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Dentin remineralization via adhesive containing amorphous calcium phosphate nanoparticles in a biofilm-challenged environment

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ABSTRACT

Objectives: The remineralization of dentin at a bonded interface would help to strengthen the bonded interface and inhibit secondary caries, and would prolong the longevity of restoration. The aim of this study was to investigate the remineralization of demineralized human dentin in a dental biofilm environment via an adhesive containing nanoparticles of amorphous calcium phosphate (NACP).

Methods: Dentin demineralization was promoted by subjecting samples to a *Streptococcus mutans* acidic biofilm for 24 h. Samples were divided into a control group, a commercial fluoride-releasing adhesive group, and an NACP adhesive group. All samples were subjected to a remineralization protocol consisting of 4-h exposure per 24-h period in brain heart infusion broth plus 1% sucrose (BHIS) followed by immersion in artificial saliva for the remaining period. The pH of BHIS after 4-h immersion was measured every other day. After 10 days, the biofilm was assessed for colony-forming unit (CFU) count, lactic acid production, live/dead staining, and calcium and phosphate content. The mineral changes in the demineralized dentin samples were analyzed by transverse microradiography, hardness measurement, X-ray diffraction characterization, and scanning electron microscopy.

Results: The NACP adhesive achieved acid neutralization, decreased biofilm CFU count, decreased biofilm lactic acid production, and increased biofilm calcium and phosphate content ($P < 0.05$). The NACP adhesive group had higher remineralization value than the commercial fluoride-releasing adhesive group ($P < 0.05$).

Conclusions: The NACP adhesive was effective in remineralizing dentin lesions in a biofilm model. Its ability to protect bond interface, inhibit secondary caries, and prolong the longevity of restoration is promising.

Clinical significance: Using NACP-containing adhesives could be recommended because of the protective ability of its hybrid layer even under a biofilm-challenged environment.

1. Introduction

Dental caries, or tooth decay, is a major widespread chronic disease [1]. Filling cavities with composite for tooth restoration is beneficial and popular [2]. However, the dentin-composite bonded interface has been reported to be the weak link in the restoration [3]. A reliable adhesion is crucial for the long-term success of tooth restoration [4–6]. In order to prolong the longevity of dentin restorations, it is important to identify strategies to protect the dentin-composite bond.

During dentin bonding procedures, the adhesive monomers infiltrate the demineralized collagen fibrils to form a hybrid layer (HL) [4–6]. Exposed collagen fibrils that have not been covered by adhesive monomers can be damaged by enzymes and oral bacterial-produced acids, resulting in the degradation of HL [7]. Therefore, protecting HL from external risk factors is a key strategy to strengthen restoration-dentin bonds. Minerals can play an indispensable role in protecting HL,

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so remineralization of the denuded HL is an effective approach to strengthen the stability of resin-dentin bonds. Remineralization is a natural process for caries lesions and is considered to be an effective repair process [1]. Nevertheless, natural remineralization could be far from sufficient to protect HL and combat the progress of demineralization in an acid-producing bacterial environment [1]. Thus, developing effective remineralization strategies could help to protect dentin-composite bond and improve the success of restoration.

One of the effective strategies to promote dentin remineralization is to incorporate calcium phosphate particles into adhesives [8]. Adhesive containing nanoparticles of amorphous calcium phosphate (NACP) have been developed as discussed recent studies [9–12]. Because of their small particle size, NACP readily flowed with adhesive into dentinal tubules to form resin tags [10,11]. NACP could release calcium and phosphate ion "smartly", an increasing number of calcium and phosphate ions could be released at a cariogenic low pH [10]. Additionally, NACP adhesive could increase solution pH from 4 to above 5.5 rapidly, which could tilt the balance toward dentin remineralization [13]. NACP was also reported to have bacteria inhibition properties [13]. NACP adhesive effectively remineralized dentin lesions in an in vitro cyclic artificial saliva/lactic acid study [14]. NACP-containing nanocomposite even achieved an enamel remineralization effectiveness that was fourfold that of a commercial fluoride-releasing composite [15]. However, dental biofilm, as one of the critical biological factors in caries formation, should not be overlooked in the assessment of caries preventive agents prior to in situ or clinical studies [16], and there has been no report on dentin remineralization properties of NACP-containing adhesive in a dental biofilm environment. Thus, it would be necessary to evaluate the dentin remineralization abilities of NACP-containing adhesive in a biofilm model.

Accordingly, the aim of this study was to investigate the effects of NACP adhesive, and a commercial fluoride-releasing adhesive on dentin remineralization in a biofilm-challenged environment for the first time. It was hypothesized that (1) NACP adhesive would increase calcium and phosphate ion concentrations in biofilm, neutralize the acids produced by bacteria, inhibit bacteria, and achieve remineralization of dentin lesions; and (2) NACP adhesive would achieve greater remineralization on dentin lesions than other groups in the biofilm-challenged environment.

2. Materials and methods

2.1. Synthesis of NACP

NACP ($Ca_3[PO_4]_2$) was synthesized via a spray-drying technique as reported previously [13,17]. Briefly, calcium carbonate and dicalcium phosphate were dissolved into an acetic acid solution. Final calcium and phosphate ionic concentrations were 8 mmol/L and 5.333 mmol/L, respectively. The calcium/phosphate molar ratio of the solution was 1.5, which was the same as that of ACP. The solution was then sprayed into a heated chamber, and the dried particles were collected by an electrostatic precipitator, yielding NACP with a mean particle size of 116 nm [17].

2.2. Fabrication of adhesive samples

Scotchbond Multi-Purpose (SBMP, 3 M, St. Paul, MN, USA) adhesive was used to examine the effects of incorporation of NACP. NACP was mixed into SBMP adhesive at a mass fraction of 40%. Previous studies concluded that increasing the NACP filler level from 20% to 40% significantly increased the calcium and phosphate ion release, and adding 40% NACP into SBMP adhesive did not affect dentin bond strength [10]. Hence, SBMP adhesive containing 40% NACP was used for our experiment.

Fluoride treatment has been the cornerstone of noninvasive treatments for early caries lesions [18]. Various types of fluoride-containing products have been applied to remineralize the demineralized tooth lesions with different recommended concentrations and dosage. Hence, a commercial fluoride-releasing adhesive, Clearfil Protect Bond (PB, Kuraray Medical Inc., Tokyo, Japan), was used as a positive control, per a previous study [15].

The SBMP adhesive contained Bisphenol A diglycidyl methacrylate (Bis-GMA) and 30%–40% 2-hydroxyethyl methacrylate (HEMA), according to the manufacturer. The PB bond contained 10-methacryloyloxydecyl dihydrogen phosphate, Bis-GMA, HEMA, hydrophobic dimethacrylate, di-camphorquinone, N, N-diethanol-p-toluidine, silanized colloidal silica, and surface- treated sodium fluoride.

The commercial fluoride-releasing adhesive paste, PB, was placed into a rectangular mold ($2 \times 2 \times 12$ mm) as per previous studies [10,14], and light-cured (Triad 2000, Dentsply, York, PA, USA) for 1 min on each open side. SBMP adhesive containing 40% NACP was made into bars using the same method as previously described [10,14]. The adhesive bars were sterilized in ethylene oxide sterilizer (Anprolene AN 74i, Andersen, Haw River, NC, USA) before use.

2.3. Preparation of dentin specimens

Approved by the Institutional Review Board, extracted caries-free human molars were collected. Informed consent was obtained for experimentation with human objects. The cleaned teeth were stored in 0.5% thymol at 4 °C, and they were stored no longer than 4 weeks before use.

Each tooth was cut at the cement-enamel junction using a low-speed water-cooled diamond saw (Minitom, Struers, Copenhagen, Denmark) to prepare a thickness of 3.5 ± 0.5 mm dentin disk. Enamel on the prepared disk surface was removed using 120-grit carbide polishing papers under running water. The samples were then protected with acrylic resin. The surfaces of the dentin disks were polished with 800, 1,200, and 2,400-grit carbide polishing papers under running water. The prepared dentin disks were ultrasonicated with an ultrasonic cleaner (FS20, Fisher Scientific, Pittsburgh, PA, USA) in distilled water for 10 min to remove the smear layer caused by the polishing process. Hardness of all the prepared dentin samples was tested by a Vickers hardness tester (MMT-X7A, Matsuzawa, Japan) with a diamond indenter under a 25-gf load for 10 s [19]. Five indentations were made in each sample. Only dentin specimens in the range from 0.6 GPa to 0.8 GPa were collected for the following experiment. The collected specimen surfaces were partly painted with two layers of acid-resistant nail varnish, which left an exposed 4×4 mm^2 window. The dentin specimens were stored in calcium-free phosphate buffered saline (PBS) at 4 °C before use. Before the following experiment, the dentin samples were sterilized in ethylene oxide sterilizer (Anprolene AN 74i, Andersen).

2.4. Artificial caries biofilm formation

First, the *Streptococcus mutans* (*S. mutans*) UA159 bacteria were cultured overnight in brain-heart infusion broth (BHI; Difco, Sparks, MD, USA) at 37 °C anaerobically (90% N_2, 5% CO_2, 5% H_2). The resulting bacterial suspension was then adjusted to an optical density of 0.5 at 600 nm, and diluted 100-fold with fresh BHI with 1% sucrose (BHIS) for the following experiment [20].

The sterile dentin specimens were placed in 24-well plates containing 2 mL of *S. mutans* suspension as previously mentioned [16,21]. *S. mutans* is one of the generally acknowledged cariogenic bacteria. The samples were incubated at 37 °C anaerobically (90% N_2, 5% CO_2, 5% H_2) for 24 h to create a demineralization lesion. After 24 h, dentin demineralized lesions beneath biofilm were formed, which was proved by the transverse microradiography (TMR) analysis results during our pre-experiment (Fig. 3a). The supernatant was removed, and the biofilm formed on the dentin samples was removed using pipette tips under aseptic conditions except for the exposed dentin area [21].

2.5. Remineralization/demineralization cycle protocol

Each dentin sample with biofilm was transferred into a 5-mL Eppendorf tube under the aseptic conditions. The dentin specimens with biofilm were randomly divided into three groups and treated as described in the next paragraph.

(1) Each dentin sample with biofilm underwent the entire remineralization protocol without any treatment, to serve as a negative control [14].
(2) Each dentin sample with biofilm was placed in contact with three PB bars 2 × 2 × 12 mm in size [14]. Three bars were used because when immersed in 1-mL solution, there would be a specimen volume/solution volume ratio of 0.14/1, per a previous study [13].
(3) Three 40% NACP SBMP adhesive bars were placed on the dentin specimen as described in (2) [14].

These three groups are denoted Control, NaF, and NACP. Twelve specimens were examined for each group (n = 12). A 5-mL Eppendorf tube was used to store each sample, which was immersed in 1 mL of a solution as described in the next paragraph.

An artificial saliva solution was prepared by dissolving 1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 130 mmol/L KCl, and 20 mmol/L 4-(2-hydroxyethyl-)-1-piperazineethanesulfonic acid (HEPES), adjusting pH to 7.0 with potassium hydroxide (1 mmol/L) [22]. It was filtered using a cylinder membrane filter before use to remove any bacteria. Each day, each sample with biofilm of the aforementioned three groups was immersed in 1 mL of fresh artificial saliva for 20 h, and then in sterilized BHIS for 4 h at 37 °C anaerobically (90% N_2, 5% CO_2, 5% H_2) [21]. The 4-h duration in BHIS showed a degree of correlation with oral conditions with respect to the accumulated food cycling in a 24-h period orally [16,21]. The everyday medium change was done under aseptic conditions and we rinsed the specimens with sterile 0.9% normal saline at every medium refreshment point, to remove the unattached bacterial cells. This cyclic immersion treatment was repeated for 10 days (d). Fig. 1 shows the entire experimental process.

2.6. Acid neutralization

At 1, 3, 5, 7, 9, and 10 d, the pH of BHIS broth after 4 h immersion was measured. The pH was monitored by Orion Dual Star, pH/ISE Benchtop (Thermo Scientific, Waltham, MA, USA).

2.7. Lactic acid measurement of biofilm after 10 days

The samples were taken out at the end of cyclic immersion, rinsed in cysteine peptone water, and transferred to a 24-well plate. Buffered peptone water (BPW) with 0.2% sucrose was added into each well and kept for 3 h at 37 °C anaerobically, in order to measure the amount of lactic acid production of the biofilm. An enzymatic method was used to analyze the lactate [23]. The absorbance of collected BPW solution for each sample was measured at 340 nm via a microplate reader (Multiskan Go, Thermo Scientific). The lactate production was calculated according to the standard curve of the standard lactic acid (Supelco Analytical, Bellefonte, PA, USA).

2.8. Colony-forming unit counts of biofilm after 10 d

Biofilm was scraped/resuspended from the bottom of the dentin samples into tubes with 1 mL sterile PBS using pipette tips and repeated pipetting [24]. Serially diluted samples were then plated onto BHI agar plates. After a 2-day incubation, the colonies were counted.

2.9. Live/dead bacteria staining of biofilms

Dentin samples with biofilms were rinsed with sterile 0.9% normal saline to remove any non-adherent bacteria, and then stained using Baclight Live/dead bacterial viability kit (Molecular Probes, Eugene, OR, USA). Live bacteria were stained by SYTO 9 to produce a green fluorescence, and dead bacteria were stained by propidium iodide. Confocal imaging was performed using an Olympus FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan) with 60× oil immersion objective lens. The image collection gates were set at 500–540 nm for SYTO 9 and 570–670 nm for propidium iodide. The 3-dimensional reconstructions were performed with Imaris 7.2.3 (Bitplane, Zürich, Switzerland) [25].

2.10. Biofilm calcium and phosphate content measurement

Biofilm was removed and transferred into tubes. 0.5 M HCl (0.3 mL per 10 mg biofilm wet weight) was added into each tube, in order to extract the calcium and phosphate ions from the collected biofilm. The tubes were subsequently shaken at 30 rpm for 3 h at room temperature [16]. The calcium and phosphate ion concentrations were measured via a spectrophotometric method (Multiskan Go, Thermo Scientific) using known standards and calibration curves, as was done in previous

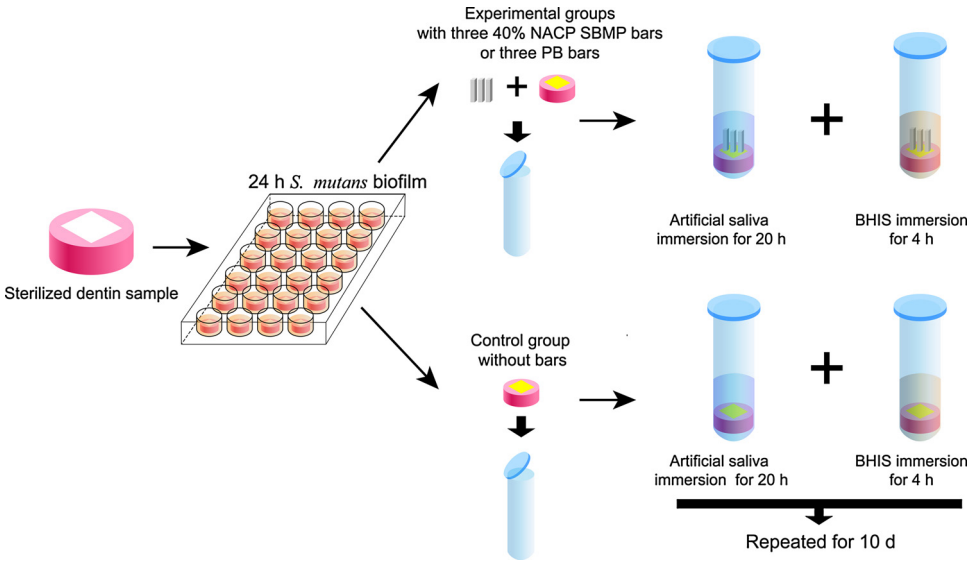

Fig. 1. Schematic illustration showing how the entire experimental process was performed.

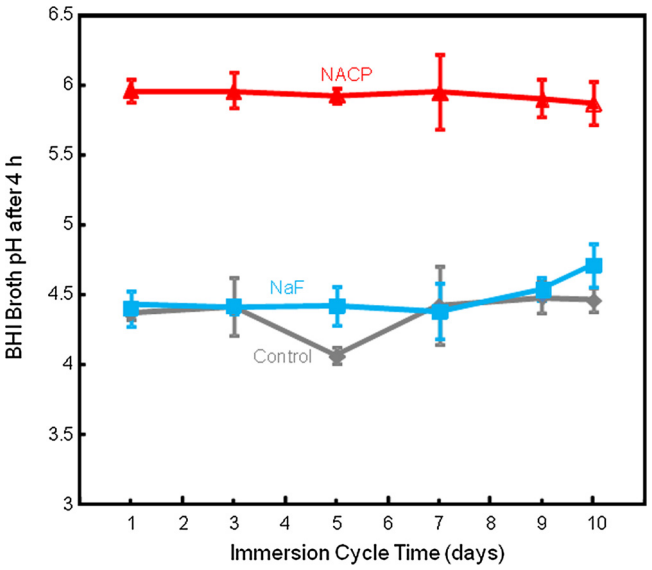

Fig. 2. The pH of BHIS solution after 4-h immersion at 1, 3, 5, 7, 10 days (mean ± SD, n = 6). In the NACP group the pH increased to almost 6, whereas the pH of NaF and control groups remained at approximately 4.4–4.5.

studies [26,27].

2.11. TMR analysis

Dentin specimens were cut into sections approximately 300 μm thick using a diamond-coated band saw, vertically to the windows that were exposed to the cyclic immersion treatment. The sections were then polished into slices of approximately 100 μm thick, ensured by a digital micrometer (Mitu-toyo, Tokyo, Japan). Slices were fixed on Plexiglass slides in a TMR sample holder (Inspektor Research Systems BV, Amsterdam, Netherlands). Then the slices were microradiographed alongside an aluminum calibration step-wedge with a monochromatic CuK X-ray source (Philips, Eindhoven, Netherlands) operated at 20 kV and 20 mA and an exposure time of 25 s. The lesion depth, mineral loss, and mineral content at selected depths were examined by imaging software (Transversal Microradiography Software 2006, Inspektor Research Systems BV). Six slices were analyzed from each dentin specimen, and five traces within areas exposed to cyclic immersion treatment were measured on each slice.

Remineralization in the dentin lesion that occurred during cyclic immersion treatment is calculated as: Remineralization R = $(M_{Before}$-$M_{After})$ / M_{Before}, where M_{Before} is mineral loss in dentin disk before remineralization protocol, and M_{After} is mineral loss in the same area of the lesion after remineralization protocol [15,19,28].

2.12. Hardness measurement

The hardness of dentin was measured for the three groups after the remineralization/demineralization cycle. The measuring steps were the same as described in 2.3. In addition, hardness of healthy untreated dentin and dentin after 24-h biofilm but without the remineralization/demineralization cycle, were also measured as comparative controls [19].

2.13. X-ray diffraction (XRD) characterization

XRD (Ultima IV, Rigaku, Japan) was carried out before and after the remineralization/demineralization cycle for three groups. Additionally, an untreated dentin disk was measured to obtain a spectrum of the intact dentin as control [29].

2.14. Scanning electron microscopy (SEM) examination

Dentin samples before and after the 10-d remineralization/demineralization cycle were ultrasonicated with an ultrasonic cleaner (FS20, Fisher Scientific, Pittsburgh, PA, USA) in distilled water to remove the bacterial biofilm. Dentin samples were then sputter-coated with gold and examined via SEM (Inspect F50, FEI, USA) [19].

2.15. Statistical analysis

Statistical analyses were conducted with SPSS software version 21.0 (SPSS, Inc., IBM, Chicago, IL, USA). Kolmogorov-Smirnov test was used to check the normal distribution of data. One-way analysis of variance was conducted to detect the significant effects of the variables. Student-Newman-Keuls multiple comparison tests were performed at a value of $P = 0.05$.

3. Results

3.1. Acid neutralization

The dentin specimens with biofilm of three groups (Control, NaF, and NACP) were immersed in artificial saliva for 20 h, and in BHIS for 4 h, every day during the remineralization/demineralization cycle. The BHIS after 4-h immersion for each sample was collected, of which the pH is plotted in Fig. 2 (mean ± SD). For NaF and Control group, the pH decreased to approximately 4.4–4.5. In contrast, for the NACP group, the pH was close to 6.0. The pH showed a slight decrease at the end of the cycle. During the whole cycle, the pH values of BHIS after 4-h immersion for NACP group are significantly higher than the pH values of the groups without NACP ($P < 0.05$).

3.2. Lactic acid production, viable count, live-dead bacteria staining and calcium, phosphate content of biofilm after 10 d

Figs. 3 and 4 show the results of the biofilm characterization. The lactic acid production (Fig. 3A) in the NACP group (2.76 ± 0.14 mmol/L) was similar to that in the NaF group (3.03 ± 0.21 mmol/L), and significantly lower than the control group (7.61 ± 1.14 mmol/L).

The value of log colony-forming unit (logCFU) count (Fig. 3B) in the Control group was 5.8 ± 0.1, whereas the values in the NaF and NACP groups decreased to 5.4 ± 0.14 and 5.5 ± 0.1, respectively.

Live/dead staining images of biofilms on dentin samples in three groups are shown in Fig. 4. Live bacteria were stained green, and dead bacteria were stained red. The Control group had a thick biofilm coverage of primarily live bacteria (Fig. 4A). The NaF or NACP group had a thinner biofilm coverage of primarily live bacteria, with a little increase in red staining (Fig. 4B, C), indicating that NaF or NACP had relatively weak bacteria inhibition abilities, which was consistent with the lactic acid production and CFU results in our present study.

The NACP treatment significantly increased the biofilm calcium and phosphate content (Fig. 5A, B). The biofilm calcium contents in the Control group and NaF group were 23.83 ± 3.11, 24.73 ± 1.71 μmol/g wet weight, respectively, whereas the biofilm Ca content in the NACP group was raised to 34.43 ± 1.89 μmol/g wet weight. The biofilm P contents in the Control group and NaF group were 7.20 ± 1.20 and 7.60 ± 1.12 μmol/g wet weight, respectively, whereas the biofilm P content in the NACP group soared to 17.28 ± 1.96 μmol/g wet weight.

3.3. TMR analysis

The Control group showed further demineralization during cyclic demineralization/remineralization. Dentin in the NaF group presented lower demineralization than the Control group, which meant that NaF could inhibit the progress of demineralization. However, the NaF group

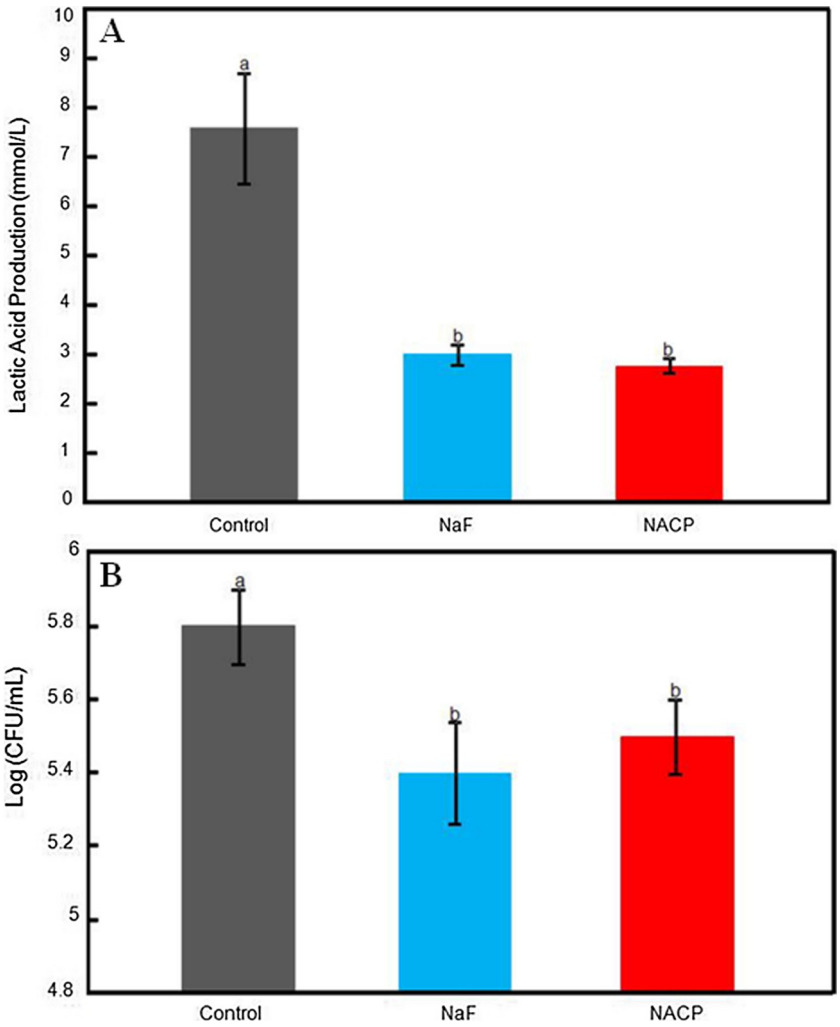

Fig. 3. (A) The lactic production of biofilm in the three groups (mean ± SD, n = 6). (B) The log (CFU/mL) of biofilm in the three groups (mean ± SD, n = 6). Dissimilar letters indicate significantly different values ($P < 0.05$).

had no noticeable remineralization. In contrast, there was significant remineralization in dentin for the NACP group after the cyclic immersion treatment (Fig. 6).

The average mineral profiles of dentin samples after remineralization/demineralization cycle or without cycle are shown in Fig. 7, and Table 1 shows the values of mineral loss and lesion depth. Specimens in the Control group resulted in higher mineral loss and lesion depth than those after 24-h biofilm without remineralization/demineralization cycle. Specimens in the NaF group resulted in lower mineral loss and lesion depth than the Control group, but resulted in still higher mineral loss than that after 24-h biofilm demineralization; no significance in lesion depth was observed in the NaF group compared with that after 24-h biofilm demineralization. In contrast, NACP induced lower mineral loss and lesion depth after the remineralization/demineralization cycle than after 24-h biofilm demineralization. The remineralization value (Fig. 8) for the NACP group (mean ± SD) was 34.25 ± 3.86%, whereas the remineralization value for the NaF and the Control group was -27.00 ± 12.06%, -69.50 ± 9.88%, respectively.

3.4. Hardness analysis

Dentin hardness of the three groups after remineralization/demineralization cycle is plotted in Fig. 9 (mean ± SD, n = 6). The hardness of sound dentin was 0.62 GPa. After 24-h biofilm, it decreased to 0.4 GPa. For NaF and Control groups, dentin hardness decreased to 0.33 GPa, and 0.27 GPa, respectively, after the remineralization/demineralization cycle. For NACP group, dentin hardness increased to 0.49 GPa, higher than NaF and Control groups.

3.5. XRD characterization

The XRD patterns of the regenerated crystals on dentin surfaces are shown in Fig. S1. After the remineralization/demineralization cycle, significant characteristic hydroxyapatite (HA) diffraction peaks, (211), (300), (004), appeared in the NACP-treated sample, which indicated that the newly regenerated minerals were mainly HA, like intact dentin.

3.6. SEM examination

Representative SEM images are shown in Fig. S2. Dentinal tubules were nearly empty with only a small number of minerals for the dentin after 24 h biofilm etched before the remineralization/demineralization cycle and for the Control group (Fig. S2A, S2B). However, the dentin surfaces in NACP group were characterized by the precipitation of minerals within the dentinal tubules (Fig. S2D), which was more significant than the NaF group (Fig. S2C).

4. Discussion

The current study determined the effects of NACP adhesive on

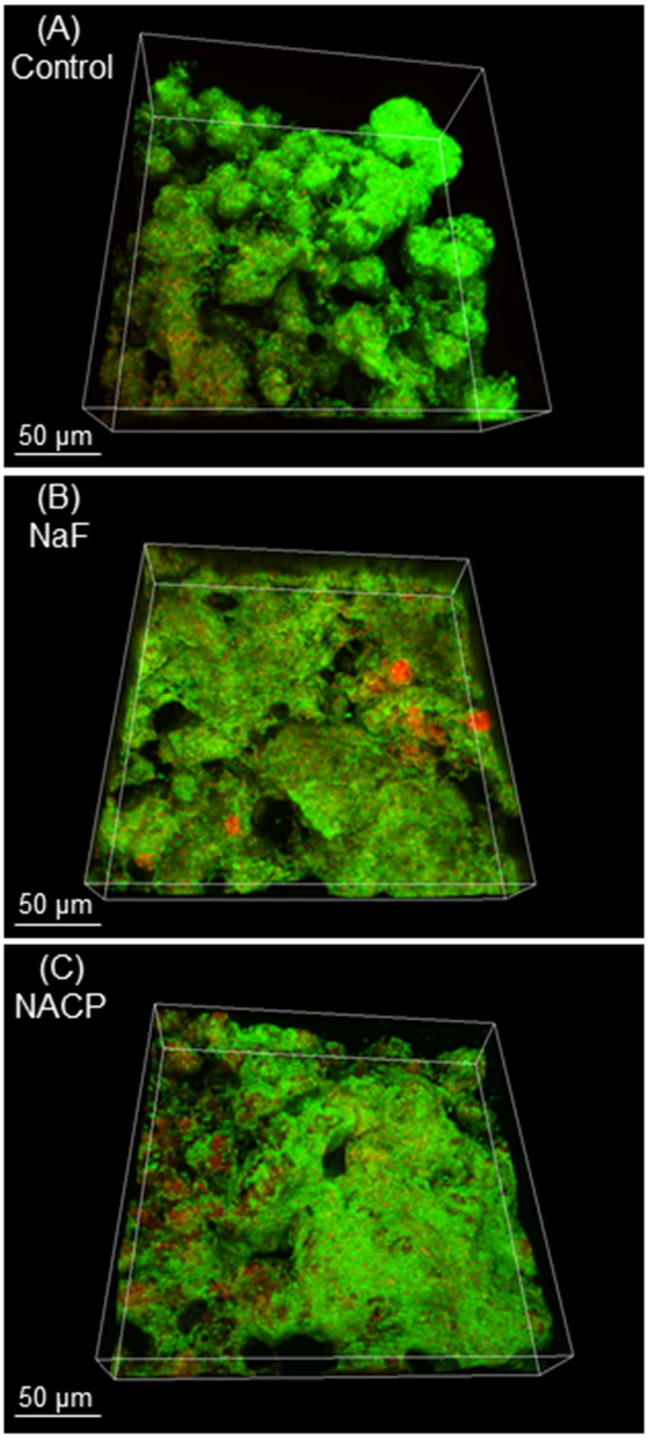

Fig. 4. Live/dead bacterial staining images. The green fluorescence represented the live bacteria, and the red one indicated the dead. (A) The Control group had a thick biofilm coverage of primarily live bacteria. (B, C) The NaF/NACP group had a thinner biofilm coverage of primarily live bacteria, with a little increase in red staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

dentin remineralization in an *S. mutans* biofilm environment for the first time. The NACP adhesive neutralized the acid, decreased the lactic acid production and CFU count, increased the biofilm calcium and phosphate ion concentration, and promoted dentin remineralization in the cyclic artificial saliva/BHI treatment with biofilm. The commercial fluoride-releasing adhesive inhibited further demineralization compared with the control group, whereas the NACP adhesive approach achieved remineralization of the biofilm-demineralized dentin. The NACP adhesive presented the best remineralization effectiveness in our experiment.

The HL acts as the connection between dentin and composite, and plays a vital role in dentin bonding. However, the weak link in tooth composite restoration continues to be its bonded interface [3]. Plaque forms on the tooth composite bonded interface, and bacterial invasion may occur because of microcaps at the tooth-restoration interfaces. Acids produced by bacteria can result in secondary caries, and lead to the failure of composite restoration [30,31]. The neonatal minerals in the HL can resist bacterial invasion better due to its acid-neutralizing ability [7]. In addition, endogenous matrix metalloproteinases (MMPs) may invade at the tooth-composite bonded interface when there are microcaps, which may result in the degradation of collagen fibrils in the HL [32]. However, the neonatal minerals can protect collagen fibrils from MMPs [33]. Therefore, the remineralization of dentin at the tooth composite bonded interface is crucial to increase restoration longevity.

Using materials to facilitate dentin remineralization can be an effective and promising strategy to enhance the longevity of dentin-composite bonds. After a sucrose rinse, the dental plaque pH can decrease to 4 to 4.5, which may cause demineralization. Raising pH to above 5.5 would be effective to resist demineralization and make remineralization possible, along with increasing the calcium and phosphate ion concentration [13,34]. According to previous studies [13,17,35], NACP acts as a promising remineralization agent and can even increase calcium and phosphate concentration at cariogenic low pH levels, when they are most needed to combat demineralization caused by acids produced from bacteria [10].

A previous study concluded that NACP-containing adhesive could facilitate dentin remineralization in a cyclic artificial saliva/lactic acid environment [14]. However, previous studies overlooked the important challenge of biofilm in the oral environment and did not explore the remineralization of NACP-containing adhesive in a biofilm environment. Biofilm should be taken into account when assessing the treatment efficacy of adhesive containing remineralizing agents because it is difficult to remove completely at the tooth-composite bond.

S. mutans acts as one of the main cariogenic bacteria, and several previous studies have assessed the efficacy of anticaries agents in in vitro *S. mutans* single-species biofilm models to simulate the real dental plaque environment [36,37]. Thus, this study evaluated the remineralization effectiveness of NACP-containing adhesive in an *S. mutans* biofilm-challenged model that better simulated the oral environment, filling a void in previous studies. An artificial saliva/BHIS cycle was developed, which is different from those biofilm models previously mentioned, where the acids produced in the biofilm continuously acted as the driving force toward demineralization. According to the TMR results, demineralization occurred to the control group compared with the baseline after the artificial saliva/BHIS cycling. Artificial saliva is widely-used remineralization solution [38,39], therefore, BHIS acted as the driving force toward demineralization. Additionally, the pH value for artificial saliva was adjusted to 7.0 during solution preparation, the pH value for BHIS after 4-h immersion in the control group was approximately 4.4 (Fig. 2), which also explained the deduction that BHIS acted as the driving force toward demineralization. The artificial saliva/BHIS cycle every day in our study displayed a degree of correlation with oral conditions with respect to food cycling (by BHIS exposures) and biofilm reformation during the remineralization in artificial saliva [21]. It has been demonstrated in the Stephen curve that a pH below 5.5 exists in dental plaque for approximately 30 min after intake of food [40]; hence, the model used in this study simulated an accelerated fluid-challenged model. Artificial saliva instead of saliva collected from healthy adult donors was used during the remineralization process, following previous remineralization studies [38,39], also in case the bacteria in saliva interfered with the *S. mutans* single-species biofilm or the salivary proteins and glycoproteins interfered with the remineralization effectiveness of NACP/NaF [41]. In the

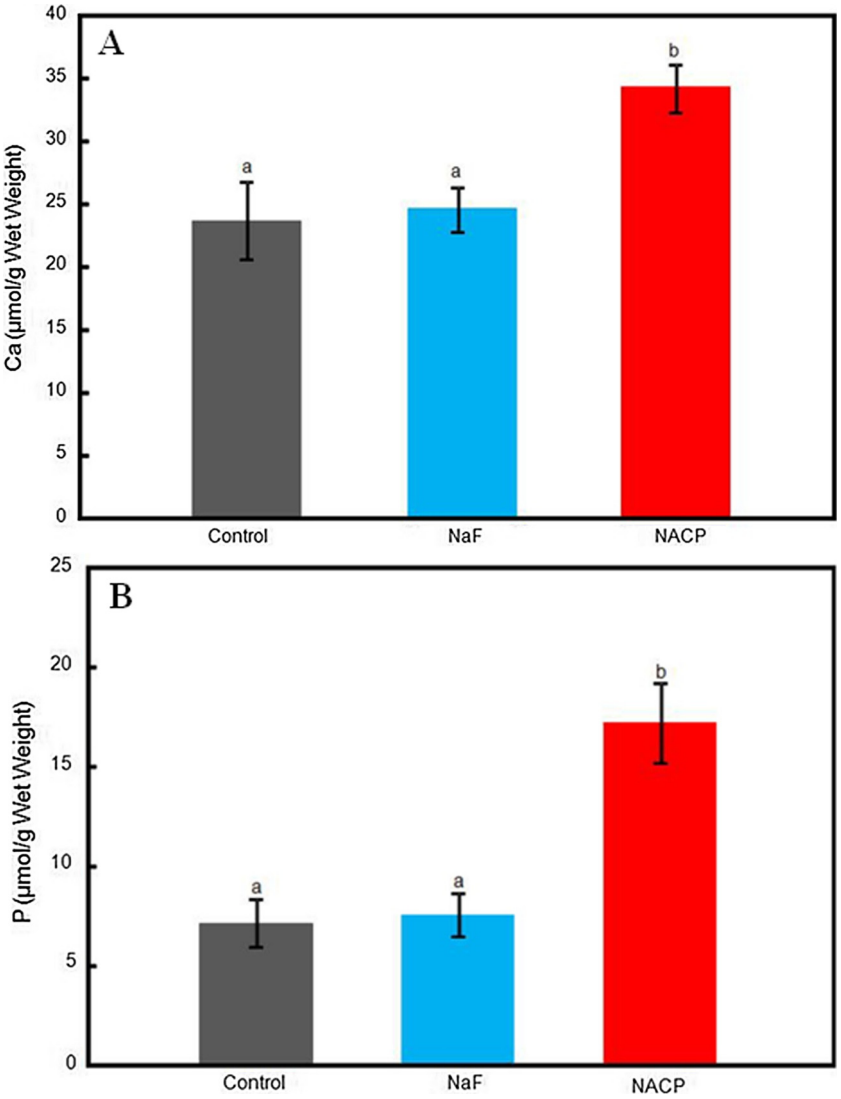

Fig. 5. (A) The calcium content of biofilm in three groups (mean ± SD, n = 6). (B) The phosphate content of biofilm in three groups (mean ± SD, n = 6). Dissimilar letters indicate significantly different values ($P < 0.05$).

current study, a simplified *S. mutans* biofilm model was used, but the ecology of caries is rather complex, and involves more than *S. mutans* alone. Therefore, demonstrating the effectiveness of NACP-containing adhesive in multi-species biofilm or human saliva microcosm biofilm models may be a crucial next step.

In the current study, the NACP group had decreased biofilm lactic acid production and CFU count compared to the control group; these findings show that NACP also had bacteria inhibition abilities. A previous study verified its bacteria inhibition abilities via agar disk-diffusion test and CFU counting [13]. The alkaline environment caused by NACP might inhibit bacteria growth [42].

However, scanning electron microscope micrographs showed that the *S. mutans* cells on the NACP-containing resin were not observed to have broken membranes or separated wall bands [13], which indicated that the bacteria inhibition abilities of NACP were relatively weak. Future studies may consider incorporating other antibacterial materials with NACP into adhesive.

TMR analysis has become one of the reliable and preferred methods studying demineralization and remineralization since a linear relationship between microradiographic measurement of enamel mineral loss and cross-sectioned hardness profiles was found in 1983 [15,35,43]. TMR is considered as the gold standard for tooth mineral density assessment in vitro [44]. The TMR analysis results concluded that the NACP adhesive approach achieved remineralization of the biofilm-demineralized dentin, whereas the commercial fluoride-releasing adhesive only inhibited further demineralization in comparison with the control group. Dentin hardness measurement is a mechanical method reflecting demineralization and remineralization degrees, the morphology of mineral crystals can be examined via SEM. These measurement methods are widely used in demineralization and remineralization studies [14,41,45,46]. NACP adhesive achieved dentin hardness recovery and tubule occlusion in our study, which confirmed its dentin remineralization effectiveness. NACP showed great prospects in realizing dentin remineralization in future clinical practice. The acid neutralization results and the results of the lactic acid production, CFU count, live-dead staining, and calcium and phosphate content of biofilm after 10 days all accounted for the good remineralization efficacy of NACP-containing adhesive. It is considered that it would be beneficial for combating tooth demineralization to increase fluid pH to 5.5 or higher; a lower pH would lead to demineralization [13,47]. The acid neutralization results demonstrated that the NACP-containing adhesive had the ability to raise the fluid pH to 5.5 or higher, a quality that the commercial fluoride-releasing adhesive lacked. This contributed to the good remineralization properties of NACP-containing adhesive. The NACP-containing adhesive had the ability to raise the fluid pH to 5.5 or higher because NACP-containing adhesive had a high level of PO_4^{3-}

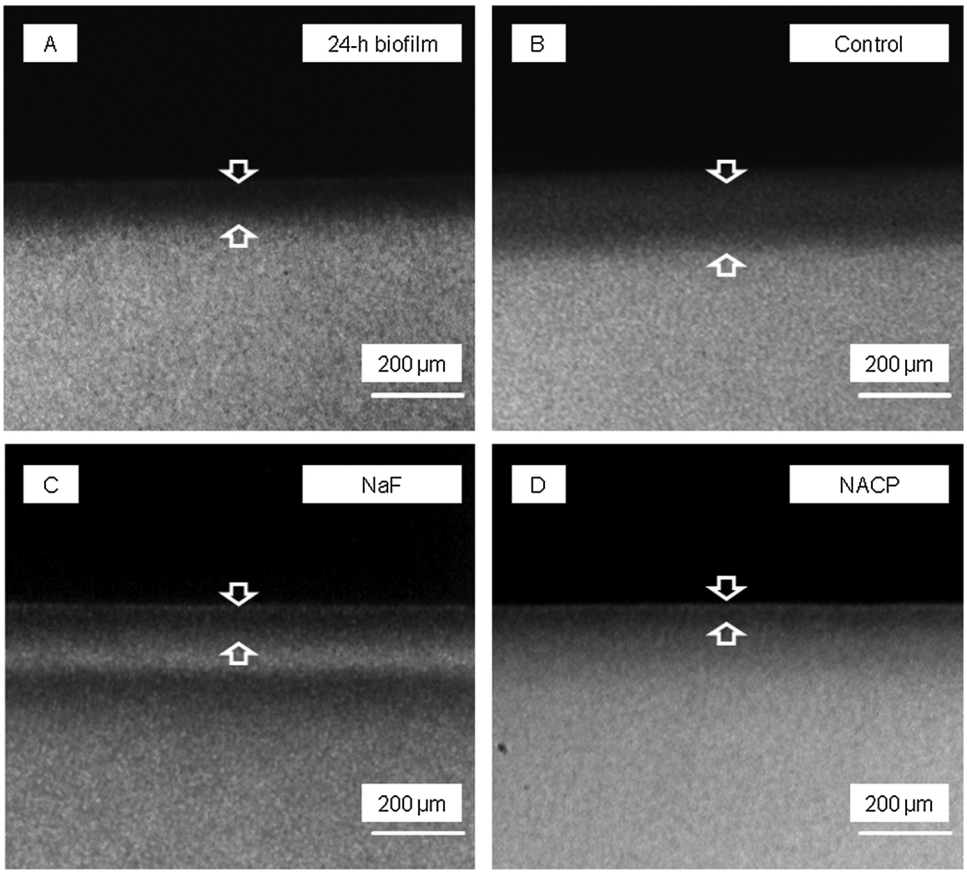

Fig. 6. Representative examples of microradiographs of dentin lesions (arrows). (A) There were significant dentin lesions after 24-h biofilm production. (B) There was further demineralization in dentin in the control group. (C) There was little remineralization in dentin in the NaF group. (D) There was significant remineralization of dentin in the NACP group.

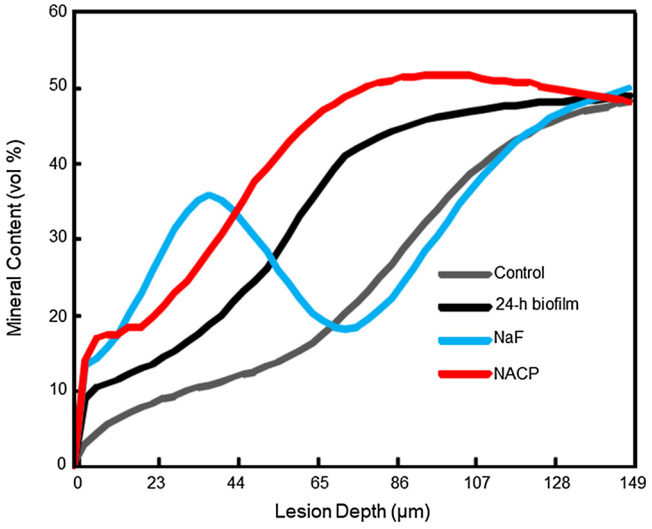

Fig. 7. Average mineral content profiles of the three groups or after 24-h biofilm production without following the experiment.

Table 1
Mineral loss and lesion depth values after different treatments.

Treatment	Mineral Loss (vol.%▪μm)	Lesion Depth(μm)
24-h biofilm without treatment	2230.00 ± 239.86[a]	111.20 ± 6.19[e]
Control	3778.75 ± 618.95[b]	137.42 ± 19.16[f]
NaF	2864.44 ± 335.71[c]	102.84 ± 39.96[e]
NACP	1460.00 ± 330.92[d]	69.08 ± 5.78[g]

All values are presented as means ± SD. The different letters indicate statistically significant differences ($P < 0.05$).

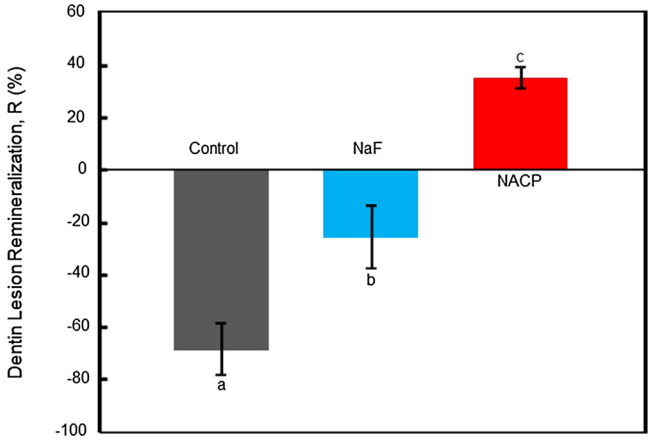

Fig. 8. Remineralization of human dentin lesions after a 10-day cycle. Dissimilar letters indicate significantly different values ($P < 0.05$). The control group showed further demineralization, the NaF group showed less demineralization than the control group, and the NACP group showed remineralization. Remineralization of dentin lesion in the control, NaF, and NACP groups was -69.50 ± 9.88%, -27.00 ± 12.06%, 34.25 ± 3.86%, respectively (mean ± SD, n = 6).

release, which could readily bind to H^+. A decrease of H^+ in fluid could result in an increase of fluid pH value [10,13]. Additionally, providing calcium and phosphate ion release would tilt the balance between tooth remineralization and demineralization to remineralization [34]. The calcium/phosphate content of biofilm after 10 days showed that the NACP-containing adhesive increased calcium/phosphate concentration in the biofilm via release of these two ions, which could not be realized in the NaF group. This also contributed to the good remineralization properties of NACP-containing adhesive. In addition, the results of CFU

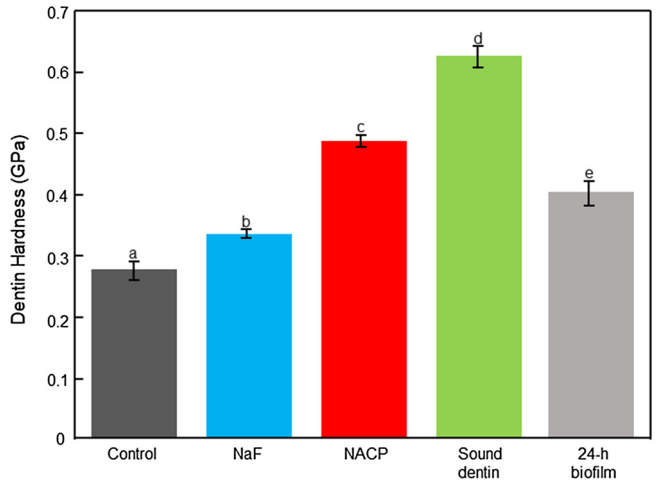

Fig. 9. Dentin hardness (mean ± SD, n = 6). Dentin hardness was measured after the remineralization/demineralization cycle for: Control, NaF and NACP groups. Hardness of healthy untreated dentin and dentin after 24-h bacterial biofilm cultivation was also measured as controls. Dissimilar letters indicate significantly different values ($P < 0.05$).

count, lactic acid production and live-dead staining of biofilm after 10 days indicated that NACP had bacteria inhibition abilities to some extent, the acids produced by biofilm bacteria could be reduced, and the dissolution of neonatal mineral could be reduced, which, as well, contributed to the good remineralization properties of NACP-containing adhesive.

Dentin remineralization techniques are classified into “top down” and “bottom up” processes [48]. The present study used an NACP-containing adhesive as a “top down” technique, the demineralized lesion is exposed to a Ca and P ion supersaturated solution and mineral precipitation starts over existing seed crystals [8]. In “bottom up” processes, based on the non-classical crystallization pathway, non-collagenous proteins (NCPs) or their biomimetic analogues serve as the regulation of calcium phosphate phases, which includes the sequestration of calcium and phosphate ions into ACP, known as stabilizing ACP, and the templating of mineral nucleation and growth in intrafibrous and interfibrous spaces, while dentin collagen matrix serves as a mineral deposition template [8,49]. The polyanionic domains of these proteins contain polycarboxylic acid and phosphate function groups which can help to bind to collagen substrates, then calcium adsorption and mineral nucleation occur through the anionic sites of the proteins [49]. In the present study, an NCP or its analogue was not included because the aim of this study was to investigate the NACP adhesive and a commercial fluoride-releasing adhesive on dentin remineralization in a biofilm-challenged environment, the addition of NCPs or their biomimetic analogues could interfere with the remineralization effectiveness of the NACP adhesive or the commercial fluoride-releasing adhesive alone. Although an ACP stabilizer is difficult to apply to adhesive, it would be interesting to add an ACP stabilizer with NACP in other forms of application in future studies, to achieve high order arrangement remineralization.

5. Conclusions

Within the limitations of our in vitro model, an NACP adhesive was shown, for the first time, to remineralize demineralized dentin in an *S. mutans* biofilm environment. Its remineralization abilities were superior to a commercial fluoride-releasing adhesive in a 10-day cyclic artificial saliva/BHIS regimen. The NACP adhesive is promising for restoration bonding that can protect bond interface, combat secondary caries, and enhance the longevity of restoration.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdent.2019.103193.

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