Effect of poly (γ-glutamic acid)/tricalcium phosphate (γ-PGA/TCP) composite for dentin remineralization in vitro

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The poly (γ-glutamic acid)/tricalcium phosphate (γ-PGA/TCP) composite was fabricated as a novel biomineralization material function in preventing caries. Demineralized bovine dentin specimens were prepared and randomly divided into 5 groups (i. α-TCP, ii. γ-PGA, iii. γ-PGA/TCP, iv. CPP-ACP, and v. deionized water) and subjected to 14 days of pH cycling. Remineralization ability was evaluated by lesion depth, mineral loss and microhardness. The morphology of dentin depositions was observed with scanning electron microscope (SEM), the crystal structure was determined by X-ray diffraction (XRD), and the wettability was tested by contact angle measurements. ANOVA revealed specimens treated by γ-PGA/TCP presented the statistically least lesion depth (p<0.01) and mineral loss (p<0.001), and the highest hardness (p<0.001). SEM revealed prominent intra- and inter-tubular precipitates in both γ-PGA and γ-PGA/TCP groups. The XRD patterns of the deposition structures in all groups were similar to those of sound dentin, and the contact angle of water decreased after γ-PGA/TCP treatment.

Keywords: Dentin remineralization, Poly (γ-glutamic acid), α-tricalcium phosphate, pH cycling

INTRODUCTION

According to the Fourth Chinese National Oral Health Survey in 2015, the prevalence of dental caries among 5-year-old children was 71.9%, with a mean DMFT of 4.24, and only 4.1% of decayed primary teeth received restoration11. In addition to the lack of dental resources, a high failure rate of restoration in primary teeth hinders the treatment of early childhood caries (ECC)9, which has become an important public health issue and may affect permanent teeth as well as the health and well-being of children9. Current philosophies have emphasized the minimal intervention of caries, and valid prevention strategies to arrest active caries and promote remineralization have been reported8. Various reagents, such as NaF, silver diamine fluoride (SDF)5,6, and calcium phosphate (CaP)7, have been used in clinical trials or in studies, among which SDF has proved to be effective, although staining of arrested carious lesions remains a concern9.

Dentin is composed of 70% mineralized dentin matrix and 20% organic phase (which comprises >85% type I collagen, with the remaining part including glycoproteins and non-collagenous proteins (NCPs)) by weight9. NCPs are believed to control apatite nucleation, crystal growth, and phase in the process of biomineralization10. However, it is difficult to obtain purified NCPs, which has inspired scientists to make use of analogues of NCPs to promote dentin biomineralization11. It is believed that in the presence of NCPs or their analogues, the metastable precursor, can be directed towards the gap zones of the collagen matrix and subsequently be converted to thermodynamically stable crystalline phases10. In this process, intrafibrillar remineralization is achieved on the surface of dentin11. Researchers have applied NCP analogues to induce dentin remineralization, generally, highly acidic or phosphorylated proteins are preferred11, such as polyacrylic acid14 and sodium trimetaphosphate15.

Poly(γ-glutamic acid) (γ-PGA) is an anionic polypeptide rich in carboxyl groups (-COOH)10 and has been extensively studied as a biomaterial due to its excellent hydrophilicity, biodegradability, and biocompatibility17,18. It has been found that consecutive glutamyl residues play vital roles in some NCPs as nucleating agents or in bridging collagen and hydroxyapatite (HA) in bone matrix19,20. Zhang and co-workers have revealed that γ-PGA may facilitate homogeneous mineralization of a porous polymeric scaffold in vitro21.

CaPs have been widely studied and used in bone regeneration due to their excellent osteoconductivity, biocompatibility and bioactivity7,22. Tricalcium phosphate (TCP), a precursor to HA23, possesses relatively high solubility24, great potential in dentin remineralization25,26, and the capability to bond strongly with mineralized tissue7. TCP also presents lattice defects, allowing for crystal modification27. In the different allotropic forms of TCP, α-TCP releases almost 10 times calcium than β-TCP under neutral pH conditions7. Based on the unique advantages of γ-PGA as a biomimetic analogue of NCP, and α-TCP as an
additional source of calcium, the composite material was fabricated for the purpose of inducing inter- and intra-tubular remineralization of demineralized dentin in vitro, and the possible mechanism was also discussed.

MATERIALS AND METHODS

Materials
γ-PGA (MW=100 kD, Nanjing Saitaisi Biotech, China) was diazylized against deionized water and lyophilized. α-TCP (Shanghai Macklin Biochemical, China) was filtered against deionized water after settling statically for 24 h and the retained liquid was used in the following experiment. CPP-ACP paste (Tooth Mousse, GC, Tokyo, Japan) was undiluted in use.

Preparation of the γ-PGA/TCP composite
To a 2% (wt/vol) γ-PGA solution in deionized water was added 5% (wt/vol) of α-TCP; then, the mixture was stirred at 80°C for 3 h. The obtained slurry was allowed to settle statically under ambient conditions for 24 h. The precipitates were filtered, and the suspension liquid was obtained and used in the following study.

Preparation of dentin slice
Fresh bovine incisors were collected, and soft tissues were cleaned using a sharp blade. All teeth were stored in a 0.1% thymol solution at 4°C before use.

The buccal and lingual portions of the cervical roots were cut and prepared into 80 dentin blocks (5×3×2 mm) on a cutting machine (Accutom-50, Struers, Copenhagen, Denmark). The sample surfaces were ground with SiC abrasive paper from 220# to 4000# grit on a polishing machine (Tegramin preparation system, Struers) until a flat surface was observed under the microscope at 10× magnification. Then, the dental slices were cleaned in an ultrasonic bath with deionized water for one hour to remove the smear layer. Finally, the entire surfaces of the slices were sealed with acid-resistant nail polish (Revlon, New York, USA), except for a 5×3 mm window on the polished dentin.

Artificial carious lesion formation
Artificial carious lesions were chemically created by immersing the samples in a demineralization solution (2.2 mM CaCl₂, 2.2 mM KH₂PO₄, 50 mM lactic acid, pH 4.0) for 72 h at 37°C; initial lesions with a depth of approximately 180 µm were observed.

pH cycling
After demineralization, 65 specimens were randomly divided into five groups and subjected to the following treatments with 13 blocks in each group: 5% α-TCP group, 2% γ-PGA group, γ-PGA/TCP group (prepared as above), 10% CPP-ACP group (positive control), and deionized water group (negative control). All dentin blocks were submitted to a pH cycle at 37°C with alternation of the demineralization (1.5 mM CaCl₂, 0.9 mM KH₂PO₄, 50 mM lactic buffer, pH 5.0, 8 h) and remineralization solutions (1.5 mM CaCl₂, 0.9 mM KH₂PO₄, 130 mM KCl, 20 mM HEPES, pH 7.0, 16 h) for 14 days. During the pH cycling, before/after incubation with the demineralization solution, dentin surfaces were treated with experimental reagents or deionized water for 5 min. In group D, CPP-ACP paste was applied with soft brush (0.01 g/tooth). In other groups, dentin slices were immersed into solutions (5 mL/group).

Characterizations
1. Concentration of Ca & P, and pH of α-TCP and γ-PGA/TCP solutions
The remaining total concentrations of calcium and phosphorus in the α-TCP and γ-PGA/TCP solutions were respectively measured by calcium assay kit using arsenazo III method and phosphorus assay kit using phosphomolybdate method (Beckman Coulter, Brea, CA, USA) with AU5800 automatic biochemical analyzer (Beckman Coulter). And pH of solutions was tested with the pH meter (Mettler Toledo, Zurich, Switzerland).

2. X-ray diffraction (XRD)
The XRD pattern of α-TCP and the γ-PGA/TCP composite was recorded on an XRD system (Empyrean, PANalytical, Almelo, the Netherlands) in the 2θ range of 20° to 60°, with a step of 0.02° and a dwell time of 40 s for each step. The samples were freeze-dried and powdered before testing.

The surface and subsurface structures of dentin after pH cycling were also characterized by XRD. The 2θ range was from 24° to 45°, with a step of 0.02° and a dwell time of 200 s for each step. In addition, a sound dentin slice and a demineralized dentin sample were measured as controls.

3. Fourier transform infrared (FTIR) spectroscopy
The infrared absorption peaks of α-TCP and γ-PGA/TCP were recorded on an FTIR spectrometer (Equinox 55, Bruker Optics, Karlsruhe, Germany). The spectra were collected in the range of 4,000–750 cm⁻¹ at 4 cm⁻¹ resolution with a total of 64 scans. The samples were freeze-dried and powdered before testing.

4. Scanning electron microscope (SEM)
A field emission SEM (JSM-6330F, JEOL, Tokyo, Japan) was used to observe the microstructure of α-TCP and the γ-PGA/TCP composite. The freeze-dried samples were prepared and gold-coated before testing.

A thermal field emission environmental SEM (Quanta 400F, FEI, Eindhoven, the Netherlands) was used to evaluate the morphology of dentin surfaces and their fractured edges after remineralization in vitro. All samples were dried in a desiccator and sputter-coated with gold before examination.

5. Transmission electron microscopy (TEM)
TEM (JEM-2010HR, JEOL) with a system operating at 200 kV was used to characterize the size and morphology of α-TCP and γ-PGA/TCP. The undried samples were dispersed in ethanol by sonication for 15 min. A 10 µL pipette was used to collect a drop of the suspension, and
the drop was applied on carbon-coated copper grids and dried in a drying cabinet for 30 min before testing.

6. Surface microhardness
Measurements were carried out on a Vickers microhardness tester (DuraScan-20, Struers) with a load of 50 g for 10 s at 10× magnification. Three random points on each slice were subjected to measurement. Before pH cycling, the surface microhardness (n=35) was measured at the baseline to select slices with HV=35±3 (kgf/mm²) for further experiments. After demineralization and a fortnight of pH cycling, the slices were measured again and recorded as H₁ and H₂.

7. Micro-CT
After the formation of the artificial caries and 14 days of pH cycling, the dentin slices were scanned on a micro-CT system (µCT 50, Scanco Medical, Zurich, Switzerland). The X-ray source was operated at 70 kV and 114 µA using 360° rotation. The voxel size used during scanning was 20 µm, with an exposure time of 1,614 ms for each specimen. A region of interest (ROI) of 100×100 µm selected in the cross-section of each dentin slice was used to measure the mineral density (MD) below the exposed surface. MD (gHAP•cm⁻³) was calibrated with phantoms and calculated as the following equation²⁹:

\[\text{MD} = (\text{Gray value} \times 0.1834) - 35.778²⁹\]

The lesion depth (LD) was defined as the distance from the immediate surface to the point where the MD was 95% of the sound tissue, and the sound dentin was set as 48 vol% mineral³⁰,³¹. Mineral loss (ΔZ, vol% • μm) was calculated by subtracting the lesion MD value from the sound dentin MD value.

8. Contact angle
The contact angle of the water droplet on the surface of the dentin was measured using the sessile drop method with a 30 μL drop of distilled water using the Drop Shape Analysis System (DSA 100, Kruss, Hamburg, German). All teeth slices were air-dried before testing. Primarily, the dentin blocks were examined to obtain the baseline. Afterwards, the slices were randomly treated with α-TCP, γ-PGA, or γ-PGA/TCP for 60 s (n=3), dried, and tested. The data were analyzed by one-way ANOVA (α=0.05).

RESULTS
Concentration of Ca & P, and pH of α-TCP and γ-PGA/TCP solutions
As Table 1 presented, the total calcium and phosphorus concentrations of the γ-PGA/TCP composite were higher than α-TCP solution. Both of the materials were slightly alkaline.

XRD and FTIR analyses of the γ-PGA/TCP composite
XRD patterns of the investigated materials are presented in Fig. 1A. In the diffraction form of α-TCP, the spectrum suggested the existence of two crystalline phases: α-TCP and calcium-deficient hydroxyapatite (CDHA) in the obtained material³² and the ratio of α-TCP and CDHA was approximately 3:7. The γ-PGA/TCP composite showed the similar peaks in the graph, but broader and less intense than those of α-TCP.

Table 1 Concentration of Ca & P, and pH of α-TCP and γ-PGA/TCP solutions

| Characterization | α-TCP | γ-PGA/TCP |
|------------------|-------|-----------|
| Ca (mmol/L)      | 0.60  | 2.00      |
| P (mmol/L)       | 0.12  | 0.36      |
| pH               | 7.66  | 7.96      |

Fig. 1 XRD (A) and FTIR (B) patterns of α-TCP, γ-PGA, and γ-PGA/TCP.
The FTIR (Fig. 1B) spectra exhibited the strongest peaks at 1,039.47 cm\(^{-1}\) (\(\nu_3\) stretching) in \(\alpha\)-TCP, which corresponds to the vibration of phosphate groups (1,124–997 cm\(^{-1}\)). In addition, the spectra possess an absorption band at 869.75 cm\(^{-1}\) which arise from HPO\(_4^{2-}\), indicating the presence of CDHA. For \(\gamma\)-PGA/TCP, this characteristic peak appeared at 1,026.96 cm\(^{-1}\) and was weaker than that for TCP. In the spectrum of \(\gamma\)-PGA, the peaks at 1,614.15 and 1,392.38 cm\(^{-1}\) indicated the amide I and amide II bands, and the peak at 3,452.01 cm\(^{-1}\) was the signal from lattice water and hydroxyl groups (3,550–3,200 cm\(^{-1}\)). \(\gamma\)-PGA/TCP also had unambiguous peaks of -NH\(_2\) groups, which occurred at 1,587.15 and 1,402.02 cm\(^{-1}\), verifying the existence of amino acid residues. These results strongly suggested the phase transformation of \(\alpha\)-TCP.

Microstructure of \(\alpha\)-TCP and the \(\gamma\)-PGA/TCP composite
The morphological characteristics of the materials were observed under SEM. In the low-power field of \(\alpha\)-TCP, unevenly sized particles that formed aggregates were observed (Fig. 2A). To compare with, those in the \(\gamma\)-PGA matrix were more uniform, dispersed and smaller in size (Fig. 2C). In the high-power field, \(\alpha\)-TCP showed an acicular structure (Fig. 2B), while the \(\gamma\)-PGA/TCP composite exhibited a massive texture (Fig. 2D).

The TEM images of \(\alpha\)-TCP and \(\gamma\)-PGA/TCP displayed great differences in both shape and size in Fig. 3. \(\alpha\)-TCP tended to be square-like, while the composite appeared as granules. Moreover, the margin of \(\alpha\)-TCP was sharp, but that of \(\gamma\)-PGA/TCP appeared less distinct. As measured by TEM, the particle sizes of \(\alpha\)-TCP and \(\gamma\)-PGA/TCP were approximately 194.50 and 104.82 nm, respectively.

SEM micrographs of dentin
The SEM micrographs presented in Fig. 4F illustrated that few minerals remained on the superficial demineralized dentin, and dentin tubules were clearly visible. After 2 days of pH cycling, the dentin surfaces of group \(\alpha\)-TCP exhibited substantial precipitation with uneven sizes. However, at the high magnification view (upper-right corner), \(\alpha\)-TCP appeared so large

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**Fig. 2** SEM images comparison of \(\alpha\)-TCP and \(\gamma\)-PGA/TCP composite.
(A, B): \(\alpha\)-TCP at different enlargement factor, (C, D): \(\gamma\)-PGA/TCP at different enlargement factor

**Fig. 3** TEM characterization of \(\alpha\)-TCP (A) and \(\gamma\)-PGA/TCP particles (B).

**Fig. 4** SEM micrographs of dentin surfaces after 2 days of pH cycling.
A: \(\alpha\)-TCP appeared large particles that were excluded outside the tubules. B: \(\gamma\)-PGA induced homogeneous and diminutive crystals to enter the tubules (arrow). C: Pebble-like granules deposited on the surface and interior tubules in group \(\gamma\)-PGA/TCP (arrow). D: In group CPP-ACP, no obvious precipitates into the tubules were observed. E: The control group showed the minerals were scattered and rare with no distinct entry to tubules. F: Clearly visible dentin tubules after demineralization.
particles that were excluded outside the tubules (Fig. 4A). For γ-PGA, the crystals deposited on dentin were more homogeneous and diminutive, which could easily enter the dentin tubules (white arrows in Fig. 4B). The slices that were treated with γ-PGA/TCP (Fig. 4C) and CPP-ACP (Fig. 4D) both showed pebble-like granules on the surface. There was obvious interior deposition for the former, but failed to observe it for the latter. The depositions were scattered and rare with no distinct entry to tubules in the control group (Fig. 4E).

Figure 5 shows SEM images of layers of particles on dentin surfaces after a fortnight of pH cycling. Compared to sound dentin, which was occlusive on tubules orifices (Fig. 5F), samples treated with α-TCP were filled with massive agglomerates of spherical grains (Fig. 5A). At the high enlargement view (upper-right corner), the morphology of the precipitates was similar to that shown earlier for α-TCP (Fig. 2B). The morphology of superficial precipitates appeared porous in group γ-PGA (Fig. 5B) and scree-like in γ-PGA/TCP (Fig. 5C), with interconnected granules resembling “corn on the cob”. The dentin tubules of both groups were blocked off by precipitates. Dental slices treated with CPP-ACP exhibited sand-like particles paved on the surface; however, the dentin tubules were still empty (Fig. 5D). In the control group, the surface was covered by larger and more heterogeneous crystallites (Fig. 5E) compared to group γ-PGA.

Figure 6 presents SEM images of the fractured surfaces of dentin. In group α-TCP, thick sediments were observed to have inhomogeneously precipitated into intratubular dentin (white arrow in Fig. 6A), but intertubular dentin appeared vacant. γ-PGA/TCP showed rod-like substances that blocked the dentin tubules and
were well distributed (Fig. 6C) compared to those with α-TCP. In group γ-PGA, the dentin tubules were filled with homogeneous tiny acicular precipitates overlaid layer by layer (Fig. 6B). In addition, prominent mineral deposition could be observed in intra- and intertubular dentin in both groups (white stellates in Figs. 6B and C). The punctiform precipitates in peritubular in the CPP-ACP group appeared more indistinct and unevenly distributed; in addition, ambiguous sheet-like minerals were observed in the intertubular dentin (Fig. 6D). The negative control group lacked mineral deposition among most tubules except for some protuberances, and no clear mineralization occurred at intertubular dentin either (Fig. 6E).

**Microhardness of dentin**
The Vickers microhardness evaluation of the dentin slabs in each group is presented in Table 2. There was no difference between the microhardness of dentin slices after demineralization ($p=0.718$). After two weeks of pH cycling, the microhardness values of the γ-PGA/TCP group significantly increased the most ($p<0.001$). Among all groups, α-TCP, γ-PGA, and CPP-ACP presented no significant difference from each other ($p>0.05$). All the experimental groups exhibited higher microhardness value compared to that of the control group ($p<0.01$).

**Micro-CT results of dentin**
Table 3 shows that after demineralization, the groups did not differ significantly in terms of LD (181.71±4.60 µm, $p=0.751$) and ΔZ (1,763.54±34.31, $p=0.842$). After pH cycling, all the groups achieved remineralization effects since the LD and ΔZ decreased. Regarding the LD, there was no significant difference between γ-PGA and γ-PGA/TCP, which both exhibited the lowest value ($p=0.05$), while the values of CPP-ACP, α-TCP, and the control group increased in that order. On the other hand, the mineral loss of the specimens treated with γ-PGA/TCP was statistically less than that of the other groups ($p<0.001$); γ-PGA and CPP-ACP showed no significant difference, followed by α-TCP and the control group.

**XRD spectra of dentin**
As shown in Fig. 7, the XRD spectra represented both the structure of the surface and the subsurface of the dentin slices after 14 days of pH cycling. Compared to demineralized dentin, which exhibited no obvious diffraction peaks, the sound dentin showed extremely sharp and intense peaks apatitic peaks at 2θ=25.95°, 31.88°, 32.96°, and 39.97°, which are the characteristic peaks of HA for the 002, 211, 300 and 400 planes, respectively. Among all spectra, groups B and D exhibited relatively higher peaks at the 211 and 300 planes, which suggested more HA deposited on the dentin surface. Compared with group C, however, group A and E exhibited broader breadths and weaker peaks at 002 plane, indicating lower crystallinity.

**Water contact angle of dentin**
Figure 8 shows that the dental surface contact angle of water was in the order control>γ-PGA/TCP>γ-PGA>α-TCP, and all the groups differed from each other

### Table 2  Surface microhardness of dentin slices after demineralization (H₁) and pH cycling (H₂).

| Group          | H₁ (kgf/mm²) (mean±SD) | $p$-value | H₂ (kgf/mm²) (mean±SD) | $p$-value |
|----------------|------------------------|-----------|------------------------|-----------|
| α-TCP          | 4.32±1.22*             |           | 9.88±0.95b             |           |
| γ-PGA          | 4.85±0.72*             |           | 9.65±1.09b             |           |
| γ-PGA/TCP      | 4.13±1.46* $p=0.718$   |           | 12.95±1.89c $p<0.001$  |           |
| CPP-ACP        | 4.35±0.96*             |           | 10.75±1.40b             |           |
| H₂O            | 4.03±1.28*             |           | 7.18±1.07d             |           |

* $p$-value of the comparisons using ANOVA (lower case letters indicate statistically significant differences between groups, $α=0.05$).

### Table 3  Lesion depth and mineral loss of each group after 14 days of pH cycling.

| Group           | Lesion depth(um) Mean±SD | $p$-value | Mineral loss (%•um) Mean±SD | $p$-value |
|-----------------|--------------------------|-----------|-----------------------------|-----------|
| TCP             | 107.22±5.34*             |           | 976.57±83.63a               |           |
| γ-PGA           | 89.05±4.99b               |           | 836.73±118.70b              |           |
| γ-PGA/TCP       | 85.71±6.00c $p<0.001$    |           | 707.06±47.51c               | $p<0.001$ |
| CPP-ACP         | 96.67±6.09*               |           | 855.08±37.36b               |           |
| H₂O             | 122±2.98d                |           | 1,101.58±87.06d             |           |

* $p$-value of the comparisons using ANOVA (superscript letters indicate statistically significant differences between groups, $α=0.05$).
Fig. 7 XRD spectra of sound dentin, demineralized dentin, and dentin after pH cycling.
A: $\alpha$-TCP, B: $\gamma$-PGA, C: $\gamma$-PGA/TCP, D: CPP-ACP, E: deionized H$_2$O, DM: demineralized dentin, Sound: sound dentin

Fig. 8 Contact angle of water on dental surfaces of treated groups.
All the groups were significantly different with each other ($p<0.05$). *indicates $p<0.01$

significantly by LSD comparison ($p<0.05$). In other words, the wettability increased when using these materials.

DISCUSSION

From the XRD and FTIR spectrum of materials characterization, it suggested the main presence of CDHA in $\alpha$-TCP. It is known that $\alpha$-TCP is metastable under the room temperature and able to transform into CDHA in water medium according to the following reaction:

$$3 \alpha\text{-Ca}_3(PO_4)_2+H_2O\rightarrow\text{Ca}_9(PO_4)_5(HPO_4)OH.$$  

On this occasion, it might play an active role in the biominerilization.

In this research, bovine teeth were used instead of human teeth because it is easier to obtain homogenous specimens. The cervical diameter of the tubules in bovine dentin was 3.63±0.06 $\mu$m, which was not significantly different from that of human dentin (3.29±0.14 $\mu$m). In addition, the formation of the lesion baseline and the effects of remineralization between bovine and human teeth were not apparently different. The pH cycling model was applied to explore the chemical reactions that occurred in dentin, simulating the physiological environment of a high cariogenic diet. Such a model is simplified, reliable and reproducible in simulating the oral system. In addition, commercial product MI paste (CPP-ACP, without F) was included as a positive control due to its similar mechanism in biominerilization.

The change in mechanical property was assessed by Vicker’s hardness measurements, and the extent of mineral precipitation was evaluated by LD and mineral loss. A previous study confirmed the high correlation between MD and hardness. That is, the more minerals are deposited, the higher hardness is obtained. This study found that all groups achieved remineralization effects, while the experimental groups displayed better results than did the negative control. Among all the groups, the $\gamma$-PGA/TCP group presented the statistically smallest value of both LD and mineral loss, and the highest surface hardness value. Combined with the SEM images, depositions occurred not only on the dentin surface but also at inter- and intratubular dentin. Despite similar circumstances observed in the $\gamma$-PGA group, the MD and microhardness achieved were inferior. This result was probably due to the high density of the rod-like distribution of $\gamma$-PGA/TCP, which enhanced the hardness more than the acicular-shaped $\gamma$-PGA crystallites did. The positive control, CPP-ACP, exhibited moderate mineral loss, LD and hardness, while the SEM images revealed blurry precipitates in the peritubular and intertubular dentin. For $\alpha$-TCP, although no homogeneous depositions in peritubular dentin or noteworthy depositions in intertubular dentin were found, the difference in microhardness was not significantly different from that of the $\gamma$-PGA and CPP-ACP groups. The superficial apatite accumulated in the $\alpha$-TCP group might have impacted the microhardness value. The apatite structure from all treatments determined by XRD analysis was approximate to that of native dentin. It was found that the negative control group showed weak remineralization after pH cycling in this study. The same phenomenon was observed in other studies. Researchers have presented a possible explanation with self-assembly theory, indicating that type I collagen has complex and orderly structures that
play an active role in guiding minerals infiltration and nucleation[11].

Based on the results above, γ-PGA/TCP has the most promising potential in the remineralization process compared to that of other agents for several possible reasons. First, γ-PGA is rich in -COOH groups, which has been widely verified in vitro to trigger apatite nucleation and induce mineral deposition, and is known to have outstanding affinity with Ca2+ [41-43]. By comparison, the slices treated with deionized H2O did not show prominent intra- and inter-tubular remineralization. According to the increasing concentrations of Ca and P in the γ-PGA/TCP solution compared to α-TCP solution, we hypothesize that this result may be because γ-PGA stabilized Ca2+ and PO43− in solution, following the Polymer-Induced Liquid Precursor (PILP) process to enable the infiltration of mineral precursors into the crosslinking interspace of collagen matrices, mineralizing the dentin. Moreover, α-TCP did not achieve ideal effects as shown above, and the remineralization process only occurred in the superficial part of the artificial lesion heterogeneously. According to previous studies, the existence of γ-PGA could disperse and narrow the size of α-TCP [44], which would be consistent with the TEM images presented in this research. From the comparison between the particle sizes and tubeule diameter, it was probably easier for γ-PGA/TCP to reach the deeper sites within dentin tubules and diffuse into the interfibrillar gap due to the blunt shape and smaller size, which allowed induction of both peritubular and intertubular remineralization. In addition, the contact angle measurements showed that γ-PGA/TCP increased the wettability of water on the dental surface, which enhanced the dentin surface energy to an extent [45], providing more “open access” for solution to enter.

Nonetheless, a limitation of this research is that the experimental conditions are different from those of the oral cavity of humans. It is well known that dental caries are related to undisturbed microbial biofilm growth on the tooth surface, influenced by environmental conditions including tooth location, pH, pO2, and so on [46]. The artifical caries generated by acid corrosion and experiments conducted in vitro cannot completely simulate the oral environment, but it is still meaningful when exploring the performance of new materials. In addition, from the remarkable result demonstrated above by the γ-PGA/TCP treatment, there are reasons to believe that intrafibrillar mineralization occurred in dentin. Based on apatitic MD recovery determined by the micro-CT results, we hypothesize that the PILP process was operative in the remineralization of artificial dentin caries. The increase in hardness also indicates intrafibrillar mineralization, which is crucial to the mechanical properties [47]. Moreover, in a previous study, Price et al. verified the theory of “mineralization by inhibitor exclusion”, indicating that macromolecules (>40 kDa) favored fibril mineralization [48]. The γ-PGA (100 kDa) used in this research supports this mechanism. In the next step, it will be certified whether γ-PGA/TCP is able to promote dentin remineralization in vivo, and the intrafibrillar remineralization phenomenon in type I collagen will also be examined, which is defined as the process of minerals filling the gap zones of the collagen fibrils [49].

**CONCLUSION**

γ-PGA/TCP is able to promote inter- and intratubular dentin remineralization, and is a potential candidate in remineralization therapy for early childhood caries.

**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

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