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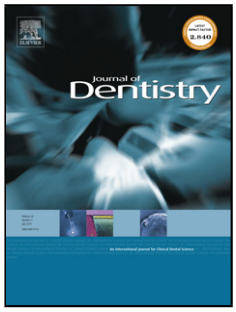

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Enamel remineralization via poly(amido amine) and adhesive resin containing calcium phosphate nanoparticles

Yuan Gao[1,2], Kunneng Liang[1,2], Michael D. Weir[2], Jianghong Gao[2,3], Satoshi Imazato[4], Franklin R. Tay[5], Christopher D. Lynch[6], Thomas W. Oates[2], Jiyao Li[1*], Hockin H. K. Xu[2,7,8*]

[1]State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Cariology and Endodontics, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China;
[2]Department of Advanced Oral Sciences and Therapeutics, University of Maryland School of Dentistry, Baltimore, MD 21201, USA;
[3]Clinical Research Center of Shaanxi Province for Dental and Maxillofacial Diseases, Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, China;
[4] Department of Biomaterials Science, Osaka University Graduate School of Dentistry, Osaka, Japan;
[5]The Dental College of Georgia, Augusta University, Augusta, GA, USA;
[6]Restorative Dentistry, University Dental School and Hospital, University College Cork, Wilton, Cork, Ireland;
[7]Center for Stem Cell Biology & Regenerative Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA;
[8]Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD, 21201, USA

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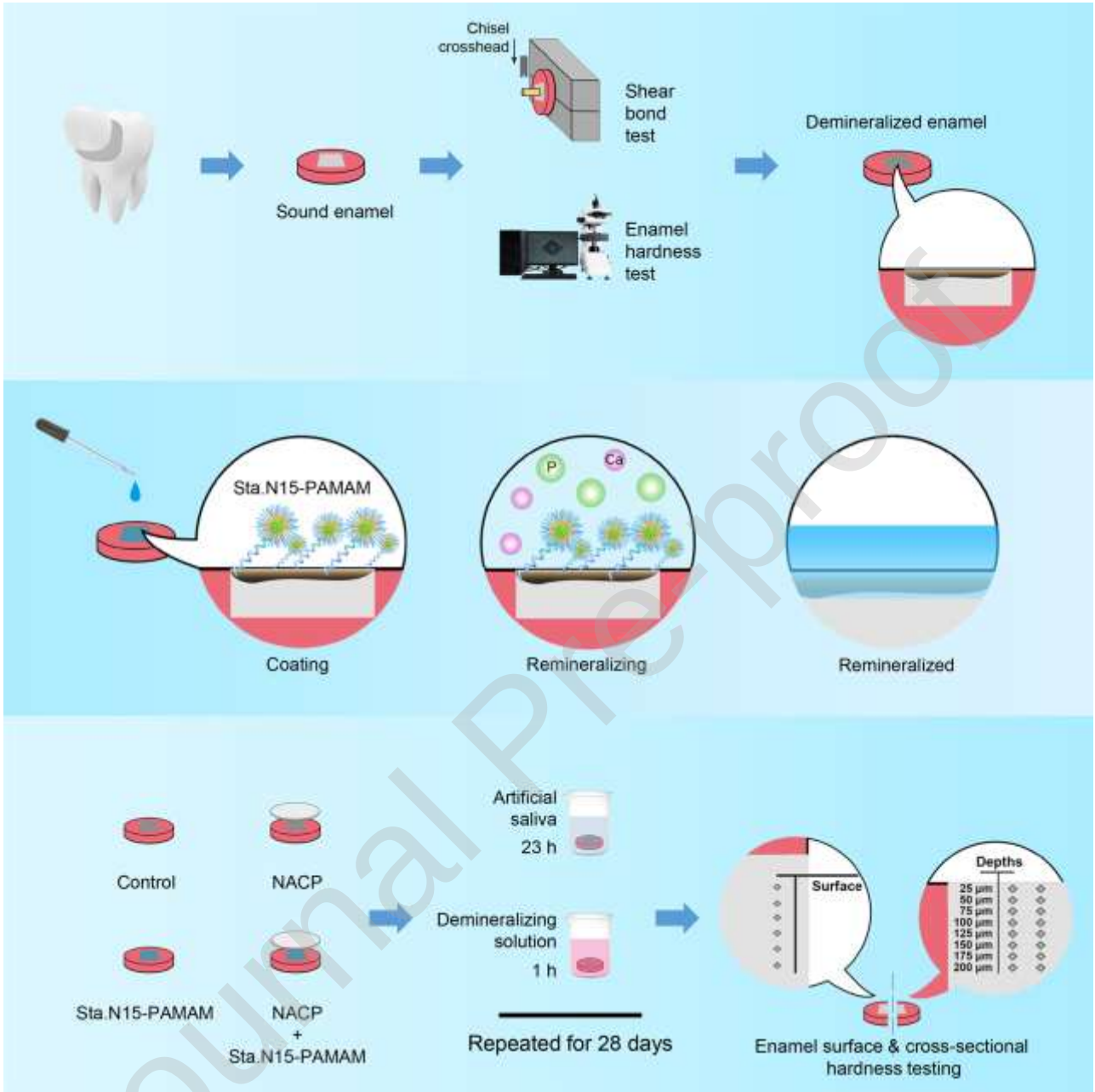
***Correspondence:** Dr. Hockin Xu, email: hxu@umaryland.edu; Dr. Jiyao Li, email: jiyaoliscu@163.com.

Short title: Enamel remineralization via SN15-PAMAM and NACP adhesive

Graphical abstract



Highlights

- Enamel remineralization induced by salivary statherin protein-inspired SN15-PAMAM and adhesive containing NACP in a cyclic artificial saliva/demineralizing solution for the first time.

- The novel SN15-PAMAM could bond to HA stronger than PAMAM-NH_2, and the NACP adhesive yielded a similar shear bond strength to commercial adhesive, and released high levels of Ca and P ions.
- The novel SN15-PAMAM + NACP adhesive method could achieve 90% higher enamel remineralization of the artificial caries than the control under acid challenge.

Abstract

Objectives: The objective of this study was to investigate enamel remineralization using salivary statherin protein-inspired poly(amidoamine) dendrimer (SN15-PAMAM) and adhesive containing nanoparticles of amorphous calcium phosphate (NACP) in a cyclic artificial saliva/demineralizing solution for the first time.

Methods: The enamel shear bond strengths of NACP adhesives were measured compared to commercial adhesive (Scotchbond Multi-Purpose, 3M). Adhesive disks containing NACP were tested for calcium (Ca) and phosphorus (P) ions release. Four groups were tested: (1) enamel control, (2) enamel with NACP, (3) enamel with SN15-PAMAM, and (4) enamel with SN15-PAMAM + NACP. The specimens were treated with cyclic artificial saliva/demineralizing solution for 28 days. The remineralized enamel specimens were examined by surface and cross-sectional hardness test.

Results: NACP adhesive yielded a similar shear bond strength to commercial control (Scotchbond Multi-Purpose, 3M). NACP adhesive released high levels of Ca and P ions. At 28 days, the enamel hardness of SN15-PAMAM + NACP group was 2.89 ± 0.13 GPa, significantly higher than that of control group (1.46 ± 0.10 GPa) ($p < 0.05$). SN15-PAMAM + NACP increased the enamel cross-sectional hardness at 28 days; at 25 μm, enamel

cross-sectional hardness was 90% higher than that of control group ($p < 0.05$).

Significance: The novel SN15-PAMAM + NACP adhesive method could achieve 90% higher enamel remineralization of the artificial caries than the control under acid challenge for the first time. This method is promising for use after tooth cavity preparation, or as a coating on enamel with white spot lesions (WSLs) for prevention, to reduce secondary caries, prevent caries procession and protect tooth structures.

Key words: Enamel remineralization, statherin protein poly(amidoamine) dendrimer, adhesive, pH cycling, nano-calcium phosphate, enamel hardness.

1. Introduction

Dental enamel is the hardest tissue of the human body. It comprises approximately 96% of carbonated hydroxyapatite (HA) mineral [1], which was more than dentin (~70%), bone (~70%) and cementum (45%) [2, 3]. Enamel is relatively stable with a dynamic equilibrium between demineralization and remineralization at the tooth-pellicle and plaque-saliva interfaces [4]. However, enamel is susceptible to be permanently damaged by caries caused by acid-producing bacteria[5-8]. This leads to a subsurface enamel lesion with partial demineralization, which is clinically manifested as white spot lesion (WSLs). Although this lesion may be remineralized under appropriate natural conditions, when acid challenge is severe, natural remineralization is insufficient [9]. It would be highly desirable to promote the remineralization of early enamel lesions before they progress to become cavities.

Resin composites are the main material for tooth cavity restorations due to their esthetics, direct-filling capability and enhanced performance [10]. Composites are bonded to tooth structure through adhesives [11-14]. The bonded interface is considered the weak link

in the restoration [11], and secondary (recurrent) caries at the margins is a main reason for restoration failure [15]. Therefore, there is a need for the development of bond protection and remineralization strategies via the adhesive resin.

Recently, poly (amido amine) dentrimers with amino terminal groups (PAMAM-NH_2) have been utilized in biomimetic processes for hard tissue remineralization [16-21]. They can absorb calcium (Ca) and phosphorus (P) ions and provide nucleation sites and mineralization template for HA [21-25]. The dendrimers consist of a central core with a branching structure and numerous peripheral multifunctional groups [26, 27], and can be modified and designed for various functions [28]. However, the binding of PAMAM-NH_2 to HA was shown to be weak [29].

Natural salivary-acquired pellicle (SAP) may provide a new strategy to improve the binding of PAMAM-NH_2 to HA. SAP is a layer of proteins, provided by salivary glands, that permanently coats the surface of oral tissues and helps maintain the integrity of the teeth [30]. Even after a complete cleaning of the teeth, SAP can be firmly formed on the surface of the teeth within several minutes. The capability of SAP to adsorb onto HA is due to the existence of several saliva proteins, including statherin and acidic proline-rich proteins [31]. Statherin, a major contributor to SAP [31], is an acidic phosphoprotein, which is a thin proteinaceous film adsorbed on tooth surfaces to protect enamel from demineralization during an acid attack [32]. It consists of 43 amino acid residues. The N-terminal 15-amino-acid residue of statherin, known as SN15 (which refers to DpSpSEEKFLRRIGRFG, where pS denotes a phosphorylated serine), exhibited an especially high adsorption ability to HA [33, 34, 35]. Therefore, SN15 is promising to enhance the binding strength between HA and PAMAM.

In the present article, we report a DpSpSEEKFLRRIGRFG-functionalized PAMAM-NH_2 (referred to as SN15-PAMAM) to mimic the adsorption function of statherin to HA. Unlike previous studies [29, 36], the present study decorated PAMAM-NH_2 with the original SN15 for the first time.

Recently, computer simulation showed that Ca and P ion in solutions could stabilize the bonding structure of SN15, hence SN15 could be adsorbed more strongly onto HA [37]. In previous studies, calcium phosphate (CaP)-releasing resins were developed for caries-inhibition [38-40]. Nanoparticles of amorphous calcium phosphate (NACP) were incorporated into adhesive resins [41-45]. Due to the small particle sizes, NACP adhesive released high levels of Ca and P ions [43], and rapidly neutralized acids to protect tooth structures [46, 47]. In addition, previous studies investigated the combination of PAMAM and NACP adhesive to induce dentin remineralization [46-48]. However, because of the weak binding of PAMAM-NH_2 to HA [29], it may take more than an hour to adsorb PAMAM to HA [46, 48], which is inconvenient for clinical usage. Furthermore, the previous studies tested dentin remineralization, with no investigation on enamel remineralization [46-48].

To date, there has been no report on combining nucleation template with NACP for enamel remineralization. As enamel comprises more HA minerals than dentin, it is necessary to enhance the interfacial binding between PAMAM-NH_2 and HA by using HA-affinitive SN15 peptide~~2~~. A continuous Ca and P ion-releasing environment could further reinforce this bonding [37], thereby promoting the remineralization [46]. Therefore, it would be beneficial to combine SN15-PAMAM with an NACP-containing adhesive to achieve an effective enamel remineralization.

The objective of this study was to develop an enamel remineralization strategy via SN15-PAMAM and NACP-containing adhesive for the first time. The effects of SN15-PAMAM alone, NACP adhesive alone, and SN15-PAMAM + NACP adhesive combination, on enamel remineralization were investigated in a cyclic artificial saliva/demineralizing solution treatment. It was hypothesized that: (1) SN15-PAMAM could be adsorbed more strongly than PAMAM onto HA to remineralize the demineralized enamel in a pH-cycling regimen; (2) NACP adhesive could increase Ca and P ion concentrations, neutralize the acid and promote enamel remineralization; and (3) SN15-PAMAM + NACP adhesive would achieve much greater remineralization in the demineralized enamel than either SN15-PAMAM or NACP adhesive alone. The null hypotheses for this study were that: (1) SN15-PAMAM and PAMAM would have no difference in the binding capability to HA; (2) NACP adhesive could not caause enamel remineralization; (3) SN15-PAMAM + NACP adhesive, SN15-PAMAM and NACP adhesive would have no significant difference in the remineralization of the demineralized enamel.

2. Materials and methods

2.1. Synthesis of statherin N-15 peptide-modified PAMAM

The synthesis of PAMAM dendrimers was described previously [16, 49]. Briefly, it included a two-step interactive sequence to produce amine-terminated structures. First, iterative sequencing involved the alkylation with methyl acrylate (MA), followed by amidation with excess 1, 2-ethylenediamine (EDA). The alkylation step produced ester-terminated intermediates called “half-generations”. The second step involved amidation of

esterterminated intermediates with a large excess of EDA to produce amine terminated intermediates that were called "full-generations". The fourth generation is a sphere macromolecule with more functional ending groups, which supplies more nucleation locations to absorb Ca and P ions during remineralization. In this article, the term "PAMAM" refers to the fourth generation of PAMAM-NH_2 (G_4-PAMAM-NH_2).

The peptide modification of PAMAM followed a previous study [29, 36]. G_4-PAMAM-NH_2 was dissolved in anhydrous trichloromethane ($CHCl_3$) in a round bottom flask, to which triethylamine and acryl chloride were added dropwise under stirring. The mixture was allowed to react for 24 h in dry nitrogen to complete the acylation reaction. The product solution was filtrated and dialyzed against deionized water for three days, followed by lyophilization to obtain the product acryloyl-PAMAM-NH_2 (yield: 85%). Acryloyl-PAMAM-NH_2 and dimethylphenylphosphine were dissolved in deionized water. Then, the fluorenylmethyloxycarbonyl protecting group (Fmoc)-protected DpSpSEEKFLRRIGRFGC peptide (Bootech BioScience &Technology Company, Shanghai, China) was dissolved in deionized water and added to the reaction mixture dropwise under stirring for 24 h. The resulting mixture was dialyzed and lyophilized to obtain the Fmoc-protected DpSpSEEKFLRRIGRFGC-PAMAM-NH_2. Finally, the product was dissolved in a mixture containing NH_3•H_2O, $C_4H_8O_2$ and NaOH to remove the Fmoc. The mixture was stirred for 24 h and dialyzed against deionized water for three days. Finally, DpSpSEEKFLRRIGRFGC-PAMAM-NH_2 (referred to as SN15-PAMAM) was collected via lyophilization with a material yield of approximately 90%.

2.2. Quartz crystal microbalance with dissipation (QCM-D)

The QCM-D measurements were performed with a qCell T Q2 (3T analytick, 3T GmbH & Co. KG, Germany), using a peristaltic pump at a flow rate of 60 μL/min at 25 °C. A detailed description of the technique and its basic principles can be found elsewhere [50]. Briefly, the electrodes of HA-coated quartz chips were mounted in the chamber. The frequency changes (Δf) upon dendrimer adsorption were detected in a gaseous environment. Δf reflects the mass changes onto the sensor surface, which can be converted to surface mass density values. After Δf was stabilized in PBS, the PBS was replaced with a series of concentrations of dendrimer dissolved in PBS, and the measurements were performed until dendrimer adsorption reached equilibrium. Finally, the samples were rinsed with PBS to evaluate its adsorption capability.

2.3. Processing of NACP-containing adhesive resin

NACP [$Ca_3(PO_4)_2$] were synthesized via a spray-drying technique. Briefly, calcium carbonate ($CaCO_3$, Fisher, Fair Lawn, NJ) and dicalcium phosphate ($CaHPO_4$, Baker Chemical, Phillipsburg, NJ) were dissolved into an acetic acid solution to obtain Ca and P ion concentrations of 8 and 5.333 mmol/L, respectively. The solution was sprayed into a heated chamber to evaporate the distilled water and volatile acid. The dried particles were collected by an electrostatic precipitator, yielding NACP with a mean particle size of 116 nm [39, 40].

For the bonding agent, the primer contained pyromellitic glycerol dimethacrylate (PMGDM) (Esstech, Essington, PA) and 2-hydroxyethyl methacrylate (HEMA) (Esstech, Essington, PA) at a mass ratio 3.3/1, with 50% acetone solvent (all mass fractions) [42, 51]. The adhesive contained PMGDM and ethoxylated bisphenol A dimethacrylate (EBPADMA)

(Sigma-Aldrich, St, Louis, MO) at a 1:1 mass ratio [42, 44]. It was rendered light-curable with 1% phenylbis (2,4,6- trimethylbenzoyl) phosphine oxide (Sigma–Aldrich). Then 10% of HEMA and 5% of bisphenol A glycidyl dimethacrylate (BisGMA) (Esstech) were added [42, 44]. This adhesive is referred to as "PEHB." PEHB adhesive was filled with a mass fraction of 30% NACP to form a cohesive paste, based on a preliminary study. NACP filler levels ≥ 40% were not used due to a decrease in the bond strength in previous study [44]. The adhesive paste was placed into a round disk mold of approximately 8.5 mm in diameter and 0.6 mm in thickness, and light-cured (Triad 2000, Dentsply, York, PA) for 1 min on each side.

2.4. Preparation of enamel samples

Extracted caries-free human molars were obtained from the dental school clinics following a protocol approved by the *** Institutional Review Board. Teeth were cleaned and stored in 0.1% thymol solution at 4 °C before use. The crown enamels were cut off using a diamond saw (Isomet, Buehler, Lake Bluff, IL), and cut into enamel squares of 4 × 4 mm with a thickness of 1.0 mm. Each enamel was then embedded in a self-curing acrylic resin block (Lang Dental Manufacturing Co., Wheeling, IL) and ground flat with wet 320-, 600- and 1200-grit silicon carbide papers (Extec, Enfield, CT). The caries-like subsurface enamel lesions was created by immersing each enamel block, with the polished enamel surface exposed, in 2 mL of an acidic solution for 72 h at 37 °C (Fig. 1). The acidic solution consisted of 8.7 mmol/L $CaCl_2$, 8.7 mmol/L KH_2PO_4, 0.05 ppm F from NaF, and 75 mmol/L acetic acid (pH = 4.0). This solution had been demonstrated, in a previous study, of being able to produce subsurface enamel demineralization in 3 days [39, 52].

2.5. Enamel shear bond testing

In previous studies [44, 46, 47], shear bond strengths were tested to evaluate the bond strengths. Hence, the present study used the same shear bond strength test to enable the comparison with previous studies. Sound enamel samples were etched with 37% phosphoric acid gel (3M Unitek) for 30 seconds (s) and rinsed with water, then it was dried with air stream until a chalky appearance was observed. The primer was applied and rubbed in for 10 s, and the solvent was removed with air. The adhesive was applied and photo-cured for 10 s (Optilux-VCL401, Demetron, Danbury, CT). A composite stub was manufactured using an Ultradent jig (Ultradent, Salt Lake City, UT). The central opening was filled with a composite (TPH, Caulk/Dentsply, Milford, DE) and photo-cured for 1 min. The bonded samples were immersed in water at 37 °C for 1 day. Then the enamel shear bond strength, S_D, was measured following previous studies [41, 43]. Briefly, a chisel was held parallel to the composite-enamel interface and loaded via a Universal Testing Machine (MTS, Eden Prairie, MN) at 0.5 mm/min until the bond failed (Fig. 1). S_D was calculated as: $S_D = 4\ P/(\pi d^2)$, where P is the load at failure, and d is the diameter of the composite [41, 43]. Twelve measurements were made for each group. Scotchbond Multi-Purpose (SBMP, 3M, St. Paul, MN) served as the commercial control.

2.6. Cyclic demineralization and remineralization treatment

The demineralized enamel specimens were randomly divided into four groups and treated as described below.

(1) Control group. Each demineralized enamel specimen was coated with 100 μL of

distilled water and then air dried, to serve as a control.

(2) NACP. Each enamel square was placed in contact with a NACP adhesive disk described above, when immersed in 11.5 mL solution, this would yield a composite/solution volume ratio of 3.0 mm^3/mL, the same as that in a previous study [53].

(3) SN15-PAMAM. Each demineralized enamel was coated with 100 μL of 1 mg/mL SN15-PAMAM solution, which was maintained on enamel for 30 min, and then the specimen was rinsed with distilled water to remove any loose SN15-PAMAM. The 100 μL was used because it could cover the enamel surface.

(4) SN15-PAMAP + NACP. Enamel was coated with 100 μL of SN15-PAMAP solution, and then a NACP adhesive disk was placed on enamel specimen as in (3) (Fig. 1).

Six specimens were tested for each group (n = 6). A 15 mL centrifuge tube was used to store each sample which was immersed in 11.5 mL of the following solution. An artificial saliva was prepared by dissolving, in water, 1.5 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and adjusting to pH 7.0 with KOH (1 mmol/L) [54]. This solution mimicked human saliva, which can supply Ca and P ions for remineralization [54]. In addition, the demineralizing solution consisted of 133 mmol/L NaCl, 50 mmol/L lactic acid, and adjusting to pH 4 [40, 46]. Each day, each specimen was immersed in 11.5 mL of fresh artificial saliva for 23 hours (h), and then in 11.5mL of the demineralizing solution for 1 h at 37 °C [39, 46, 52]. The 1-h duration in the demineralizing solution approximated the accumulated acid challenge times in 24-h period

orally [39, 52]. This treatment was repeated for 28 days.

2.7. Ca and P ion concentration measurement

A sodium chloride (NaCl) solution (133 mmol/L) was buffered to pH 4 with 50 mmol/L lactic acid, and to pH 7 with 50 mmol/L HEPES [40]. A previous study used a composite volume/solution of 3.0 mm^3/mL [53]. In the present study, a disk of approximately 8.5 mm in diameter and 0.6 mm in thickness were immersed in 11.5 mL solution, yielding the same specimen volume/solution of 3.0 mm^3/mL. The immersion times were: 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, and 28 days. At each time, the 11.5 mL solution was completely removed and replaced by fresh NaCl solution. The Ca and P ion concentrations in the collected solution was measured via a spectrophotometric method (DMS-80 UV-visible, Varian, Palo Alto, CA, USA), according to established standards and calibration methods [53, 55].

2.8. Enamel hardness measurement

A hardness tester (Shimadzu HMV-2000, Kyoto, Japan) was used to measure enamel hardness with a Vickers diamond indenter at a load of 25 g and a dwell time of 5 s [56]. In addition, hardness of sound enamel, and enamel after demineralization, were also measured as comparative controls. Six indentations were made in each enamel, and six samples were tested for each group, yielding 36 indentations per group at each time period.

After 28-day immersion, each sample block was cut in half using a diamond saw (Isomet, Buehler, Lake Bluff, IL). One was used for surface hardness testing, and the other was used for cross-sectional hardness testing.

The cross-sectional surfaces were serially polished to 4000-grit, to ensure the surface to be parallel to the platform of the hardness tester (Shimadzu HMV-2000, Kyoto, Japan). A Vickers indenter was used to measure the hardness at a force of 25 g and a dwell time of 5 s. Six indentations for each lane from the external side to the internal side were made at 25, 50, 75, 100, 125, 150, 175, and 200 μm depth, with two lanes for each sample (Fig. 1).

2.9. Statistical analysis

Statistical analyses were performed by the SPSS 19.0 (Chicago, IL, USA) and GraphPad Prism (San Diego, CA, USA). All data were checked for normal distribution with the Kolmogorov–Smirnov test. One-way and two-way analyses of variance (ANOVA) were applied to detect the significant effects of the variables. Tukey's multiple comparison tests were used at a p value of 0.05.

3. Results

3.1. Adsorption capability of SN15-PAMAM on HA

QCM-D was employed to evaluate the adsorption of SN15-PAMAM on HA-coated quartz chips, in comparison with the original PAMAM-NH_2. QCM-D could correlate a change in the frequency of the quartz crystal resonance overtones (Δf) to the adsorbed dendrimer mass. After the frequency was stabilized in phosphate-buffered saline (PBS) solution, the dendrimer solution was introduced to the measurement chamber to evaluate its adsorption, followed by rinsing with PBS solution.

As shown in Fig. 2 A and B, upon the addition of 0.05 mg/mL dendrimer, a rapid

increase in adsorbed mass was observed, reaching saturation at 145.9 ng/cm^2 for PAMAM-NH_2 and 193.8 ng/cm^2 for SN15-PAMAM. This indicated that the adsorbed mass of SN15-PAMAM on HA chips was higher than that of PAMAM-NH_2. After the adsorbed mass was stabilized, a PBS solution was injected to wash the samples, and there was a small decrease in the adsorbed mass (40.8 ng/cm^2 for PAMAM-NH_2 and 33.2 ng/cm^2 for SN15-PAMAM), corresponding to a desorption of dendrimers. However, for dendrimer concentrations equal to or higher than 0.1 mg/mL, the increase in the adsorbed mass due to dendrimer adsorption became smaller, when compared to that at 0.05 mg/mL.

3.2. Enamel shear bond strength

Dentin shear bond strengths are plotted in Fig. 3 (mean ± sd; n = 6). For the PEHB bonding agent, the NACP filler levels of 0 (24.83 ± 2.59 MPa), 20% (26.01 ± 4.22 MPa) and 30% (23.06 ± 3.88 MPa) had no significant effect on enamel bond strength ($p > 0.1$). PEHB with 30% NACP had a bond strength similar to that of commercial control (27.16 ± 5.23 MPa) ($p > 0.1$).

3.3. Ca and P ion concentration measurement

The Ca and P ion concentrations in the NaCl solution are plotted in Figure 4 (mean ± sd; n = 6). The released ion concentrations are plotted as the total accumulative ion concentration versus time. After 28 days, the adhesive released Ca ions about 4.16 mmol/L in pH 4 solution and about 2.03 mmol/L in pH 7 solution, released P ions to concentrations of about 2.55 mmol/L and 0.90 mmol/L in pH 4 and pH 7 solutions, respectively. The Ca and P ion release

was much faster in pH 4 solution than in pH 7 solution.

3.4. Enamel surface hardness testing

Enamel hardness is plotted in Figure 5 (mean ± sd; n = 36). The hardness of sound healthy enamel was 3.43 GPa. After acid-etching, the hardness decreased to 1.86 GPa. After being immersed in cyclic artificial saliva/demineralizing solution for various time periods, the hardness of control group decreased due to the further demineralization of enamel. At 28 days, NACP group had higher enamel hardness than SN15-PAMAM ($p < 0.05$). The SN15-PAMAM + NACP group increased the enamel hardness to 2.65 GPa at 10 days, and to 2.89 GPa at 28 days, which were the highest among all groups ($p < 0.05$).

3.5. Enamel cross-sectional hardness testing

The hardness at 25-200 μm depths from the enamel surface is plotted in Figure 6 (mean ± sd; n = 12). Control group showed further demineralization. The blue line represents SN15-PAMAM + NACP which had the highest hardness ($p < 0.05$) at above 150 μm. Especially at 25 μm and 50 μm depths, the SN15-PAMAM + NACP group achieved 2.90 ± 0.11 GPa and 2.64 ± 0.14 GPa, respectively. They were about 2-fold those of the control (1.53 ± 0.16 GPa and 1.45 ± 0.16 GPa), respectively. There was no significant difference between all groups at below 175 μm. These results demonstrate that the SN15-PAMAM + NACP combination method reduced the enamel lesion depth.

4. Discussion

The present study investigated SN15-PAMAM, NACP adhesive, and SN15-PAMAM + NACP for enamel remineralization in a cyclic artificial saliva/demineralizing solution treatment for the first time. The SN15-PAMAM + NACP adhesive strategy successfully induced enamel lesion remineralization. The hypotheses were proven that the NACP adhesive alone achieved only a low level of remineralization of the demineralized enamel; SN15-PAMAM could not induce enough remineralization under the challenge of the acidic condition; and the novel SN15-PAMAM + NACP method yielded the greatest enamel remineralization. The SN15-PAMAM + NACP approach increased the enamel hardness to 2.89 GPa, vs. the 1.46 GPa of the control group. Moreover, after 28 days, SN15-PAMAM + NACP significantly increased the enamel cross-sectional hardness at above 150 μm depth from the surface, which was higher than the other groups ($p < 0.05$). At 25 μm depth from the surface, the enamel cross-sectional hardness was 90% higher than that of the control group. The results rejected the null hypotheses.

When the dendrimer concentrations were equal to or higher than 0.1 mg/mL, the increase in the adsorbed mass due to dendrimer adsorption became smaller, compared to that observed at 0.05 mg/mL. This phenomenon could be a result of the limited surface area. After the addition of 0.05 mg/mL dendrimer, the surface of HA-coated quartz chip could provide a limited number of additional sites for dendrimers adsorption. It was also noted that SN15-PAMAM attained a maximum value in a shorter time. This phenomenon could indicate that its binding to HA was stronger than PAMAM-NH_2, partly due to the highly anionic SN15 unit which could mediate strong electrostatic interactions with HA. Moreover, as a zwitterionic polymer, SN15-PAMAM could possess charged cationic groups and anionic

groups, which could form intermolecular electrostatic interactions among SN15-PAMAM dendrimers, leading to the higher adsorbed amount and higher stability of the coating. Thus, the first null hypotheses was rejected.

The Ca and P ion release was much faster at pH 4 than at pH 7. This "smart" feature could induce more ion release at a cariogenic pH condition when such ions would be most needed to combat tooth demineralization. When the cariogenic bacteria produce acids to demineralize the tooth, this NACP adhesive could release the Ca and P ions and neutralize the acid to increase the pH to above 5 to minimize demineralization [57]. However, the NACP adhesive or SN15-PAMAM each alone failed to induce enough remineralization of the demineralized enamel in a cyclic artificial saliva/demineralizing solution. SN15-PAMAM can remineralize the enamel in artificial saliva, especially with the presence of Ca and P ions. However, when immersed in demineralizing solution, the lack of Ca and P ions in the solution may influence the stability of its bonding structure [37]. Moreover, SN15-PAMAM could not neutralize the acids, which could dissolve the minerals induced in artificial saliva. For NACP adhesive, it released less Ca and P ions in artificial saliva, which could only induce a low level of remineralization(thus rejecting the second null hypotheses), with inefficiency of using Ca and P ions without the needed nucleation templates. In the demineralizing solution, NACP could increase the ion release and neutralize the acid to increase the pH to a safe level [46, 57].

In contrast, the SN15-PAMAM + NACP adhesive method induced the most remineralization, and showed the greatest enamel hardness recovery. The SN15-PAMAM + NACP synergy achieved triple benefits: (1) strong binding to HA; (2) excellent nucleation

templates; and (3) a Ca and P ion source. SN15 peptide can bond to HA surface strongly via salt-bridge adsorption and electrostatic adsorption [35, 37], and reduce HA demineralization [58]. NACP adhesive released Ca and P ions for remineralization [43], and rapidly neutralized acids in the demineralizing solution [46, 47]. PAMAM can absorb Ca and P ions and provide nucleation sites and mineralization template for the remineralization [21, 23-25]. Moreover, the α-helix in SN15, which is essential for its binding capacity [59], is more stable in a Ca/P solution [37]. In addition, the Ca ions around SN15 can form equilateral triangle, which resembles the structure formed by Ca^{2+} in the HA (001) crystal face, to be the initial stage for HA nucleation [37], thus promoting the remineralization. The significant differences between the groups led to the rejection of the third null hypotheses.

When immersed in artificial saliva, SN15-PAMAM could fulfill its nucleation function. PAMAM macromolecules immobilized on the demineralized enamel could rapidly absorb Ca and P ions to form minerals in the demineralized enamel, yielding much greater remineralization than NACP adhesive alone. When immersed in the demineralizing solution, NACP adhesive rapidly released Ca and P ions and raised the pH to favor remineralization. The Ca and P ions could stabilize the bonding structure of SN15 as well. Because of the NACP adhesive is rechargeable and can be repeatedly recharged to re-release the ions [44], its release capability could recover when being re-immersed in the artificial saliva, with potential to provide long-term remineralization.

In previous studies, the undecorated PAMAM-NH_2 and NACP adhesive were used for dentin remineralization [46, 47]. The present study represents the first report on modifying the PAMAM and using NACP adhesive to remineralize enamel. PAMAM in

artificial saliva can restrain the biomineralization process of mineral crystals, similar to the inhibition of $CaCO_3$ formation by free dendrimer in an aqueous solution [60]. Although the binding between PAMAM-NH_2 and HA was weak [29], dentin was a porous biomaterial [61, 62] and adsorbed more PAMAM into dentinal tubules. This could help retain the remineralization capability of PAMAM-NH_2, especially after coating for one hour which was inconvenient for clinical usage. In contrast, enamel has a smooth and dense surface [63]. Therefore, it would be necessary to enhance the interfacial binding strength between PAMAM-NH_2 and HA by HA-affinitive SN15 peptide in order to induce remineralization after coating for a short time. Moreover, previous studies using the undecorated PAMAM to remineralize the etched enamel performed the remineralizing process in a continuous Ca and P condition [16, 64]; they did not test the pH-cycling treatment which could mimic the oral conditions. In the present study, the NACP adhesive could rapidly neutralize acids while being in the demineralizing solution, which provided synergistic effects with SN15-PAMAM in the pH-cycling condition to cause remineralization to enamel.

Regarding potential clinical applications, there is a major need in dentistry to improve the longevity of the composite-dentin bond, as well as to inhibit caries and protect tooth structures for patients with WSLs. Several bioactive salivary proteins, including lysozyme, lactoferrin, and lactoperoxidase, alone or in combinations, have been incorporated into oral health care products such as dentifrices, mouthrinses, moisturizing gels, and chewing gums [65, 66]. Therefore, it would be meaningful to further develop salivary statherin-bioinspired peptide for anti-caries clinical applications. The present study demonstrated that the novel SN15-PAMAM + NACP approach was a promising method to meet this need. The

SN15-PAMAM + NACP resin materials could be used after tooth cavity preparation, or could be coated on the enamel with WSLs to reduce secondary caries and protect tooth structures. Further studies need to explore the long-term results testing timer periods much longer than 28 days, as well as the remineralization efficacy under conditions that more closely simulate the oral environment.

5. Conclusions

This study developed the SN15-PAMAM + NACP method for enamel remineralization for the first time, and investigated the remineralization of enamel lesions in a cyclic artificial saliva/acid environment. SN15-PAMAM or NACP adhesive alone failed to induce enough remineralization of the demineralized enamel. In contrast, SN15-PAMAM + NACP yielded the greatest remineralization of enamel lesions, increasing the enamel hardness to 2.89 GPa, while the control group had only 1.46 GPa. Furthermore, the enamel cross-sectional hardness at 25 μm depth for SN15-PAMAM + NACP group was nearly 2-fold that of control group. Therefore, the novel SN15-PAMAM + NACP adhesive method is promising for applications to remineralize tooth lesions, increase the hardness, protect tooth structures at the margins, and improve the longevity of the restoration-tooth interface to inhibit secondary caries.

Conflict of Interest Statement:

The authors declare no conflict of interest.

Acknowledgments

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Figure Captions

Fig. 1. Schematic diagram illustrating the workflow of this study, including sample preparation, test of shear bond strength, enamel hardness testing and cross-sectional hardness analysis.

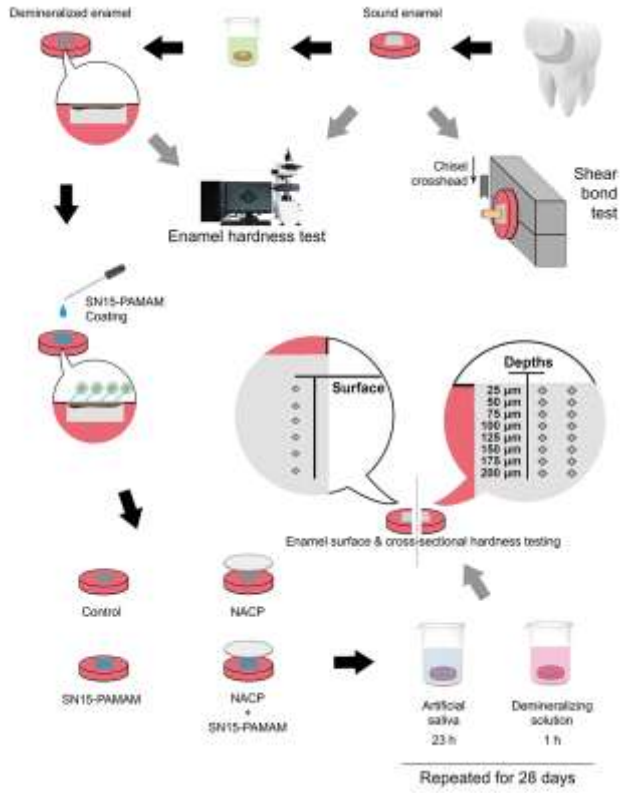

Fig. 2. QCM-D results of the dendrimer adsorption behavior on the surface of HA-coated quartz chios. The adsorbed mass density of original PAMAM-NH_2 **(A)** and SN15-PAMAM **(B)** at different concentrations. SN15-PAMAM adsorbed faster than original PAMAM-NH_2.

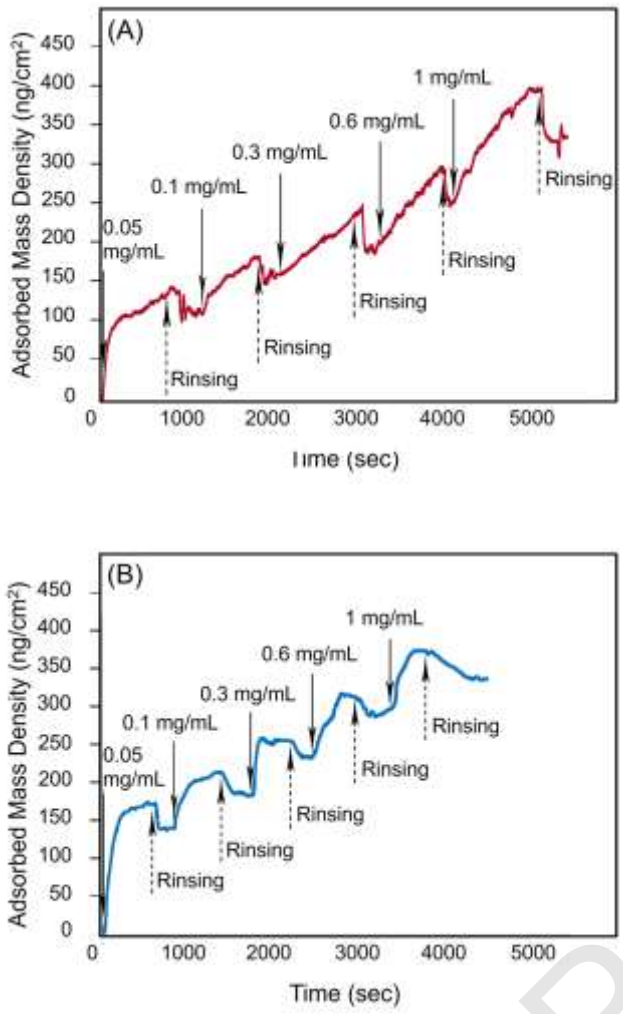

Fig. 3. Enamel shear bond strength of four groups (mean ± sd, n = 12), tested after storage in distilled water for 1 day. Horizontal line indicates values that are not significantly different from each other.

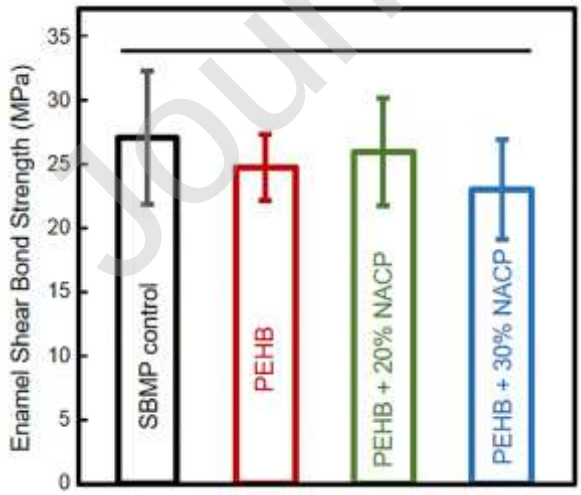

Fig. 4. Ca and P ion concentrations (mean ± sd, n = 12) for 28 days. **(A)** Ca ion concentration in pH 4 and pH 7 solutions. **(B)** P ion concentration in pH 4 and pH 7 solutions. The released ion concentration was plotted as total accumulative ion concentration versus time.

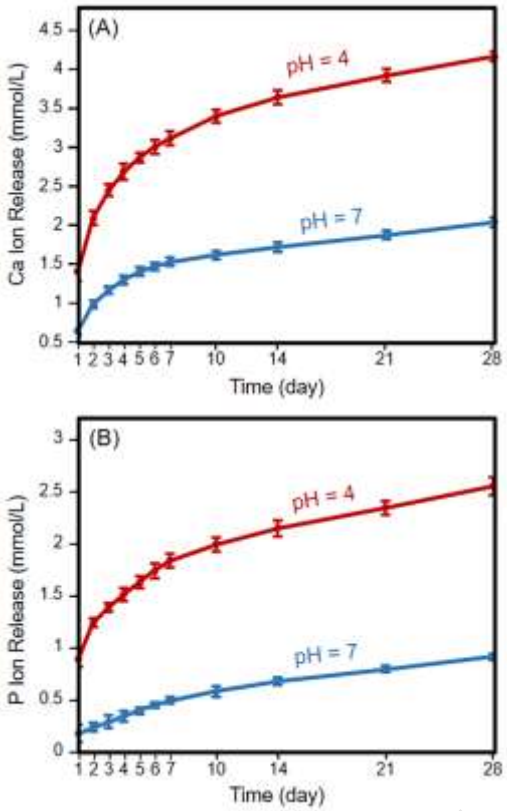

Fig. 5. Hardness of demineralized enamel after 10 and 28 d of artificial saliva/demineralizing solution cyclic treatment for: Control group, NACP group, SN15-PAMAM group, and SN15-PAMAM + NACP group (mean ± sd, n = 12). Hardness of healthy enamel and demineralized enamel were also measured as comparative controls. The NACP filler ratio was 30%. Dissimilar letters indicate significantly different values ($p < 0.05$).

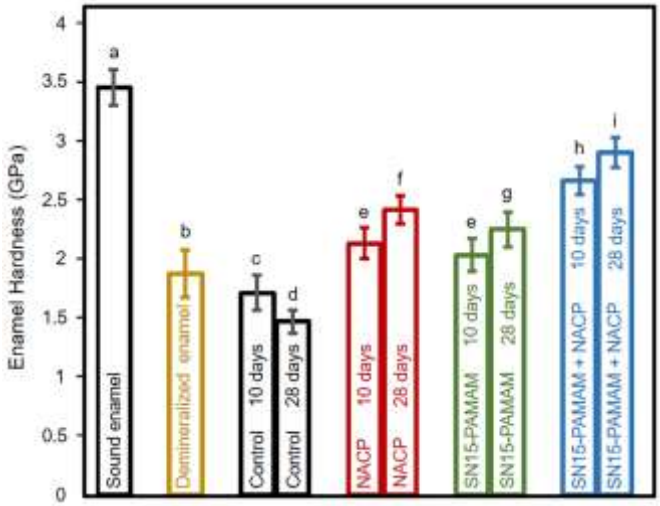

Fig. 6. Enamel cross-sectional hardness testing according to different groups and testing depths (mean ± sd, n = 12), respectively at 25, 50, 75, 100, 125, 150, 175, 200 μm depth. Control group showed further demineralization. SN15-PAMAM + NACP group gained significantly higher remineralization ($p < 0.05$), while NACP group and SN15-PAMAM group showed less remineralization.