Effect of carboidiimide on thermal denaturation temperature of dentin collagen

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ABSTRACT

Objectives. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) has been shown to cross-link dentin type I collagen. Increased cross-linking usually elevates the glass transition temperature of polymers. The aim of this study was to evaluate the cross-linking reaction promoted by EDC in different aqueous concentrations by measuring the thermal denaturation temperature (T_d) of human dentin collagen.
Methods. The T_d of dehydrated collagen and of insoluble dentin matrix collagen immersed in 0.5 M or 1 M EDC aqueous solution for different treatment times was obtained using a Differential Scanning Calorimeter (DSC). Specimens were also analyzed by Energy Dispersive X-Ray Spectroscopy.
Results. EDC-treated dentin collagen showed a significantly higher T_d than the untreated specimens when immersed in either 0.5 M EDC or 1 M EDC for 10 min or longer ($p < 0.05$). EDC-treated dentin collagen showed an increase of sulfur and chloride, not detectable in EDC-untreated dentin specimens. Conversely, the relative amount of carbon, nitrogen and oxygen was not modified by treatments.
Significance. EDC-treated dentin collagen showed a higher T_d than the untreated control at all tested concentrations and immersion times. A higher T_d can be considered an indirect indicator of a more resistant and highly cross-linked collagen network. More data are needed to confirm these results.

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1. Introduction

Dentin adhesive systems have greatly improved in the last decades. However, the degradation of the collagen matrix at the resin–dentin interface after resin bonding is still a potential drawback that adversely affects the longevity of the bonded interface [1]. Acid-etching procedures used in etch-and-rinse adhesive techniques expose the dentin collagen matrix and activate endogenous matrix-metalloproteinases (MMPs) [2,3], which are usually inactive in mineralized dentin and bone [4,5]. In addition to MMPs, mineralized dentin contains cysteine cathepsins (in particular cathepsin K) [6,7] that is also involved in collagen degradation within the hybrid layer. MMPs and cathepsins act synergistically and gradually destroy the exposed collagen fibrils within the hybrid layer, when sub-optimally infiltrated by the adhesive resin [8,9]. This degradation process may cause the loss of resin–dentin bond strength and failure of the adhesive interface [1,10,11].

Type I collagen represents the 90% of the dentin organic matrix, determining its biomechanical properties and functional integrity [12]. Collagen is stabilized through the formation of native pyridinium cross-links [13], which reduce its solubility and impart high tensile properties to the molecule. Such intramolecular cross-links are formed over time during the maturation process of tissues or in response to a disease and strengthen aggregated forms of collagen fibrils [14]. Lysine and hydroxylysine residues contained in collagen telopeptides are implicated in the cross-linking process [15]. The oxidative deamination of lysine or hydroxylysine in telopeptides, followed by the formation of pyridinium bonds [13] increase the number of covalent inter- and intramolecular collagen cross-links [16,17] in natural cross-linking of collagen over time. Later modifications of the collagen matrix have important structural and mechanical consequences, and the degradation of cross-links can lead to severe loss of tissue properties [18]. Collagen cross-linking is considered an effective approach to enhance the insolubility and resistance of the demineralized dentin matrix to endogenous enzymes [19–21] and to obtain a stable hybrid layer with improved mechanical strength [22]. It has been shown that the stability of collagen fibrils can be artificially increased by pretreatment of acid-etched dentin with cross-linking agents [22]. Cross-linking collagen increases its resistance to collagenases by preventing unwinding of the triple elix [23]. Various compounds have been studied [24–26]. Glutaraldehyde and grape-seed extracts [19,27] have been proposed as cross-linking agents, even though glutaraldehyde can be toxic [28]. As an alternative, 0.1% riboflavin applied to acid-etched dentin followed by UVA light exposure for 2 min prior to resin-bonding was proposed to inhibit dentin MMPs and increase the hybrid layer stability by creating cross-links [29]. In previous studies, the application of 0.1 or 1% riboflavin to demineralized dentin treated with UVA or a dental blue light doubled the ultimate tensile strength of collagen and reduced the amount of hydroxyproline release after bacterial collagenase challenge [30]. Carbodiimide compounds represent the most popular and versatile agents for cross-linking the carboxylic moieties bonded to the side chains of collagen. In particular, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), an imide-based zero-length cross-linking agent, has been proved to be effective for cross-linking dentin collagen and altering the three-dimensional structure of endogenous collagen-degrading enzymes [22] (Fig. 1 [31]). Thus, EDC is able to inhibit the enzymatic degradation of exposed collagen fibrils within the hybrid layer, thereby improving bond durability over time.

Thermal stability of type-I collagen in connective tissues [32,33] and dentin [26] [34–36] has been previously investigated by differential scanning calorimetry (DSC), a sensitive tool for monitoring the thermal denaturation process of heated collagen. During heating of collagen, hydrogen bonds are destroyed and the thermally induced structural transitions of the collagen network [37] can be monitored by DSC by noting the temperature associated with an endothermic DSC peak. Thermal energy is absorbed when collagen is converted to gelatin. This is analogous to the glass transition temperatures of synthetic peptides.

Thus, the aim of this study was to assess the effect of EDC on the thermal stability of dentin collagen. The null

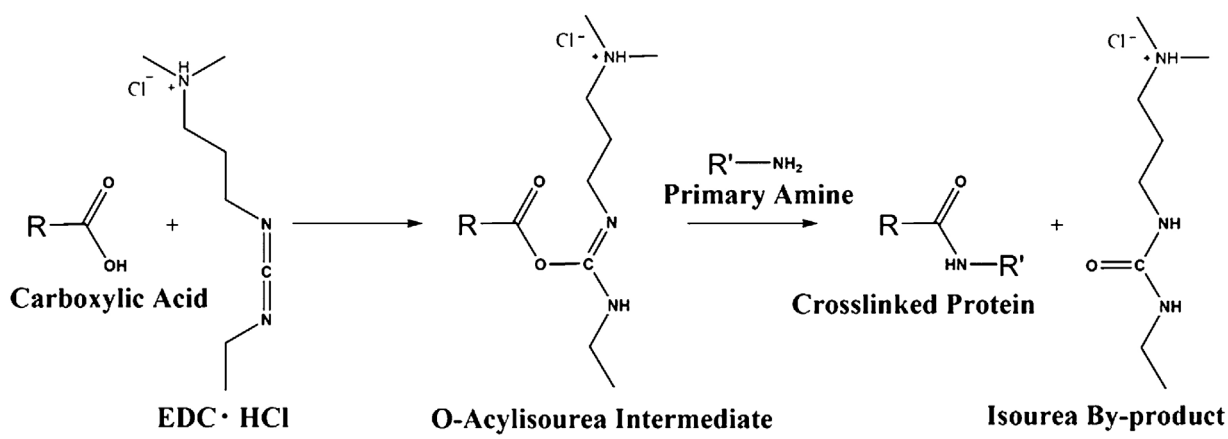

Fig. 1 – Chemical mechanism of collagen cross-linking promoted by EDC. EDC first reacts with free carboxylic acid moiety of a peptide, generating the O-acylisourea intermediate, an unstable compound. The intermediate quickly reacts with a primary amine group of another peptide forming an amidic bond. The chemical reaction leads to the formation of isourea by-product (*R and R′ indicate aliphatic moieties of the aminoacids*) [31].

hypotheses tested were that (1) there is no difference in the thermal degradation resistance of dentin collagen after treatment with an EDC water solution; (2) the effect of EDC is unrelated to concentrations and exposure times.

2. Materials and methods

2.1. Dentinal collagen preparation

Dentin slabs (1 mm thick, 6 mm in diameter) were obtained from human third molars collected after patient's informed consent was obtained under a protocol approved by the University of Trieste (Italy), stored in 0.5% chloramine in water at 4 °C and used within 1 month after extraction. Each specimen was immersed in 10% H_3PO_4 solution for 24 h to completely demineralize its collagen, and then carefully rinsed in MilliQ water in agitation for at least other 24 h.

To verify the complete demineralization of dentin, the intensity of the phosphate mineral peaks was monitored by Fourier Transform Infrared (FTIR) spectroscopy (Nicolet 6700, Thermo Scientific, Milan, Italy). A preliminary FTIR spectrum of each specimen was acquired before the immersion in 10% H_3PO_4 solution and correlated to a spectrum acquired after the demineralization process. Each dentin slab was placed on the diamond crystal of a horizontal attenuated total reflectance (ATR) stage positioned in the optical compartment of a FTIR spectrophotometer at room temperature. Infrared (IR) spectra were obtained between 4000 and 525 cm^{-1} at 6 cm^{-1} resolution. The complete dissolution of the mineral was verified by the disappearance of the most evident mineral peak (PO_4^{3-} at 1004 cm^{-1}) on both sides of each disk. To ensure that all the depth of the specimens was demineralized, 5 additional dentin disks were perpendicularly cut and analyzed with the ATR-FTIR along the cut, i.e. the disk thickness.

2.1.1. Differential Scanning Calorimeter analysis (DSC)

The tested cross-linking agent was N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, (EDC) (FM = 191.70 g/mol, CAS number 25952-53-8, Sigma–Aldrich,) in aqueous solution at different concentrations: *Group 1*: demineralized dentin collagen treated with 0.5 M EDC; *Group 2*: demineralized dentin collagen treated with 1 M EDC; *Group 3*: untreated demineralized dentin control.

Each demineralized dentin collagen slab was radially sectioned in six parts and dried in anhydrous atmosphere with SiO_2. One specimen was analyzed with a differential scanning calorimeter (DSC, Q10 TA Instruments, New Castle, DE, USA) without EDC treatment, to obtain the thermal denaturation temperature (T_d) of untreated dehydrated collagen (control). The other five pieces of each slab were kept immersed in the assigned EDC solution for 10, 20, 30 or 60 min (T_{10}, T_{20}, T_{30}, T_{60} respectively), and then rinsed for 15 min in demineralized water under agitation, dehydrated and analyzed with the DSC to obtain the T_d of EDC-treated anhydrous collagen. Five repetitions for each test condition were made.

Data were statistically analyzed with a one-way ANOVA and Tukey's post hoc test and *t*-test for repeated measures at $\alpha = 0.05$.

2.2. Energy Dispersive X-Ray Spectroscopy (EDS)

Both control and EDC-treated specimens were analyzed to obtain detailed information of the elemental composition of both treated and untreated dentin using a field emission environmental Scanning Electron Microscope (FEI Quanta 200, FEI Hillsboro, Oregon, USA) equipped with EDAX X-ray energy dispersive spectrometer (EDAX Inc., Mahwah, NJ, USA).

Briefly, freshly EDC-treated and dehydrated dentin slabs were placed onto the microscope's aluminum specimen stubs, previously covered with a conductive carbon adhesive disk (TAAB Ltd, Berks, England). The microscope was used in low vacuum mode at 30 kV accelerating voltage, at a magnification of 10,000×. The spectrometer unit was equipped with an ECON (Edax Carbon Oxygen Nitrogen) 6 Ultra-Thin Window X-ray detector, which is able to detect any chemical element over the atomic number 5 (boron) of the periodic table of elements. The analysis system was controlled by dedicated analysis software (Genesis, EDAX Inc., Mahwah, NJ, USA). Each specimen was measured three times with a time count of 100 s, and an amplification time of 51 μs and results were expressed as a percentage in relation to the total counts collected.

EDS results were analyzed with the Mann–Whitney U-test (level of significance, $p < 0.05$). The level of significance was adjusted according to the Bonferroni's correction.

3. Results

3.1. Differential Scanning Calorimeter analysis

Representative DSC thermograms of the tested specimens are shown in Fig. 2. Endothermal peaks associated with the helix-coil transition due to thermal disruption of hydrogen bonds, which stabilize the collagen triple helix, were detectable in all specimens.

The T_d of untreated and EDC-treated demineralized dentin collagen specimens are summarized in Table 1. EDC-treated dentin collagen showed a significantly higher T_d than the untreated one when 0.5 M EDC and 1 M EDC were applied for 10 min or longer ($p < 0.05$).

3.2. EDS analysis

The complete dentin demineralization of specimens was confirmed by the EDS analysis, in which no calcium could be detected. In Table 2, the relative amount of each detectable element is reported for specimens after 20 min and 60 min of EDC cross-linking. A significant increase of sulfur groups was detectable in specimens treated with both 0.5 M and 1 M EDC solutions at the longest tested EDC incubation time (60 min) ($p < 0.05$). Additionally, EDC-treated specimens showed a significant presence of chloride (completely absent in controls) ($p < 0.05$), especially at T_{60}. On the contrary, the relative amounts of nitrogen and oxygen were not significantly modified by EDC treatments ($p > 0.05$). A great amount of carbon was found in all specimens, but it was not affected by EDC application ($p > 0.05$).

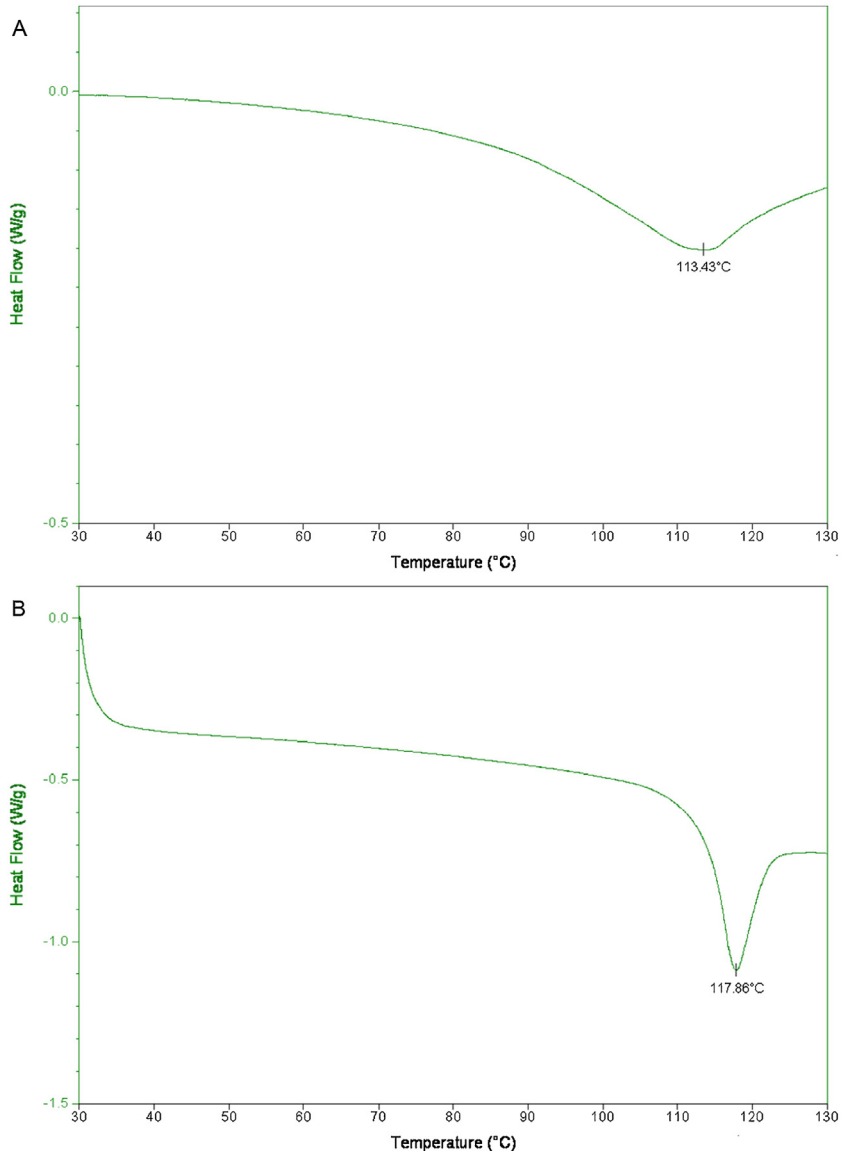

Fig. 2 – Illustrative DSC thermograms of a control specimen (A) and an EDC-treated one (B): the endothermal peak represents the thermal degradation temperature of collagen.

4. Discussion

The first test hypothesis tested that the use of EDC aqueous solutions would not modify the dentin collagen T_d and the composition of dentin matrix was rejected. The second tested hypothesis that EDC-induced modifications are unrelated to EDC concentration and treatment time was also rejected as both EDC concentrations increased the T_d at all application times.

An increased T_d can be considered an indirect indicator of a more highly cross-linked collagen network, thus the results

Table 1 – Means and standard deviation of denaturation temperature (T_d) of collagen with EDC in aqueous solution expressed as °C.

Group	T_{10} (°C)	T_{20} (°C)	T_{30} (°C)	T_{60} (°C)
Group 1:EDC 0.5 M	119.8 (8.5)^{bA}	122.8 (3.2)^{bA}	120.3 (3.6)^{bA}	120.0 (5.1)^{bA}
Group 2: EDC 1 M	120.9 (3.2)^{bA}	127.5 (6.9)^{cB}	125.2 (3.6)^{bB}	123.5 (2.5)^{bB}
Group 3: Control		111.7 (3.3)^{a}		

Different lowercase superscript letters indicate statistically significant differences between groups (in columns) at each interval ($p < 0.05$). Different uppercase superscript letters indicate statistically significant differences between time intervals in each group ($p < 0.05$).

Table 2 – Means and standard deviations of EDS elemental detection. All values are expressed as percentage in relation to the total amount of detectable elements.

	Carbon	Nitrogen	Oxygen	Phosphorous	Sulfur	Chloride
0.5 M EDCT_{20}	62.22 ± 5.63^{a}	16.86 ± 3.21^{a}	19.88 ± 2.68^{a}	0.34 ± 0.04^{a}	0.40 ± 0.15^{a}	0.44 ± 0.19^{a}
0.5 M EDCT_{60}	63.78 ± 1.21^{a}	14.72 ± 0.46^{a}	18.90 ± 0.50^{a}	0.43 ± 0.09^{a}	0.87 ± 0.05^{b}	1.24 ± 0.14^{c}
1 M EDC T_{20}	63.22 ± 3.77^{a}	14.87 ± 2.05^{a}	19.82 ± 1.92^{a}	0.34 ± 0.06^{a}	0.58 ± 0.07^{a}	0.88 ± 0.16^{b}
1 M EDC T_{60}	63.37 ± 3.17^{a}	15.02 ± 2.40^{a}	18.05 ± 1.70^{a}	0.35 ± 0.02^{a}	0.64 ± 0.15^{b}	2.57 ± 0.83^{d}
Control	57.58 ± 2.07^{a}	20.29 ± 1.71^{a}	21.17 ± 0.45^{a}	0.39 ± 0.09^{a}	0.53 ± 0.11^{a}	N/A

Different lowercase superscript letters indicate statistically significant differences between groups (in columns) ($p < 0.05$).

of this study showed that 0.5 and 1 M EDC could improve the thermal resistance of dentin collagen. The results are in accordance with previous studies, which demonstrated that EDC application increased the nanomechanical properties of the dentin matrix [38] and the stability of dentin matrix and dentin–resin interfaces [26].

EDC has a very low cytotoxicity [17] and differs from other collagen cross-linkers because it promotes a direct aminoacid-to-aminoacid cross-linking without any interposition of external molecules (i.e. glutaraldehyde remains entrapped as a spacer between the fibrils forming a bridge) [27]. Carbodiimides work by activating the free carboxyl groups for direct reaction with primary amines via amide bond formation. No portion of the carbodiimide molecule becomes part of the final bond between conjugated molecules: thus EDC should not be named in literature as a "cross-linker" but more correctly as "cross-linking promoter" instead. The dentin collagen reinforcement through EDC cross-linking can increase the resin–dentin bond strength and the stability of the resin–dentin interface over time, improving its resistance to the enzymatic and/or hydrolytic degradation [17]. EDC contains a functional group with the formula RN=C=NR and reacts with the carboxylic acid moiety to form an active O-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture (Fig. 1). The primary amine forms an amide bond with the original carboxylic group, and the EDC by-product is released as a soluble urea derivative. EDC may reduce the molecular mobility of the active site or transform negatively charged ionized carboxyl groups into positively charged amides, thus inactivating the active sites of dentin proteases [26,39]. Moreover, EDC can cross-link both helical and especially telopeptide domains in collagen and may also inhibit the telopeptidase activity [3]. Increased collagen stiffness may prevent MMPs from "unwinding" collagen peptides [40]. This process is necessary for the MMPs catalytic site to cut the individual collagen peptides [1,11,41], thus EDC can also effectively inhibit MMPs activity by stiffening their substrate.

EDC pretreatment of demineralized dentin matrices has been shown to increase the mechanical properties of the dentin matrix [22] and to completely inhibit the endogenous protease activity when applied for 1 min on acid-etched dentin [34]. Previous zymographic studies showed that the pre-treatment of acid-etched dentin with a 0.3 M EDC containing conditioner could completely inhibit the collagenolytic activity of endogenous enzymes (MMPs) [26], even after adhesive application [39].

The temperature required to thermally denature collagen treated with EDC was previously investigated. Scheffel et al. [42] applied 0.5 M, 1 M or 2 M EDC on dentin collagen and used DSC to investigate its thermal properties after the EDC treatment, showing that all the EDC solutions increased the thermal stability of demineralized collagen. In another paper (Safandowska and Pietrucha [43] collagen from fish skin was treated with EDC and analyzed with DSC. These authors observed that EDC effectively cross-linked fish collagen and increased its T_d. Nevertheless, in those cited studies, dentin collagen was investigated in its hydrated condition. Hydrated collagen shows much lower T_d (e.g. 67 °C) compared to dry collagen. Bound water, bonded to specific sites of collagen, fills the intermolecular spaces [44] and stabilizes collagen [45]. In our study, the action of EDC was investigated isolating its effect from the contribution of collagen intrinsic water. It is hypothesized that in hydrated dentin the packing density of fibrillar collagen molecules is so high that prevents the replacement of interfibrillar bound water from an external agent such as adhesive monomers. To avoid the complicating effects of water, we pre-dried demineralized dentin. In this way the thermal energy needed to denature the collagen structure detected by DSC could be assigned to the chain–chain interactions only [32].

Changes in the composition of the dentin collagen matrix were also induced by EDC treatment. Carbon, nitrogen, phosphorous and oxygen were present both in the control and EDC treated specimens. The large amount of carbon in all specimens was due to the high signal derived from the carbon adhesive tape in addition to the specimen's carbon coating. EDC-treated specimens showed a significant presence of chloride, which was not detectable in the untreated demineralized group. The detection of chloride, which is a constituent of EDC, was highest in specimens incubated with EDC for the longest time of incubation (i.e. 60 min) (Table 2). Completely demineralized dentin contains 30% matrix, 70% water. When incubated with 0.5 or 1 M EDC, the EDC would diffuse into the water-filled matrix. As EDC is a hydrochloride salt, 1 M EDC would contain 1 M chloride ions, which is a very high concentration. Even though the excess EDC was rinsed from the treated disks with water for 15 min, trace amounts of EDC-hydrochloride may have remained bound to the matrix. Demineralized collagen fibrils are known to bind many compounds (e.g. chlorhexidine digluconate) that are not easily rinsed off by water [46]. Table 2 shows that when specimens were treated with 0.5 M EDC for 20 min, $0.44 \pm 0.19\%$ chloride was found; when specimens were treated for 60 min (three times longer than the 20 min specimens), the chloride levels

increased 3-fold. When dentin disks were treated with twice as much EDC (1 M), the specimens took up twice as much chloride. When the incubation time increased 3-fold, the chloride uptake increased 3-fold.

Sulfur groups were also significantly increased in specimens treated with EDC concentrations solutions at the longest tested EDC incubation time (60 min). The increase of sulfur could be explained considering the chemical composition of the dentin matrix. The role of chondroitin sulfate in the dentin structure is well known [47] and could be considered the sulfur source of the present experimental results. According to these observations, the effect of EDC on dentin proteoglycans and their constituents (sulfate glycosaminoglycans) must be further investigated.

Clinically, just before bonding adhesive resins to mineralized dentin, dentin is acid-etched with 37 wt% phosphoric acid for 15 s. This solubilizes all of the mineral phase from dentin to a depth of 5–10 μm. When EDC is used to cross-link such acid-etched dentin, it only has to diffuse 5–10 μm into the demineralized dentin to cross-link the matrix. Other studies have shown that 0.5 M EDC accomplishes that task in 60 s [42]. However, there are no quantitative analytical methods available that can measure increases in the stiffness of 5–10 μm of compliant demineralized matrix. This has forced us to utilize "macrohybrid layers" of dentin where slabs of dentin 1.0 mm (1000 μm) thick are completely demineralized prior to measuring increases in their thickness induced by cross-linkers [48].

In this study the tested EDC application times were quite long and not clinically applicable, but they were used as a proof-of-concept to determine the effect of EDC on dentin matrix T_d. Additional studies are currently on going to investigate shorter application times and find the optimal formulation and application time of EDC solutions to reinforce the dentin collagen network to stabilize the adhesive interface over time. Further studies are also needed to verify the effect of EDC on dentin components other than collagen.

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