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Cross-linked demineralized dentin maintains its mechanical stability when challenged by bacterial collagenase

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Abstract

The molecular structure, weight loss and mechanical properties of demineralized dentin of non-crosslinked/crosslinked by glutaraldehyde (GA) were investigated when being challenged by bacterial collagenase solution over time in the present study. Raman spectra proved that cross-linking occurred in demineralized dentin matrices after being treated with GA. Meanwhile, the weight of the cross-linked demineralized dentin matrices didn't change after being challenged by bacterial collagenase solution up to one week. However, the weight of non-crosslinked dentin collagen fell by almost 45% after degradation for 5 hr, and up to 100% after 19 hr. The tensile strength of demineralized dentin matrices didn't show a significant change after being crosslinked, while the stiffness of demineralized dentin matrices showed more improvement than that of non-crosslinked collagen. The toughness of demineralized dentin matrices decreased slightly after being crosslinked. Importantly, neither the tensile strength of GA-crosslinked demineralized dentin nor its stiffness changed over time in either control buffer or collagenase solution compared to that of noncrosslinked controls. These results suggested that improving the degree of cross-linking in dentin collagen could be one method to inhibit its biodegradation and further to increase the durability of dental restorations.

Keywords

dentin; collagen crosslinking; biodegradation; mechanical properties

Introduction

Dental caries is still a major concern around the world although its prevalence has declined over last 20 years¹. According to the literatures, about 70% of the 12- to 17-year-olds have caries, and 94% of all dentate adults have evidence of coronal dental caries¹. Dental restorations by composite resins have been used to treat/repair carious teeth. However, these dental restorations have a prohibitively high failure rate, and the dental restoration replacement accounts for about 70% of all operative dentistry^{2,3}. Secondary dental caries^{4,5} is thought to be one of the main reasons dental restorations fail in a relatively short time⁶. One primary reason for the secondary caries is the lack of a tight bond between the composite resin and the underline dentin. Failure of the bond allows bacterial enzymes, oral fluids and bacteria to infiltrate the spaces between the tooth and composite, undermining the restoration and leading to recurrent caries and pulpal inflammation⁷⁻⁹.

The breakdown of the bond at the dentin/adhesive/composite interfaces can be connected to the failure of current materials to develop a long-lasting seal to dentin¹⁰. At present, the interface bonding is based on micromechanical interlocking via adhesive penetration and entanglement of exposed collagen fibrils. The formation of this interface layer is called a resin-reinforced or hybrid layer (HL). The durability of the interface is directly related to the quality of the HL that usually forms in the presence of water. However, the dentin bonding via interlocking is very technique sensitive and difficult to control. It has been shown that the formation of HL is frequently not achieved under moist clinical relevant conditions^{11,12}. Generally, the collagen fibrils in the HL are also exposed. The stability and mechanical strength of the exposed/unprotected dentin collagen is often low, since it is subject to attack by water and bacterial enzymes.

To maintain the durability of dental composite restorations, it is very important to stabilize dentin collagen against degradation. However, so far efficient methods to inhibit collagen degradation and maintain the mechanical properties of dentin collagen against degradation are seldom. Chlorhexidine has been used to preserve the adhesive/dentin interface in the recent research work¹³. But there are doubts as to how long the chlorhexidine can be retained in the adhesive/dentin interface¹⁴ since chlorhexidine is difficult to be preserved in adhesive resin. Glutaraldehyde, proanthocyanidin and genipin have been used as cross-linkers to treat dentin and the effects of these cross-linkers on mechanical properties of dentin have been evaluated^{15,16}. It was reported^{15,16} that the ultimate tensile strength of dentin increased after treated with these cross-linkers. However, it is not clear that if the cross-linked dentin collagen can improve collagen degradation resistance or maintain the mechanical properties when being challenged by bacterial collagenase solution over time. The purpose of this study was to investigate the effects of collagen cross-linking on the stability and mechanical properties against degradation. The tested null hypothesis is that glutaraldehyde crosslinking of demineralized dentin matrices does not produce any resistance to collagenase challenge.

Materials and Methods

Sample preparation

Thirty-six non-carious, human third molars were collected according to the protocol approved by the UMKC adult health sciences institutional review board. After removing any remaining soft tissues, the extracted teeth were stored at 4°C in 0.9% PBS buffer with 0.002% sodium azide added to inhibit bacterial and fungal contamination (pH 7.4). A slow-speed water cooled diamond saw (Buehler Ltd., Lake Bluff, IL, USA), was used to remove the crowns and roots from the molars. The obtained dentin stubs were sectioned into dentin beams of 0.8 mm × 0.8 mm × 10 mm along the long axis of the tooth. These dentin beams were demineralized with 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 7.4) for 1 week and the EDTA solution was changed 1 time per day. Every demineralized dentin beam was washed with distilled water for 1 minute and immersed into distilled water for 2 hours. This washing process is repeated two times to make sure the EDTA has been removed thoroughly.

The half of the above dentin collagen beams were well immersed into the crosslinking solution for 1 hour at 25°C. The solution contains 10% GA crosslinking reagent in TESCA buffer (pH = 7.4) (TES: N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid; TESCA: TES+CaCl₂). The cross-linked dentin collagen slabs were washed with distilled water and immersed into distilled water for 30 min before the degradation tests.

Enzymatic biodegradation solution

First of all, the TESCA buffer solution was prepared by adding 11.5 g TES in 900 mL distilled water; 50 mg sodium azide and 53 mg CaCl_2 were also added and the pH value of the solution was adjusted with a NaOH aqueous solution to ~7.4 at room temperature. The final volume of the solution was adjusted to 1 liter with distilled water. Then, 160 mg collagenase from *Clostridium histolyticum* (EC 3.4.24.3, P/N C-0130; Sigma Chemical Co., St. Louis, MO) with an activity of 253 CDU/mg solid was dissolved in 1 liter of a TESCA buffer solution. Finally, this biodegradation solution with a 40 CDU/ml was aliquoted and stored at -20°C .

Enzymatic biodegradation process of demineralized dentin specimens

The above demineralized dentin beams were randomly selected for the biodegradation study and immersed in the above biodegradation solution for various durations in an incubator. After degradation, the beams were rinsed three times with distilled water, each rinse lasting one minute. The rest of the beams in the buffer solution were used as controls. The wet mass of the beams was measured. For each measurement, excess water of each dentin beam was removed from the surface with absorbing tissue (Kimwipes, Kimberly-Clark Global Sales, Inc, GA, USA). The mass of beams before and after degradation were monitored with the METTLER TOLEDO balance (AG285, Switzerland). The specimens could be classified into four groups: A), dentin collagen beams without GA treatment (without being challenged by the collagenase solution); B), dentin collagen beams without GA treatment (being challenged by the collagenase solution); C), dentin collagen beams with GA treatment (without being challenged by the collagenase solution); D), dentin collagen beams with GA treatment (being challenged by the collagenase solution).

Raman spectroscopy

A LabRam HR 800 Raman spectrometer (HORIBA JOBIN YVON, Longjumeau, Paris, France) using monochromatic radiation emitted by a He-Ne laser (632.8 nm) was used to collect Raman spectra. It was equipped with a confocal microscope (Olympus BX41), a piezoelectric XYZ stage with a minimum step width of 50 nm, and an air-cooled CCD detector of 1024×256 pixels. The following parameters were used: 600 grating, 140- μm confocal hole, and 100- μm slit width. Spectra were Raman-shift-frequency-calibrated using known lines of silicon. Raman spectra were acquired by focusing the laser beam through a 100X Olympus water immersion objective to a $\sim 1 \mu\text{m}$ beam diameter onto the specimens. Two consecutive scans of spectra (with 60 seconds accumulation time each) were obtained from each measurement. No post-processing of the data was performed.

Mechanical testing

Mechanical properties of the beams before and after cross-linking, or before and after enzymatic degradation were measured using SSTM-5000 mechanical tester (United Calibration Corporation, CA) with a 150 lb load cell at a speed of 0.5 mm/min after the beams were tightly and fully attached to the upper and lower grips using cyanoacrylate adhesive (Zapit, Dental Ventures of America, Corona, CA). A typical stress-strain curve obtained in this study is shown in Fig. 1. Many mechanical properties such as the ultimate tensile strength, Young's modulus and toughness can be calculated from the resulting stress-strain curve.

The ultimate tensile strength (UTS) of each specimen was measured by dividing the maximum force at the point of failure (Correspondingly, it is the peak value on a stress-strain curve) with the specimen cross-sectional area. In addition, Young's modulus (or modulus of elasticity) is defined as the ratio of the linear stress to the linear strain in the

range of stress in which Hooke's Law holds. It can be experimentally determined from the slope of a stress-strain curve generated during a tensile test as shown in Fig. 1. Specifically, the initial region is related to the straightening of the coiled fibrils. The subsequent linear region corresponds to the elastic lengthening of the fibrils, in which its slope is defined as the Young's modulus of the dentin collagen. Its value can be calculated using following formulations:

$$\sigma_e = \frac{P}{A_0} \quad \varepsilon_e = \frac{\delta L}{L_0}$$

$$\sigma_e = E \varepsilon_e \Rightarrow E = \frac{\delta \sigma_e}{\delta \varepsilon_e}$$

Where P is the load applied on the specimen. A_0 is the original specimen cross-sectional area. σ_e is the stress calculated by dividing P with A_0 . L_0 is the original specimen length. δL is the displacement after applying load P . ε_e is the strain calculated by dividing P with A_0 . E is the Young's modulus. $\delta \sigma_e$ and $\delta \varepsilon_e$ are the variants of stress and strain in the linear range of the curve respectively as shown in Fig. 1.

Toughness values were calculated by taking the area (i.e., by taking the integral) underneath the stress-strain curve as shown in Fig. 1, based on the following formulation:

$$U^* = \frac{1}{V} \int_0^L P dL = \int_0^L \frac{P}{A_0} \frac{dL}{L_0} = \int_0^\varepsilon \sigma d\varepsilon$$

Where U^* is strain energy; V is the volume of specimen. Toughness is the resistance to fracture of a specimen when strained. It is defined as the amount of energy per volume that a specimen can absorb before rupturing.

Results

Typical Raman spectra of dentin collagen beams with and without GA treatment, after being kept in a buffer solution or a degradation solution for 5 hr respectively, are shown in Fig. 2. The peaks at 1246/1270 cm^{-1} , 1450 cm^{-1} and 1667 cm^{-1} are assigned to amide III, CH and amide I from collagen. The position and intensity of these amide I and III peaks are sensitive to the molecular conformation of the polypeptide chains. The similarities of the four spectra in these regions indicate that the molecular structure of collagen beams was not noticeably altered after GA treatment and the degradation challenge. One major change in spectra was observed in the region around 1030 cm^{-1} , which is believed to be associated with GA treatment (Fig. 3). The spectra were normalized based on the peak at 1003 cm^{-1} which is assigned to C-C bond in the phenyl group. This peak is used for normalization because it is unchanged during the cross-linking treatment. In the spectra from the non-crosslinked group, the peak appeared at 1031.7 cm^{-1} is related to the C-C in pyridium ring¹⁷. However, it shifts to 1032.7 cm^{-1} in the specimens of the crosslinked group. The relative intensity of this peak is also increased after the crosslinking treatment (Fig. 3). The mean intensity ratios of the peak at ~1032 cm^{-1} to 1003 cm^{-1} from four experiment groups are listed in Table 1.

The weight loss of non-crosslinked and crosslinked demineralized dentin matrices after being kept in a buffer solution or a degradation solution for different time is shown in Fig. 4. As expected, the weight of both the non-crosslinked and crosslinked beams did not change after kept in the PBS buffer (control) solution for 5, 19 and 168 hr (Fig. 4A). To study the biodegradation resistance, the above crosslinked and non-crosslinked specimens were kept in the collagenase (degradation) solution for the same time periods. For non-crosslinked collagen, the weight loss was almost 45% after in the degradation solution for 5 hr (Fig. 4B).

However, there was no weight loss for the crosslinked collagen. At 19 hr, the weight loss for non-crosslinked collagen was 100%; no materials were left for mechanical testing. For crosslinked collagen, only minimum weight loss was observed after a long time (168 hr) immersion in the degradation solution.

Non-crosslinked and crosslinked collagen beams were also tested for the stress-strain properties after being kept in a buffer solution or a degradation solution for different time. The representative stress-strain curves obtained are shown in Fig. 5. The crosslinked dentin collagen tends to become rigid as compared to the non-crosslinked collagen. In addition, in the buffer solution, the stress-strain behavior for cross-linked collagen was similar as a function of time. However, for non-crosslinked collagen, the stress-strain behavior varied after storing in the solution for different time. In the degradation solution, the stress-strain behavior for crosslinked collagen did not change as a function of time, which was similar to that in the buffer solution. However, for non-crosslinked collagen, only the stress-strain behavior at 5 hr was measured. No collagen left for the stress-strain measurement at 19 and 168 hr.

Based on the stress-strain curves, the ultimate tensile strength (UTS), Young's modulus and toughness values were calculated, and presented in Figs. 6–8 respectively. The UTS values of non-crosslinked and crosslinked collagen were similar (~ 7 MPa) (Fig. 6A), indicating crosslinking did not affect the collagen's tensile strength. These values also did not change as a function of time in the buffer solution. In the degradation solution, however, the UTS of non-crosslinked collagen largely decreased from ~ 7 MPa to ~ 4.5 MPa after degraded for 5 hr. In comparison, the UTS of crosslinked collagen didn't change when being challenged by bacterial collagenase solution (Fig. 6B), indicating collagen crosslinking stabilized mechanical strength.

The Young's modulus values of non-crosslinked and crosslinked dentin collagen after being kept in a buffer solution or a degradation solution for different time are shown in Fig. 7. It was shown that in the buffer solution, there were no significant differences in modulus at 5 hr between non-crosslinked and crosslinked collagen. However, the modulus of non-crosslinked collagen decreased dramatically from 62.8 ± 13.2 MPa at 5 hr to 38.8 ± 8.7 MPa at 168 hr, even though storage in the buffer solution (Fig. 7A). There was no significant decrease in Young's modulus for crosslinked collagen both in the control buffer solution and the degradation solution (Fig. 6A and B). The results indicated that crosslinking enhanced collagen's mechanical stability. However, collagen crosslinking slightly affect the toughness of demineralized dentin matrices (Fig. 8). The average toughness of non-crosslinked collagen was ~ 0.6 MN/m² and the value was decreased to ~ 0.4 MN/m² after the cross-linking treatment (Fig. 8A). Both the values didn't change after storage in the buffer solution for different time. In the degradation solution, the toughness of the non-crosslinked collagen decreased from ~ 0.6 to ~ 0.3 MN/m² after 5 hr storage (Fig. 8B). However, similar to other stress-strain properties, the toughness value of cross-linked group didn't change after storage in the degradation solution for different time.

Discussion

The Raman results indicate that dentin matrices develop additional cross-links following glutaraldehyde (GA) treatment. It was shown that after immersion in the GA containing solution for 1 hr, an increase in the peak intensity at $1030\text{--}1033\text{ cm}^{-1}$ was observed (Fig. 3). According to the previous literatures^{17–20}, this peak is assigned to the C-C in a 3-hydroxypyridinium ring, which is associated with a non-reducible cross-linking formation naturally occurred in collagen. While the GA crosslinking mechanism is complicated, the crosslinker undergoes similar crosslinking reactions, which generally involve Schiff base

formation between an aldehyde of GA with the amine of (hydroxyl)lysine residue of the collagen (as illustrated in Fig. 3). This is why an increase in the peak intensity at 1030–1033 cm^{-1} (solid line) was observed as compared to the collagen without GA treatment (dashed line).

The observed peak shift (from 1031.7 to 1032.7 cm^{-1}) possibly stems from the different source of aldehyde group (For natural cross-linking, the aldehydes come from conversion of epsilon-amino groups of lysine in collagen peptides to aldehydes. However, for the cross-linking formed by GA, the aldehydes are supplied by the dialdehyde glutaraldehyde). Based on the above results, it turns out that this peak at 1030–1033 cm^{-1} becomes a rapid screening method to check the interaction between crosslinking agents and collagen. Raman spectroscopy is a very powerful tool for studying the structure and molecular interactions of many complex biomolecules. One of the great advantages is its ability to provide information about the structure and interactions of biomolecules in their microenvironments nondestructively, without homogenization, extraction or the use of dyes or other contrast-enhancing agents. This technique will be used to study the real time interactions between crosslinkers and dentin collagen in the dentin bonding in our future studies.

Crosslinked dentin matrices dramatically improved the resistance of collagen to bacterial collagenase, and maintain matrix mechanical stability during challenge by high concentrations of exogenous collagenase. These results require rejection of the test null hypothesis that glutaraldehyde crosslinking of demineralized dentin matrices does not produce any resistance to collagenase challenge. This is a very exciting result of our study. For non-crosslinked collagen, the weight loss was almost 45% after in the degradation solution for 5 hr (Fig. 4B). At 19 hr, the weight loss for non-crosslinked collagen was 100%, no materials even were left for stress-strain tests. However, there was only neglectable weight loss for the crosslinked collagen after a long time storage (168 hr). It should be pointed out that the bacterial collagenase concentration used in this study is stronger than that in the mouth. Therefore, the crosslinked dentin collagen should have sufficient resistance to withstand the digestion under in vivo conditions. These results are very promising, thus clearly validating our approach to improved bonding durability by keeping collagen fibrils from degradation.

Traditionally, dentin matrix collagen degradation was thought to be caused by oral bacteria²¹ or the protease they secreted²². Recently, It is^{23,24} reported that matrix metalloproteinases (mostly MMP-2, MMP-8 and MMP-9), a class of zinc- and calcium-dependent endopeptidases, play an important role in the degradation process of dentin matrix²⁵ and are the main enzymes to degrade dentin collagen in the early digest process. It is well known that one of an enzyme's characteristics is its high specific activity. Based on previous literature²⁶, the cleaved spot of collagen degradation occurs at a single locus between residues 775 and 776 which correspond to Gly-Ile in each α_1 chain and one Gly-Leu in each α_2 chain. As a result, the rest of peptides are degraded into small pieces, even into individual amino acids by MMPs and proteases. The key point in this degradation process is the match of the active center of enzymes with a specific position of a peptide chain. Once this match process is inhibited by different biochemical ways, collagen degradation will be blocked.

One of efficient biochemical ways is the direct deactivation of MMPs by using MMP inhibitors. For example, chlorhexidine, a non-specific MMPs inhibitor, has been widely used to inhibit endogenous collagenolytic and gelatinolytic activities of MMPs in dentin collagen. Under in vivo conditions, it was shown that chlorhexidine treated teeth exhibited normal structural integrity of the collagen network in the dentin bonding interface as compared to the progressive breakdown of the collagen fibrils identified in the control teeth.²⁷ Collagen

crosslinking could be considered as another efficient biochemical way to block collagen from the degradation. When the glutaraldehyde crosslinker is used, the pyridine aromatic rings are formed due to the reaction between amino-groups in collagen and aldehyde groups in GA²⁸. We speculate that glutaraldehyde crosslinking of dentin matrices may prevent matrix degradation by crosslinking the binding and/or active sites of endogenous dentin MMPs and by sterically blocking access of MMPs to the specific Gly-Ile peptide bond. However, the collagen degradation resistance depends on the type of cross-links formed. Recently several other cross-linkers such as genipin, and proanthocyanidin have been used to crosslink collagen^{29,30}. Based on our tests, the cross-links formed by the naturally occurring cross-linking agent genipin are not stable and unable to prevent dentin collagen from the degradation when challenged by the collagenase solution (data not shown). The degradation resistance is greatly improved by forming the more stable pyridinium-like cross-linking between collagen and glutaraldehyde.

In this study, we have shown that collagen crosslinking by GA enhances mechanical properties of demineralized dentin matrices, and its mechanical stability is maintained after challenged by the strong degradation solution. The differences in mechanical properties of collagen, between non-crosslinked and crosslinked or before and after degradation, can be clearly seen and explained in the stress-strain curves (Fig. 5). The initial region of the stress-strain curve was defined as the “toe” region³¹. The toe region is related to the un-folding of the crimp in the collagen fibrils. The following linear region corresponds to the elastic stretching of the fibril backbone. As shown in Fig. 5, the crosslinked dentin collagen turns hard as compared to the non-crosslinked collagen. Specifically, the toe region becomes shorter and its slope gets steeper after crosslinking, indicating that the stiffness in both the initial region (the un-folding of the crimp in collagen) and subsequent linear region is increased after cross-linking. The increase in stiffness is related to the formation of exogenous crosslinks between GA and amino groups of collagen. The small size of glutaraldehyde and its aggressive nature could have induced strong inter-fibril and/or inter-peptide cross-linking reactions, which enhancing the mechanical stability of collagen.

In summary, the molecular structure, weight loss and mechanical properties of demineralized dentin of non-crosslinked/crosslinked by glutaraldehyde (GA) were investigated when being challenged by bacterial collagenase solution over time in the present study. Raman spectra proved that cross-linking occurred in demineralized dentin matrices after being treated with GA. The weight of non-crosslinked dentin collagen fell by almost 45% after degradation for 5 hrs, and up to 100% after 19 hrs. However, the collagen crosslinking by GA maintains not only the mass of the demineralized dentin matrices, but also its mechanical properties after challenged by the strong degradation solution. These results suggested that improving the degree of cross-linking in dentin collagen could be one method to inhibit its biodegradation and further to increase the durability of dental restorations.

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Fig. 1.
A typical stress-strain curve of dentin collagen beams

Fig. 2.

Representative Raman spectra of demineralized dentin matrices with and without GA treatment, after being kept in a buffer solution or a degradation solution for 5 hr respectively. a) without GA before degradation; b) with GA before degradation; c) without GA after degradation; d) with GA after degradation.

Fig. 3.

Raman spectra of demineralized dentin matrices from Fig. 2 in the region of 1120–980 cm^{-1} , showing the evidence of cross-linking. The dashed and solid lines are from the beams without GA before degradation and with GA before degradation, respectively.

Fig. 4. The weight loss of non-crosslinked and crosslinked demineralized dentin matrices stored in A), control buffer solution; B), collagenase degradation solution for various times. (* indicates $P < 0.05$, $n = 12$)

Fig. 5. Typical stress-strain curves obtained from different groups, A), in control buffer solution; B), in collagenase degradation solution for various times. The solid lines are crosslinked demineralized dentin matrices and the dashed lines are non-crosslinked demineralized dentin matrices.

Fig. 6. The tensile strength values of non-crosslinked and crosslinked demineralized dentin matrices stored A), in buffer solution; B), in collagenase solution for various times. (* indicates $P < 0.05$, $n = 10-15$)

Fig. 7. The elastic modulus values of non-crosslinked and crosslinked demineralized dentin matrices stored A), in buffer solution; B), in collagenase solution for various times. (* indicates $P < 0.05$, $n = 10-15$)

Fig. 8.

The toughness values of non-crosslinked and crosslinked demineralized dentin matrices stored A), in buffer solution; B), in collagenase solution for various times. (* indicates $P < 0.05$, $n = 10-15$)

Table 1

The intensity ratios of 1030 cm^{-1} to 1003 cm^{-1} for the four experimental groups

$R_{1030/1003}$	Before degradation	After degradation
Crosslinked	0.84 ± 0.01	0.84 ± 0.03
Non-Crosslinked	0.63 ± 0.01	0.66 ± 0.02