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Remineralization of Artificial Dentin Lesions via the Polymer-Induced Liquid-Precursor (PILP) Process

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Abstract

Acid-etched dentin samples with a zone of demineralized dentin were remineralized via the polymer-induced liquid-precursor (PILP) process. Poly-L-aspartic acid was used as the polymeric process-directing agent. Samples were incubated in the mineralization solution for 1–4 weeks. Dentin samples remineralized by the PILP process presented a surface morphology very similar to the intact mineralized dentin's architecture, in contrast to samples mineralized via the conventional nucleation and growth method (without polymer additive), which led to a superficial crust of randomly organized mineral crystals. Energy dispersive x-ray spectroscopy analysis of the PILP-mineralized samples showed the presence of calcium and phosphate ions at high levels. Since no hydroxyapatite (HA) clusters were observed on the surface of the PILP-mineralized samples, we could conclude the signal was produced from the mineral embedded within the dentin matrix. TEM and diffraction analyses suggest that both intrafibrillar and interfibrillar remineralization occurred in the demineralized dentin matrix.

INTRODUCTION

Dental caries represents a major problem in dentistry despite the significant advances in preventive care. Repair of caries using resin-composite fillings is the preferred treatment. However, bonding between dentin and fillings created by adhesives fails with time due to *in vivo* degradation of demineralized collagen. A common experimental approach to improve the durability of these bonds is the remineralization of dentin *in situ*. Our group has been able to achieve intrafibrillar mineralization of a diverse group of collagen matrices using the polymer-induced liquid-precursor (PILP) mineralization process [1, 2]. The PILP process consists of the addition of anionic polymers to a supersaturated mineralization solution in order to induce an amorphous calcium phosphate precursor. The current work reported herein is focused on using this same polymer-directed process to remineralize partially demineralized human dentin substrates, where it is of interest to determine if this PILP process is capable of calcium phosphate mineral formation throughout multiple levels of the dentin hierarchical structure.

EXPERIMENTAL DETAILS

Dentin specimens were prepared from extracted non-carious human teeth. Prior to demineralization, teeth were cut into cubes with a 6-mm² exposed surface area, followed by polishing to 0.25 µm. Demineralization of dentin was carried out for 66 hours in 40 mL of 0.05M acetic acid buffered with 2.2mM Ca²⁺ and PO₄³⁻ (pH=5.0). Acid-etched dentin samples with a 150-µm-thick zone of highly demineralized dentin on top of the native mineralized dentin base were remineralized via the PILP process. In this study, the mineralization solution was prepared by mixing equal volumes of 9 mM CaCl₂ 2H₂O (Sigma, St. Louis, MO) and 4.2 mM K₂HPO₄ (Sigma, St. Louis, MO) solutions. To maintain the pH of the mineralization solution at 7.4, calcium and potassium solutions were made in Tris-buffered saline (TBS) supplemented with 0.02% (w/v) sodium azide.

Poly-L-aspartic acid sodium salt (Mw: 27,000 Da; Alamanda Polymers, Huntsville, AL) was added at a 100-µg/mL concentration to the calcium solution before mixing an equal volume of the phosphate counterion solution. Samples were incubated at 37°C under continuous stirring for 1–2 weeks. Surface and sagittal sections through the lesions prepared by focused ion beam (FIB) milling were characterized using SEM and TEM. For elemental analysis of remineralized samples, energy dispersive X-ray spectroscopy (EDS) analysis was done during SEM examination. Backscattered electron mode images and elemental mapping were obtained for further analysis of the mineral distribution. For quantitative analysis, measurement of mineral occlusion of dentinal tubules was done using the software ImageJ (NIH). Wide angle X-ray diffraction (WAXD) analysis was used to determine the crystallographic structure of the remineralized specimens.

DISCUSSION

Demineralized dentin specimens were remineralized either by the conventional crystallization process (i.e., nucleation and growth) without polymer additive or by the PILP process [3–5]. Figure 1 shows images of native dentin, demineralized dentin, and specimens remineralized via the conventional crystallization process. The initial morphology of dentin (Fig. 1A) changed after the demineralization process with the acid treatment, where a widening of the dentinal tubular orifices was observed, as seen in Figure 1B, mostly due to the absence of the peritubular dentin. When the demineralized specimen was placed in a mineralization solution without polyaspartate additive, it only provided heterogeneous nucleation sites for hydroxyapatite (HA) on the surface of the specimen. Figure 1C shows the surface of demineralized dentin treated with the conventional process (no polymer additive), where a coating of random clusters of HA crystals deposited on the surface of the collagen substrate is seen. The results for the no-polymer-containing samples were anticipated since this is the common morphology of HA precipitated by the conventional crystallization process.

In contrast to the spherulitic clusters produced with no polymer additive, the morphology of the PILP-remineralized lesions after 7 days of treatment (Figs. 2A, D-E) appears very similar to that of the original dentin (Fig. 1A). None of the PILP-remineralized dentin specimens showed evidence of surface deposits of the spherulitic type, as was the case for

the conventional mineralized samples (Fig. 1C). Instead, the intertubular and tubular collagen fibrils showed a smooth surface morphology (Figs. 2D-E), where even the radial orientation of the dentinal tubular collagen fibrils described by Weiner and Wagner [6] could be observed (Fig. 2D).

Energy dispersive X-ray spectroscopy analysis of the PILP-remineralized samples showed the presence of calcium and phosphorous at high levels when compared to the carbon peak at around 0 keV (Figs. 2B and F). EDS of the adjacent areas, which consist of native dentin covered with nitrocellulose varnish (called the control area in Fig. 2A), confirmed that the calcium and phosphorus detected by EDS was only coming from the remineralized area and not from the neighboring area because it was covered with nail polish (Fig. 2C). Since no HA clusters were observed on the surface of the PILP-mineralized samples, we could conclude the signal was produced from the mineral embedded within the collagen matrix.

The artificial lesions remineralized by the PILP process for 14 days still showed a smooth surface morphology (Figs. 2G-H) with high levels of calcium and phosphorous detected by EDS (Fig. 2I). However, mineral occlusion of dentinal tubules became evident after 14 days of PILP treatment. Although the benefits of tubular occlusion with the mineralization progress are still not well identified at this stage of the study, it is known that a common approach used to treat tooth sensitivity is to create tubular occlusion. Perhaps another possible application for our mineralization process could be as a tooth desensitizing treatment inducing mineral occlusion of dentinal tubules, a more biomimetic approach as compared to the current occlusion treatments.

To further analyze mineral distribution across the bulk specimen, SEM in backscattered electron (BSE) mode and elemental mapping analysis of the PILP-remineralized samples was performed (Fig. 3). BSE mode was used because it gives an enhanced signal for higher atomic number elements, thereby enhancing the brightness of the mineralized regions. Regions of electron-dense material were found at the intertubular areas for the 7-day dentin specimens (Fig. 3A), as well as inside the dentinal tubules for the 14-day samples (Fig. 3D). This was particularly apparent in the elemental mapping results. High levels of calcium and phosphorous were localized at the surface of the bulk sample, mostly at the intertubular dentin, with lower mineral levels inside the tubules for the 7-day samples (Fig. 3B-C); while mineral occlusion in the 14-day samples was confirmed by higher calcium and phosphorous signals localized all over the surface, including the inter- and tubular regions (Fig. 3E-F).

For the quantitative analysis of dentinal tubular occlusion, tiled-field images ($\times 500$ magnification) in the backscattered electron mode were taken from each sample. After defining the region of interest, the ImageJ software was used to determine mineral occlusion of dentinal tubules by measuring and counting the open area of dentinal tubules from threshold images. Figure 3G shows the average area of open dentinal tubules of remineralized specimens after 7 and 14 days of PILP treatment. The open surface area of dentinal tubules of remineralized specimens at 7 days was around $6.5 \mu\text{m}^2$ while the average open surface area for the 14-day samples was about $5.3 \mu\text{m}^2$. The artificial dentin lesions remineralized via PILP for 14 days yielded a significant increased in mineral occlusion of the dentinal tubules of approximately 20% compared to the 7-day sample. These results are

very promising since it opens the possibility of using our PILP process as the next generation agent to treat hypersensitivity.

The XRD patterns of the PILP-remineralized dentin at 7 and 14 days are shown in Figures 4A and 4B, respectively. After 7 days of reaction, broad peaks of hydroxyapatite were observed in the PILP-remineralized dentin at around 26° and 32° , corresponding to the (002) and the combination of overlapping (211), (112), (300) peaks, respectively [7]. This XRD pattern of the PILP-remineralized dentin exhibits similar peak widths to the XRD patterns of native bone. The (211), (112), (300) peaks of carbonated hydroxyapatite in bone are normally overlapped into one broad peak due to the extremely small crystal size and defects such as carbonate, impurities and calcium vacancies [8–13]. No difference was observed when comparing the XRD patterns of 7 and 14 days. In the case of native dentin coated by the nitrocellulose varnish (the control area), no peaks were observed (data not shown) therefore the crystallographic analysis only corresponded to the PILP-treated areas.

The surface topology, combined with EDS and XRD analyses, correlate to the presence of HA in the PILP-mineralized dentin samples after 7 and 14 days of mineralization. Furthermore, given that hydroxyapatite clusters were not observed on the surface of PILP-remineralized specimens, as it is the case of samples treated without the polymer additive, the EDS and XRD readings of calcium and phosphorous suggest that hydroxyapatite crystals are localized within (intra) and around (inter) the collagen fibrils. The overall morphology of the PILP-remineralized specimen bears a striking resemblance to native dentin (Fig. 1A), which also consists of intra- and extra-fibrillar mineral.

Bright-field TEM and selected area electron diffraction (SAED) modes were used to examine the calcium phosphate crystals present in the PILP-remineralized dentin specimens. TEM of thin remineralized dentin sections prepared by focused ion beam (FIB) milling showed a microstructure very similar to native dentin (Fig. 4C). It should be noted that the TEM samples were not stained with phosphotungstic acid or any other electron-dense substance. The fibrils in Fig. 4C showed dark contrast and looked highly mineralized. The FIB section looks similar to FIB sections of dentin shown in the literature [14]. The dark striations are presumably interfibrillar crystals, and the intrafibrillar crystals cannot be seen in this specimen. The banding pattern of collagen can become concealed when the collagen is heavily mineralized, as is often the case in our PILP system. Tay and coworkers [15] have demonstrated that a pronounced banding of the mineralized collagen is produced when a polyphosphate templating additive is used in conjunction to the acidic polymeric sequestering agent (polyacrylate in their system).

The SAED pattern of the 14-day remineralized dentin is shown in Figure 4D. This SAED pattern is very similar to that of native dentin [16], with tilting and rotational disorder creating arcs for most of the planes [3]. Because it is difficult to discern the banding pattern of the collagen (although there appears to be a weak banding pattern in the right mid-section), it is difficult to ascertain the crystallographic orientation relative to a specific collagen fibril. But the arcs in the pattern, the broad XRD peaks, and the lack of collagen banding, seem to indicate that there is a high degree of intrafibrillar mineral, in addition to the dark streaks of interfibrillar mineral.

CONCLUSIONS

Using a biogenic collagen scaffold obtained from partially demineralized human dentin, it was demonstrated that a dentin-like nanostructure (intrafibrillar mineral) and microstructure (mineralized dentin matrix) could be restored using a polymer-induced liquid-precursor (PILP) mineralization process. Up to 20% of mineral occlusion of the dentinal tubules was found using the PILP process for a period of 14 days. The overall morphology of the PILP-remineralized specimens bore a strong resemblance to the native dentin, in stark contrast to the conventional crystallization process, which led to surface precipitates. Our prior studies have focused primarily on intrafibrillar mineralization of collagen. These results demonstrate that both intrafibrillar and interfibrillar remineralization of partially demineralized dentin matrices are produced by the PILP process. Comparison of the microstructure of the PILP-remineralized dentin to native dentin suggests that the function of polyaspartate in our *in-vitro* mineralization process may emulate the role of the acidic non-collagenous proteins in dentin formation.

Although most of the analyses presented in this preliminary study were qualitative, the results are promising. Current studies are focused on the mechanical properties of the PILP remineralized dentin to determine if mechanical properties are recovered along with the remineralization of the collagen fibrils. Thus current efforts are focused on methods to improve the functional restoration of the dentin lesions. More recently, we have also been examining remineralization of natural caries, which present a more heterogeneous and challenging microenvironment.

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References

1. Gower LB, Odom DJ. *J Crystal Growth*. 2000; 210:719.
2. Dai L, Douglas EP, Gower LB. *Journal of Non-Crystalline Solids*. 2008; 354:1845.
3. Olszta MJ, Cheng X, Jee SS, Kumar R, Kim Y-Y, Kaufman MJ, et al. *Materials Science & Engineering R-Reports* 2007 July 20. 2007; 58:77.
4. Jee SS, Thula TT, Gower LB. Development of bone-like composites via the polymer-induced liquid-precursor (PILP) process: 1) Influence of polymer molecular weight. *Acta Biomaterialia*. 2010; 6:3676–3686. [PubMed: 20359554]
5. Thula TT, Svedlund F, Rodriguez DE, Podschun J, Pendi L, Gower LB. *Polymers*. 2011; 3:10. [PubMed: 22328971]
6. Weiner S, Wagner HD. *Annu Rev Mater Sci*. 1998; 28:271.
7. Jee SS, Kasinath RK, DiMasi E, Kim YY, Gower LB. *CrystEngComm*. 2011; 13:2077.
8. LeGeros, RZ. *Calcium Phosphate in Oral Biology and Medicine*. N.Y: Karger; 1991.
9. Dorozhkin SV. *Materials*. 2009; 2:399.
10. Lowenstam, HA.; Weiner, S. *On Biomineralization*. N.Y: Oxford University Press; 1989.
11. Glimcher, MJ. The nature of the mineral phase in bone: Biological and clinical implications. In: Avioli, LV.; Krane, SM., editors. *Metabolic bone disease and clinically related disorders*. Academic Press; San Diego, CA: 1998. p. 23-50.

12. Kikuchi M, Itoh S, Ichinose S, Shinomiya K, Tanaka J. *Biomaterials*. 2001; 22:1705. [PubMed: 11396873]
13. Murugan R, Ramakrishna S. *Crystal Growth & Design*. 2005; 5:111.
14. Nalla RK, Portera AE, Daraio C, Minor AM, Radmilovic V, Stach EA, Tomsia AP, Ritchie RO. *Micron*. 2005; 36:672. [PubMed: 16182542]
15. Liu Y, Kim Y-K, Dai L, Li N, Khan SO, Pashley DH, Tay FR. *Biomaterials*. 2011; 32:1291. [PubMed: 21040969]
16. Tay FR, Pashley DH. *Biomaterials*. 2007; 29:1127. [PubMed: 18022228]

Figure 1. SEM of (A) untreated, native dentin; (B) demineralized dentin; and (C) remineralized dentin with the conventional process (no polymer additive), showing only surface deposition of apatite clusters on the top of the demineralized collagen substrate.

Figure 2. SEM and EDS of remineralized dentin via the PILP process for 7 and 14 days. (A) Demineralized dentin remineralized using a PILP solution (4.5 mM CaCl_2 /2.1 mM K_2HPO_4 in tris buffer with 100- $\mu\text{g/mL}$ 27KDa Polyaspartate) under stirred conditions. (B) Remineralized area shows high levels of calcium and phosphorous as detected by EDS. (C) In the control area, consisting of native dentin coated with nitrocellulose varnish, the presence of calcium and phosphorous was not detected by EDS. (D and E) SEM of PILP-remineralized dentin for 7 days shows high levels of calcium and phosphorous, as detected by EDS (F), both inside the dentinal tubules and on the intertubular dentin areas. (G thru I) SEM and EDS of PILP-remineralized dentin for 14 days. Stars indicate areas where EDS analysis was performed.

Figure 3.

Backscattered electron mode images displaying electron-dense areas found on the surface and surrounding the dentinal tubules of the specimen after 7 days (A) and 14 days (D) of mineralization. Backscattered electron mapping showing the calcium (red) and phosphorus (green) distributions across the 7-day (B, C) and 14-day (E, F) samples. (G) Mineral occlusion of dentinal tubules after remineralization of the dentin via the PILP process. Significant differences between the two time points were observed.

Figure 4.

XRD and TEM of PILP-remineralized dentin treated for 7 and 14 days. XRD curves of PILP remineralized dentin at 7 (A) and 14 (B) days. (C) Bright-field TEM micrograph of a FIB cross-section of PILP-remineralized dentin at 14 days. (D) SAED pattern of the remineralized dentin sample shown in (C).