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Poly(amido amine) and rechargeable adhesive containing calcium phosphate nanoparticles for long-term dentin remineralization

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ABSTRACT

Objectives: The objective of the present study was to investigate long-term dentin remineralization via the combination of poly(amido amine) (PAMAM) with a novel rechargeable adhesive containing nanoparticles of amorphous calcium phosphate (NACP).

Methods: The NACP adhesive was immersed in lactic acid at pH 4 to exhaust its calcium (Ca) and phosphate (P) ion release, and then recharged with Ca and P ions. Dentin samples were pre-demineralized with 37% phosphoric acid, and then divided into four groups: (1) dentin control, (2) dentin treated with PAMAM, (3) dentin with recharged NACP adhesive, (4) dentin with PAMAM + recharged NACP adhesive. In group (2) and (4), the PAMAM-coated dentin was immersed in phosphate-buffered saline with vigorous shaking for 77 days to accelerate any detachment of the PAMAM macromolecules from the demineralized dentin. Samples were treated with a cyclic remineralization/demineralization regimen for 21 days.

Results: After 77 days of fluid flow challenge, the immersed PAMAM still retained its nucleation template function. The recharged NACP adhesive possessed sustained ion re-release and acid-neutralization capability, both of which did not decrease with repeated recharge and re-release cycles. The immersed PAMAM with the recharged NACP adhesive achieved long-term dentin remineralization, and restored dentin hardness to that of healthy dentin.

Conclusions: The PAMAM + NACP adhesive completely remineralizes pre-demineralized dentin even after long-term fluid challenges and provides long-term remineralization to protect tooth structures.

Clinical significance: The novel PAMAM + NACP adhesive provides long-term bond protection and caries inhibition to increase the longevity of resin-based restorations.

1. Introduction

Resin composites and adhesives are popular for restoring tooth cavities because of their esthetics, direct-filling ability and enhanced performance [1,2]. Nevertheless, the bonded interface is the weak link in these restorations [3]. Secondary (recurrent) caries at the restorative margins is the major reason for dental restoration failure [4]. Replacement of failed restorations accounts for 50–70% of all restorations [5–7].

Strong and durable adhesion to the tooth is important for the success of the restoration [8–10]. Dentin bonding involves infiltration of adhesive resin monomers into the demineralized dentin collagen fibrils to produce a hybrid layer (HL) [8–10]. In the oral cavity, the demineralized collagen matrix may be damaged by enzymes, oral bacteria and fluids, thus degrading the HL [11,12]. Because minerals play an important role in protecting the HL, remineralization of the resin-sparse regions of the HL is an effective strategy to improve the stability of resin-dentin bonds. Remineralization is an effective repair process for

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carious lesions [13–17]. However, natural remineralization via saliva is too weak to protect the HL and is insufficient to reverse the caries process when bacterial acid challenge is severe [13]. Accordingly, much research efforts have been devoted to develop novel strategies to improve the remineralization of tooth structures [18–22].

An important approach uses nucleation template materials to absorb calcium (Ca) and phosphorus (P) ions more effectively [23–27]. Poly (amido amine) (PAMAM) dendrimers possess excellent nucleation template functions. Dendrimers prepared from poly(amido amine) are highly-branched polymers with a central core, internal cavities, branch structures and ending functional groups [28]. For example, carboxylic-terminated PAMAM (PAMAM-COOH) attracted Ca and P ions in collagen fibrils to produce intrafibrillar minerals [29]. Hydroxyl-terminated PAMAM (PAMAM-OH) facilitated remineralization and induced deep dentinal tubule occlusion [30]. In addition, amine-terminated PAMAM (PAMAM-NH_2) promoted mineral precipitate in the demineralized dentin [31]. Phosphate-terminated PAMAM (PAMAM-PO_3H_2) induced dentin remineralization in the oral environment of mice [32].

Another method for remineralization is the development of adhesive resins containing calcium phosphate (CaP) filler particles [33]. Adhesives containing nanoparticles of amorphous calcium phosphate (NACP) released large amounts of Ca and P ions, and rapidly neutralized acids and raised the pH [34], both of which promoted remineralization [35–37]. Due to their small particle size, NACP readily flow with the adhesive into dentinal tubules to form resin tags and release Ca and P ions to remineralize the remnants of carious tissues in prepared tooth cavities [35–37]. Resins containing NACP produced remineralization *in vitro* that was four-fold greater than that of a commercial fluoride-releasing material, and reduced caries to 1/3 that of a control composite in a human *in-situ* model [38,39].

A previous study by the authors showed that the combined use of PAMAM and NACP adhesive (PAMAM + NACP adhesive) had triple benefits of acting as nucleation templates, providing ion release, and acid-neutralization. This approach induced strong dentin remineralization in a cyclic remineralization/demineralization regimen [40]. However, the results also showed that the Ca and P ion release and acid-neutralization capability of NACP adhesive decreased with longer immersion time. The oral cavity is full of fluids with a continuous flow, which decreases the effects of NACP as well as PAMAM. The fluids can detach the PAMAM macromolecules from dentin, thus removing the nucleation templates [31]. Hence, there is a need for the development of a PAMAM + NACP adhesive strategy that is effective for dentin remineralization even with fluid challenge. To date, there is no report on the effect of PAMAM + NACP adhesive on dentin remineralization under long-term fluid immersion and shaking conditions.

PAMAM binds firmly to demineralized dentin because of the size-exclusion features of the collagen matrix and electrostatic interactions, which are important for the dendrimer to fulfill its nucleation template capability in the oral fluid-flowing environment [31,41]. A new rechargeable NACP adhesive was developed recently to provide long-term ion release [42]. The resin consisted of pyromellitic glycerol dimethacrylate (PMGDM), ethoxylated bisphenol A dimethacrylate (EBPADMA), 2-hydroxyethyl methacrylate (HEMA) and bisphenol A glycidyl dimethacrylate (BisGMA), which was filled with NACP [42]. This new NACP adhesive could be repeatedly recharged to re-release Ca and P ions, thus providing long-term remineralization capability. Accordingly, it is highly desirable to combine PAMAM with the rechargeable NACP adhesive to achieve long-term dentin remineralization with fluid immersion and shaking challenges.

The objectives of the present study were: (1) to develop a long-term dentin remineralization strategy that is effective even after prolonged fluid challenges; and (2) to investigate remineralization via combining PAMAM after fluid immersion and shaking together with the rechargeable NACP adhesive. The hypotheses tested were: (1) PAMAM function as nucleation templates even after severe fluid immersion and shaking treatment; (2) The ion-exhausted and then recharged NACP adhesive maintains its acid-neutralization and Ca and P ion release capability; (3) PAMAM after fluid challenge and shaking, and NACP adhesive after ion exhaustion and recharge, are capable of full remineralization of pre-demineralized dentin and restore its hardness to that of healthy dentin.

2. Materials and methods

2.1. PAMAM synthesis and rechargeable NACP adhesive fabrication

PAMAM dendrimers were synthesized using the classical method of Tomalia [28]. The third generation of PAMAM-COOH (G3-PAMAM-COOH) was commercially obtained from Chenyuan Dendrimer Tech Company (Weihai, China). The G3-PAMAM-COOH was used because previous studies showed that it effectively facilitated mineral deposition in the demineralized human teeth [29,43]. In the present article, the term “PAMAM” refers to G3-PAMAM-COOH. A PAMAM solution (10 mg/mL) was prepared by dissolving 100 mg of dry PAMAM powder in 10 mL of deionized water [29,43].

A spray-drying technique was used to synthesize NACP, following the method employed in a previous study [44], yielding NACP with a mean particle size of 116 nm. To fabricate the adhesive, PMGDM (Esstech, Essington, PA, USA) and EBPADMA (MilliporeSigma, St, Louis, MO, USA) were mixed in a 1:1 mass ratio, which was subsequently rendered light-curable with 1% phenylbis (2,4,6-trimethylbenzoyl) phosphine oxide (Sigma–Aldrich). Then, 10% HEMA (Esstech) and 5% BisGMA (Esstech) were added, and this PMGDM-EBPADMA-HEMA-BisGMA adhesive was referred to as “PEHB” [42]. The NACP were mixed into PEHB adhesive at a mass fraction of 40% to obtain a PEHB + NACP adhesive paste, which was then placed into a 2 × 2 × 12 mm rectangular mold [45,46]. The mass fraction of 40% was used because our previous studies showed that adding 10–40% NACP into PEHB adhesive did not negatively affect the dentin bond strength, and increasing the NACP filler level from 10% to 40% greatly increased the Ca and P ion release [34,40]. Specimens were light-cured (Triad 2000, Dentsply, York, PA, USA) for 1 min on each open side.

2.2. Initial Ca and P ion release from virgin PEHB + NACP adhesive

A 133 mmol/L of NaCl solution was buffered to pH 4 with 50 mmol/L lactic acid, which simulated a cariogenic condition (referred to as “lactic acid solution”). Three PEHB + NACP adhesive bars of 2 × 2 x 12 mm were immersed in 50 mL of lactic acid solution, yielding an adhesive volume per solution of 2.9 mm^3/mL, as reported in a previous study [46]. The Ca and P ions released from PEHB + NACP adhesive were determined at 1, 3, 5, 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days. At each time interval, 0.5 mL aliquots were removed and replaced by fresh solution. The collected aliquots were analyzed for Ca and P ion concentrations using a spectrophotometric method (DMS-80 UV–vis, Varian, Palo Alto, CA, USA) with known standards and calibration curves [42].

2.3. Remineralization after a long period of fluid challenge

Tooth collection was approved by the University of Maryland Baltimore Institutional Review Board. Caries-free human third molars were obtained from the dental school clinics. Dentin squares of approximately 4 × 4 x 1 mm were prepared by cutting perpendicularly to the longitudinal axis of the tooth at 4 mm above the cementoenamel junction using a diamond-coated saw (Buehler, Lake Bluff, IL, USA). The dentin squares were first demineralized with 37% phosphoric acid for 15 s [47]. The demineralized dentin specimens were divided into four groups:

(1) Control group. Each dentin specimen was coated with 100 μL of water for 1 h [29]. Each specimen was immersed in phosphate-buffered saline (PBS, pH 7.4) and shaken on a mixer (Analog Vortex Mixer,

Fisher Scientific, Waltham, MA, USA). After 77 days, the dentin specimens were removed from the PBS and served as negative control.

(2) PAMAM group. Each dentin sample was coated with 100 μL of PAMAM solution for 1 h, then washed with water to remove any unattached PAMAM [29]. To test whether fluid immersion and shaking challenge decreased the nucleation template capability of PAMAM, the PAMAM-coated dentin was immersed in PBS with a heavy shaking action as described in (1). The PBS immersion and shaking were much more severe than the fluid flowing in the oral environment, which accelerated any detachment of PAMAM from dentin. The 77 days of PBS immersion and shaking approximated the time of oral fluid challenge for more than 1 year. These dentin specimens were referred to as "immersed-PAMAM-coated dentin".

(3)PEHB + NACP adhesive group. After 70 days of immersion in lactic acid at pH 4, the PHEB + NACP adhesive bars were collected and stored in 100 mL of fresh lactic acid for another 7 days to ensure that the ion release was exhausted [48]. These exhausted bars were used for Ca and P ion recharge. The Ca ion recharge solution contained 100 mmol/L of $CaCl_2$ and 50 mmol/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in water. The P ion recharge solution contained 60 mmol/L of K_2HPO_4 and 50 mmol/L of HEPES buffer in water. Both the Ca and P recharge solutions were adjusted to pH 7.0 with 1 mmol/L of KOH [42]. Three exhausted bars were immersed into 5 mL of Ca recharge solution and shaken for 1 min. These specimens were then washed with water, air dried, and immersed in 5 mL of P recharge solution with a shaking action for another 1 min [42]. The recharge was repeated three times. This immersion and shaking process aimed to simulate the mouth-rinsing process [42]. Each dentin specimen was placed in contact with three recharged PEHB + NACP bars. This was because when immersed in 1 mL solution, this would yield a resin volume per solution of 0.14, the same as that in a previous study [34].

(4)PAMAP + PEHB + NACP adhesive group. The immersed-PAMAM-coated dentin was prepared as described in (2). The ion-exhausted and recharged PEHB + NACP was obtained as described in (3). The immersed-PAMAM-coated dentin was placed in contact with three recharged PEHB + NACP bars, as described in (3). A 1.5 mL conical vial was employed for storing each specimen, immersed in 1 mL of the solution as described below.

Artificial saliva was prepared with1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 130 mmol/L KCl, 1.0 mmol/L NaN_3 and 20 mmol/L HEPES, and adjusting to pH 7.0 with 1 mmol/L of KOH [49]. Each day, each specimen from the aforementioned four groups underwent cyclic remineralization/demineralization treatment by immersing in 1 mL of fresh pH 7 artificial saliva for 23 h, and then in 1 mL of pH 4 lactic acid for 1 h [40]. The 1 h approximated the accumulated intraoral acid challenge times per day [38,50]. The procedure was repeated for 21 days. Bars containing NACP were first recharged each day, as described in (3), prior to the artificial saliva/lactic acid immersion treatment.

2.4. Acid neutralization and Ca and P ion concentration measurements

The pH values of artificial saliva and lactic acid solution of the four groups were monitored at 1, 3, 5, 7, 10, 14 and 21 days, using a combination pH electrode (Orion, Cambridge, MA). For each time period, the Ca and P ion concentrations in both the artificial saliva and lactic acid solutions were measured by removing 1 mL of the solution and replacing with 1 mL of fresh solution. The collected solutions were analyzed for ion concentrations as described in Section 2.2 [42].

2.5. Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS)

To examine both the dentin occlusal surface and longitudinal section after 21 days of cyclic remineralization/demineralization, each dentin specimen was cut into two equal halves along its midline, using a diamond saw under water cooling. The occlusal and longitudinal sections were sputter-coated with gold and examined with SEM (JEOL 5300, Peabody, MA, USA). Energy dispersive spectroscopy (INCA350, Oxford, UK) was used to analyze the elemental compositions of the dentin specimens.

2.6. Dentin hardness measurement

Dentin hardness was measured for the four groups after cyclic remineralization/demineralization for 21 days. In addition, the hardness of healthy dentin, and dentin after acid-etching but without undergoing the remineralization/demineralization regimen, were measured as comparative controls. A hardness tester (Tukon 2100B, Instron, Canton, MA, USA) was employed using a Vickers diamond indenter with a load of 20 g and 10 s dwell time [51]. The hardness results showed that the PAMAM + PEHB + NACP adhesive group produced remineralization and greatly increased the hardness of demineralized dentin at 21 days, but the value was still lower than that of healthy dentin. To investigate how long it would take for the PAMAM + PEHB + NACP adhesive to completely restore the hardness of the demineralized dentin to that of healthy dentin, the specimens were further treated with cyclic remineralization/demineralization regimen for another 14 days (yielding a total of 35 days). Six indentations were made in each dentin specimens, with six specimens per group per time point.

2.7. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

At 21 days, each dentin specimen was analyzed using ATR-FTIR (NICOLET iS10, Thermo Scientific, USA). Infrared spectra in the range of 800 to 1800 cm^{-1} were recorded and analyzed with the OMNIC 8 software (Nicolet, Madison, WI, USA).

2.8. Statistical analysis

All experimental data were checked for normal distribution and quality of variances with the Kolmogorov–Smirnov test and modified Levene test, respectively. One-way and two-way analyses of variance (ANOVA) were performed to detect the significant effects of the variables. Tukey's multiple comparison tests were used for post-hoc comparisons. Statistical significance for all tests was set at $\alpha = 0.05$.

3. Results

The initial ion release results from the virgin PEHB + NACP adhesive are shown in Fig. 1. The Ca and P ion concentrations increased with time, reaching a plateau at about 49–56 days. There was minimal ion release from 56 days to 70 days. The specimens were continuously immersed in a pH 4 solution for 7 more days to ensure complete exhaustion of the ion release. The exhausted adhesive specimens were then recharged to examine the re-release Ca and P ions for long-term remineralization.

Fig. 2 plots the pH values of the four groups. The pH was always maintained at around 7.0 when specimens were immersed in artificial saliva, because of the strong buffering capacity of HEPES, (data not shown). While immersed in lactic acid solution, the control group and PAMAM group both had a stable pH of ˜4. For the PEHB + NACP and PAMAM + PEHB + NACP groups, pH values increased to pH 5.6 at 15 min, and to pH 6.2 at 1 h. This was similarly observed from 1 to 21 days.

Fig. 2C plots the time it took to raise the pH to above 5.5 for the lactic acid solution with an initial pH of 4. For the PEHB + NACP and PAMAM + PEHB + NACP groups, the recharged PEHB + NACP adhesive rapidly raised the pH from the cariogenic pH 4 to a safe pH of ≥ 5.5. From 1 to 21 days, it took about 11 min to raise the pH from 4 to 5.5. There was no significant decrease in the acid-neutralization

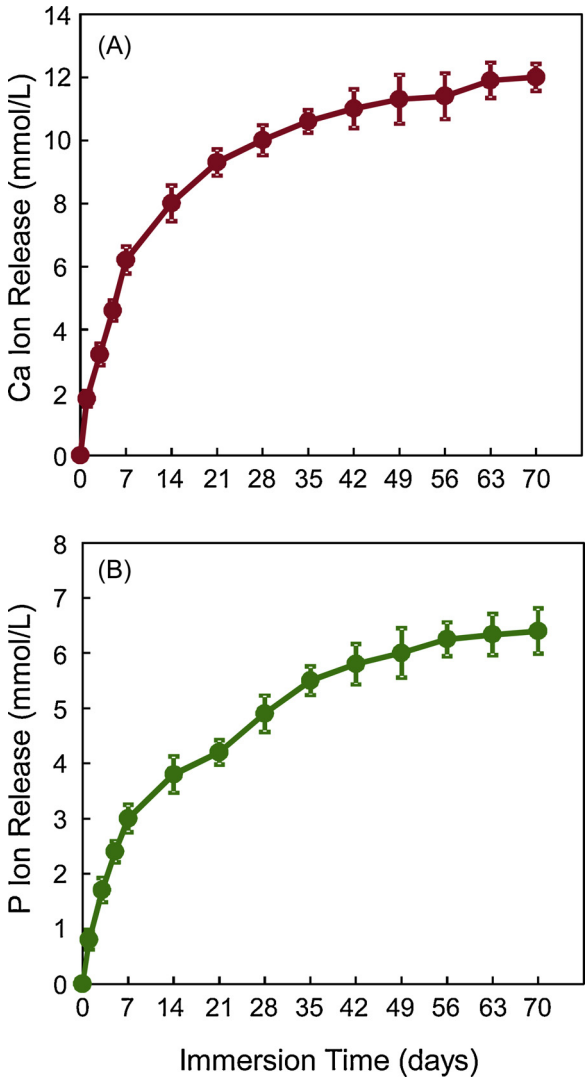

Fig. 1. Initial Ca and P ion release from virgin PEHB + NACP adhesive. (A) Cumulative Ca, and (B) P ion concentrations (mean ± SD; n = 6).

capacity with increasing time from 1 to 21 days. In contrast, the control and PAMAM groups both failed to neutralize acids, with pH maintained at ˜4.

The ion concentrations of the four tested groups are plotted in Fig. 3. For artificial saliva (Fig. 3A and B), the Ca and P ion concentrations of the control group were around 1.5 mmol/L and 0.9 mmol/L, respectively, which were consistent with the original intrinsic levels in the solution. The PAMAM group had lower Ca and P ion concentrations than the control group because the PAMAM molecules absorbed some of the ions. The PEHB + NACP and PAMAM + PEHB + NACP groups had much higher ion concentrations than the control and PAMAM groups ($p < 0.05$), due to Ca and P ion re-release from the recharged PEHB + NACP adhesive. For the lactic acid solution (Fig. 3C and D), the Ca and P concentrations were almost zero for the control group and PAMAM group because the solution had no intrinsic ions. In contrast, the PEHB + NACP and PAMAM + PEHB + NACP groups had high Ca and P concentrations (Ca: 2.7 mmol/L, P: 1.5 mmol/L), due to the ion re-release from the recharged PEHB + NACP adhesive.

Fig. 4 shows typical SEM micrographs of dentin occlusal section perpendicular to the dentinal tubules. Control dentin had little mineral precipitation on its surface, and all the tubules were fully open (Fig. 4A1, A2). In contrast, after 77 days of PBS immersion and shaking treatment, PAMAM macromolecules were still retained and absorbed Ca and P ions to regenerate minerals on dentin surface and in the dentinal tubules (Fig. 4B1, B2). The recharged PEHB + NACP adhesive alone also slightly induced mineral re-deposition, with some mineral deposits in the tubules (Fig. 4C1, C2). Importantly, the PAMAM + PEHB + NACP group facilitated the greatest mineral deposition within the demineralized dentin, which was covered with large amounts of newly-generated minerals (Fig. 4D1, D2).

Representative SEM images of the dentin longitudinal section parallel to the dentinal tubule axis are shown in Fig. 5. Control dentin had exposed collagen fibrils in the tubules (Fig. 5A1, A2). The immersed-PAMAM macromolecules had some mineral regeneration in the dentinal tubules (Fig. 5B1, B2). The recharged PEHB + NACP adhesive alone induced small amounts of mineral deposition within the tubules (Fig. 5C1, C2). Remarkably, the immersed-PAMAM + recharged PEHB + NACP group regenerated the most minerals in the tubules, which completely occluded the tubules (Fig. 5D1, D2).

The EDS maps are inserted in Figs. 4 and 5. The results showed that control dentin had very weak Ca and P element peaks. In contrast, moderate Ca and P element peaks were observed in PAMAM and PEHB + NACP groups. Remarkably, PAMAM + PEHB + NACP group had the strongest Ca and P peaks, suggesting that PAMAM + PEHB + NACP facilitated the most mineral re-deposition.

The dentin hardness results are plotted in Fig. 6(A, B). The hardness of healthy dentin was 0.55 GPa. After the treatment of acid-etching, the hardness decreased to 0.33 GPa. After the cyclic remineralization/demineralization treatment, the hardness of control dentin decreased to 0.27 GPa at 21 days, and 0.25 GPa at 35 days. In contrast, PEHB + NACP group maintained dentin hardness, which was 0.34 GPa at 21 days, and 0.35 GPa at 35 days. The PAMAM group produced higher dentin hardness than that of the PEHB + NACP group, which was 0.4 GPa and 0.44 GPa at 21 and 35 days, respectively. The PAMAM + PEHB + NACP group achieved the greatest increase in dentin hardness, raising the hardness to 0.47 GPa at 21 days, and 0.52 GPa at 35 days, which became stantistically similar to that of healthy dentin ($p > 0.1$). These results demonstrate that PAMAM + PEHB + NACP produced complete refilling of the pre-demineralized dentin with mineral precipitation to regain the hardness of normal dentin.

The results of FTIR are presented in Fig. 6C. The intensities of phosphate peaks at 950-1150 cm^{-1} in the infrared spectra were in accordance with the results of EDS, which suggested that mineral re-deposition efficacy was in the order: PAMAM + PEHB + NACP > PAMAM > PEHB + NACP > Control. These results confirmed that the PAMAM + PEHB + NACP group facilitated the greatest mineral regeneration in the pre-demineralized dentin.

4. Discussion

The present study represents the first report that the ion-exhausted and recharged NACP adhesive, with PAMAM after fluid immersion and shaking, completely regenerated minerals in the pre-demineralized dentin and raised the hardness back to that of healthy dentin. The HL plays an important role in the bonding of dentin [8–10], and the composite-dentin bonded interface is the weak link in the restoration [3]. Micro-gaps often form at the composite-dentin interfaces, which allow the bacteria and enzymes to invade the HL [52,53]. Bacterial acids demineralize the tooth structures, causing secondary caries [52,53]. Endogenous matrix metalloproteinases (MMPs) degrade resin-sparse collagen fibrils in the HL, causing bonding failure [54]. Therefore, dentin crystallite re-deposition is a promising approach to prolong the longevity of composite-dentin bonding [55], because the re-generated minerals occupy the nanometer-sized voids such as nano-leakages in the HL [55]. The minerals also prevent MMPs from degrading the collagen fibrils in the HL. Hybrid layers with re-deposited minerals are more stable in the oral cavity, and may be able to resist and neutralize biofilm acid challenges [56].

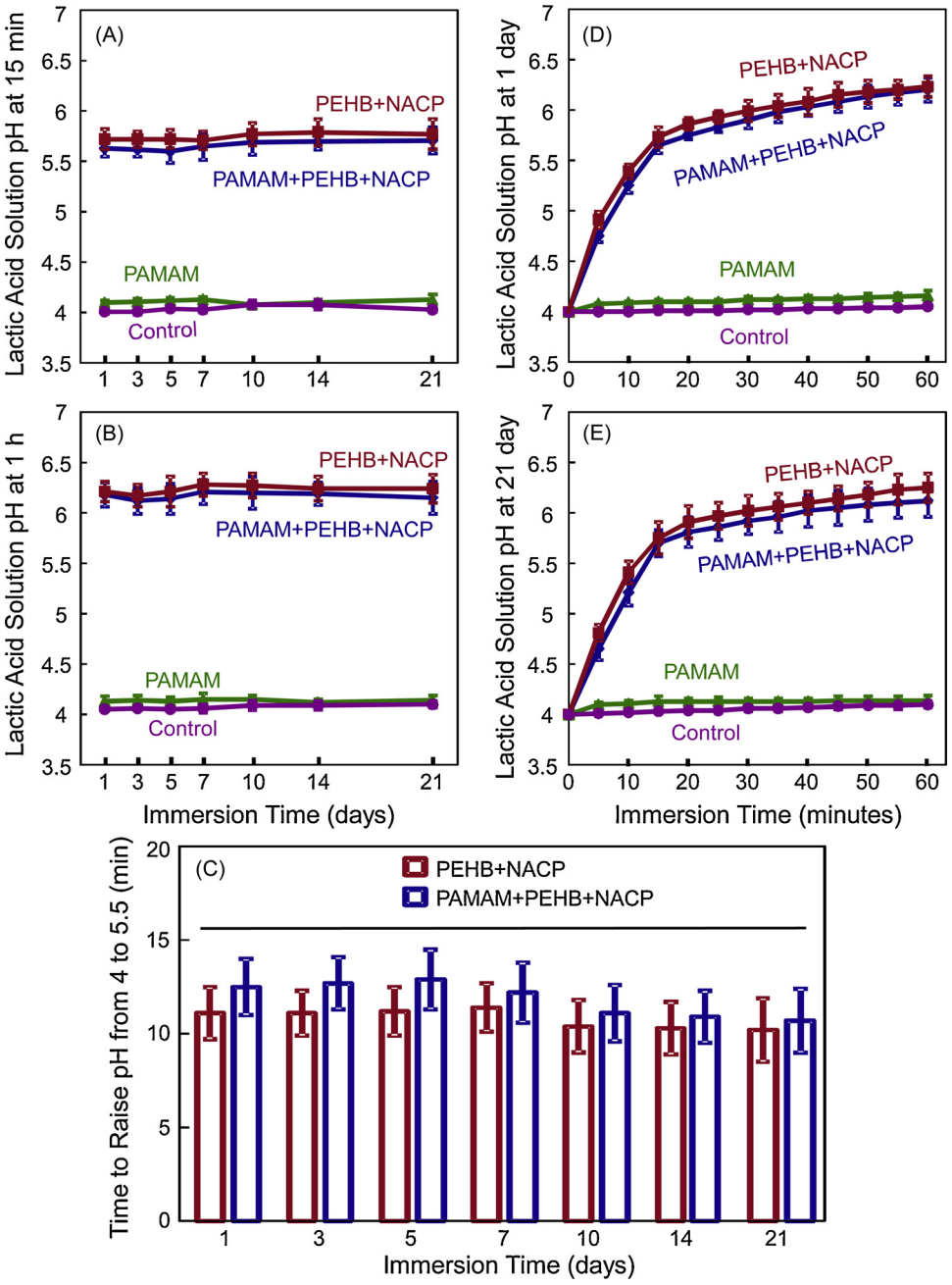

Fig. 2. Acid neutralization. The pH value of lactic acid solution was measured after the sample was immersed for: (A) 15 min, (B) 1 h, and (C) the time it took to raise the pH from 4 to 5.5 (mean ± SD; n = 6) from 1 to 21 d. The pH of lactic acid solution was continuously monitored after the sample was immersed within 1 h at: (D) 1 d, and (E) 21 d (mean ± SD; n = 6). PEHB + NACP and PAMAM + PEHB + NACP groups neutralized acids challenge and raised the pH. Importantly, the acid neutralization ability of the two groups did not diminish with repeated recharge cycles even though a fresh solution was used to immerse the sample every day, indicating a long-term acid-neutralization efficacy. PAMAM and control groups did not neutralize acids, and their pH stayed at 4.

Unfortunately, the demineralized dentin cannot promote strong remineralization because it is mainly consists of a collagen matrix, with only weak nucleation capability [20,56]. Therefore, two strategies were applied to promote dentin remineralization: coating nucleation template materials on the surface of demineralized dentin, and increasing Ca and P concentrations of the remineralization solution [55]. In the authors' previous study, the combination of PAMAM with NACP adhesive produced triple benefits of providing superior nucleation templates, ions release and acid-neutralization [40]. The combination facilitated mineral re-deposition with the demineralized dentin in a cyclic remineralization/demineralization regimen. However, that study also showed that the acid-neutralization and ion release abilities of NACP adhesive decreased over time [40]. In addition, the nucleation template function of PAMAM macromolecules after long periods of fluid challenge was not investigated in the previous study.

Most tooth remineralization studies examined the short-term or immediate remineralization effects. For example, a novel oligopeptide was coated on the demineralized dentin surface for several minutes, and then immersed into a saliva-like solution to determine its immediate remineralization capability [57]. In another study, researchers employed polyacrylic acid (PAA) as the ACP sequestration analog, and used polyvinylphosphonic acid (PVPA) as nucleation template to examine the remineralization effect of PAA/PVPA on collagen fibrils within 3 days [58].

Most dental restorations do not suffer bonding failures within 1 year of placement. In addition, recurrent caries does not occur immediately after the composite is placed, and the process of recurrent caries also usually takes years [59]. Therefore, short-term remineralization is not enough, and long-term remineralization is needed. However, there has been no report to date on the long-term remineralization efficacy of the remineralization materials.

In the present study, an accelerated fluid challenge model was developed and used to investigate the long-term remineralization efficacy of PAMAM + NACP adhesive. Two issues should be noted. First, the 77

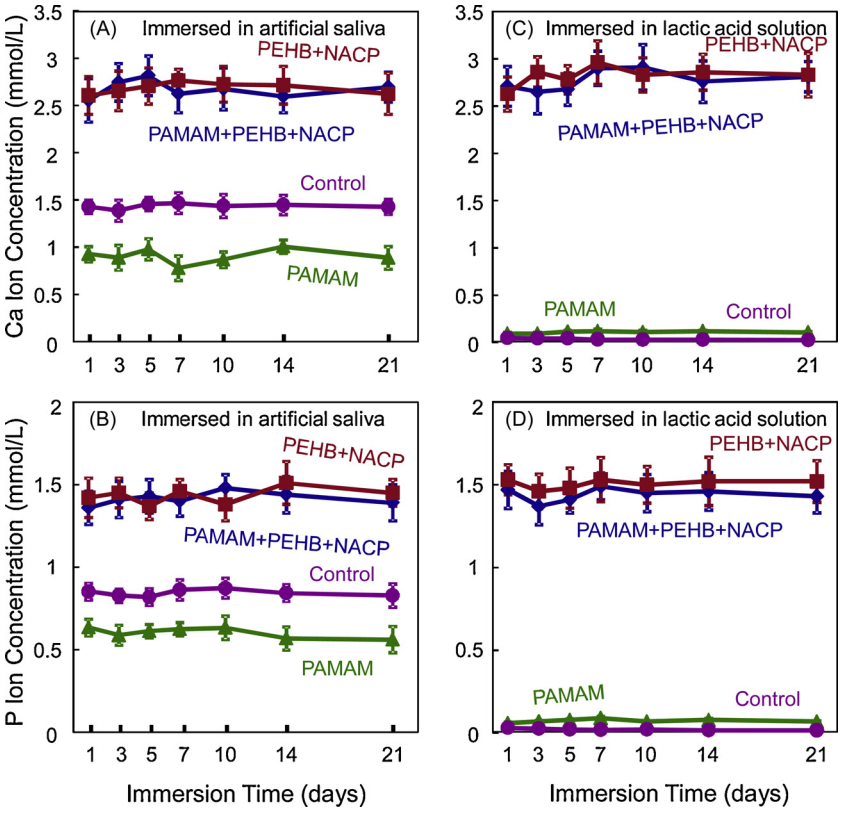

Fig. 3. Ion concentrations (mean ± SD; n = 6). Ca and P concentrations of (A, B) artificial saliva solution, and (C, D) lactic acid. PEHB + NACP and PAMAM + PEHB + NACP showed much higher Ca and P ion concentrations than control and PAMAM, due to the ion re-release from the recharged PEHB + NACP adhesives. The ion concentrations of artificial saliva in PAMAM group were lower than those in control group, suggesting that PAMAM absorbed Ca and P ions. For PEHB + NACP and PAMAM + PEHB + NACP groups, the ion re-release did not diminish with repeated recharge cycles even though a fresh solution was used every day, indicating long-term ion recharge and re-release.

days of PBS immersion and shaking created a much more severe fluid-flowing environment than the natural oral environment. The 77 days of PBS immersion and shaking represented an accelerated model to potentially detach PAMAM molecules from the demineralized dentin. The resting saliva flow rate is only 0.3 to 0.65 mL/min in the oral cavity [60], and other severe fluid flows such as drinking and gargle typically do not last for more than 30 min per day. The PBS immersion with a strong and constant shaking action in the present study was much more severe than the natural fluid challenge in the oral cavity. Therefore, the 77 days of PBS immersion with continuous shaking action would be equivalent to severe fluid challenge times that would take more than a year to accumulate orally. Second, the 77 days of pH 4 lactic acid solution immersion exhausted the Ca and P ion release of PEHB + NACP adhesive. Since the pH of local plaque in the oral environment decreasing to approximate 4 would last for only a few minutes after each meal or sugar intake, 77 days of pH 4 lactic acid solution immersion approximated low pH time accumulations in more than 1 year orally [42]. Therefore, in the present study, 77 days of PBS immersion and shaking for PAMAM-coated dentin, and 77 days of pH 4 lactic acid immersion for PEHB + NACP adhesive, constituted an accelerated fluid-challenge model that simulates the condition of PAMAM and PEHB + NACP adhesive being fluid-challenged in the oral environment for more than a year. Hence, this model enabled the investigation of long-term dentin remineralization of PAMAM + PEHB + NACP adhesive. The hypotheses were validated that PAMAM macromolecules fulfilled its nucleation template function even after long period of fluid challenge; the ion-exhausted and then recharged PEHB + NACP adhesive re-released Ca and P ions and neutralized acid challenges; and the combination of immersed-PAMAM with recharged PEHB + NACP adhesive achieved complete dentin remineralization, returning the pre-demineralized dentin hardness to that of healthy dentin.

After 21 days of cyclic remineralization/demineralization regimen, the control group showed further demineralization. When immersed in artificial saliva for 23 h each day, demineralized dentin alone could only induce minimal mineral deposition because of its weak nucleation template function [20,47]. When immersed in pH 4 lactic acid solution for 1 h, the demineralized dentin were continuously being acid etched, resulting in further demineralization.

The binding strength of PAMAM to the demineralized dentin is important to sustain its long-term mineral re-deposition capability. In the present study, the PAMAM group yielded moderate dentin re-deposition, which suggested that most PAMAM macromolecules were still attached in the demineralized dentin even after 77 days of severe fluid challenge. Therefore, PAMAM macromolecules firmly attach to the demineralized dentin, which is dependent on the electrostatic interactions and size-exclusion features of the collagen fibrils [29,41]. The immersed-PAMAM retained cationic Ca ions through electrostatic interactions via its outside anionic carboxylic groups during immersion in artificial saliva, which promoted mineral re-precipitation [29]. Unfortunately, PAMAM macromolecules failed to prevent dentin demineralization during the 1-h lactic acid immersion period daily, because PAMAM lacked Ca and P ions and acid-neutralization capabilities.

The ion release of traditional CaP-containing adhesive was short-term, lasting for only a couple of months [42]. Once the Ca and P ion release was exhausted, the CaP-containing adhesive cannot induce remineralization anymore. In contrast, the novel rechargeable PEHB + NACP adhesive can be repeatedly recharged to re-release Ca and P ions to provide long-term mineral precipitation within the demineralized dentin [42]. The recharge capability of the PEHB + NACP adhesive is likely due to two mechanisms. First, PMGDM can chelate with Ca ions in the recharge solution using its carboxylate groups. Second, when the ion release was exhausted, numerous sites that were previously occupied by the Ca and P ions became available for the incoming Ca and P ions from the recharge solution [46]. In the present study, the exhausted PEHB + NACP adhesive was recharged by immersing in Ca and P ion solutions, which can potentially be used as a mouth-wash solution [42]. Each day, during the 23 h of artificial saliva immersion, the recharged PEHB + NACP adhesive re-released Ca and P ions to facilitate mineral re-deposition. When immersed in the lactic acid solution for the remaining 1 h, the recharged PEHB + NACP adhesive rapidly released

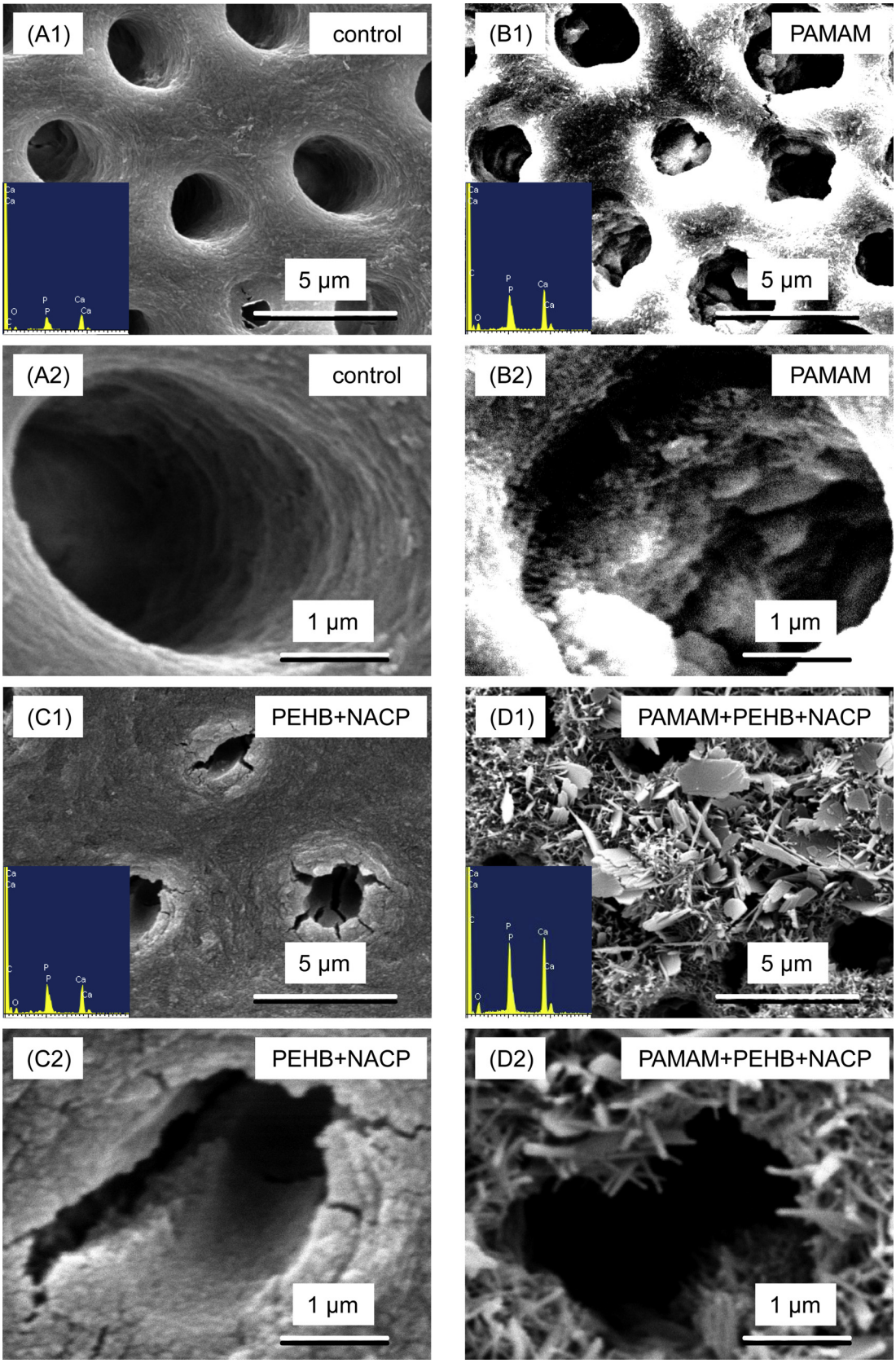

Fig. 4. Typical SEM micrographs of demineralized dentin occlusal surface perpendicular to dentinal tubule axis after 21 d cyclic remineralization/demineralization treatment: (A1, A2) control group, (B1, B2) PAMAM group, (C1, C2) PEHB + NACP group, and (D1, D2) PAMAM + PEHB + NACP group. (A1, A2) showed dentin surface with many widely open dentinal tubules. (B1, B2) showed moderate dentin remineralization. (C1, C2) showed a small amount of precipitated minerals within dentinal tubules due to remineralization. In (D1, D2), the greatest mineral crystals deposited in the demineralized dentin surface. EDS maps were inserted into (A1, B1, C1, D1).

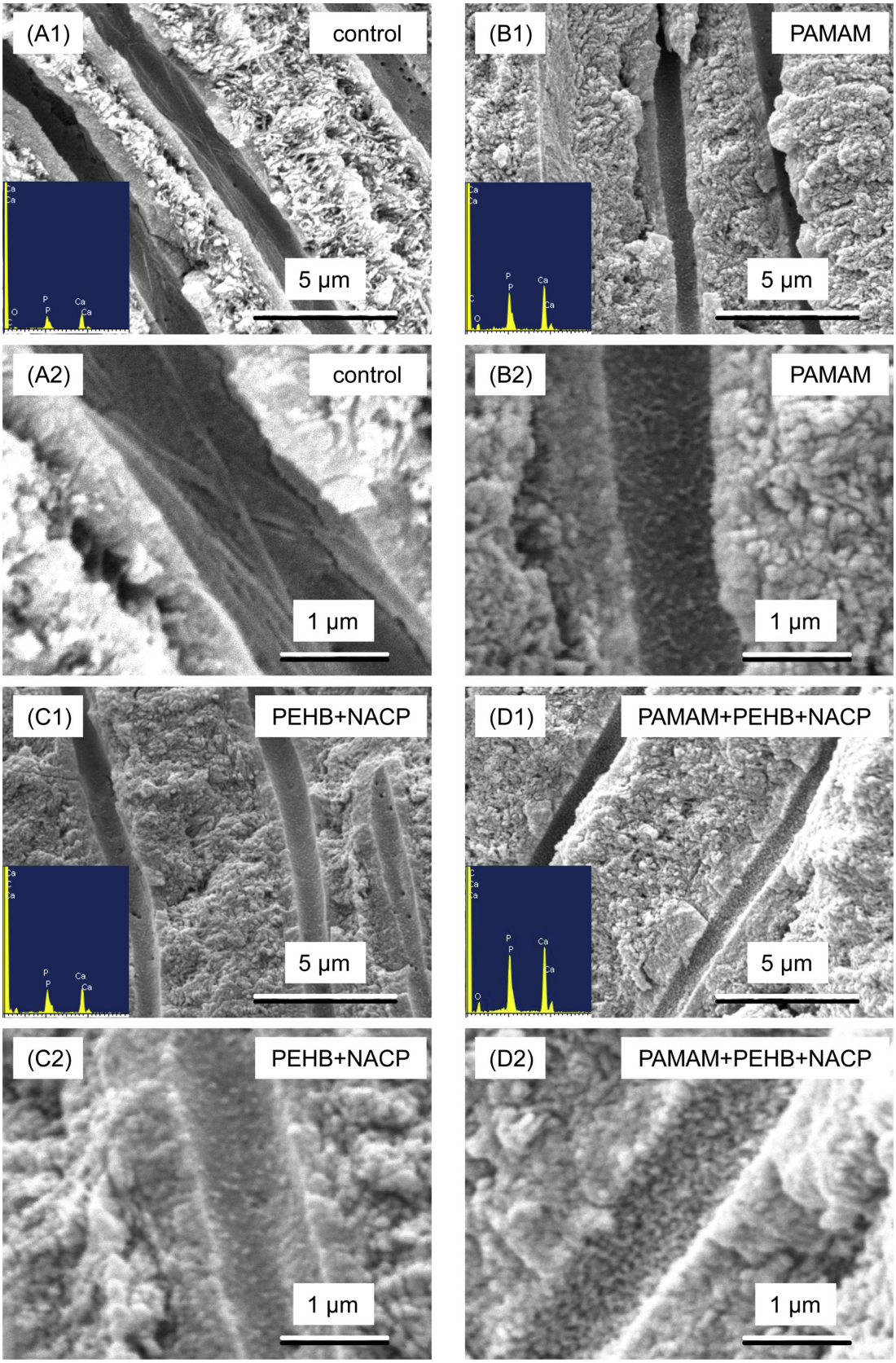

Fig. 5. SEM micrographs of demineralized dentin cross-sections parallel to the dentinal tubule axis after 21 d cyclic remineralization/demineralization treatment: (A1, A2) control group, (B1, B2) PAMAM group, (C1, C2) PEHB + NACP group, and (D1, D2) PAMAM + PEHB + NACP group. Micrographs were taken on dentin cross-sections in a subsurface region of 2–30 μm beneath the surface. In (A1, A2), dentinal tubules were full of exposed collagen fibrils. (B1-C2) showed small amount of minerals precipitate in dentinal tubules due to remineralization. In (D1, D2), the dentinal tubules were filled with dense minerals with the greatest remineralization among all groups. EDS maps were inserted into (A1, B1, C1, D1).

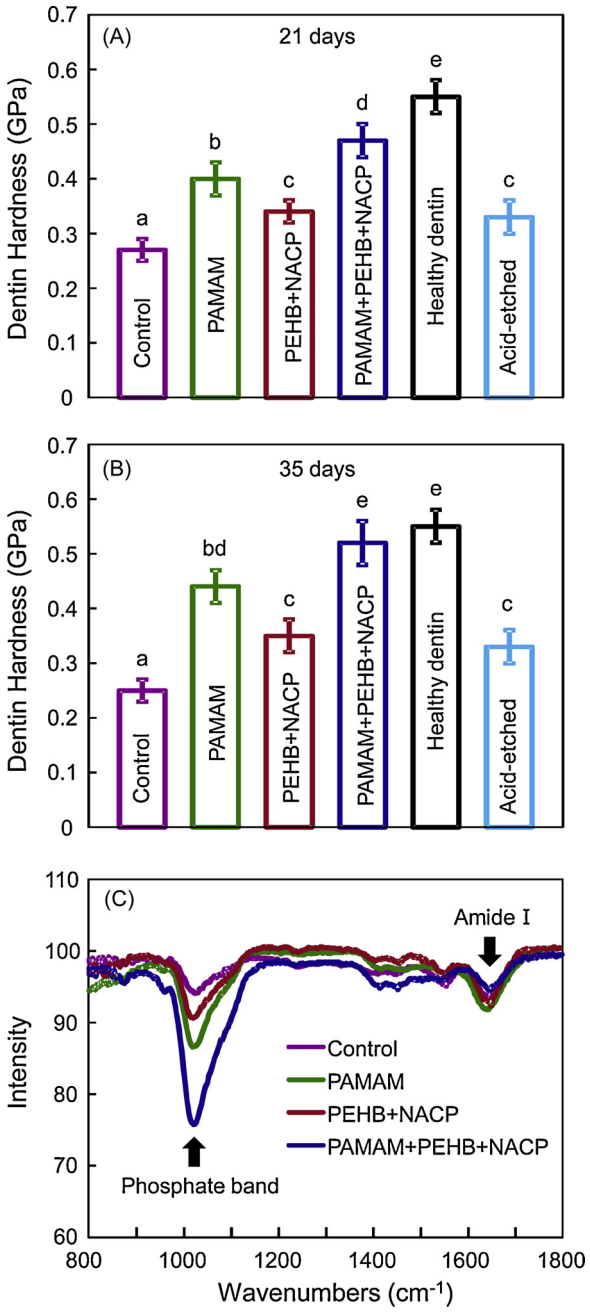

Fig. 6. Dentin hardness and ATR-FTIR spectra (mean ± SD; n = 6). Dentin hardness was measured after (A) 21 d and (B) 35 d of cyclic remineralization/demineralization treatment for: control group, PAMAM group, PEHB + NACP group, and PAMAM + PEHB + NACP group. The hardness of healthy dentin and acid-etched dentin was also measured. Dissimilar letters indicate significantly different values ($p < 0.05$). (C) ATR-FTIR spectra after 21 d of cyclic remineralization/demineralization treatment. PAMAM + PEHB + NACP had the strongest PO_4^{3-} peak in ATR-FTIR spectra, indicating that the combination of immersed-PAMAM with recharged PEHB + NACP adhesive yielded the greatest dentin remineralization.

Ca and P ions, neutralized the acid challenge, and raised the pH to above 5.5, thus helping to inhibit demineralization. Remarkably, the ion re-release and acid-neutralization capability of PEHB + NACP adhesive did not diminish with repeated recharge and re-release cycles, suggesting its long-term remineralization efficacy. In addition, since the remineralization of HL could effectively improve the long-term stability of the resin-dentin bonds, further study should investigate the long-term remineralization effect of the immersed PAMAM + recharged NACP adhesive on the HL.

These factors contributed to the results that the PAMAM + PEHB + NACP strategy achieved complete dentin mineral re-precipitation, returning the pre-demineralized dentin hardness to that of healthy dentin. Due to the synergy between the immersed-PAMAM and the ion-exhausted and then recharged PEHB + NACP adhesive, this strategy sustained long-term triple benefits: stable nucleation templates, sustained ion recharge and re-release, and acid-neutralization. Therefore, the PAMAM + PEHB + NACP strategy has the potential to provide long-term bond-protection, thus addressing the need to improve the weak-link in the restoration. Further study is needed to investigate the long-term dentin re-precipitation and bond protection of the PAMAM and PEHB + NACP adhesive strategy under oral conditions and in a human *in-situ* model.

5. Conclusions

The present study developed an accelerated fluid challenge model and investigated the long-term remineralization of PAMAM + PEHB + NACP adhesive on demineralized dentin. After a long period of fluid challenge, the immersed-PAMAM retained its nucleation template function and promoted remineralization. The ion-exhausted and recharged PEHB + NACP adhesive possessed long-term Ca and P ion release and acid-neutralization capabilities, which did not decrease with time. The immersed PAMAM with the recharged PEHB + NACP adhesive completely remineralized dentin and fully restored the hardness of the pre-demineralized dentin to that of healthy dentin. This novel approach of PAMAM + PEHB + NACP adhesive is promising to protect the HL in the long-term, strengthen the bonded interface and inhibit recurrent caries, thus prolonging restoration longevity.

Conflict of interest statement

The authors declare no conflict of interest.

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