (Cross-linking pyridinium/phenyl: XPB T0–1.02; XPB 5–0.98; EDC+XPB T0–1.03; EDC+XPB T5–1.16; CSE T0–1.18; CSE T5–1.02; EDC+CSE T0–1.19; EDC+CSE T5–1.19). Additional peaks assigned to EDC could not be identified.

DISCUSSION

The present study demonstrated for the first time that EDC pretreatment retained significantly higher bond strength values, and decreased gelatinolytic activity, even after 5 years of accelerated aging in artificial saliva. Cross-linking of the demineralized collagen matrix by EDC was evident from the chemical profile of the HL and the underlying dentin. Hence, the three null hypotheses have to be rejected.

Endogenous dentin enzymatic activity undoubtedly contributes to the degradation of the HL over time (Breschi et al. 2018). MMPs inhibitors and cross-linkers have been extensively studied as strategies to preserve the integrity of the HL (Breschi *et al*. 2018). An advantage of

cross-linkers over MMP inhibitors is that they possess a dual protection role. On one hand, they cross-link and reinforce collagen molecules, and on the other, they inactivate MMPs (Bedran-Russo *et al*. 2008, 2010, 2014; Cova *et al*. 2011; Mazzoni et al. 2013; Tjäderhane et al. 2013a, 2013b; Breschi e*t al*. 2018; Comba *et al*. 2020). Specifically, EDC reacts with the carboxyl group on the C-terminus of one polypeptide chain, forming an intermediate which reacts with the amino group on the N-terminus of a neighboring polypeptide chain. The aforementioned chemical reaction produces a stable covalent bond and leaves urea as byproduct (Tezvergil-Mutluay *et al*. 2012). Direct reaction with primary amines occurs via the formation of amide bonds, which was reflected in the micro-Raman results of the present study (Figure 4). Amide III peaks are highly-sensitive to the orientation of the collagen fibrils (Shepherd *et al*. 2015). In the present study, changes were identified in the amide III peaks in all EDC-treated groups. Furthermore, the second peak of the amide III doublet was less well-defined in the CSE subgroup at T5, and was almost undetectable in the XPB subgroup at T5, likely representing gradual degradation of a non-cross-linked collagen fibrillar matrix by endogenous collagenolytic enzymes over time (Shepherd *et al*. 2015). Further, higher pyridinium/phenyl band ratio is associated with a greater extent of collagen cross-liking (Daood *et al*. 2013; Toledano *et al.* 2015). In the present study, this ratio was not influenced by EDC pretreatment at T0 but was maintained at the same level at T5, while in the control groups it dropped over time (Figure 4). The authors hypothesize that even though EDC did not initially influence pyridinium within the N- and C-terminal telopeptides, it preserved these functionalities over time. Since EDC is a zero-length cross-linker (Hwang *et al*. 2011), additional peaks assigned to EDC could not be identified. Moreover, EDC activation only results in intra-helical and inter-helical cross-links but not inter-microfibrillar cross-links (Zeeman *et al*. 1999). Although the aforementioned cross-links are sufficient to promote stability of the collagen fibrils and their enzymatic resistance (Zeeman *et al*. 1999), the changes

in the collagen structure were discrete (Vidal et al. 2016), and therefore not detectable by spectroscopic analysis.

Apart from cross-linking collagen, EDC also interacts with the extracellular dentin matrix by inactivation of MMPs, probably through conformational changes. Cross-linking of the catalytic or non-catalytic portion of an MMP by EDC disables the recognition and cleavage of the substrate, or unraveling of the triple helix of a collagen molecule (Busenlehner and Armstrong 2005; Mazzoni *et al*. 2017, 2018). Such a property has been reported by others directly after bonding and after aging the bonded specimens for one year in artificial saliva (Tjäderhane, *et al*. 2013b; Scheffel *et al*. 2014; Mazzoni *et al*. 2018; Comba *et al*. 2019).

The results of Raman spectroscopy and *in-situ* zymography (Figures 3,4) support the bond strength results. Pretreatment with EDC preserved resin-dentin bond strength in the etch-and-rinse group for at least 5 years (length of the present study). This finding further reinforces the previous reports that EDC enhances longevity of resin-dentin bonds and increases resistance of the collagen matrix to degradation (Bedran-Russo *et al*. 2010; Tezvergil-Mutluay *et al*. 2012; Ryou *et al*. 2016; Mazzoni *et al*. 2014, 2017). In those studies, the maximum aging time used was one year. This EDC-based *in-situ* bioengineering is likely to be extremely stable.

In the present study, EDC was more efficient in bond strength preservation and enzyme silencing when applied with an etch-and-rinse adhesive, probably due to differences in the application mode and properties of the adhesive. In etch-and-rinse adhesive systems, dentin is etched, rinsed and then pretreated with the EDC aqueous solution, enabling the cross-linker to interact freely with collagen molecules, with an abundance of collagen substrate to cross-link and exposed MMPs to inactivate (Mazzoni *et al*. 2006). We speculate that during the course of aging, EDC may still exert its cross-linking potential in the resin-sparse, water-rich layer of collagen fibrils at the bottom of the HL.

Compared with an etch-an-rinse adhesive, separate EDC pretreatment associated with the use of a self-etch adhesive is conducted after placement of the self-etching primer on dentin. EDC may not be as efficient in diffusing through a bed of partially-demineralized collagen fibrils that has been infiltrated with a mixture of water and resin. Moreover, CSE Bond contains 10-methacryloyloxydecyl dihydrogen phosphate, an acidic phosphate monomer with the purported ability to bind chemically to dentin (Van Meerbeek *et al*. 2011). The HLs created with this system are thinner since the penetration of the acidic monomer is shallower, with better dentin hybridization compared to an etch-and-rinse adhesive (Breschi *et al*. 2004). In addition, there is likely to be less exposed MMPs for the EDC to inactivate and less denuded collagen fibrils to cross-link, as also demonstrated in the SEM failure analysis, with dentin tubules occluded by smear layer, compared to the etch-and-rinse groups, which presented open dentin tubules and exposed sparse denuded collagen fibrils (Figures 1,2). Furthermore, although thorough evaporation has been performed during bonding procedures, the authors cannot exclude the risk of water remnants after the use of the separate EDC aqueous primer over the CSE Primer. The remaining water can hamper polymerization and render the adhesive layer more prone to hydrolytic degradation due to plasticization and swelling, also confirmed in the Raman analysis. This could have minimized the beneficial effect of EDC on collagen structure preservation, possibly causing lack of differences in bond strength after aging in the CSE group (Mazzitelli et al. 2008; Cadenaro et al. 2018). Moreover, the ISO/DTS 11405 recommended 600-grit silicone-carbide paper was demonstrated to produce thinner smear layers compared to burs (Armstrong et al. 2017), which increases the dentin etching and priming efficacy, and could have influenced the results.

The present EDC-priming protocol appears to be clinically feasible, considering that EDC is a compound with low transdentinal cytotoxicity (Scheffel *et al*. 2015) and that it prolongs the adhesive procedure for merely one minute. Clinical trials are necessary to confirm the

effectiveness of EDC in the preservation of resin-dentin bonds over time. Incorporation of EDC as a component of dentin adhesive systems may result in further simplification of the clinical application procedure, making it more appealing to dental practitioners.

Cross-linking of completely/partially demineralized dentin with EDC preserves resin-dentin bond strength after 5 years of accelerated aging in artificial saliva when an etch-and-rinse adhesive is used. This is probably through collagen cross-linking and inactivation of endogenous enzymatic activity. This simple and non-toxic primer should be considered for use in clinical practice.

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Author contributions

Tatjana Maravic: Contributed to study design, acquisition, analysis and interpretation of data, drafted the manuscript.

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Allegra Comba: Contributed to analysis and interpretation of data, critically revised the manuscript.

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Lorenzo Breschi: Substantially contributed to conception and design, analysis and interpretation of data, drafted and critically revised the manuscript.

Annalisa Mazzoni: Contributed to conception and design, analysis and interpretation of data, and critically revised the manuscript.

All authors gave their final approval and agree to be accountable for all aspects of the work.

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Table 1. Results of microtensile test at T0 and T5 (n=8 teeth per group). Data are expressed in MPa (mean ± standard deviation). The percentages of failure modes among the different groups are included in the table. T0: Data obtained after 24 h of storage at 37 °C. T5: Data obtained after 5 years of aging in artificial saliva at 37 °C.

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

Different superscript upper-case letters indicate differences ($p<0.05$) within the columns.
Different superscript lower-case letters indicate differences ($p<0.05$) within the rows.
A – adhesive failure; CC – cohesive failure in composite; CD – cohesive failure in dentin; M – mixed failure.

FIGURES

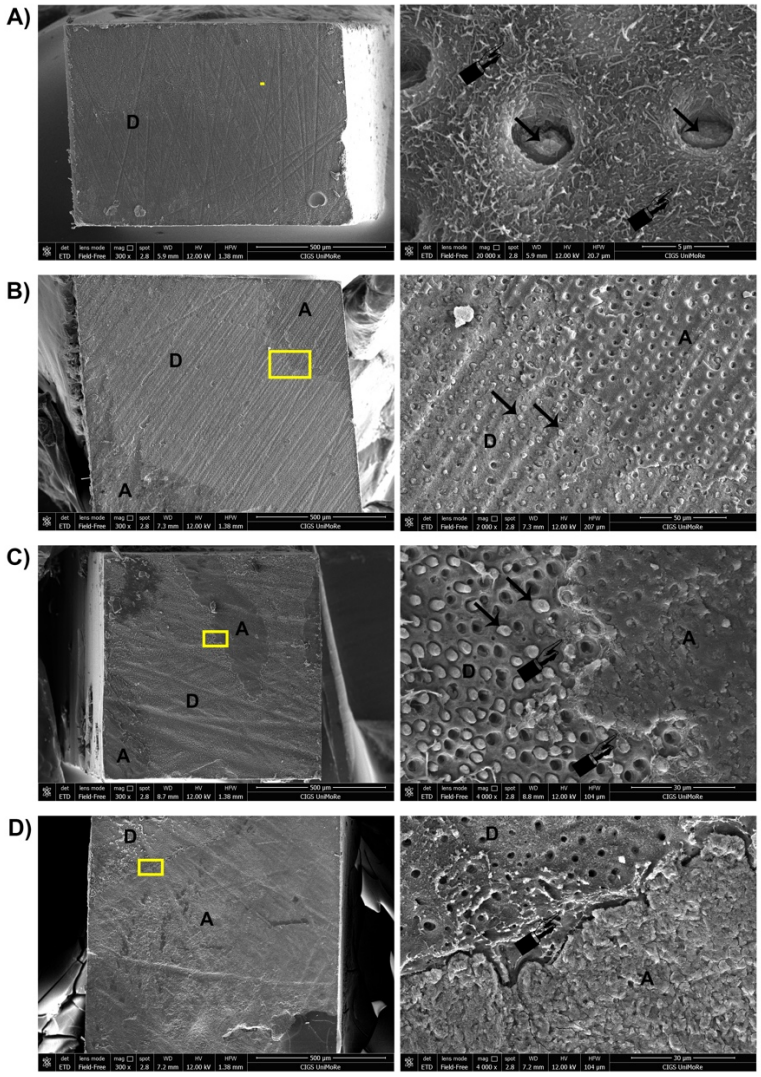

Figure 1. FEI-SEM micrographs of the adhesive interfaces of fractured μTBS sticks (the dentin side) for the XP Bond (XPB) groups (left – view of the whole adhesive surface; right – enlarged view of the area marked with the yellow selection): **A**) XPB T0 group – dentin surface after adhesive failure. Denuded collagen fibrils and a small number of resin tags can be identified within the dentin tubules (bars: 500 μm - left, 5 μm - right); **B**) EDC XPB T0 – mixed failure with a fine layer of resin adhering to the dentin surface (bars: 500 μm - left, 50 μm - right); **C**) XPB T5 – mixed failure with visible dentin tubules containing resin tags and sparse denuded collagen fibrils (bars: 500 μm - left, 30 μm - right); **D**) EDC XPB T5 – mixed fracture with the majority of the adhesive interface covered by resin. The dentin portion is free of resin tags, with sparse denuded collagen fibrils (bars: 500 μm - left, 30 μm - right). Abbreviations: D – dentin; A – adhesive resin; black arrow – resin tag in the dentinal tubule; pointer – denuded collagen fibrils; EDC – carbodiimide pretreated group; T0 – 24 h aging; T5 – 5 years aging.

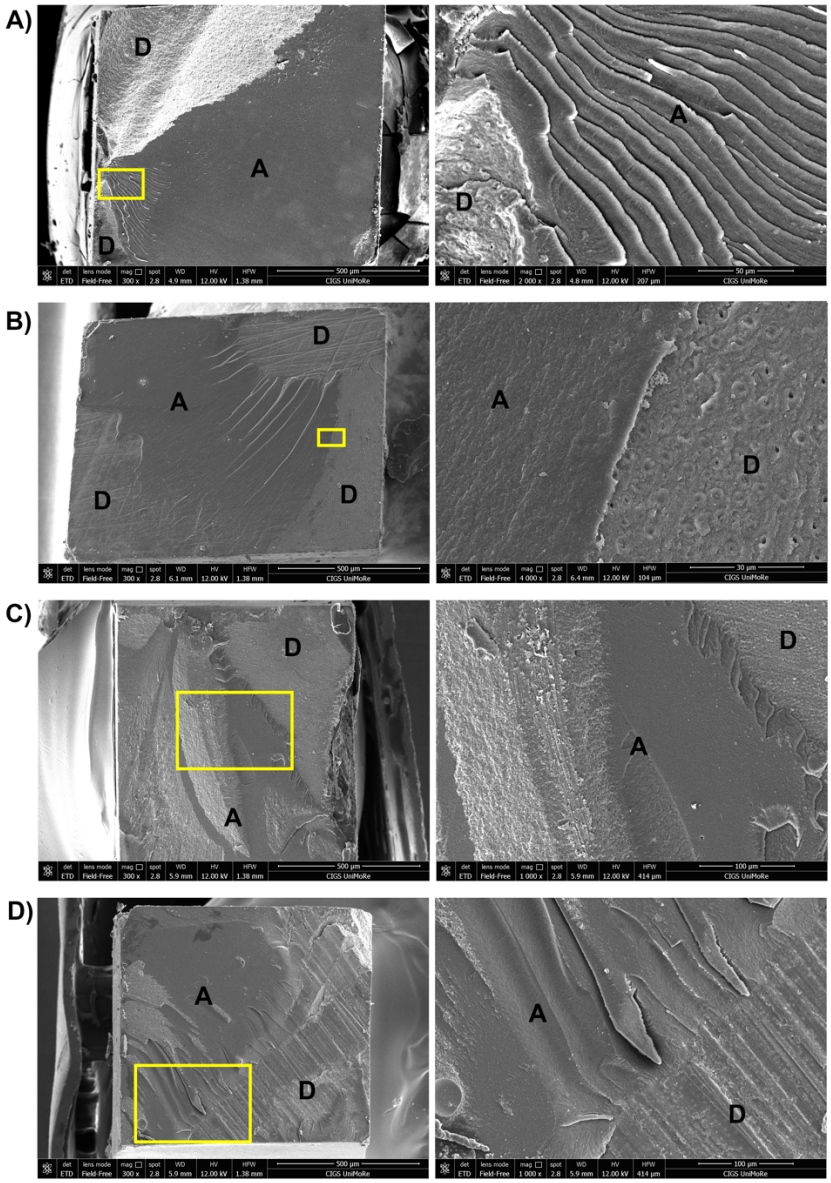

Figure 2. FEI-SEM micrographs of the adhesive interfaces of fractured μTBS sticks (the dentin side) for the Clearfil SE Bond (CSE) groups (left – view of the whole adhesive surface; right – enlarged view of the area marked with the yellow selection): **A**) CSE T0 group – mixed failure. Flat resin surface with chevron marks (river lines) of the fractured resin created during crack propagation. Side chipping of the dentin is evident. Dentin tubules are occluded by a smear layer (bars: 500 μm - left, 50 μm - right); **B**) EDC CSE T0 – mixed failure with a thicker layer of resin adhering to the dentin surface. Dentin tubules are occluded by a smear layer (bars: 500 μm- left, 30 μm - right); **C**) CSE T5 –mixed failure with a layer of resin adhering to the dentin surface. Dentin tubules occluded by a smear layer (bars: 500 μm - left, 100 μm - right); **D**) EDC CSE T5– mixed failure. Dentin tubules occluded by a smear layer (bars: 500 μm - left, 100 μm - right). Abbreviaitons: D – dentin, A – adhesive resin; EDC – carbodiimide pretreated group; T0 – 24 h aging; T5 – 5 years aging.

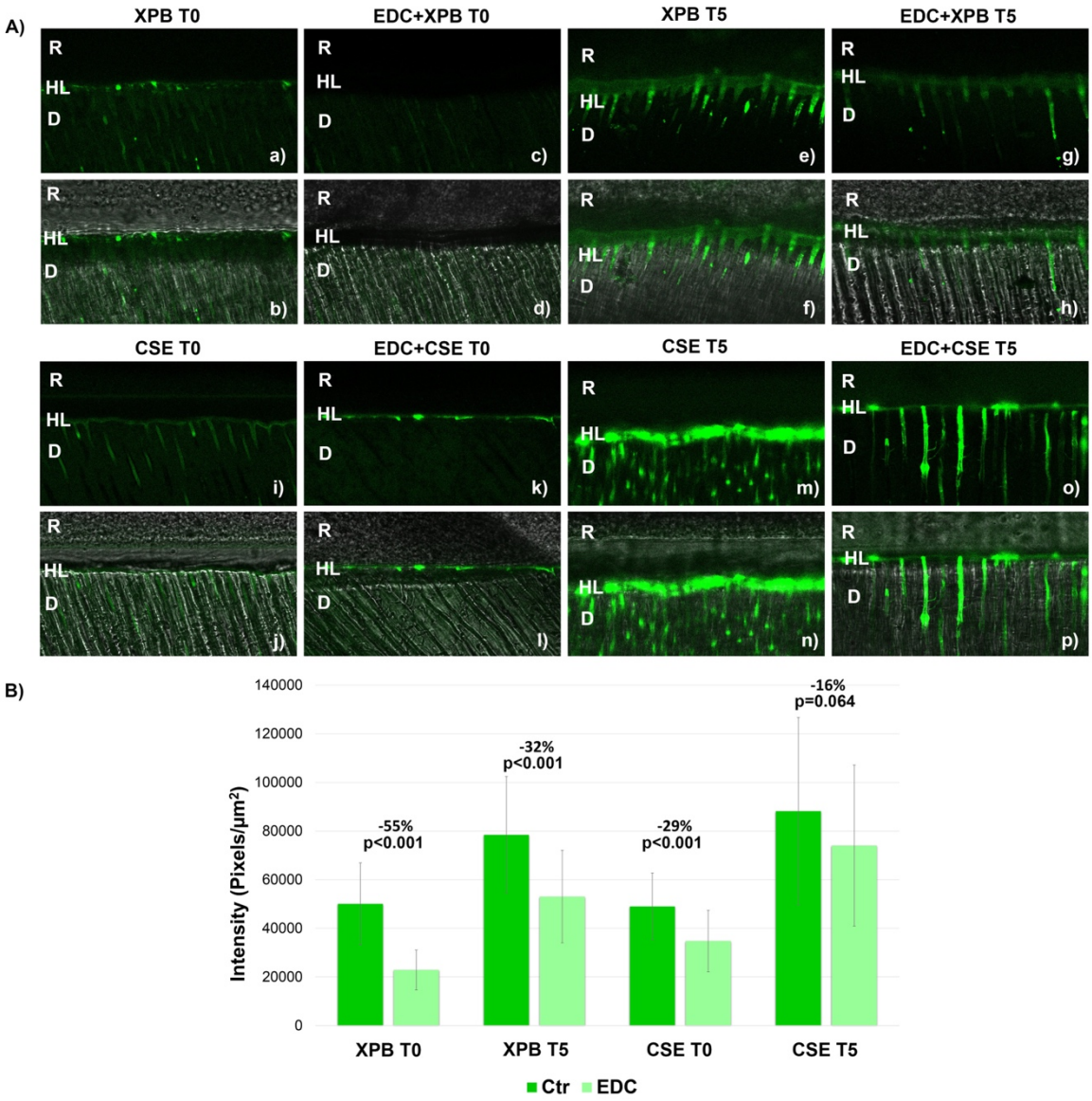

Figure 3. A) Resin-dentin interfaces incubated with quenched fluorescein-labeled gelatin. (a,c,e,g,i,k,m,o) Images acquired in the green channel showing fluorescence in dentinal tubules and within the HL of the tested groups. Fluorescence is lower in the groups treated with EDC primer and at T0 compared with the aged specimens; (b,d,f,h,j,l,n,p) Image obtained by merging the differential interference contrast (DIC) image (showing optical density of the resin-dentin interface) and the image acquired in the green channel. Abbreviations: XPB - XP Bond; CSE – Clearfil SE Bond; EDC – carbodiimide D - dentin; HL – hybrid layer; R – resin composite; T0 – 24 h aging; T5 – 5 years aging; **B**) Quantification of the gelatinolytic activity within the resin-dentin interfaces of the tested groups.

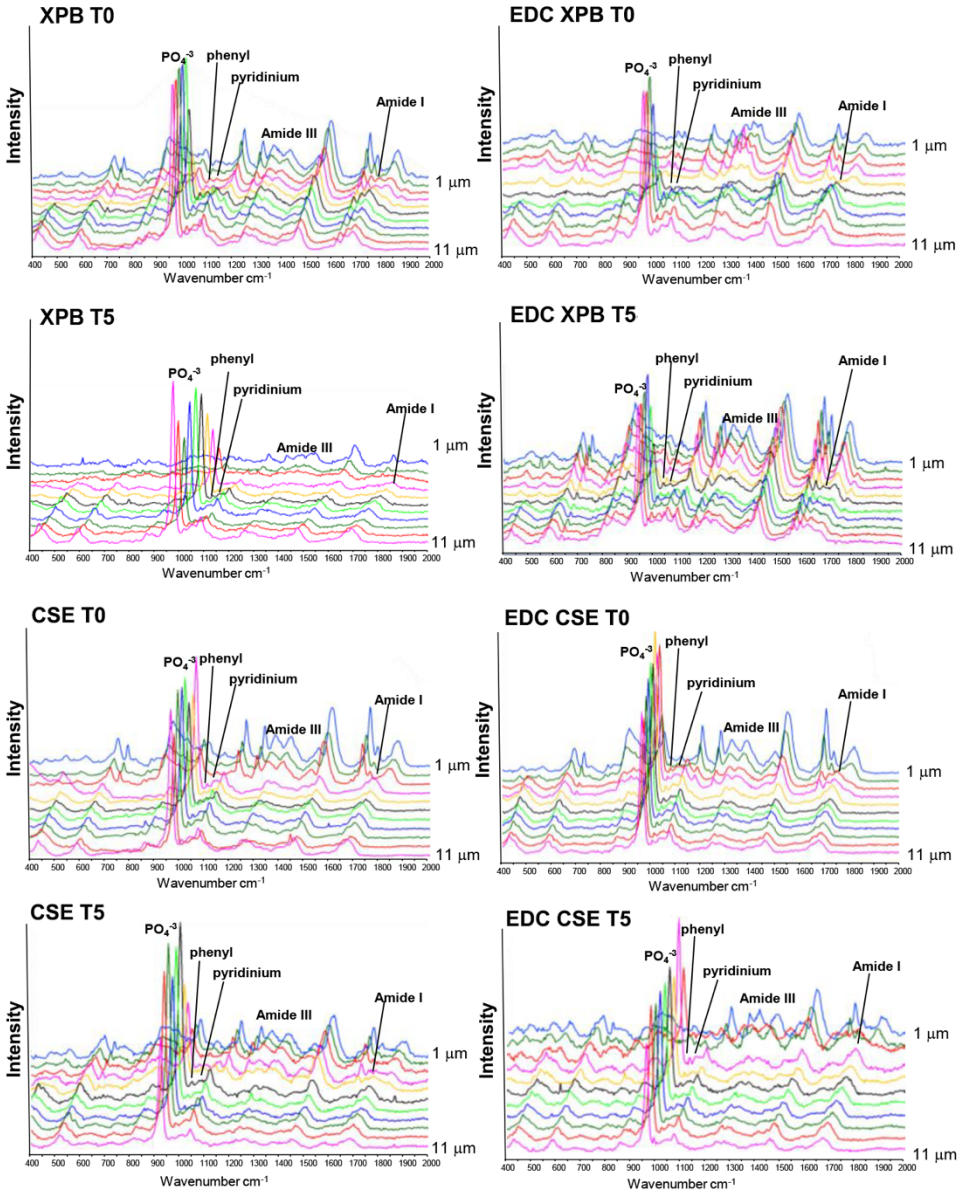

Figure 4. Micro-Raman line-spectra acquired at the adhesive-dentin interface created by XPB (XP Bond) and CSE (Clearfil SE Bond) without and with the application of the carbodiimide (EDC) primer (EDC XPB and EDC CSE) immediately (T0) and after 5 years of accelerated aging (T5). Each spectrum was acquired from the middle of the hybrid layer (top spectra) to the underlaying dentin (bottom spectra). The relative peaks associated with methacrylate monomers, mineralized and demineralized dentin in the hybrid layer were observed in both groups. Changes were identified at amide III peaks (1243-1248 cm^{-1} and 1273-1278 cm^{-1}) for all EDC-treated groups. The band ratios between intensity of pyridinium ring and phenyl vibrations (1032 cm^{-1}/1003 cm^{-1}) were used for evaluation of the cross-linking effect of EDC. No difference was detected for EDC treatment of each adhesive subgroup at T0. This ratio was not reduced at T5 for the EDC groups when compared with their respective ratio at T0 for both adhesives.