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Effect of calcium phosphate nanocomposite on *in vitro* remineralization of human dentin lesions

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ABSTRACT

Objective. Secondary caries is a primary reason for dental restoration failures. The objective of this study was to investigate the remineralization of human dentin lesions *in vitro via* restorations using nanocomposites containing nanoparticles of amorphous calcium phosphate (NACP) or NACP and tetracalcium phosphate (TTCP) for the first time.

Methods. NACP was synthesized by a spray-drying technique and incorporated into a resin consisting of ethoxylated bisphenol A dimethacrylate (EBPADMA) and pyromellitic glycerol dimethacrylate (PMGDM). After restoring the dentin lesions with nanocomposites as well as a non-releasing commercial composite control, the specimens were treated with cyclic demineralization (pH 4, 1 h per day) and remineralization (pH 7, 23 h per day) for 4 or 8 weeks. Calcium (Ca) and phosphate (P) ion releases from composites were measured. Dentin lesion remineralization was measured at 4 and 8 weeks by transverse microradiography (TMR).

Results. Lowering the pH increased ion release of NACP and NACP-TTCP composites. At 56 days, the released Ca concentration in mmol/L (mean ± SD; n = 3) was (13.39 ± 0.72) at pH 4, much higher than (1.19 ± 0.06) at pH 7 ($p < 0.05$). At 56 days, P ion concentration was (5.59 ± 0.28) at pH 4, much higher than (0.26 ± 0.01) at pH 7 ($p < 0.05$). Quantitative microradiography showed typical subsurface dentin lesions prior to the cyclic demineralization/remineralization treatment, and dentin remineralization *via* NACP and NACP-TTCP composites after 4 and 8 weeks of treatment. At 8 weeks, NACP nanocomposite achieved dentin lesion remineralization (mean ± SD; n = 15) of (48.2 ± 11.0)%, much higher than (5.0 ± 7.2)% for dentin in commercial composite group after the same cyclic demineralization/remineralization regimen ($p < 0.05$).

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Significance. Novel NACP-based nanocomposites were demonstrated to achieve dentin lesion remineralization for the first time. These results, coupled with acid-neutralization and good mechanical properties shown previously, indicate that the NACP-based nanocomposites are promising for restorations to inhibit caries and protect tooth structures.

1. Introduction

Approximately 166 million tooth cavity restorations were placed in 2005 in the Unites States [1], with at least half of the posterior direct restorations using composites [2]. Composites are increasingly used because of their excellent esthetics and direct-filling capability [3–7]. Advances in resin compositions, filler particles and the resin–filler interface have improved the composite properties [8–11]. However, the lifetime of composite restorations is limited by inferior properties such as polymerization shrinkage/stress formation, fracture, abrasion and wear resistance, and marginal leakage [2,12–15]. Marginal leakage can result in the formation of secondary caries, the main reason for composite restoration failures [16–20].

A promising approach to combat caries is to use composites containing calcium phosphate (CaP) particles. These composites have been shown to release calcium (Ca) and phosphate (P) ions and remineralize tooth lesions *in vitro* [21–23], *in situ* in the oral environment [24], and *in vivo* in human volunteers [25]. Mineral growth in tooth lesions can be stimulated by increasing the calcium and phosphate concentrations within the lesion to levels greater than those existing in oral fluids. Indeed, enamel subsurface lesions were remineralized by a CPP-ACP solution [26]. In this approach, CPP-ACP was included in a sugar-free chewing gum to control dental caries *via* active remineralization and salivary stimulation. Additionally, ACP was added to sealants to release supersaturating levels of calcium and phosphate ions, driving the solution thermodynamics toward formation of apatite [22]. A drawback with previous CaP composites for dental restorations was that these composites used traditional CaP particles and had low mechanical properties, which were inadequate for bulk restoratives [21,22].

Recent studies reported novel nanocomposites containing CaP and CaF_2 nanoparticles with sizes of about 50–100 nm [27]. Nanoparticles of amorphous calcium phosphate (NACP) with a size of 116 nm were synthesized *via* a spray-drying technique [28]. Nanocomposites containing NACP are advantageous because of the small size and high surface area of the nanoparticles [27]. A previous study showed that the NACP nanocomposite had mechanical properties 2-fold those of traditional CaP composites [28]. The NACP nanocomposite neutralized acid attacks, while commercial controls failed to neutralize the acid [29]. In addition, composites containing CaP nanoparticles released substantially more ions than that with micrometer-sized particles at the same filler level [30], and CaP nanocomposites possessed much higher strength, fracture toughness, and wear resistance than traditional CaP composites [27]. Recently, NACP nanocomposites were shown to remineralize lesions in human enamel in an *in vitro* model [23]. Additionally, NACP nanocomposite was shown to reduce caries in enamel in a human *in-situ* model [24]. Enamel remineralization was partially enabled by the presence of residual seed mineral crystals, which resulted in apatite mineral formation from the diffusion of calcium and phosphate ions into the carious lesion [31]. However, the previous studies focused on enamel without testing the effect of NACP nanocomposite on dentin [23,24].

Dentin contains 70% carbonated apatite, 20% organic matrix (mostly collagen) and 10% water [31]. When dentin lesions form, the mineral phase is damaged and may be destroyed. As the carious attack progresses, the collagen fibers are exposed and degraded, leading to a decrease in the mechanical properties of dentin [32]. In demineralized dentin, unlike enamel, there are fewer residual mineral seed crystals present, which may make it more difficult to remineralize dentin compared to enamel. Clinically, treatment of carious dentin lesions is dependent on the depth of the lesion. In shallow to moderate lesions, the carious material can be completely removed and restored with composite, amalgam or glass ionomer. In asymptomatic deep lesions, where there is a risk of pulp exposure but restoration of tooth function is possible, partial removal of the carious dentin may be considered the clinically conservative approach. The treatment can involve an attempt to remineralize the demineralized dentin by either indirect pulp treatment or stepwise caries removal. In indirect pulp treatment, most of the carious lesion is removed and the finished cavity preparation is lined with a remineralizing material (calcium hydroxide, resin-modified glass ionomer, *etc.*) and the final restoration is placed to provide a good seal. Stepwise caries removal is a 2-step process, requiring removal of less carious dentin, followed by an interim placement of glass ionomer cement to aid in remineralization. After several months, remineralization is assessed and, if successful, a permanent restoration is placed [25]. While CaP nanocomposites were shown to release more Ca and P ions and possess much better mechanical properties than traditional CaP composites [23,24,27–30], the remineralization of dentin caries *via* nanocomposite containing NACP has yet to be reported.

Accordingly, the objective of this study was to investigate the remineralization of dentin lesions in human teeth *in vitro via* nanocomposites containing NACP and NACP plus micron-sized tetracalcium phosphate (TTCP) particles. It was hypothesized that: (1) the cyclic demineralization/remineralization regimen would fail to remineralize dentin lesions when restored with the commercial composite; (2) the new NACP and NACP-TTCP nanocomposites would successfully regenerate the mineral lost in dentin; (3) dentin

remineralization *via* NACP and NACP-TTCP nanocomposites would increase with increasing time from 4 to 8 weeks.

2. Materials and methods

2.1. Synthesis of ACP nanoparticles

NACP ($Ca_3[PO_4]_2$) were formed *via* a spray-drying technique [33]. Briefly, a solution was prepared by adding 1.5125 g of glacial acetic acid (J.T. Baker, Phillipsburg, NJ) into 500 mL of distilled water. Then, 0.8 g of calcium carbonate ($CaCO_3$, Fisher, Fair Lawn, NJ) and 5.094 g of dicalcium phosphate anhydrous (DCPA, J.T. Baker) were dissolved into the acetic acid solution. The final Ca and P ionic concentrations were 8 mmol/L and 5.333 mmol/L, respectively. This yielded a Ca/P molar ratio of 1.5, the same as that for ACP. This solution was sprayed through a nozzle (PNR, Poughkeepsie, NY) that was situated on a spray chamber with heated air flow. The water/volatile acid were evaporated into the dry, heated column. The dried particles were collected by an electrostatic precipitator (AirQuality, Minneapolis, MN). The collected powder was examined with X-ray diffractometry (XRD, DMAX2200, Rigaku, Woodlands, TX). This method produced NACP with a mean size of 116 nm [28].

2.2. TTCP and glass fillers

TTCP ($Ca_4[PO_4]_2O$) was synthesized from a solid-state reaction between $CaHPO_4$ and $CaCO_3$ (Baker Chemical, Phillipsburg, NJ), which were mixed and heated at 1500 °C for 6 h in a furnace (Thermolyne, Dubuque, IA) [34,35]. The mixture was quenched to room temperature and ground in a blender (Dynamics Corp., New Hartford, CT). The powder was then sieved to obtain TTCP particles with sizes of 1.5–60 μm, with a median of 16 μm. This TTCP powder was then ground in 95% ethanol with a ball-mill (Retsch, Newtown, PA) for 24 h. The particle size distribution was measured *via* a sedimentation method with the use of a centrifugal particle analyzer (SA-CP3, Shimazu, Kyoto, Japan) as described in a previous study [36]. This yielded a particle size range of 0.2–3.0 μm, with a median of 0.8 μm. In addition, for mechanical reinforcement, barium boroaluminosilicate glass particles with a median diameter of 1.4 μm (Caulk/Dentsply, Milford, DE) were used because it is a typical dental glass filler similar to those in a hybrid composite (TPH, Caulk/Dentsply). The glass particles were silanized with 4% 3-methacryloxypropyltrimethoxysilane and 2% *n*-propylamine (mass %) [37,38].

2.3. Resin composite fabrication

The resin consisted of ethoxylated bisphenol A dimethacrylate (EBPADMA, Sartomer Co., West Chester, PA) and the acidic pyromellitic glycerol dimethacrylate (PMGDM, Esstech, Essington, PA), each at a mass fraction of 49.5%. This was photoactivated with 0.2% camphorquinone and 0.8% ethyl 4-*N*,*N*-dimethylaminobenzoate. Two experimental composites were formulated: (1) 40% NACP + 20% silanized glass + 40% resin (referred to as NACP nanocomposite); (2) 40% NACP + 20% TTCP + 40% resin (referred to as NACP-TTCP nanocomposite). Both had a total filler mass fraction of 60%. Additionally, a non-releasing commercial hybrid composite (TPH, Caulk/Dentsply) was included as a control. For mechanical properties, a glass ionomer Fuji II LC (GC America, Alsip, IL) served as a control. Fuji II LC is indicated for Class III and Class V restorations, restoration of primary teeth, and core buildup applications. Fuji II LC is a two part, powder/liquid system. Specimens were made using the manufacturer's suggested powder/liquid ratio of 3.2/1. In addition, a resin-modified glass ionomer Vitremer (3M, St. Paul, MN) also served as a control. Vitremer consisted of fluoroaluminosilicate glass and a light-sensitive, aqueous polyalkenoic acid. Its indications include Class III, V and root-caries restorations, Classes I and II restorations in primary teeth, core-buildup, and orthodontic cement. A powder/liquid mass ratio of 2.5/1 was used according to the manufacturer. Each material was placed into 2 × 2 × 25 mm molds, photo-cured (Triad 2000, Dentsply, York, PA; light intensity at specimen location was approximately 110 mW/cm^2) for 1 min on each open side, and incubated for 24 h at 37 °C before immersion, as described below.

2.4. Mechanical testing

For immersion and mechanical testing, a sodium chloride solution (133 mmol/L) was buffered to two pH values: pH 4 with 50 mmol/L lactic acid, and pH 7 with 50 mmol/L HEPES. For each material, the cured specimens were randomly divided into two groups and immersed in the pH 7 and pH 4 solutions, respectively, at 37 °C for 1 day or 4 weeks. Each group was immersed in 200 mL of solution in a sealed polyethylene container. For the 4 weeks groups, the solution was changed every week. For the pH 4 groups, the pH was adjusted with lactic acid to be at pH 4.

A computer-controlled Universal Testing Machine (5500R, MTS, Cary, NC) was used to fracture the specimens in three-point flexure with a 10 mm span at a crosshead speed of 1 mm/min. The specimens were wet and not dried, and were fractured within a few minutes after being taken out of the solution. Flexural strength (S) was calculated as: $S = 3P_{max}L/(2bh^2)$, where P is the fracture load, L is span, b is specimen width and h is thickness. Elastic modulus (E) was calculated as: $E = (P/d)(L^3/[4bh^3])$, where load P divided by displacement d is the slope in the linear elastic region.

2.5. Calcium and phosphate ion release

A sodium chloride (NaCl) solution (133 mmol/L) was buffered to two different pHs: pH 4 with 50 mmol/L lactic acid, and pH 7 with 50 mmol/L HEPES. Following previous studies [39,40], three specimens of approximately 2 × 2 × 12 mm were immersed in 50 mL of solution at each pH, yielding a specimen volume/solution of 2.9 mm^3/mL. This compared to a specimen volume per solution of approximately 3.0 mm^3/mL in a previous study [41]. For each solution, the concentrations of Ca and P ions released from the specimens were measured at 1, 3, 7, 14, 21, 28, 35, 42, 49 and 56 days (d). At each time, aliquots of 0.5 mL were removed and replaced by fresh solution. The aliquots were analyzed for Ca and P ions *via* a spectrophotometric method (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using known standards and

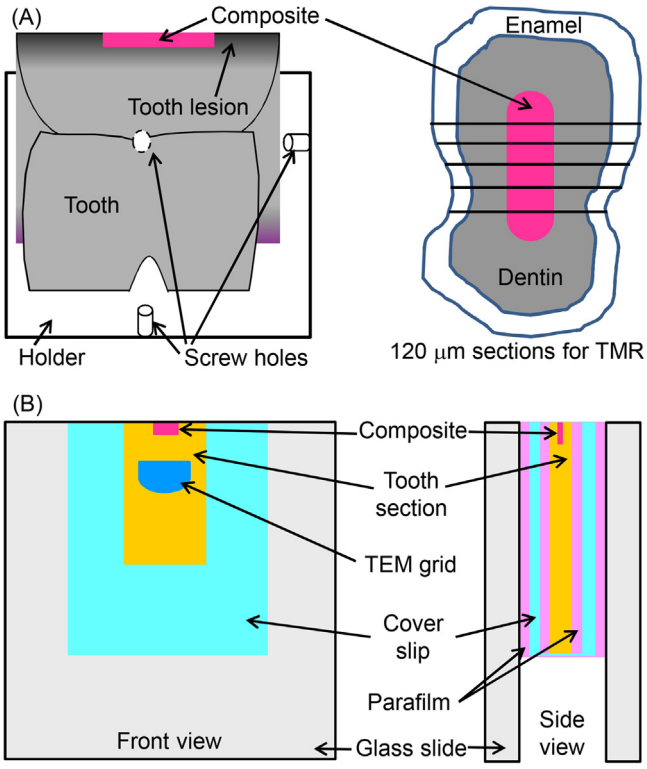

Fig. 1 – Schematic of experimental setup for the dentin demineralization study. (A) Cavity preparation and tooth sectioning. (B) Assembly of tooth section between glass slides to be used in the cyclic demineralization/remineralization treatment.

calibration curves [21,41]. The released ions were reported in cumulative concentrations.

2.6. Tooth section preparation

This study (Protocol #: 29749) received an exemption from the University of Maryland Institutional Review Board, as it was deemed not human subjects research. Teeth were collected from clinics and had no personal information of the donors. Twenty caries-free teeth were disinfected in a 0.005% promodyne solution for 4 h. The roots of each tooth were removed at a location approximately 5 mm below the cemento-enamel junction and embedded with composite resin (Fig. 1A). The upper third of the crown was removed to expose the dentin surface and a single cavity with dimensions 6 × 3 × 1 mm was machined into the dentin surface under constant water irrigation. The enamel surface was coated with varnish and the dentin surface was exposed to 25 mL of demineralizing solution (8.7 mmol/L $CaCl_2$, 8.7 mmol/L KH_2PO_4, 0.05 ppm NaF, 75 mmol/L acetic acid, pH 4.0—adjusted with KOH) for 48 h. Each cavity was filled with either NACP composite, NACP-TTCP composite, or TPH control without the use of a bonding agent and light cured for 2 min (Triad 2000, Dentsply International, York, PA, USA). Five teeth were restored for each of the above conditions. Each restored tooth was wet cut into sections approximately 120 μm thick using a diamond blade (Buehler, Lake Bluff, IL) on a Buehler Isomet low-speed saw at the highest speed setting. Additionally, unrestored teeth were also sectioned and acted as a negative control. 3–6 tooth sections were collected from each restored tooth. To assist in the alignment of the "before" and "after" microradiographic images, 200-mesh Maxtaform Copper/Rhodium transmission electron microscopy (TEM) grids (Electron Microscopy Sciences, Hatfield, PA) were cut under a stereomicroscope into rows encompassing at least one full grid and adhered to each tooth section.

2.7. Transverse microradiography

Contact microradiographs of the tooth sections before treatment were produced on holographic film (Integraf LLC, Kirkland, WA, USA) exposed for 30 min to Cu Kα radiation (80 kV, 3 mA; Faxitron Model #43855A, Hewlett Packard, McMinnville, OR, USA) according to a previous study [42]. An aluminum step-wedge was used to estimate the mineral density [43].

2.8. Assembly of specimens and pH cycling experiment

After the initial contact microradiographs were taken, the tooth sections were assembled for the cyclic demineralization/remineralization protocol by sandwiching between layers of parafilm and a plastic coverslip and then wrapped again with parafilm and sandwiched between glass microscope slides as depicted in Fig. 1B, with the restoration edge exposed.

The demineralizing solution consisted of: 3.0 mmol/L $CaCl_2$, 1.8 mmol/L K_2HPO_4, 0.1 mol/L lactic acid, mass fraction 1% carboxymethylcellulose, and a pH of 4.0 (adjusted with KOH) [22]. The remineralizing solution consisted of 1.2 mmol/L $CaCl_2$, 0.72 mmol/L K_2HPO_4, 2.6 μmol/L F, 50 mmol/L HEPES buffer (pH 7.0 adjusted with KOH) [22]. 20 mL of fresh demineralizing or remineralizing solution was used per specimen for each immersion with continuous magnetic stirring. The specimens were immersed in demineralizing solution for 1 h and remineralizing solution for 23 h at 37 °C. This was repeated for 8 weeks. Contact microradiographs were retaken and the remineralization after treatment was determined.

2.9. Microradiography analysis

The developed film was fixed to a glass slide and observed under an optical microscope (Olympus BX50F, Olympus, Japan). Digital images were captured with a digital microscope camera (RGB/YC/NTSC, Microimage Video Systems, Boyerstown, PA, USA) with an intensity resolution of 256 gray levels and a horizontal spatial resolution of 1.25 μm/pixel. Digitized images were analyzed with the ImageJ software (NIH, Bethesda, MD). This is illustrated in Fig. 2. A rectangular selection the width of one square of the copper grid was made between gridlines and perpendicular to the tooth surface; starting in sound dentin, passing through the demineralized lesion, and extending outside the tooth surface. The "Plot Profile" option was then used to average pixels in a line and to generate and plot the grayscale profile from the exterior to the interior of the tooth which has a direct correlation to mineral density. The profiles collected before and after pH cycling were plotted and aligned *via* the TEM grids and normalized by using the aluminum step-wedge

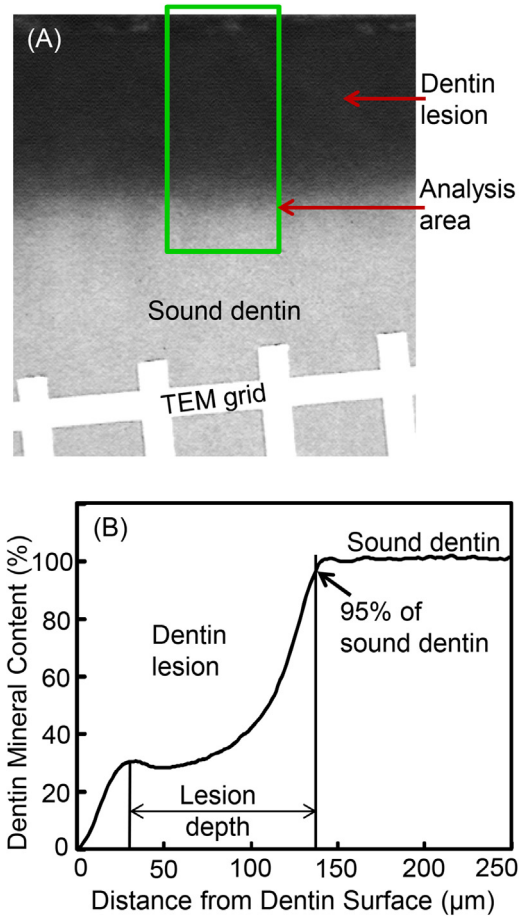

Fig. 2 – Transverse microradiographic (TMR) imaging technique for dentin. (A) A typically prepared tooth section, and (B) a corresponding plot profile determined from the grayscale intensities of the TMR image.

as a standard [43]. Changes in lesion depth (L_d, defined as the distance in μm from the point of peak mineral density in the surface layer to the point where mineral content reaches 95% of sound dentin), mineral loss (ΔZ; volume fraction % × 1.25 μm) were compared for each imaged area before and after the treatment. The percent change in mineral content given by the integrated ΔZ values across the depth of each lesion before and after treatment was calculated according to equation to yield remineralization [22–24,41–43]: $\Delta(\Delta Z)\% = [(\Delta Z_{before} - \Delta Z_{after})/(\Delta Z_{before})] \times 100$. The remineralization Δ(ΔZ) obtained for all image areas of NACP composite, NACP-TTCP composite, and TPH was used to indicate remineralization or further demineralization of the lesions as a result of pH-cycling treatment.

One-way and two-way ANOVA were performed to detect the significant effects of the experimental variables. Tukey's multiple comparison test was used to compare the measured data at a p value of 0.05.

3. Results

The mechanical properties (mean ± sd; n = 6) of the materials are plotted in Fig. 3: flexural strength after immersion for 1 day and 4 weeks in solutions with (A) pH 4 and (B) pH 7, and elastic modulus after immersion for 1 day and 4 weeks in solutions with (C) pH 4 and (D) pH 7. TPH had the highest strength. NACP composite, NACP-TTCP composite, Fuji II LC and Vitremer in general had similar strengths. There was a moderate decrease in strength from 1 day to 4 weeks of immersion. Fuji II LC had the highest elastic modulus. NACP composite, NACP-TTCP composite and Vitremer had similar elastic moduli. These results demonstrate that NACP and NACP-TTCP matched the mechanical properties of Vitremer after immersion for 1 day and 4 weeks in solutions at pH 4 and pH 7.

Fig. 4 plots Ca and P ion release for NACP nanocomposite and NACP-TTCP composite (mean ± sd; n = 3). Two-way ANOVA showed significant effects of solution pH and immersion time, with a significant interaction between the two variables ($p < 0.05$). Lowering the pH increased the ion release. At 56 days, Ca concentration for NACP-TTCP was (13.39 ± 0.72) mmol/L at pH of 4, much higher than (1.19 ± 0.06) mmol/L at pH 7 ($p < 0.05$). At 56 days, P ion concentration was (5.59 ± 0.28) mmol/L at pH of 4, much higher than (0.26 ± 0.01) mmol/L at pH 7 ($p < 0.05$). A similar trend was seen for NACP nanocomposite. There was no difference in Ca or P ion release between NACP and NACP-TTCP nanocomposites ($p > 0.1$).

Transverse microradiographs of dentin sections exhibited significant demineralization before the cyclic demineralization/remineralization treatment (Fig. 5). After 4 and 8 weeks of the cyclic demineralization/remineralization, there was remineralization of dentin lesion *via* NACP and NACP-TTCP nanocomposites. The tooth cavity restored with TPH showed little change in the dentin lesion over the 8 weeks, while the unfilled control dentin lesion showed further demineralization. These trends can be seen more clearly in the mineral density curves in Fig. 6, which shows representative examples for each of the groups tested. The average depth of demineralization in the tooth sections was measured (mean ± SD; n = 25) to be (157 ± 13) μm. Remineralization occurred in the NACP and NACP-TTCP groups, with little change in the TPH-restored cavities, and further demineralization in unfilled dentin control.

Fig. 7 illustrates the percent remineralization calculated from the mineral density curves in Fig. 5 (mean ± SD; n = 15). At 4 weeks, significant remineralization was seen in NACP group (22.3 ± 10.3)% and NACP-TTCP group (25.0 ± 10)% ($p > 0.1$). There was minimal remineralization in TPH (4.3 ± 6.6)% ($p < 0.05$). At 8 weeks, remineralization increased further for NACP (48.2 ± 11)% and NACP-TTCP (43.2 ± 12.6)%, while there was little change in TPH (5.0 ± 7.2)%. At both 4 and 8 weeks, there was no significant difference between NACP and NACP-TTCP nanocomposites. In comparison, the untreated dentin lesion control exhibited significant mineral loss at 4 weeks (−55.7 ± 20.3)% and 8 weeks (−100.66 ± 35.8)%.

4. Discussion

This study demonstrated excellent remineralization of human dentin lesions *via* NACP-based nanocomposites *in vitro* for the first time. The hypotheses were proven that the cyclic demineralization/remineralization regimen failed to remineralize

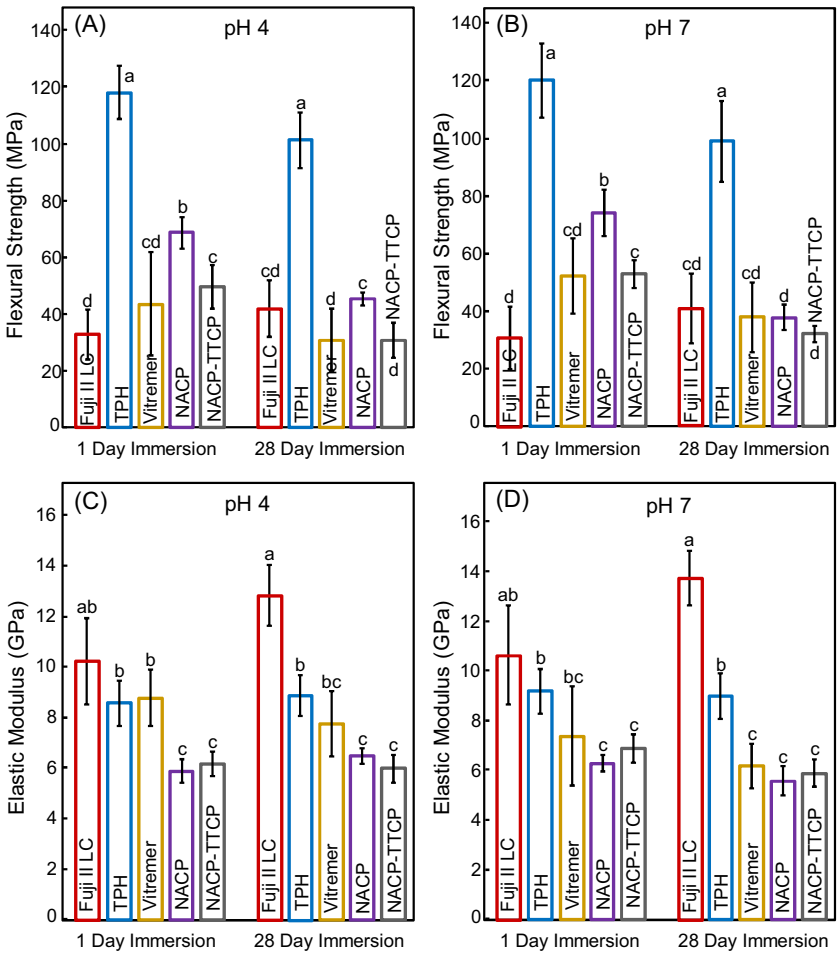

Fig. 3 – Mechanical properties (mean ± sd; n = 6). (A, B) Flexural strength, and (C, D) elastic modulus, after immersion for 1 day and 4 weeks in solutions with pH 4 and 7, respectively. For flexural strength, values indicated by different letters are significantly different from each other ($p < 0.05$). For elastic modulus, values indicated by different letters are significantly different from each other ($p < 0.05$). NACP and NACP-TTCP matched the mechanical properties of Vitremer.

dentin lesions when restored with a commercial composite control, that the new NACP and NACP-TTCP nanocomposites regenerated the minerals lost in dentin, and that the dentin remineralization increased with time, doubling the remineralization from 4 to 8 weeks. Teeth are under constant cycles of demineralization (where tooth mineral begins dissolving at low local pH) and remineralization or repair (conditions that favor deposition of new mineral). This balance between demineralization and remineralization will dictate whether carious lesions progress to the point of needing clinical treatment or the lesion can stabilize or remineralize. The ideal clinical outcome is to minimize the progression of carious lesions so that tooth structure can be preserved and restoration of the tooth is unnecessary. Therefore, the new NACP-based nanocomposites, capable of reversing and remineralizing dentin lesions, represent a novel and promising approach in caries inhibition.

The flexural strength of the new NACP and NACP-TTCP nanocomposites were similar to those of Fuji II LC and Vitremer controls. The elastic moduli of NACP and NACP-TTCP nanocomposites were similar to that of Vitremer. Mechanical properties were slightly decreased after 4 weeks of immersion, compared to those at 1 day. Immersing at pH 4 and 7 had little difference. According to the manufacturer, the indications for Vitremer include Class III, V and root-caries restorations, Classes I and II restorations in primary teeth, and core-buildup. The fact that the new NACP and NACP-TTCP nanocomposites had mechanical properties matching those of Vitremer indicates that they may also be suitable for these applications, with the added benefit of remineralization to inhibit caries. The appearance of NACP and NACP-TTCP nanocomposites were whitish, translucent and capable of being photo-cured, and they may be suitable for anterior and posterior restorations, which require further investigation. Indeed, a previous study showed that the NACP composite successfully remineralized enamel lesions and had a remineralization capability that was four times that of a fluoride releasing commercial control [23]. This was likely because NACP provided calcium and phosphate ions needed to cause remineralization.

Although caries exist in both enamel and dentin, the differences in the structure affect both demineralization and remineralization. Enamel consists of 96% hydroxyapatite mineral, 3% water and 1% organics [44]. Enamel is in constant

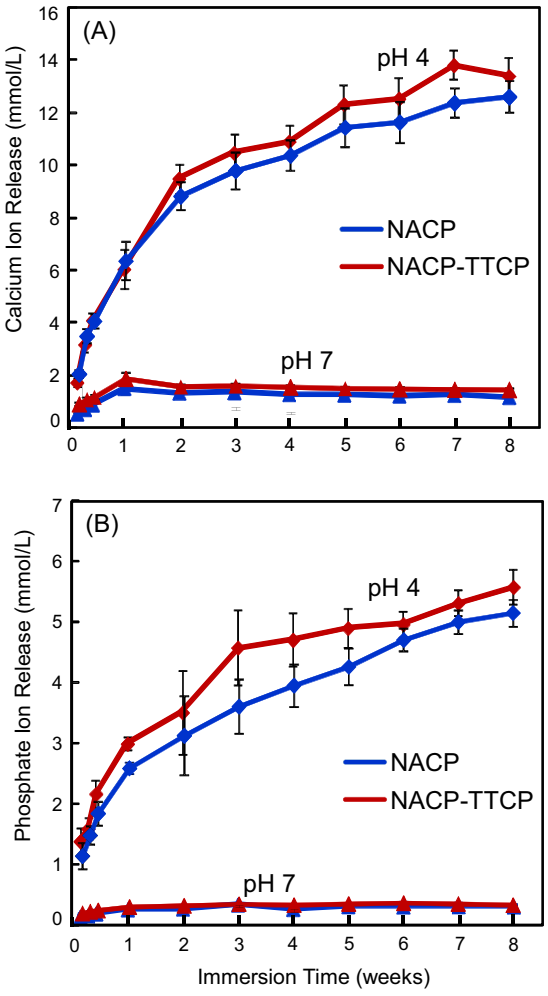

Fig. 4 – Calcium (Ca) and phosphate (P) ion release from two composites: the nanocomposite containing 40% NACP, and the nanocomposite containing 40% NACP and 20% TTCP. (A) Ca and (B) P ion releases (mean ± sd; n = 3). Lowering the pH substantially increased the amount of ion release.

contact and chemical equilibrium with saliva. Dissolution of the hydroxyapatite mineral can occur when the oral pH decreases to 5.5 or lower, and the equilibrium is tipped toward demineralization. However, Ca and P ions in saliva act as a buffer to neutralize the acid. As the pH is increased, and in the presence of Ca and P ions, the equilibrium can be shifted toward remineralization [44]. Even after demineralization, hydroxyapatite "seed" crystals are present in the remaining enamel structure. Upon exposure to supersaturated concentrations of Ca and P ions during a remineralization regimen, these crystals provide locations at which precipitation can occur and where new minerals can form.

In contrast, dentin consists of 70% hydroxyapatite, 20% organic matrix and 10% water. The organic matrix is predominantly made up of collagen fibrils, but also contains non-collagenous proteins (NCPs) and glycoproteins, which play a key role in dentin demineralization and remineralization. Dentin demineralization is similar to enamel demineralization in that initially, the carious lesion develops by dissolution of the mineral. However, once the mineral phase is dissolved, the collagen fibers are exposed and can be destroyed by continued contact with acidic salivary fluid [32]. Clinically, dentin demineralization can be classified in two groups: (1) caries-infected dentin, which is not physiologically remineralizable and is characterized by extensive demineralization, degeneration of collagen fibers and negative odontoblastic processes; and (2) caries-affected dentin, which is physiologically remineralizable and is characterized by intermediate demineralization, sound collagen fibers and active odontoblastic processes [32]. Unlike enamel, the structure of dentin is highly ordered and complex. The collagen fibrils provide an ordered scaffold for mineral deposition, which stiffens and strengthens the collagen to ultimately form a matrix of highly mineralized collagen fibers. Because of this complexity, remineralization of dentin lesions continues to be a challenge.

Dentin remineralization techniques can be classified as either "top down" or "bottom up" processes [31]. In top-down techniques, the carious lesion is exposed to a supersaturated solution of calcium and phosphate ions and mineral precipitation occurs over existing seed crystals in the mineral phase [31]. The source of calcium and phosphate ions can be from a gel, solution, or – as is the case in the work presented here – a CaP-containing composite [21–25]. Unlike enamel, however, caries-infected dentin contains very few seed crystals where mineral precipitation can arise, hence spontaneous nucleation of minerals cannot occur on the collagen matrix. Caries-affected or partially-demineralized dentin does have more residual seed crystals and effective remineralization can occur. In situations where dentin demineralization has progressed significantly, researchers are using the "bottom up" approach to remineralization. The bottom up approach does not rely on seed crystals to provide nucleating sites for crystal growth. Instead, the dentin collagen matrix serves as a template for mineral deposition in the presence of naturally-occurring non-collagenous proteins (NCPs) or biomimetic analogs of these proteins which serve as nucleators [45]. For example, biomimetic molecules were added to an amorphous calcium phosphate system that penetrated the demineralized collagen matrix and transformed into apatite nanocrystals [45,46]. In another case, polyvinylphosphonic acid (PVPA) or sodium trimetaphosphate (STMP) were used as biomimetic analogs to bind to dentin collagen matrix to further attract amorphous calcium phosphate to collage matrix [47,48]. Other NCP analogs or nucleating agents have been investigated as well, including synthetic peptides [49], casein-phosphopeptide with amorphous calcium phosphate (CPP-ACP) [26] and chitin [50], and have shown promising results for remineralizing the demineralized dentin.

The present study investigated the use of NACP-containing nanocomposites for the remineralization of partially-demineralized dentin. Previous studies indicated that calcium phosphate (CaP)-containing resins had the potential to remineralize carious lesions and could be used as pit and fissure sealants [22] or restoration-supporting lining materials [21]. Their relatively low mechanical properties made them inadequate for stress-bearing applications [51]. Dental composites consist of a polymerizable resin matrix,

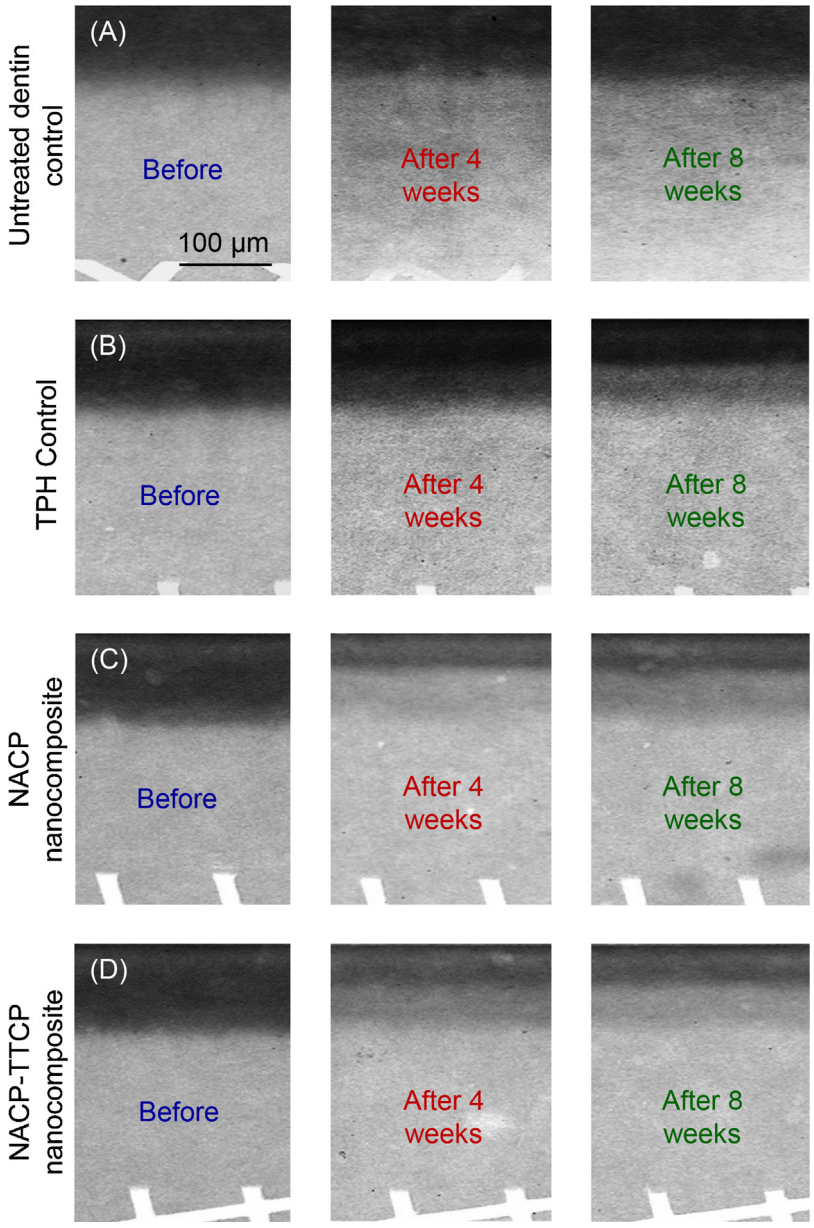

Fig. 5 – Representative microradiographs of dentin lesions before and after the cyclic demineralization/remineralization regimen. The left column, before, refers to the initial dentin demineralization created in the acidic solution. The middle column is after 4 weeks of the cyclic demineralization/remineralization regimen. The right column is after 8 weeks of the cyclic demineralization/remineralization regimen.

fillers, and matrix-filler interfaces. Advances in these areas have led to the use of posterior and hybrid composites in stress-bearing restorations [2,12–15]. However, these composites do not release calcium and phosphate ions. On the other hand, CaP-based composites cannot be used in stress-bearing restorations. It is desirable, therefore, to develop composites with a combination of improved mechanical properties and CaP ion release for remineralization potential. CaP-based nanocomposites were recently developed that greatly improved the strength, fracture toughness, and wear resistance compared with traditional CaP composites [27,37,52]. NACP nanocomposite remineralized demineralized human enamel *in vitro*, with a remineralization that was 4-fold that of a commercial fluoride-releasing composite in a 30-day cyclic demineralization/remineralization regimen [23]. A human *in situ* study with NACP nanocomposite demonstrated the effectiveness in reducing caries development in a 14 day study [24]. The present study advances this research to show that human dentin lesions can also be remineralized and that the lost mineral was regenerated by NACP-based nanocomposite.

Dental caries is a dietary carbohydrate-modified bacterial infectious disease [53,54]. Acidogenic bacteria ferment carbohydrates and produce organic acids, with biofilm pH dropping into the cariogenic region [55]. To simulate *in vivo* pH cycles, previous studies tested cyclic

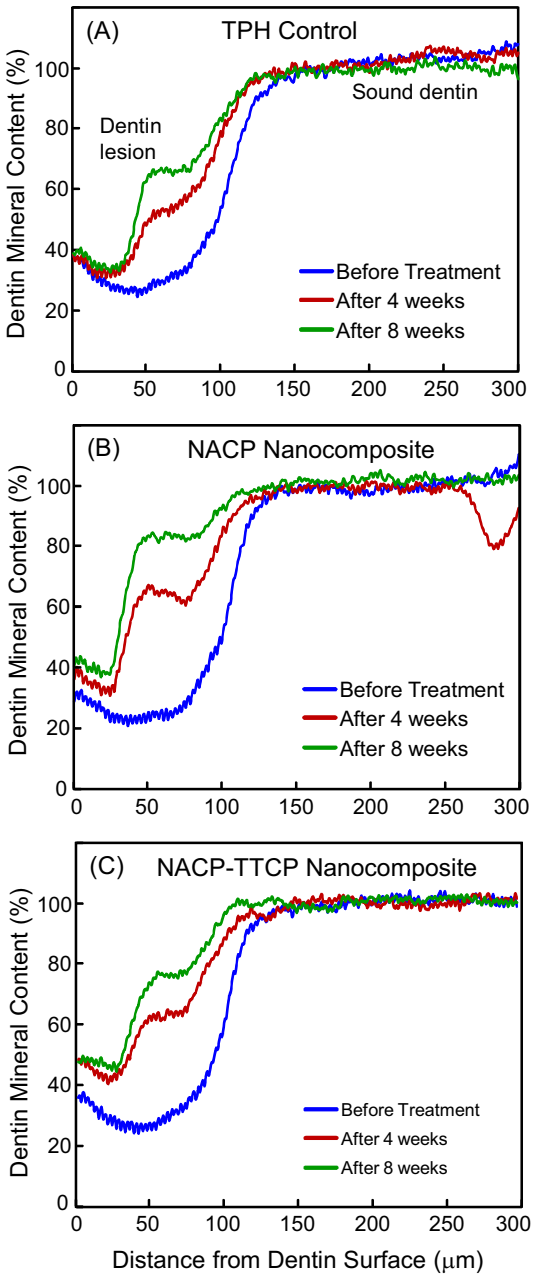

Fig. 6 – Representative mineral profiles of human dentin lesions before, after 4 weeks and after 8 weeks of the cyclic demineralization/remineralization regimen. (A) No-composite dentin control, (B) dentin lesion with the non-releasing commercial composite control, (C) dentin lesion with NACP nanocomposite, and (D) dentin lesion with NACP-TTCP nanocomposite. Dentin lesions without a composite showed further mineral loss in the demineralization/remineralization regimen. Dentin lesions with NACP nanocomposite and NACP-TTCP nanocomposite had great increases in mineral content.

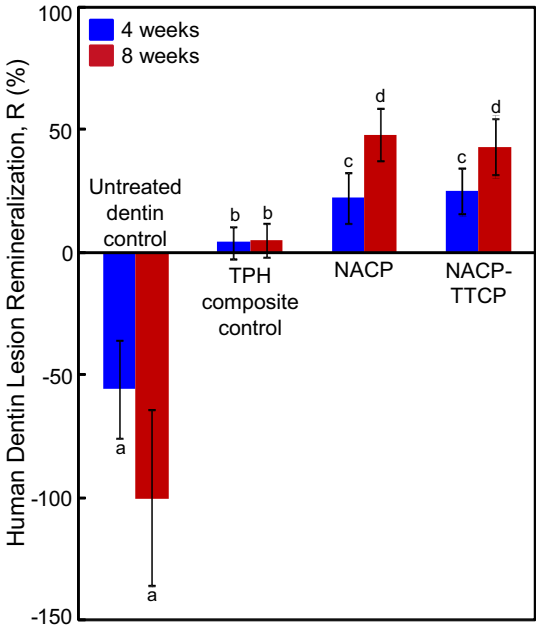

Fig. 7 – Remineralization (mean ± SD; n = 15) of human dentin lesions in the cyclic demineralization/remineralization regimen *in vitro*. Dentin lesions without composite had further mineral loss over time. Dentin lesions restored with commercial composite had little remineralization after 8 weeks of demineralization/remineralization regimen. Remineralization with NACP and NACP-TTCP nanocomposites had the highest values, and was doubled with increasing time from 4 to 8 weeks.

demineralization/remineralization *in vitro* [22,56], with demineralization solution at pH 4, and remineralization solution at pH 7. It has been shown in the current study (Fig. 2) for NACP-TTCP nanocomposites and previous studies [27,28] for NACP nanocomposites that Ca and P ion release is pH dependant and significantly greater at cariogenic pH. During the 1-h demineralization cycle at pH 4, the NACP and NACP-TTCP nanocomposites released more Ca and P ions, indicating that they could reduce dentin demineralization. However, the actual dentin remineralization likely occurred during the thermodynamically-favorable remineralization cycle. Remineralization of dentin under the non-releasing hybrid composite (TPH) was minimal during the 8 week study. This suggests that: (1) the 1-h demineralization and 23-h remineralization regiment, which could potentially occur orally with saliva containing Ca and P ions, was insufficient to remineralize the dentin lesions; and (2) the release of ions from NACP-based nanocomposites was the major contributor to the dentin remineralization in this study.

Ca and P ions can help prevent demineralization. For example, dentifrice with calcium was effective in reducing enamel demineralization [57]. Calcium in mouth rinses or chewing-gums remineralized enamel lesions [58]. Remineralization was achieved via solutions containing Ca and P [59]. Ca and P ion release from NACP nanocomposite was greatly increased when pH was decreased from 7 to 4, hence the NACP nanocomposite was "smart" and greatly increased the ion release at a cariogenic pH 4 when these ions were most needed to inhibit caries [28].

The current study is an example of the top-down method of dentin remineralization. Calcium and phosphate ions are

released from the NACP-based nanocomposites and infiltrate the partially-demineralized dentin. In Fig. 5, the progress of remineralization from 0 weeks to 4 weeks to 8 weeks can be observed as beginning at the demineralized dentin/sound dentin interface. It is anticipated that seed crystals exist at this interface and the apatite mineral can precipitate effectively at this location. As remineralization proceeds, then newly formed mineral develops from the demineralized dentin/sound dentin interface and moves toward the surface, depositing mineral on the remaining interfibrillar collagen. NACP nanocomposite remineralized dentin lesions by 48.2% after 8 weeks and NACP-TTCP composites remineralized dentin by 43.2% after 8 weeks. Although it was anticipated that the addition of another source of calcium and phosphate (TTCP) would provide an enhancement of remineralization, that did not occur. Instead, the remineralization of dentin with NACP alone or with NACP-TTCP was similar statistically. Ca and P ion release (Fig. 4) also showed little difference in ion concentrations, hence the NACP and NACP-TTCP composites had similar ion concentrations available for dentin remineralization. This may indicate that there may exist a threshold CaP filler level in the resin (40% CaP in the present study) above which an additional increase in CaP filler level does not increase the Ca and P ion release, possibly because the ion release is already maximized and the ion concentrations could suppress the driving force for more release. Further study is needed to investigate why the composite with 40% NACP + 20% TTCP had similar ion release to that with 40% NACP. Nonetheless, the excellent remineralization of NACP-based nanocomposites, combined with the acid neutralization and good mechanical properties reported earlier [28,29], indicates that they are highly promising for caries-inhibiting restorations. It should be noted that the present study focused on the effects of CaP composite without using an adhesive. Separate studies formulated adhesives containing NACP for remineralization [60,61]. In addition, rechargeable NACP composite and NACP adhesive were developed for long-term Ca and P ion release [61,62]. Further studies are needed to investigate the tooth structure remineralization efficacy using NACP composite and NACP adhesive with recharge capabilities.

5. Conclusions

NACP-based nanocomposites were shown to effectively remineralize the demineralized human dentin *in vitro* for the first time. A cyclic demineralization/remineralization regimen treatment for 8 weeks failed to remineralize the dentin lesions restored with a commercial composite. In contrast, the novel NACP-based nanocomposites achieved substantial dentin lesion remineralization, and the extent of dentin remineralization doubled when the time was increased from 4 to 8 weeks. This remineralization was related to the NACP-based nanocomposites' capacity to release high levels of Ca and P ions, acid-neutralizing properties, and ability to substantially increase the ion release at cariogenic low pH when these ions would be most needed to combat caries. The new NACP-based nanocomposites are promising for restorations that can remineralize tooth lesions to protect the tooth structures and inhibit secondary caries.

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