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Remineralization of demineralized dentin using a dual-analog system

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Abstract

Objectives ––—Improved methods are needed to remineralize dentin caries in order to promote conservation of dentin tissue and minimize the surgical interventions that are currently required for clinical treatment. Here we test the hypothesis that bulk substrates can be effectively mineralized via a dual-analog system proposed by others, using a tripolyphosphate (TPP) “templating analog” and a poly(acrylic acid) (PAA) or poly(aspartic acid) (pAsp) “sequestration analog,” the latter of which generates the polymer-induced liquid-precursor (PILP) mineralization process studied in our lab.

Material & Methods ––—Demineralized human dentin slices were remineralized with and without pre-treatment with TPP, using either PAA or pAsp as the PILP process-directing agent. A control experiment with no polymer present was used for comparison.

Results ––—No mineralization was observed in any of the PAA groups. In both the pAsp and no polymer groups, TPP inhibited mineralization on the surfaces of the specimens but promoted mineralization within the interiors. Pre-treatment with TPP enhanced overall mineralization of the pAsp group. However, when analyzed via TEM, regions with little mineral were still present.

Conclusion ––—PAA was unable to remineralize demineralized dentin slices under the conditions employed, even when pre-treated with TPP. However, pre-treatment with TPP enhanced overall mineralization of specimens that were PILP-remineralized using pAsp.

Keywords

Caries; Collagen; Dentin; Mineralization; PILP

1. Introduction

Due to the short longevity of many dental restorations, researchers are exploring biomimetic methods to restore dentin lesions to their natural state.[1–10] Our laboratory has discovered a

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Conflict of Interest Statement

The authors declare no conflict of interest.

way to mineralize collagen fibrils with intrafibrillar hydroxyapatite using the polymer-induced liquid-precursor (PILP) process.[11–13] In this process, the unmineralized collagen and anionic polypeptides (*e.g.* polyaspartic acid, pAsp) are placed in solutions with supersaturated concentrations of calcium and phosphate ions. The acidic polypeptide acts as a process-directing agent and sequesters ions to induce/stabilize nanodroplets of a liquid-like, amorphous calcium phosphate precursor phase. These nanodroplets (which are an ion-enriched phase) infiltrate collagen fibrils, perhaps by capillary action[11] or the Gibbs-Donnan effect.[14] After infiltration, the precursor phase solidifies into amorphous calcium phosphate and then crystallizes into nanocrystals of hydroxyapatite that are embedded within and aligned parallel to the collagen fibril axis.[11, 15] This intrafibrillar mineral is similar to that found in native dentin.[16–18]

When the PILP process was used to remineralize artificial dentin caries, full mineral density recovery was obtained. However, nanoindentation revealed that the modulus (stiffness) was not fully restored near the surfaces of the lesions, with values ~50% that of native dentin.[16] We hypothesize that the mechanical properties were not restored due to preferential infiltration of PILP droplets into the interiors of collagen fibrils, resulting in small, intrafibrillar crystals. In native dentin, interfibrillar crystals (those between collagen fibrils) are also present and are believed to augment mechanical properties due to their location and larger size.[17, 19, 20]

Tay and colleagues have also performed work with collagen mineralization, both on individual fibrils and within dentin artificial lesions.[5, 21–23] Their system using poly(acrylic acid) (PAA) after a collagen pre-treatment with tripolyphosphate (TPP) has shown promise. They refer to this approach as a dual-analog system, where the TPP serves as a “templating analog”, mimicking the highly phosphorylated domains within many non-collagenous proteins, while the PAA is referred to as the “sequestration analog” because it contains repeated carboxylic acid side chains that mimic the acidic domains on many non-collagenous proteins, which are thought to play a role in stabilizing amorphous calcium phosphate precursors. They argue that although the sequestration analog alone can lead to high amounts of intrafibrillar mineral, the mineral is not organized in a “hierarchical” way as is frequently seen in native dentin.[24]

This paper conducts experiments on demineralized dentin slices, borrowing conditions developed by Tay *et al.,*[21] in the hopes of optimizing our current mineralization methods. We hypothesized that their reported “hierarchical mineralization” might lead to a more uniform penetration depth into the dentin by first creating the higher amount of mineral in the gap zones before it then spreads and forms an interfibrillar mineral cement.[16, 25] TPP pre-treatment was explored in conjunction with mineralization using either PAA or pAsp as the “sequestration analog”. The only group that showed promise was TPP pre-treatment with subsequent remineralization using pAsp. However, neither PILP alone nor PILP with TPP pre-treatment resulted in full remineralization throughout the substrates.

2. Material and Methods

2.1 Sample Preparation

Human third molars were sliced parallel to the occlusal surfaces using an IsoMet wet, circular saw (Buehler, Lake Bluff, IL) with a diamond blade. Slices ~300 μm thick were demineralized in 1 L of 0.5 M EDTA in 0.5 M Tris buffer under constant stirring at 25 °C for six days. The specimens were then rinsed in DI water for 30 minutes 3x and then for 24 hours with constant shaking.[13, 16] They were then remineralized immediately or lyophilized for further characterization.

2.2 Tripolyphosphate (TPP) Pre-Treatment

Following similar protocols to Dai *et al.*,[21] half of the specimens were pre-treated with sodium tripolyphosphate (Fisher) solution prior to remineralization by dissolving 0.106 and 0.245 M TPP in DI water. The pH was adjusted to 7.4 via HCl addition. Just prior to remineralization, the specimens were placed in 300 mL of either low TPP (0.106 M) or high TPP (0.245 M) solution while shaking for 1 hour. Specimens were then rinsed in 300 mL of DI water while shaking for 30 minutes prior to placement into mineralization solutions.[21]

2.3 Remineralization

50 mM Tris buffer solution was prepared with 0.9% NaCl and 0.02% sodium azide. The solution was then divided in half and 9 mM $CaCl_2$•$2H_2O$ was added to one half, while 4.2 mM K_2HPO_4 was added to the other half. The solutions were filtered separately using 0.22 μm PES filters and 100 μg/mL 27 kDa pAsp (n=200, Alamanda Polymers) or 2000 μg/mL PAA (1.8 kDa, Sigma) was added to the solution containing $CaCl_2$. The two solutions were mixed together, creating a final mineralization solution of Tris buffer with 4.5 mM calcium, 2.1 mM phosphate, and either 50 μg/mL pAsp or 1000 μg/mL PAA, or no polymer as a control. Three samples were added to 500 mL of mineralization solution per group. The solutions were stirred at 150 rpm at 37°C for two weeks, after which the specimens were rinsed in DI water 3x for 30 minutes while continually shaking. They were then flash-frozen in liquid nitrogen and lyophilized overnight before characterization.[12, 26]

2.4 X-Ray Diffraction (XRD)

XRD is a technique to determine crystal structure and, in these types of substrates, to differentiate apatitic crystals from other calcium phosphate minerals. Freeze-dried samples were analyzed with a Panalytical XPert Powder by placement atop an amorphous glass slide. Samples were scanned from 10° to 60° (2Θ) with a step size of 0.01° and a dwell time of 10 s/step using Cu Kα x-rays (λ = 1.54 Å).

2.5 Scanning Electron Microscopy (SEM) & Energy Dispersive X-Ray Spectroscopy (EDS)

Freeze-dried samples were mounted onto aluminum stubs with double-sided copper tape. For the interior of the specimens, a razor blade was used to fracture the samples. The mounted samples were sputter-coated with amorphous carbon before analysis with a JEOL-6400 SEM. EDS spectra were obtained to determine the elemental composition of the surface of the sample.

2.6 Thermogravimetric Analysis (TGA)

TGA measures the mass of a sample as it is heated. In these experiments, the mass decreases as the collagen within the sample combusts and complete collagen combustion occurs by 600 °C, at which point only mineral residue is left. TGA was performed under nitrogen up to 600 °C at a heating rate of 20 °C/min. using a TA Instruments-Q5000. Sample masses were ~5 mg.

2.7 Transmission Electron Microscopy (TEM)

Sections of samples were prepared via focused ion beam (FIB) with a Strata-DB235 Dual-Beam (FIB/SEM) instrument. To access different depths below the surface, slices were wet sanded with 1200 grit sandpaper and wet sonicated to remove buildup within the tubules. FIBed sections were then analyzed with a JEOL-2010F TEM.

3. Results

Figure 1A shows x-ray diffraction patterns of native, demineralized, and remineralized dentin. There is little difference between the low and high TPP groups. The groups with patterns most similar to dentin were pAsp, with and without pre-treatment with TPP, with broad hydroxyapatite peaks indicating nano-sized/impure crystals. The PAA samples contained little to no mineral, as confirmed by SEM micrographs with EDS spectra (data not shown). SEM micrographs of native and demineralized dentin are shown in Figure 1B-D. Figure 1B shows the surface of native dentin. Not many tubules are visible due to mineral occlusion. However, when demineralized, the tubules can be seen (Figure 1D). In the interior of native dentin, tubules can be seen, but individual collagen fibrils are not apparent due to extrafibrillar mineral (Figure 1C). When demineralized, however, some individual fibrils can be seen in the interior (Figure 1E).

Figure 2A-F shows SEM micrographs of dentin remineralized without polymer. Without TPP treatment, there appears to be large mineral deposits both on the surface and interior, but EDS suggests the collagen is not highly mineralized (Figure 2A&B). Pre-treatment with either TPP solution inhibited remineralization on the surface but enhanced it on the interior (Figure 2C-F). The sharp XRD peaks in the TPP pre-treated groups (Figure 1A) likely indicates the presence of larger, extrafibrillar crystals in the crusty regions (*e.g.*, top of Figure 2F).

When remineralized with pAsp, the surfaces of the slices were covered in a mineral crust, with many tubules occluded, similar in microstructure to native dentin (Figure 2G vs. Figure 1B). The interiors were similar to native dentin microstructures, but appeared less mineralized, as confirmed by EDS (Figure 2H vs. Figure 1C). When pre-treated with TPP, the specimens did not exhibit thick mineral crusts on the surfaces (the tubules were not as occluded), but EDS shows the surfaces were highly mineralized (Figure 2I&K). The interiors of these specimens exhibited similar microstructures to native dentin and were more highly mineralized than the specimens without TPP pre-treatment (Figure 2J&L). Thermogravimetric analysis revealed mineral contents between 51 and 64 wt.%, with TPP pre-treatment enhancing overall remineralization (Table 1). Since 0.245 M TPP pre-treated

samples exhibited mineral amounts closest to native dentin (70 wt.%), TEM analysis was performed on these specimens and compared with specimens remineralized using pAsp alone.

Figure 3A&B shows TEM micrographs of native dentin prepared via FIB. Remineralization via pAsp with or without 0.245 M TPP yielded surfaces with similar nanostructures to native dentin (Figure 3C&F vs. Figure 3A&B). In both groups, crystals did not seem preferentially located within gap zones. At 60 μm below the surface, little mineral was present (Figure 3D&G). At 100 μm below the surface, the nanostructures were similar to native dentin, but the crystals appear much thicker (Figure 3E&H).

4. Discussion

A high degree of remineralization was achieved using 27 kDa pAsp, with and without pre-treatment with TPP. However, little to no mineralization was detected using 1.8 kDa PAA, even with TPP pre-treatment. Because mineralization solutions containing pAsp precipitated within several days and those containing PAA did not precipitate after 14 days, the likely explanation as to why mineralization did not occur when using PAA is because nucleation was overly inhibited in these systems. Given the low molecular weight of the PAA, it is possible that some of these small polymer molecules entered the collagen fibrils and reduced the Gibbs-Donnan effect, which is presumably what promotes intrafibrillar mineral formation.[14, 27] Likewise, polymer molecules remaining in solution overly inhibited extrafibrillar mineral formation. Tay *et al.* place their collagen substrates on Portland cement blocks during mineralization, which would continuously replenish ions in the mineralization solution (and also introduce other ions into the system).[28–30] Due to this difference, and that our experiments were performed on bulk specimens instead of individual fibrils like some of Tay's work, the mineralization conditions may need adjusting for this polymer type and molecular weight.

TPP pretreatment of collagen prior to pAsp PILP-mineralization enhanced overall mineralization (Table 1). This could be because TPP pre-treatment seemed to inhibit mineralization on the surfaces of the substrates, leaving tubules open through which PILP droplets could penetrate (Figure 2I-L). It is likely that the TPP concentrations at the surfaces of the specimens are greater than in the interiors of the specimens. TPP could be inhibiting mineral formation by chelating Ca^{2+} ions and keeping them from associating with free phosphates. Another reason for the enhancement of mineralization using TPP pre-treatment could be that TPP molecules associated with the amine groups on the collagen, where they then chelated Ca^{2+} to promote nucleation and growth of hydroxyapatite crystals within the fibrils. Thus, TPP could be acting as the proposed "templating analog", even though enhanced gap-zone banding was not observed. This may lead to higher amounts of mineral than substrates remineralized with PILP alone (Table 1), but apparently only within those regions with the optimal amount of TPP. There were still regions ~60 μm below the surface that showed very little mineral (Figure 3G). This is in accord with our prior modulus mapping studies that found a dip in modulus at an intermediate depth into the lesion.[25] This suggests diffusion limited entry of TPP as well as PILP nanodroplets is creating this non-uniformity of remineralization depth, so alternative methods of delivery may be needed.

5. Conclusions

Poly(acrylic acid) was unable to effectively remineralize demineralized dentin slices. Although not reported here, simulated body fluid and higher ionic strength solutions were also tested with the addition of PAA. All of these systems failed to produce noticeable mineral in bulk substrates. Tripolyphosphate pre-treatment enhanced the PILP remineralization of demineralized dentin slices. However, neither PILP alone nor PILP with TPP pre-treatment was able to fully remineralize these substrates at all depths over the study period. Although the PILP with TPP pre-treatment did not remineralize the lesions uniformly, this system shows promise in increasing mineral content and should be further studied as a possible way to effectively remineralize artificial dentin lesions.

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References

1. Forss H, Widström E, Reasons for restorative therapy and the longevity of restorations in adults. Acta Odontol Scand 2004;62:82–86. [PubMed: 15198387]
2. Li J, Yang JJ, Li J, Chen L, Liang K, Wu W, Chen X, Li J, Bioinspired intrafibrillar mineralization of human dentine by PAMAM dendrimer. Biomater 2013;34:6738–6747.
3. Wang T, Yang S, Wang L, Feng H, Use of Multifunctional Phosphorylated PAMAM Dendrimers for Dentin Biomimetic Remineralization and Dentinal Tubule Occlusion. Roy Soc Ch Adv 2015;5:11136–11144.
4. Sun J, Chen C, Pan H, Chen Y, Mao C, Wang W, Tang R, Gu X, Biomimetic promotion of dentin remineralization using L-glutamic acid: inspiration from biomineralization proteins. J Mater Chem B 2014;2:4544–4553.
5. Niu L-N, Zhang W, Pashley DH, Breschi L, Mao J, Chen J-H, Tay FR, Biomimetic remineralization of dentin. Dent Mater 2014;30:77–96. [PubMed: 23927881]
6. Kim J, Arola DD, Gu L, Kim YK, Mai S, Liu Y, Pashley DH, Tay FR, Functional biomimetic analogs help remineralize apatite-depleted demineralized resin-infiltrated dentin via a bottom–up approach. Acta Biomateriala 2010;6:2740–2750.
7. Vollenweider M, Brunner TJ, Knecht S, Grass RN, Zehnder M, Imfeld T, Stark WJ, Remineralization of human dentin using ultrafine bioactive glass particles. Acta Biomateriala 2007;3:936–943.
8. Chen Z, Cao S, Wang H, Li Y, Kishen A, Deng X, Yang X, Wang Y, Cong C, Wang H, Zhang X, Biomimetic Remineralization of Demineralized Dentine Using Scaffold of CMC/ACP Nanocomplexes in an In Vitro Tooth Model of Deep Caries. PLoS ONE 2015.
9. Cao Y, Mei ML, Xu J, Lo ECM, Li Q, Chu CH, Biomimetic mineralisation of phosphorylated dentine by CPP-ACP. J Dentistry 2013;41:818–825.
10. Lausch AJ, Quan BD, Miklas JW, Sone ED, Extracellular Matrix Control of Collagen Mineralization In Vitro. Adv Funct Mater 2013;23:4906–4912.
11. Olszta MJ, Cheng X, Jee SS, Kumar R, Kim Y-Y, Kaufman MJ, Douglas EP, Gower LB, Bone structure and formation: A new perspective. Mater Sci Eng R 2007;58:77–116.
12. Jee S-S, Thula TT, Gower LB, Development of bone-like composites via the polymer-induced liquid-precursor (PILP) process. Part 1: Influence of polymer molecular weight. Acta Biomateriala 2010;6:3676–3686.

13. Thula TT, Rodriguez DE, Lee MH, Pendi L, Podschun J, Gower LB, In vitro mineralization of dense collagen substrates: A biomimetic approach toward the development of bone-graft materials. Acta Biomater 2011;7:3158–69. [PubMed: 21550424]
14. Niu L-n, Jee SE, Jiao K, Tonggu L, Li M, Wang L, Yang Y-d, Bian J-h, Breschi L, Jang SS, Chen J-h, Pashley DH, Tay FR, Collagen intrafibrillar mineralization as a result of the balance between osmotic equilibrium and electroneutrality. Nat Mater 2017;16:370–378. [PubMed: 27820813]
15. Jee SS, Kasinath RK, DiMasi E, Kim Y-Y, Gower L, Oriented hydroxyapatite in turkey tendon mineralized via the polymer-induced liquid-precursor (PILP) process. Cryst Eng Comm 2011;13:2077–2083.
16. Burwell AK, Thula-Mata T, Gower LB, Habeliz S, Kurylo M, Ho SP, Chien Y-C, Cheng J, Cheng NF, Gansky SA, Marshall SJ, Marshall GW, Functional Remineralization of Dentin Lesions Using Polymer-Induced Liquid-Precursor Process. PLoS ONE 2012;7.
17. Bertassoni LE, Habelitz S, Kinney JH, Marshall SJ, Marhsall GW, Biomechanical Perspective on the Remineralization of Dentin. Caries Res 2009;43:70–77. [PubMed: 19208991]
18. Weiner S, Traub W, Bone structure: from angstroms to microns. FASEB J 1992;6:879–885. [PubMed: 1740237]
19. Liu Y, Thomopoulos S, Chen C, Birman V, Buehler MJ, Genin GM, Modelling the mechanics of partially mineralized collagen fibrils, fibres and tissue. J R Soc Interface 2014;11.
20. Nikolov S, Raabe D, Hierarchical Modeling of the Elastic Properties of Bone at Submicron Scales: The Role of Extrafibrillar Mineralization. Biophys J 2008;94:4220–4232. [PubMed: 18310256]
21. Dai L, Qi Y, Niu L, Liu Y, Pucci CR, Looney SW, Ling J, Pashley DH, Tay FR, Inorganic-Organic Nanocomposite Assembly Using Collagen as a Template and Sodium Tripolyphosphate as a Biomimetic Analog of Matrix Phosphoprotein Crystal Growth & Design 2011;11:3504–3511. [PubMed: 21857797]
22. Gu L-s, Kim YK, Liu Y, Takahashi K, Arun S, Wimmer CE, Osorio R, Ling J-q, Looney SW, Pashley DH, Tay FR, Immobilization of a phosphonated analog of matrix phosphoproteins within cross-linked collagen as a templating mechanism for biomimetic mineralization. Acta Biomaterialia 2011;7:268–277. [PubMed: 20688200]
23. Gu L, Kim YK, Liu Y, Ryou H, Wimmer CE, Dai L, Arola DD, Looney SW, Pashley DH, Tay FR, Biomimetic Analogs for Collagen Biomineralization. J Dent Res 2011;90:82–87. [PubMed: 20940362]
24. Liu Y, Kim Y-K, Dai L, Li N, Khan SO, Pashley DH, Tay FR, Hierarchical and non-hierarchical mineralisation of collagen. Biomater 2011;32:1291–1300.
25. Saxena N, Cremer MA, Dolling ES, Nurrohman H, Habelitz S, Marshall GW, Gower LB, Influence of fluoride on the mineralization of collagen via the polymer-induced liquid-precursor (PILP) process. Dent Mater 2018;34:1378–1390. [PubMed: 29935767]
26. Thula TT, Svedlund F, Rodriguez DE, Podschun J, Pendi L, Gower LB, Mimicking the Nanostructure of Bone: Comparison of Polymeric Process-Directing Agents. Polymers 2011;3:10–35. [PubMed: 22328971]
27. Toroian D, Lim JE, Price PA, The size exclusion characteristics of type I collagen - Implications for the role of noncollagenous bone constituents in mineralization. J Biol Chem 2007;282:22437–22447. [PubMed: 17562713]
28. Asgary S, Eghbal MJ, Parirokh M, Ghoddusi J, Kheirieh S, Brink F, Comparison of Mineral Trioxide Aggregate's Composition with Portland Cements and a New Endodontic Cement. J Endodontics 2009;35:243–250.
29. Chang S-W, Yoo H-M, Park DS, Oh T-S, Bae K-S, Ingredients and cytotoxicity of MTA and 3 kinds of Portland cements. J Korean Acad Conserv Dent 2008;33:369–376.
30. Reyes-Carmona JF, Felippe MS, Felippe WT, Biomineralization Ability and Interaction of Mineral Trioxide Aggregate and White Portland Cement With Dentin in a Phosphate-containing Fluid. J Endodontics 2009;35:731–736.

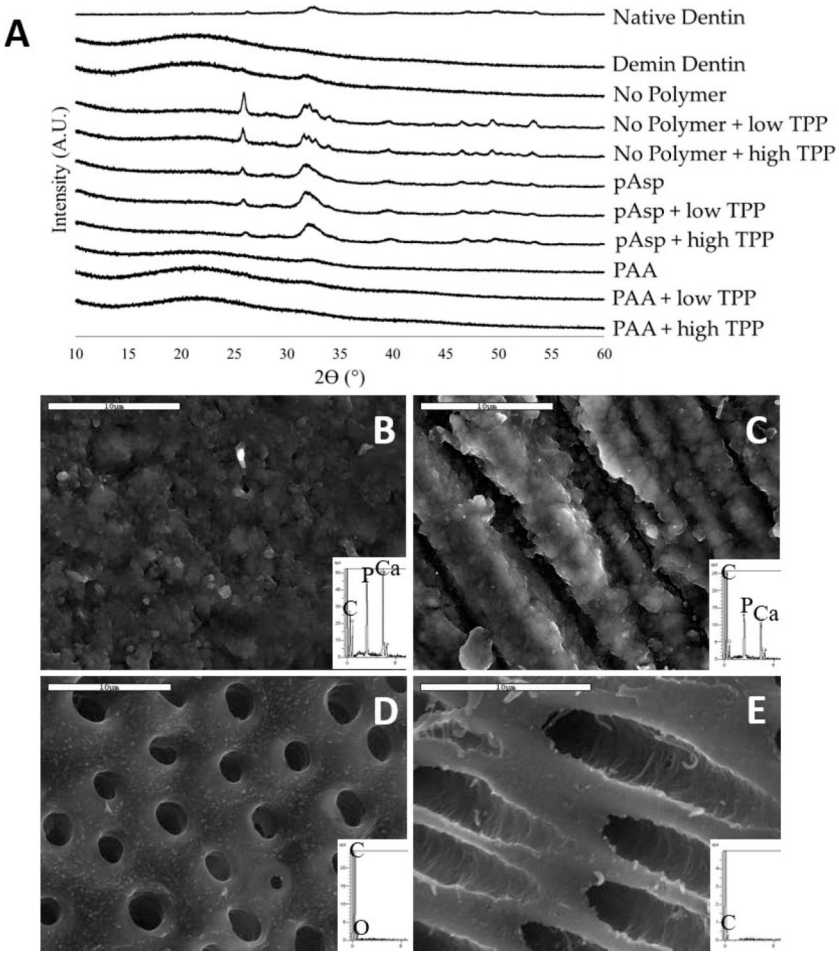

Figure 1.

X-ray diffraction patterns (A) and SEM micrographs (B-E) of native, demineralized, and remineralized dentin slices. B) SEM of surface of native dentin, C) interior of native dentin, D) surface of demineralized dentin, and E) interior of demineralized dentin.

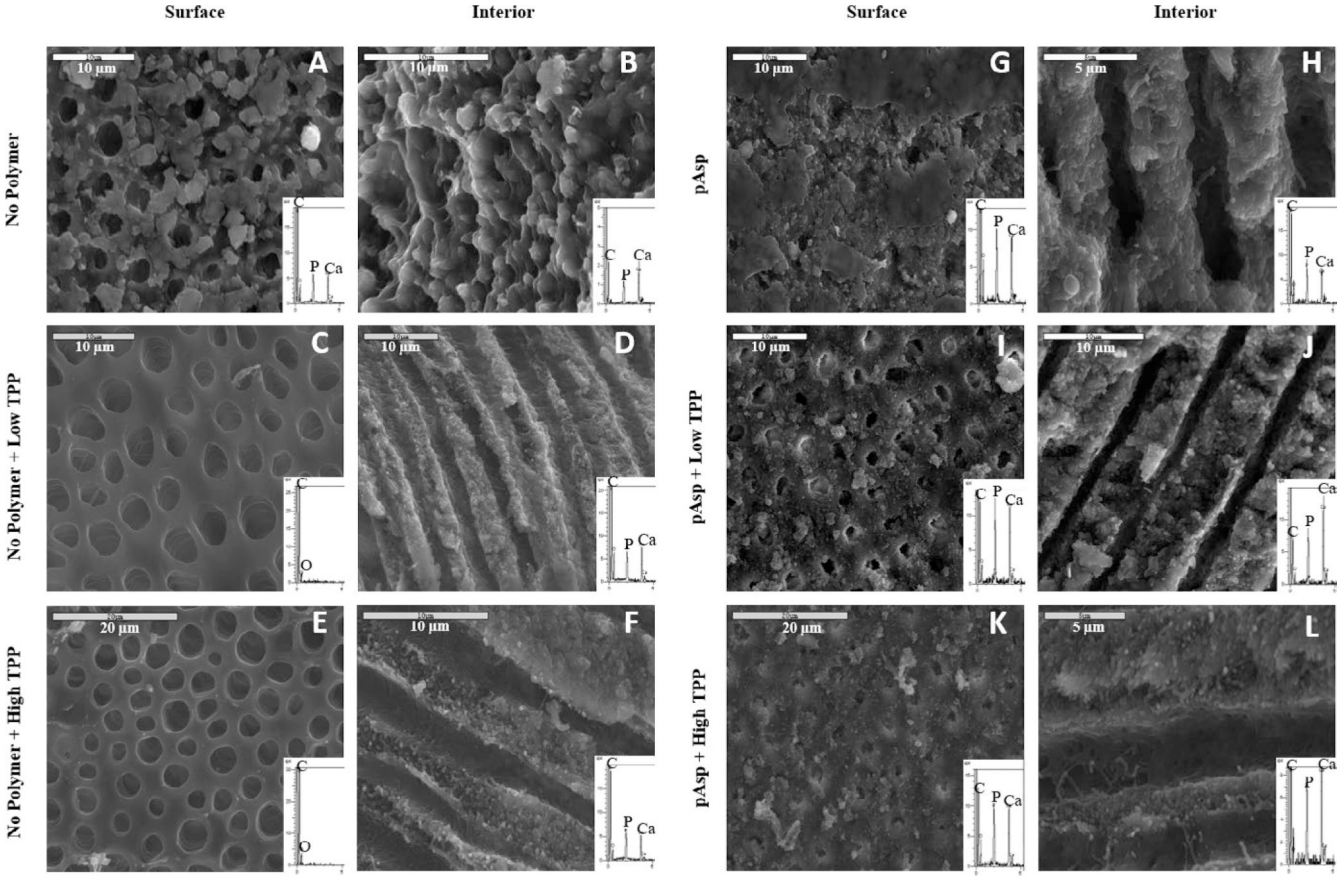

Figure 2.

Influence of TPP pre-treatment on dentin slices remineralized with either no process-directing agent (A – F) or with pAsp-PILP solution (G – L). SEM micrographs of A) Surface- no pAsp or TPP, B) interior- no pAsp or TPP, C) surface- no pAsp and 0.106 M TPP, D) interior- no pAsp and 0.106 M TPP, E) surface- no pAsp and 0.245 M TPP, and F) interior- no pAsp and 0.245 M TPP. G) Surface- pAsp and no TPP, H) interior- pAsp and no TPP, I) surface- pAsp and 0.106 M TPP, J) interior- pAsp and 0.106 M TPP, K) surface- pAsp and 0.245 M TPP, and L) interior- pAsp and 0.245 M TPP.

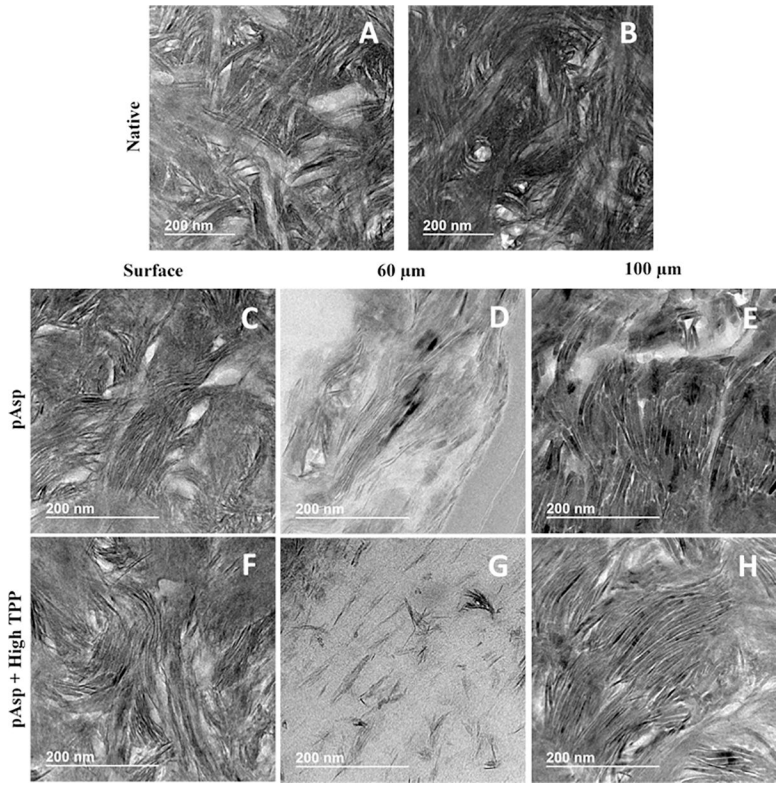

Figure 3.

TEM micrographs of native dentin (A & B) and demineralized dentin remineralized in pAsp-PILP solution (C – F), with and without 0.245 M TPP pre-treatment. C) Surface- no TPP, D) 60 µm below surface- no TPP, E) 100 µm below surface- no TPP. F) Surface- with TPP, G) 60 µm below surface- with TPP, and H) 100 µm below surface- with TPP.

Table 1.

Mineral weight percentages determined by ash weight from thermogravimetric analysis. All values are ± 2.2 wt.%

Sample	Mineral Amount
pAsp	51.9 wt.%
pAsp + high TPP	63.4 wt.%
pAsp + low TPP	61.3 wt.%