

# Crosslinking Improve Demineralized Dentin Performance and Synergistically Promote Biomimetic Mineralization by CaP\_PILP

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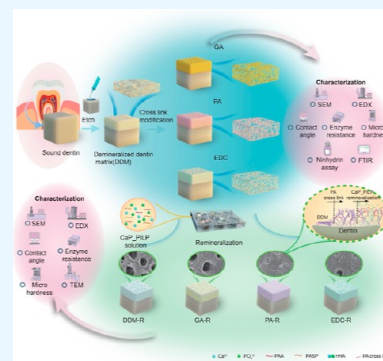
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**ABSTRACT:** Objective: to explore the effects of optimal crosslinking (chemical treatment) on demineralized dentin matrix and the possible synergism with calcium phosphate polymer-induced liquid precursor (CaP-PILP) bionic remineralization (physical treatment), and offer benefit to the clinic of resin–dentin bonding and dentin hypersensitivity. Methods: demineralized dentin was treated with glutaraldehyde (GA), carbodiimide (EDC), and procyanidin (PA) for crosslinking, followed by CaP-PILP biomimetic remineralization. The morphology, surface mechanical and physio-chemical properties, and enzymatic resistance were evaluated regardless of the modification. Results: the collagen fibers appeared morphologically complete with higher surface microhardness and characteristic peaks of amide I–III bands were visible after GA, PA, and EDC crosslinking. Collagen collapse and dissolution were seen in untreated demineralized dentin with enzyme attack, while the collagen fiber structure remained intact in GA- and PA-treated specimens. The lamellar mineral phase was visible at 2 days and the dentin tubules were almost completely enclosed at 4–6 days after PA crosslinking and mineralization. However, demineralized collagen fibers and open tubules were still visible between the dentinal tubules on day 8 in the control group. Conclusion: the structure integrity, enzyme resistance, and mechanical properties of the collagen fiber network could be significantly preserved by GA and PA crosslinking than EDC and no treatment. While, strongest synergistic effects were observed in PA on bionic remineralization by CaP-PILP, and further significantly improve the quality and shorten the duration of mineralization. These findings would be beneficial for dental clinical practice of resin–dentin bonding and dentin hypersensitivity.



## ■ INTRODUCTION

Demineralization is a multifactorial process that may extend from enamel to the dentin and cause collagen fiber degradation by the action of endogenous enzymes and hydrolysis.<sup>1-3</sup> This presents a challenge for composite resin bonding and dentin hypersensitivity.

Physical crosslinking methods, including atmospheric plasma, dehydrothermal or ultraviolet treatment, enhance the bond strength of resin–dentin hybrid layers but require special equipment and specific conditions.<sup>4–9</sup> Chemical crosslinking methods involve dentin modification using natural or synthetic crosslinking agents.<sup>10,11</sup> Carbodiimide (EDC) and procyanidin (PA) could antagonize endogenous collagenase through covalent bond formation or chelation to improve bond strength as previously reported.<sup>12–14</sup>

Biomimetic mineralization of the demineralized dentin also prevents collagen fiber degradation by encapsulating them. Studies have aimed to shorten the time of bionic remineralization and improve the quality of the hybrid layer by modifying the mineralization fluid composition.<sup>15–18</sup> The non-classical crystallization pathway theory suggests that collagen fibrils act as a template for biomimetic remineralization, while non-collagenous proteins stabilize amorphous

calcium phosphate, allowing internal and external fibrillar mineralization.<sup>19–21</sup>

GA is a molecule with high affinity for the free primary amine group of amino acids, with two aldehyde groups. These two aldehyde groups are mainly related to lysine and hydroxylysine residues on type I collagen  $\epsilon$ -amino components react to form Schiff groups, thus modifying dentin collagen fibers.<sup>10,16</sup> Gu et al. reported that crosslinking increased the agglomeration density inside and outside the collagen protofibrils and improved the molecular sieve properties of type I collagen.<sup>22</sup> Chen et al. demonstrated that the crosslinking agent glutaraldehyde (GA) may also induce calcification.<sup>16</sup> However, GA is highly cytotoxic and its clinical applications are limited. PA-modified casein phosphopeptide-amorphous calcium phosphate and tricalcium phosphate mineralization liquids have been investigated for the treatment

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of root caries.<sup>18</sup> EDC is a commonly used synthetic chemical crosslinking agent in tissue engineering but its use for bionic remineralization of demineralized dentin has not been reported. Few studies have investigated the use of crosslinking agents combined with bionic remineralization in demineralized dentin.

How to break through the obsolete application and better utilize the potential of cross-linking agents themselves, and pay attention to their subsequent possible synergistic effects on biomimetic remineralization, is generally ignored in existing studies. Considering the template role of collagen fibrils, crosslinking may improve the calcium phosphate polymer-induced liquid precursor (CaP-PILP) bionic remineralization of dentin. CaP used in this experiment based on the nonclassical mineralization crystallization theory, PILP stabilizes ACP through anionic polymers such as polyacrylic acid (PAA) and polyaspartic acid (PASP), and further induces the formation of mineral crystals in collagen fibers in high concentration calcium phosphate solution.<sup>23,24</sup> This study investigated the synergistic effects of natural, artificial, and classical crosslinkers (PA, EDC, and GA, respectively) on biomimetic remineralization to enhance dentin matrix stability.

## MATERIALS AND METHODS

**Dentin Specimen Preparation.** The study was approved by the Biomedical Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB-202164068) and written informed consent was obtained from the participants. Healthy third molars extracted from patients aged 18–40 years were collected from the Oral and Maxillofacial Surgery Department. The teeth were cleaned with saline and immediately placed in 0.5% aqueous chloramine-T solution at 4 °C for use within 1 month of extraction. The teeth were embedded and fixed in molds using red compound. Uniformly-sized dentin sections were created under water irrigation using a low-speed saw (Isomet 1000, Buehler Ltd., USA). Dentin surfaces were polished sequentially using 120, 200, 400, and 600 grit silicon carbide papers (Panda, Beijing East New Grinding Tools Co., Ltd., China) for 20 s each under flowing water.

The dentin specimens were demineralized using 10% phosphoric acid and rinsed using deionized water by repeated centrifugation and lyophilized using a freeze dryer (FreeZone, LABCONCO, USA).

**Specimen Crosslinking Treatment.** The dentin specimens were randomly divided into four groups ( $n = 20$ ) based on the surface treatment. The demineralized dentin matrix (DDM) group was not treated, while the other three groups were crosslinked for 3 min in the dark using 5% GA,<sup>25</sup> 6.5% PAs,<sup>26</sup> and 0.3/0.12 M 1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide/*N*-hydroxy succinimide (EDC/NHS),<sup>25,27</sup> respectively.

**Crosslinking Combined with Biomimetic Remineralization Modification.** The DDM group was modified by mineralization and was then known as the DDM-remineralization (DDM-R) group. Dentin specimens from the remaining three groups were placed in 1.5 mL Eppendorf tubes containing equal amounts of the mineralization solution, which were sealed and incubated at 37 °C. The specimens were then tested at 2, 4, 6, and 8 days (Table 1).

The CaP-PILP mineralization fluid was prepared as follows:<sup>28,29</sup> 0.1 M CaCl<sub>2</sub> was mixed with 0.3 g/mL of PASP (molecular weight = 6–8 kDa) to form solution A; 0.1 M

**Table 1. Subgroups and Abbreviations<sup>a</sup>**

treatment	abbreviation	remineralization period (days)	remineralization time groups
biomimetic remineralization only	DDM-R	2, 4, 6, 8	DDM-R2, DDM-R4, DDM-R6, DDM-R8
GA + biomimetic remineralization	GA-R	2, 4, 6, 8	GA-R2, GA-R4, GA-R6, GA-R8
PA + biomimetic remineralization	PA-R	2, 4, 6, 8	PA-R2, PA-R4, PA-R6, PA-R8
EDC + biomimetic remineralization	EDC-R	2, 4, 6, 8	EDC-R2, EDC-R4, EDC-R6, EDC-R8

<sup>a</sup>GA: glutaraldehyde; PA: procyanidin; EDC: carbodiimide.

Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O was mixed with 0.3 g/mL of poly acrylic acid (PAA, average molecular weight = 450 kDa) and 0.15 g/mL poly-aspartic acid, and magnetically stirred overnight to form solution B. Solution B was added dropwise to solution A while constantly stirring it. Finally, the pH of the solution was adjusted to 7.4 using 10 M NaOH.

**Scanning Electron Microscopy.** The dentin specimens before and after remineralization were dehydrated using gradient ethanol (25% ethanol for 10 min, 50% ethanol for 10 min, 75% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 30 min), dried using an automatic critical point dryer (K850 Quorum, UK), and sputter-coated with gold palladium for 60 s. Scanning electron microscopy (SEM) (S-4800, Hitachi, Tokyo, Japan) was used to observe the microstructure at an acceleration voltage of 5 kV. Energy dispersive X-ray spectrometry (EDX) (Flash 6130, Burker, Japan) was optionally used to evaluate the surface calcium and phosphorus peaks.

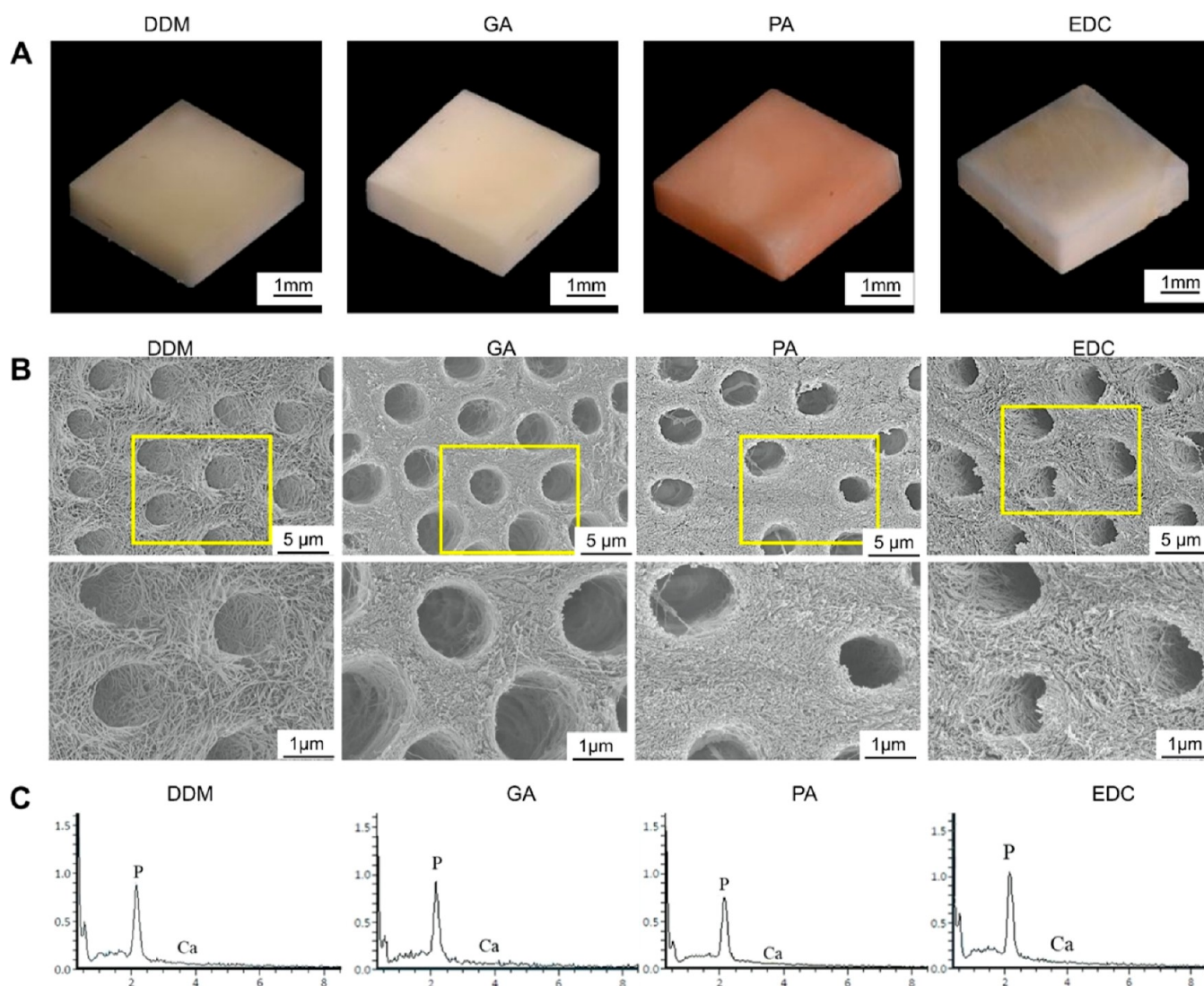
**Hydrophilic Property Analysis.** A video contact angle meter (OCA 15EC, Dataphysics, Germany) was used to measure the hydrophilicity of the demineralized dentin surface before and after remineralization for the groups. The contact angle was measured immediately after a 1 μL water drop touched the lyophilized dentin; the calculations were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Surface Microhardness.** A Venus automatic microhardness tester (Tukon 2500-6, Wilson, USA) was used to assess the demineralized dentin specimens before and after remineralization. An indenter with a 100 g load was held for 10 s to form an indentation and its diagonal length was measured microscopically (40×) to obtain the Vickers hardness value.

**Infrared Spectroscopy.** The lyophilized, demineralized dentin powder before and after remineralization was washed thrice by centrifugation with deionized water. Then, it was analyzed using Fourier transform infrared spectroscopy (Bruker, USA) for functional group analysis in the range of 400–4000 cm<sup>-1</sup> (32 scans).

**Free Amino Content.** The reaction of ninhydrin with primary amine groups of collagen was assessed using the absorption spectroscopy method to determine the free amino content. Specimens before remineralization were heated with ninhydrin solution for 20 min and the optical absorbance was measured at 570 nm using a microplate reader and known concentrations of glycine. The amount of free amino groups was proportional to the optical absorbance of the solution.

**Anti-enzymatic Degradation Properties.** Dry weight loss and the hydroxyproline (HYP) contents of the dentin



**Figure 1.** Morphology characteristics of demineralized dentin specimens with/without cross-linking. (A) Gross view; (B) microscopic appearance; (C) EDX spectrum of Ca and P.

samples after collagenase exposure were used to assess resistance to enzymatic digestion.

The collagenase solution with an enzyme concentration of 1 g/L (125 U/mL) was prepared by dissolving type I collagenase in Tris-HCl buffer (50 mM, pH = 7.4), adding  $\text{CaCl}_2$  pellets (5 mM), and stirring with a magnetic mixer for 2 h.

The specimens before and after remineralization were hydrated with deionized water for 24 h in 1.5 mL Eppendorf tubes and weighed on a microbalance ( $W_0$ ). Then, they were dried in a vacuum oven for 24 h. After adding 1 mL of 0.1% collagenase solution, the solution was placed in a constant temperature shaker at 37 °C for 48 h. Enzymatic digestion was followed by centrifugation and the upper layer of the solution was collected. The specimens were rinsed, dried, and weighed again in a vacuum drying oven ( $W_1$ ). Dry weight loss ( $L$ ) was calculated as follows:  $L (\%) = (W_0 - W_1)/W_0 \times 100\%$ . Specimen morphologies were observed using SEM.

The HYP content was quantified using a HYP assay kit (A0302-1, Nanjing Jiancheng Bioengineering Institute, China), and the optical absorbance was measured using a 550 nm microplate reader (Enspire, PerkinElmer, Waltham, MA, USA).

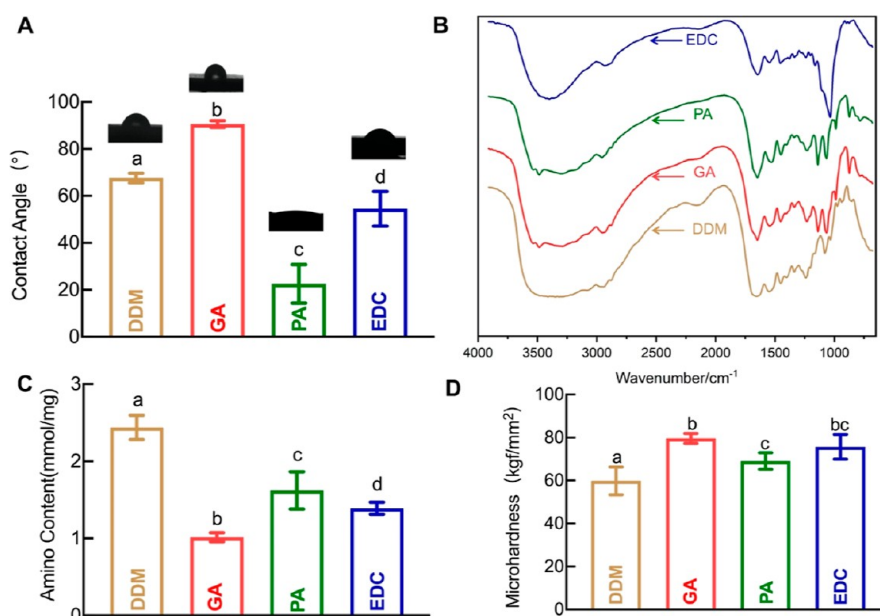
**Transmission Electron Microscopy.** The samples from each group were mineralized for 2 or 8 days and observed using transmission electron microscopy (TEM) (JEM-1400, JEM, Japan). The samples were washed using deionized water, sonicated for 1 min to remove surface debris, and rinsed thrice in phosphate-buffered saline for 10 min. The samples were fixed with 1% osmium acid at 4 °C for 2 h, rinsed thrice with double-distilled water for 10 min, dehydrated using gradient alcohol, replaced with propylene oxide, and trimmed after polymerization with pure resin embedding to produce ultrathin sections on copper mesh. They were stained using uranyl acetate and lead citrate, and observed using TEM with an accelerating voltage of 80 kV.

**Statistical Analysis.** The results were subjected to an analysis of variance using SPSS Statistics 20.0 software (IBM Corp., Armonk, NY, USA) to compare differences between means. Fisher's least significant difference method was used for multiple comparisons with a significance level of  $\alpha = 0.05$ .

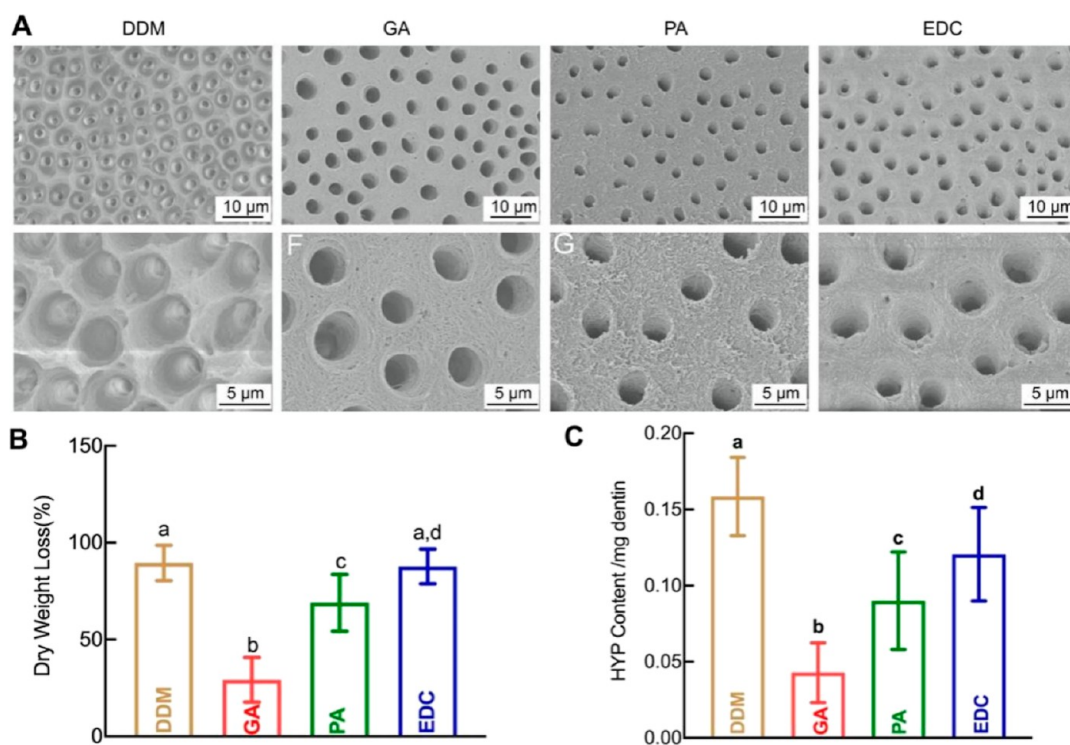
## RESULTS

**Physicochemical Properties of Crosslinked Pre-treated Demineralized Dentin. Gross Appearance.** The





**Figure 2.** Physicochemical and mechanical properties of demineralized dentin specimens with/without cross-linking. (A) Video contact angle; (B) FTIR spectra; (C) free amino content; (D) surface microhardness.



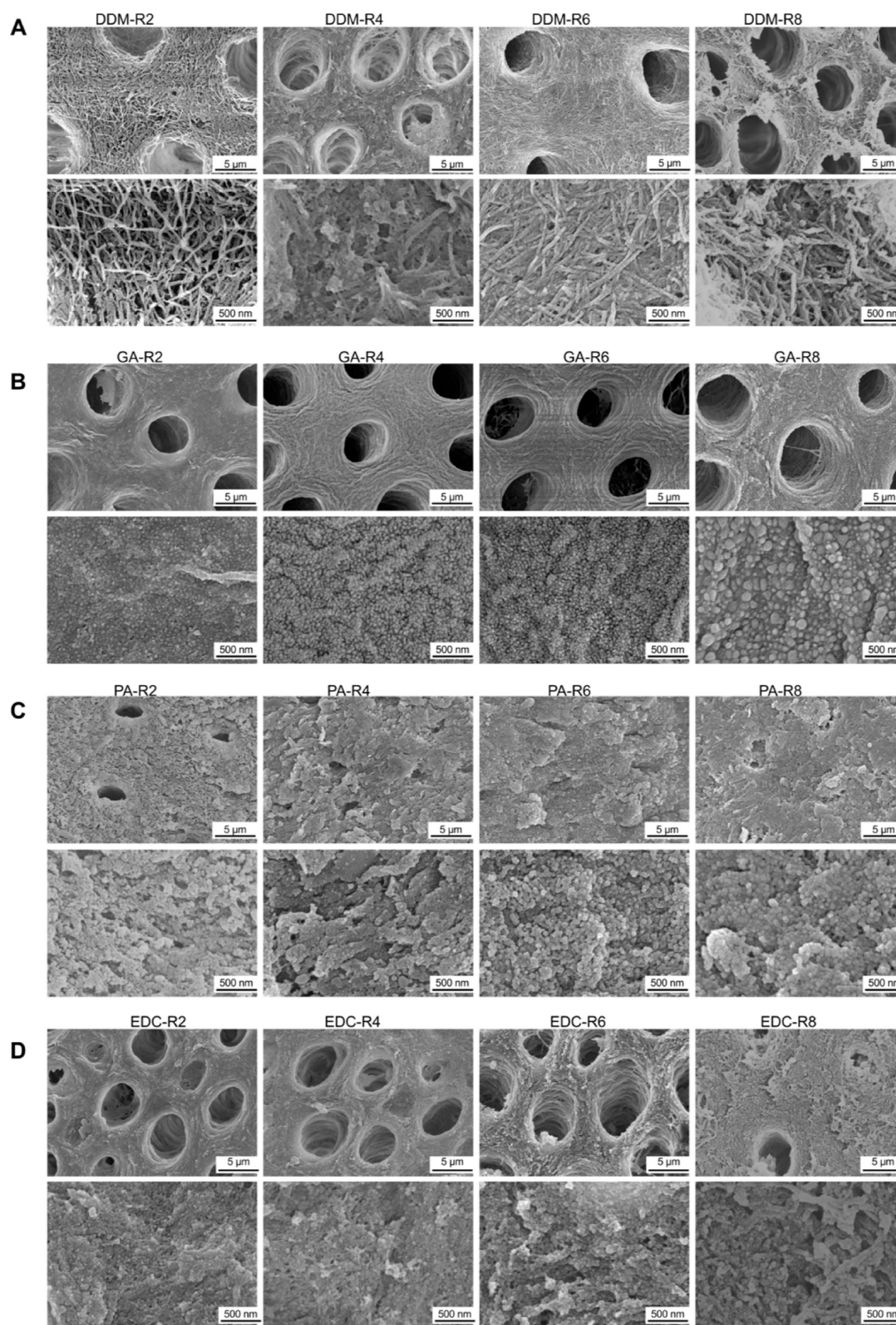
**Figure 3.** Enzymatic resistance of demineralized dentin specimens with/without cross-linking. (A) Microscopic morphology of specimens after enzyme attack; (B) dry weight loss after collagenase attack; (C) HYP content after enzyme digestion.

surface of demineralized dentin was flat and smooth regardless of crosslinking treatment. The DDM, GA, and EDC groups were light yellow, while the PA treated demineralized dentin was reddish-brown (Figure 1A).

**Microscopic Morphology.** The dentin tubules and reticulated collagen fibrils were visible on the dentin surface under SEM. The gap between the collagen fibrils was larger in the DDM group compared to the other groups, which had gaps similar to each other (Figure 1B). EDX showed that the

calcium peaks disappeared in all four groups, while there were no significant differences in the phosphorus peaks (Figure 1C).

**Hydrophilic Properties.** The representative video contact angle image and analysis of all groups were given in Figure 2A. The largest contact angle was found in the GA group, followed by the DDM and EDC groups. The mean angle was significantly influenced by different treatments; specifically, the mean angle of the PA group declined dramatically compared with the other groups.

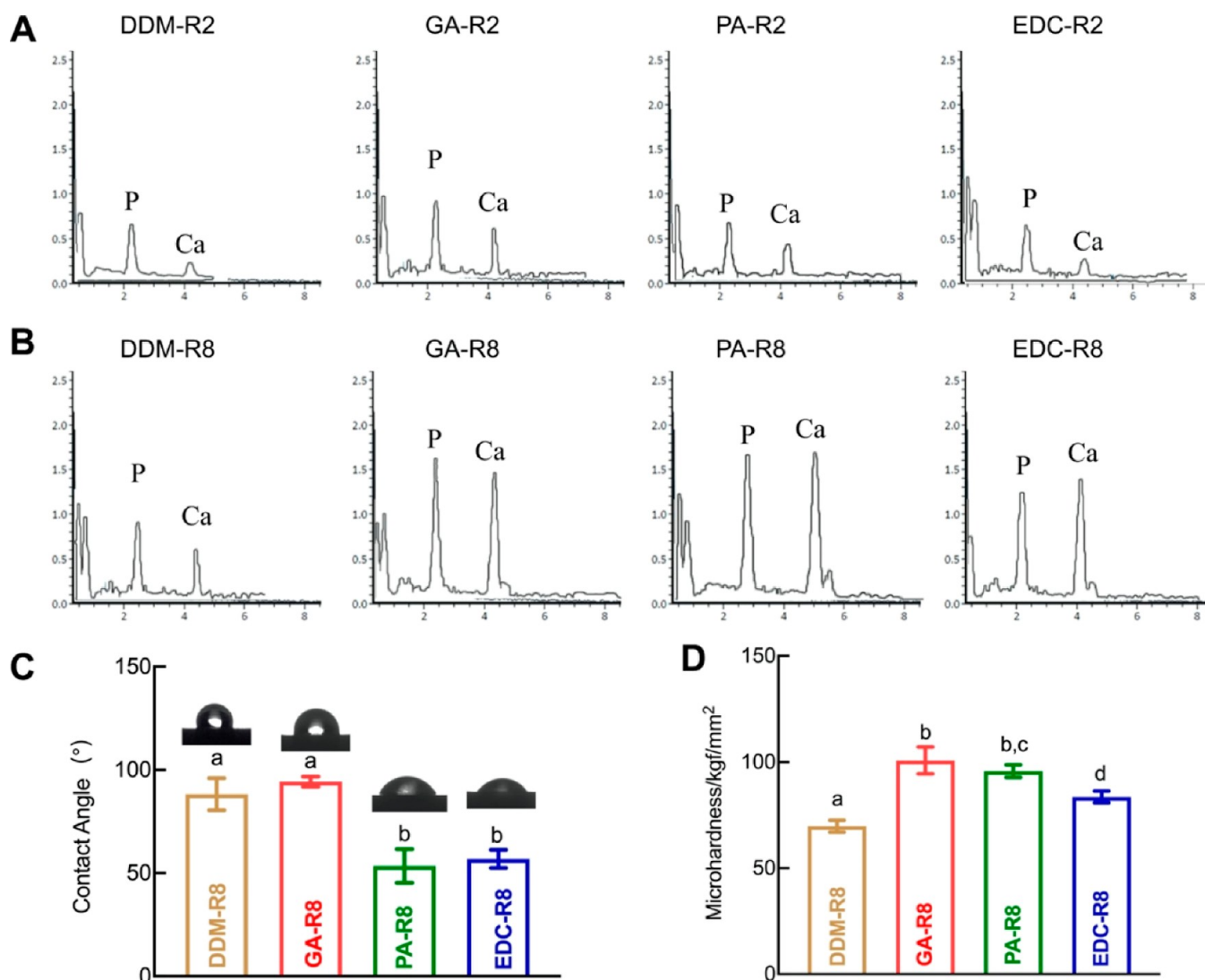


**Figure 4.** Microscopic morphology of demineralized dentin with/without cross-linking and bionic remineralization treatment with different days. (A) No treatment and remineralization (DDM-R) for 2, 4, 6, or 8 days; (B) GA cross-linking and remineralization (GA-R) for 2, 4, 6, or 8 days; (C) PA cross-linking and remineralization (PA-R) for 2, 4, 6, or 8 days; (D) EDC cross-linking and remineralization (EDC-R) for 2, 4, 6, or 8 days.

**Fourier Transform Infrared Spectroscopy Analysis.** As shown in Figure 2B, characteristic collagen peaks were visible in the infrared spectra of demineralized dentin in every treatments.  $\sim 1627\text{ cm}^{-1}$  vibrations represented C=O stretching and C–N stretching in the amide I band;  $\sim 1542\text{ cm}^{-1}$  vibrations represented C–N stretching and N–H bending in the amide II band;  $\sim 1453\text{ cm}^{-1}$  vibrations

represented C–H bending;  $\sim 1337\text{ cm}^{-1}$  vibrations represented C–H stretching;  $\sim 1231\text{ cm}^{-1}$  vibrations represented C–N stretching in the amide III band; and  $1072\text{--}883\text{ cm}^{-1}$  vibrations were caused by the characteristic  $\text{PO}_4^{3-}$  absorption peak (attributable to hydroxyapatite).

**Free Amino Content.** The amounts of free amino groups in the GA, PA and EDC groups were lower than in the DDM



**Figure 5.** Physicochemical and mechanical properties of demineralized dentin specimens with/without cross-linking and bionic remineralization treatment. (A) EDX spectrum of specimens biomimetic mineralization for 2 days; (B) EDX spectrum of specimens biomimetic mineralization for 8 days; (C) video contact angle of specimens biomimetic mineralization for 8 days; (D) surface microhardness of specimens biomimetic mineralization for 8 days.

group ( $2.43 \pm 0.13$  mmol/mg) ( $P < 0.05$ ) (Figure 2C). Meanwhile, significant difference were found among different treatment groups.

**Surface Mechanical Properties.** Dentin surface microhardness was greater in the GA, PA, and EDC groups compared to the DDM group ( $59.84 \pm 5.79$  kgf/mm<sup>2</sup>) ( $P < 0.05$ ) (Figure 2D). No statistically significant change in microhardness between the GA and EDC groups and between the PA and EDC groups were indicated.

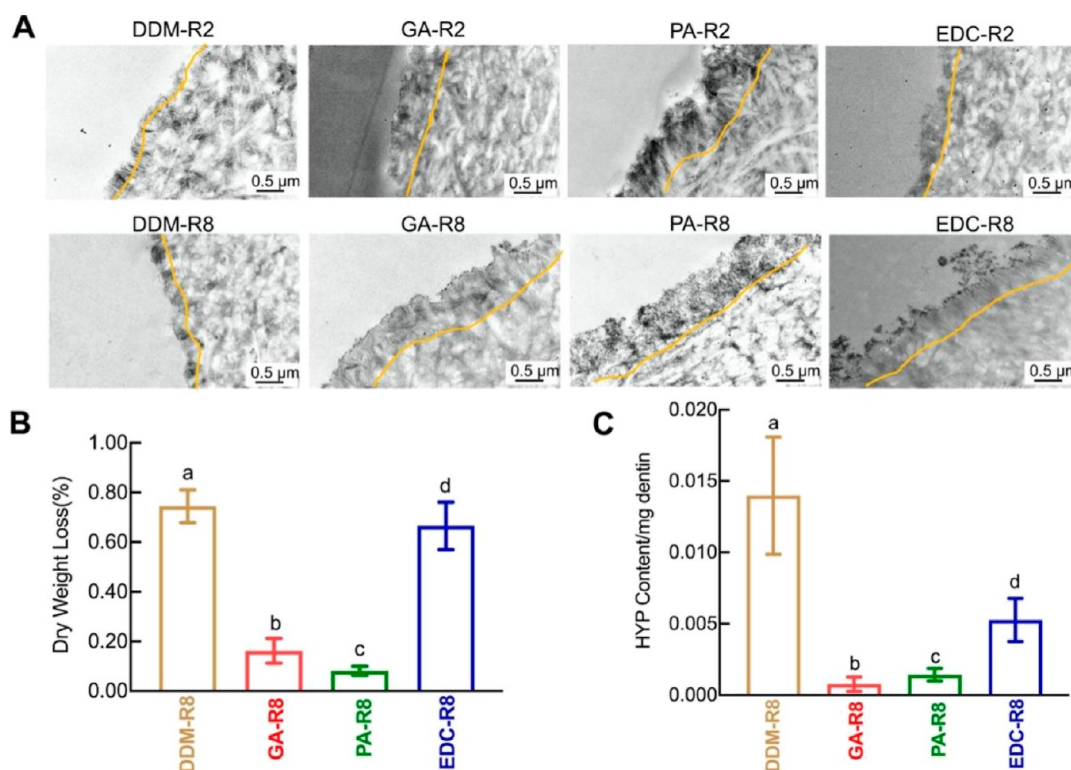
**Enzymatic Resistance. Micro Morphology.** After enzymatic digestion, SEM (Figure 3A) showed increased surface dentin tubule diameters, an oval tubule morphology, and a reduced inter-tubular distance in the DDM group compared to the crosslinked groups. In addition, the peri- and inter-tubular collagen fibers were dissolved and collapsed, and lacked a reticular structure. In the PA and GA groups, the tubules had round morphologies. In the EDC group, the tubules were oval and slightly larger than in the DDM group; collagen fibrinolysis and collapse were visible.

**Dry Mass Reduction.** Enzymatic hydrolysis caused significantly lower mass reductions in the GA and PA groups compared with the DDM group ( $89.58 \pm 7.41\%$ ) ( $P < 0.05$ ). While, no difference was found between the control and EDC treatment groups (Figure 3B).

**HYP Content.** The crosslinked groups (GA, PA and EDC group) had significantly reduced HYP contents compared to the DDM group ( $0.15 \pm 0.02$ ) ( $P < 0.05$ ) after collagenase attack (Figure 3C). Among the treatment groups, the GA group had the lowest value, followed by the PA group.

**Dentin Crosslinking Combined with Bionic Remineralization. Microscopic Morphology.** In the DDM-R group, SEM showed large gaps between the collagen fibers and the absence of fiber thickening or mineral crystals after 2 days of mineralization. The gaps became smaller but disorganized after 4 days of mineralization. After 6 days, the gaps disappeared and the collagen fibers thickened significantly. On day 8, the thickened collagen fibers remained visible and small amounts of mineral crystals were deposited (Figure 4A).





**Figure 6.** (A) Lateral morphology by TEM morphology of demineralized dentin specimens with/without cross-linking and bionic remineralization for 2 or 8 days; (B) dry weight loss and (C) Hyp content of demineralized dentin specimens with/without cross-linking and bionic remineralization for 8 days after collagenase attack.

In the GA-R group, the gaps between collagen fibers had disappeared on day 2 of mineralization and few mineralized particles were visible. The mineral density and particle size increased gradually with time. Despite the mineralization of inter-tubular collagen fibers, the tubules remained relatively clear and devoid of minerals (Figure 4B).

The mineralization pattern of the PA-R group was somewhat different from that of the GA-R group. On day 2 of mineralization, a lamellar mineral phase appeared, the tubule diameter decreased, and the tubules became slightly oval. The tubules became almost invisible on day 4 of mineralization as the lamellar mineral phase increased. The tubules were completely covered by minerals and spherical mineralized particles appeared after 6–8 days of remineralization (Figure 4C).

In the EDC-R group, larger tubule diameters were seen on days 2 and 4 of mineralization; a punctate mineral phase was observed with small, scattered mineral particles. The mineral particles increased in size and density after 6 days of mineralization. On day 8, the tubules were partially covered with minerals and had variable morphologies, but the collagen fibers had thickened significantly (Figure 4D).

**EDX Analysis.** On day 2 of mineralization, the DDM-R2 group had the lowest surface calcium and phosphorus contents, but they increased significantly over the following days. The GA-R2 group on day 2 had a calcium–phosphorus peak similar to that of the DDM-R8 group on day 8. There was no difference between the calcium and phosphorus peaks of the EDC-R2 and DDM-R2 groups on day 2, but the EDC-R8 group had a significantly higher peak on day 8 (Figure 5A,B).

**Hydrophilic Properties after Crosslinking and/or Bionic Remineralization.** The contact angles of the DDM-

R8 ( $88.27 \pm 6.33^\circ$ ) and GA-R8 ( $94.37 \pm 1.99^\circ$ ) groups after 8 days of bionic remineralization were not significantly different ( $P > 0.05$ ), but were greater than those of the PA-R8 ( $53.5 \pm 6.66^\circ$ ) and EDC-R8 groups. There were no significant contact angle differences between the PA-R8 and EDC-R8 groups ( $P > 0.05$ ) (Figure 5C).

**Microhardness Analysis after Crosslinking and/or Bionic Remineralization.** Dentin surface microhardness in the crosslinked and bionic treated groups (GA-R8, PA-R8, EDC-R8) was higher than in the DDM-R8 group ( $69.70 \pm 2.54$  kgf/mm<sup>2</sup>); the differences were statistically significant ( $P < 0.05$ ) (Figure 5D).

**Cross-Sectional Analysis after Crosslinking and/or Bionic Remineralization.** On day 2 of mineralization, the PA-R2 group showed the deepest and uneven mineralization, followed by the GA-R2 group. The DDM-R2 and EDC-R2 groups showed only a small amount of superficial mineralization. On day 8, the PA-R8 group showed the deepest and most homogeneous mineralization, followed by the GA-R8, EDC-R8, and DDM-R8 groups (Figure 6A).

**Enzyme Resistance. Dry Mass Reduction.** Compared to the DDM-R8 group, the GA-R8 and PA-R8 groups had the most significant lower dry mass reductions, followed by the EDC-R8 group ( $P < 0.05$ ) (Figure 6B).

**HYP Content.** The HYP contents were also significantly reduced in the GA-R8, PA-R8, and EDC-R8 groups compared to the DDM-R8 group ( $P < 0.05$ ) (Figure 6C).

## DISCUSSION

An exposed demineralized dentin collagen fiber network collapses as it loses mineral support. This makes the fragile collagen susceptible to degradation by endogenous or

exogenous proteases (e.g., metallo-matrix and cysteine proteases) and to aging caused by temperature and pH changes. Compromised integrity and stability of the hybrid layer may lead to the failure of resin-bonded restorations and composite resin treatments.<sup>30</sup> In the present study, demineralized dentin treated with three crosslinking agents (GA, PA, and EDC) maintained a compact three-dimensional collagen morphology, in agreement with a previous study by Liu et al.<sup>31</sup>

The compact dentin collagen network structure had enhanced mechanical properties; the microhardness values for the PA group were lower than those for the remaining crosslinked groups. This may be because the pH of the 6.5% w/v solution was not adjusted in order to maintain polyphenol activity. The original pH was around 4, which might have led to dentin surface slight demineralization. The bioactive ability of PA to interact with proline-rich proteins was pH-dependent because active polyphenols were more stable under acidic conditions.

Traditional crosslinking treatments, including GA and EDC, rely on amine deprotonation to form covalent bonds and consume amino groups to form crosslinks. The amount of free amino groups decreased significantly after GA and EDC crosslinking.<sup>32</sup> In contrast, PA crosslinking was driven by hydrophobic interactions between the phenyl ring of polyphenols and the pyrrolidine ring of proline with lower free amino group consumption.<sup>33</sup> The calcium and phosphorus contents of the dentin collagen remained unchanged after treatment, unlike thermal crosslinking, which leads to negative changes in the collagen structure.<sup>4</sup> GA crosslinking decreased the hydrophilicity because of coalescence of biological tissue fibers during crosslinking.<sup>34</sup> The hydrophilicity of PA-treated demineralized dentin was greatly enhanced. A study by He et al.<sup>35</sup> showed that the water contact angle decreases as proanthocyanidin concentration increases above 4%; this may be due to incomplete reactivity and hydrophilicity of PA.

Relaxation of the triple helix collagen structure is a prerequisite for collagenase enzymatic digestion.<sup>36</sup> Exposed collagen on the demineralized dentin surface rapidly and completely disintegrates as a result of enzymatic hydrolysis, and only the basal portion had a visible mineral phase in the DDM group. Crosslinked collagen networks degraded slightly but remained relatively intact. This was because crosslinking blocked the enzymatic active site in dentin collagen to resist degradation,<sup>37</sup> thus improving the dry mass loss and HYP content after enzymatic exposure.<sup>38</sup> Generally, GA and PA crosslinking led to better resistance to enzymatic hydrolysis, while the EDC group had greater collagen collapse and disintegration.

Therefore, we concluded that chemical crosslinking, especially using GA and PA, enhanced the properties of demineralized dentin collagen networks. Would this improvement contribute to the subsequent bionic mineralization of collagen?

The dentin matrix minerals included extra- and intra-fibrous spatial forms.<sup>39</sup> Bionic remineralization alone did not significantly improve the mechanical properties of demineralized dentin. Collapsed demineralized collagen prevented deeper penetration of inorganic minerals. However, the combination of GA/PA/EDC crosslinking and biomimetic remineralization allowed mineral deposition within 2–6 days. This was faster than the bioactive glass mineralization period of 2 weeks reported in literature,<sup>40</sup> indicating a possible synergistic effect of crosslinking and biomimetic remineraliza-

tion. PA crosslinking almost completely closed the dentin tubules within 2 days of calcification with optimal mineralization quality and the shortest realization.

Among them, GA is a compound containing two functional aldehyde groups. It has a pair of non-binding free electron pairs, forming a chelating ring for calcium phosphate crystallization.<sup>16</sup> SEM and EDX patterns showed that the biomimetic remineralization process of demineralized dentin pretreated with GA was shortened from 8 to 2 days, which was similar to the research results of Chen et al., and shortened the mineralization time of surface demineralized dentin.<sup>16</sup> By contrast in our study, completely demineralized dentin was used for biomimetic remineralization.

The demineralized dentin treated by PA showed lamellar mineralized substances, and the dentin tubules are gradually covered by minerals, which was significantly different from the collagen fibers after GA treatment. PA could promote biomimetic remineralization of demineralized dentin. The possible reasons are as follows. First, PA can chelate with calcium ions to enhance the deposition of minerals in the collagen matrix after PA biological modification; secondly, PA is a polyphenol compound containing a large amount of hydroxyphenyl, which can be used as a ligand for binding calcium ions.<sup>41</sup> It has been pointed out that GSE rich in proanthocyanidins may cause mineral deposition on the surface of the lesion, because GSE will form insoluble complexes when mixed with the remineralization solution within a certain pH range.<sup>42</sup> TEM results show that there was no obvious difference between the mineralization depth of the demineralized dentin treated by PA for 2 and 8 days, indicated that PA could help effectively shorten the mineralization time and improve the quality of mineralization.

Due to the functional groups absence of EDC to promote mineralization, EDC treatment only cross-linked demineralized dentin collagen network acts as a scaffold but still better than the no treatment demineralized dentin group.

Poor hydrolysis resistance of demineralized dentin collagen was detected with bionic remineralization alone. Meanwhile, the anti-enzymatic properties improved significantly when combined crosslinking and biomimetic remineralization treatment. It was speculated the possible reasons were that crosslinking chemically blocks the active site for the enzyme, while biomimetic remineralization leads to physical closure of the tubules. Their synergistic effect enhances the structural stability of dentin and improves the anti-enzymatic properties.<sup>27,32,37</sup>

In conclusion, we explored the improvement effects of three chemical crosslinking agents on the physicochemical properties of demineralized dentin and the synergistic effect of subsequent bionic remineralization. PA-crosslinked mineralization showed the most superior results within this study limitations. This would be of great benefit to the clinic of resin–dentin bonding and dentin hypersensitivity. However, the realization of simultaneous intra- and extra-fibrous mineralization requires further investigation.

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L.T. and L.Z. contributed equally to this research. L.T.: conceptualization, methodology, validation, formal analysis, resources, writing—original draft, reviewing & editing, funding acquisition. L.Z.: methodology, validation, formal analysis, data curation, writing—original draft. Y.L.: conceptualization, supervision, resources, writing—reviewing & editing, funding acquisition. Y.Z.: writing—review & editing, investigation. B.L.: conceptualization, investigation. M.W.: investigation.

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## Notes

The authors declare no competing financial interest.

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