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Bonding durability, antibacterial activity and biofilm pH of novel adhesive containing antibacterial monomer and nanoparticles of amorphous calcium phosphate

Yuncong Li[a,b,1], Xiaoyi Hu[a,1], Jianping Ruan[a], Dwayne D. Arola[c], Chao Ji[b], Michael D. Weir[b], Thomas W. Oates[b], Xiaofeng Chang[a,**], Ke Zhang[b,d,*], Hockin H.K. Xu[b,e,f,*]

[a] 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
[b] Department of Advanced Oral Sciences & Therapeutics, University of Maryland School of Dentistry, Baltimore, MD, 21201, USA
[c] Department of MaterialsScience and Engineering, University of Washington, Seattle, WA, 98195, USA
[d] Department of Orthodontics, School of Stomatology, Capital Medical University, Beijing, China
[e] Center for Stem Cell Biology & Regenerative Medicine, University of Maryland School of Medicine, Baltimore, MD, 21201, USA
[f] Department of Mechanical Engineering, University of Maryland Baltimore County, Baltimore County, MD, 21250, USA

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ABSTRACT

Objectives: The dentin bonding often fails over time, leading to secondary caries and restoration failure. The objectives of this study were to develop an adhesive with dimethylaminohexadecyl methacrylate (DMAHDM) and nanoparticles of amorphous calcium phosphate (NACP), and investigate the effects of storage in artificial saliva for six months on the bonding durability, antibacterial activity, ion release and biofilm pH properties for the first time.

Methods: DMAHDM was added at 5% (by mass) to Scotchbond Primer and Adhesive (SBMP). NACP was added at 10%, 20%, and 30% to SBMP adhesive. Dentin bonding durability, antibacterial activity against *Streptococcus mutans* biofilms, and calcium (Ca) and phosphate (P) ion liberation properties were investigated after 1 day and 6months of storage in artificial saliva.

Results: Dentin bond strength (n = 50) had 25% loss after 6 months of aging for SBMP control. However, SBMP + DMAHDM + 10NACP and SBMP + DMAHDM + 20NACP showed no loss in bond strength after storage in artificial saliva for 6 months. The DMAHDM + NACP incorporation method dramatically reduced the biofilm metabolic activity and acid production, and decreased the biofilm CFU by four orders of magnitude, compared to SBMP control, even after 6 months of aging ($p < 0.05$). DMAHDM + NACP had long-lasting Ca and P ion releases, and raised the biofilm pH to 6.8, while the control group had a cariogenic biofilm pH of 4.5.

Conclusions: Incorporating DMAHDM + NACP in bonding agent yielded potent and long-lasting antibacterial activity and ions liberation ability, and much higher long-term dentin bond strength after 6-month of aging. The new bonding agent is promising to inhibit caries at the restoration margins and increase the resin-dentin bonding longevity.

Clinical significance: The novel bioactive adhesive is promising to protect tooth structures from biofilm acids and secondary caries. NACP and DMAHDM have great potential for applications to a wide range of dental materials to reduce plaque and achieve therapeutic effects.

1. Introduction

Dental resin composite restores tooth defects by bonding to the tooth structures via an adhesive [1–3]. The main challenge for the adhesive is to provide durable and effective bonding to tooth hard tissues [4]. Unfortunately, it has been estimated that as much as half of dental resinous restorations have to be replaced with the main reason for replacements being secondary caries at the tooth-restoration

* Corresponding authors at: Department of Advanced Oral Sciences & Therapeutics, University of Maryland School of Dentistry, Baltimore, MD, 21201, USA.
** Corresponding author.
E-mail addresses: changxf@xjtu.edu.cn (X. Chang), tuzizhangke@163.com (K. Zhang), hxu@umaryland.edu (H.H.K. Xu).
[1] Co-first author.

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margins [5,6]. Bonding failure, microleakage and bacterial acid production lead to secondary caries. Therefore, the development of a new generation of dental adhesives with antibacterial activity and stronger and longer-lasting bonds is needed to reduce secondary caries and restoration failure rates [7].

Quaternary ammonium methacrylates (QAMs) were synthesized and incorporated into resins to kill the residual bacteria in the tooth cavity as well as to suppress new bacteria at the tooth-restoration margins. 12-methacryloyloxydodecyl-pyridinium bromide (MDPB) with antibacterial function was incorporated into self-etching primer and adhesive [8,9]. In addition, methacryloxylethyl cetyldimethyl ammonium chloride (DMAE-CB) [10], 2-methacryloxylethyl hexadecyl methyl ammonium bromide (MAE-HB) [11], and dimethylaminododecyl methacrylate (DMADDM) were developed as polymerizable cationic monomers to covalently bond with the polymer matrix to kill bacteria upon contact [12,13]. Also novel cavity disinfectant was formulated with quaternary ammonium silane [14]. Recently, dimethylaminohexadecyl methacrylate (DMAHDM) was synthesized and showed powerful antibacterial effects [15,16]. DMAHDM possessed a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) that were an order of magnitude lower than DMADDM [15]. Moreover, DMAHDM induced no drug resistance in cariogenic, endodontic and periodontal bacterial species, while chlorhexidine control showed drug resistance [17].

Another approach to enhancing the bond durability of adhesives is via remineralization [18,19]. Because of the excellent biocompatibility and bioactivity, nanoparticles of amorphous calcium phosphate (NACP) were incorporated in composites and possessed much better mechanical properties than traditional calcium phosphate composites [20]. Moreover, bonding agents containing NACP released high levels of calcium (Ca) and phosphate (P) ions to induce remineralization [12,13]. Due to their small particle sizes, NACP readily flowed with adhesive into the demineralized dentin to deliver mineral ions to promote the regrowth of apatite crystals in the interfibrillar region via classic nucleation [20,21]. Thus, NACP-containing adhesive may be highly beneficial to protect the exposed collagen, facilitate remineralization in the bonded interface, and alleviate or eliminate microleakage.

Regarding durability, the resins containing MDPB and MAE-HB maintained strong antibacterial effects after 3 or 6 months of immersion in water, due to QAM co-polymerization with resin [11,22,23]. The potent antibacterial activity of an adhesive containing 5% DMADDM and 20% NACP was also demonstrated after 6 months of water-aging, which improved the long-term dentin bond strength [12]. Even after a longer aging time of 1 year, there was no decrease in the anti-biofilm effects compared to those at 1 day, while the mechanical properties of the antibacterial composite matched those of a commercial control composite [24]. However, those studies used water as the storage medium for the long-term aging tests [12,24], without using artificial saliva. The use of artificial saliva storage would be more clinically-relevant for long-term aging tests [25,26]. In addition, the double effects of antibacterial and remineralization agents in adhesive containing DMAHDM and NACP on long-term dentin bonding durability and biofilm pH have not been reported.

The objectives of this study were to investigate the effects of artificial saliva-aging for 6 months on bonding agents containing DMAHDM and NACP for the first time. The following properties were determined: (1) dentin bond strength durability; (2) antibacterial effects after long term immersion; (3) ions liberation and biofilm pH measurements. It was hypothesized that: (1) The DMAHDM + NACP bonding agent would have no decrease in dentin bond strength from 1 day to 6 months of immersion in artificial saliva, while a commercial control would suffer a substantial loss in bond strength; (2) The antibacterial activity of DMAHDM + NACP bonding agent would be potent and not decrease from 1 day to 6 months of immersion; (3) The DMAHDM + NACP bonding agent would maintain its ions liberation ability in aging and be able to raise the biofilm pH to a safe region of > pH 6, while the commercial control would have a cariogenic biofilm pH of close to 4.

2. Materials and methods

2.1. Incorporation of DMAHDM into bonding agent

Scotchbond Multi-Purpose Adhesive and Primer (referred as "SBMP") (3 M, St. Paul, MN) was used as the parent bonding system. According to the manufacturer, SBMP adhesive contained 60–70% of bisphenol A diglycidyl methacrylate (BisGMA) and 30–40% of 2-hydroxyethyl methacrylate (HEMA), tertiary amines and photo-initiator. SBMP primer contained 35–45% of HEMA, 10–20% of a copolymer of acrylic and itaconic acids, and 40–50% water.

DMAHDM was synthesized using a modified Menschutkin reaction method where a tertiary amine group was reacted with an organo-halide [9]. A benefit of this reaction is that the products are generated at virtually quantitative amounts and require minimal purification. Briefly, 10 mmol of 2-(dimethylamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich, St. Louis, MO) and 10 mmol of 1-bromohexadecane (BHD, TCI America, Portland, OR) were combined with 3 g of ethanol in a 20 mL scintillation vial. The vial was stirred at 70 °C for 24 h. The solvent was then removed via evaporation, yielding DMAHDM as a clear, viscous liquid. DMAHDM was incorporated into SBMP primer at a mass fraction of DMAHDM/(SBMP primer + DMAHDM) = 5%. The 5% was selected following previous studies [27,28]. Similarly, DMAHDM was incorporated into SBMP adhesive at 5% mass fraction.

2.2. Incorporation of NACP into bonding agent

NACP [$Ca_3(PO_4)_2$] were synthesized via a spray-drying technique as previously described [20]. Briefly, calcium carbonate ($CaCO_3$) and dicalcium phosphate ($CaHPO_4$) were dissolved into an acetic acid solution to obtain concentrations of Ca and P ions of 8 mmol/L and 5.333 mmol/L, respectively. The solution was sprayed into a heated chamber to evaporate the water and volatile acid. An electrostatic precipitator (Air Quality, Minneapolis, MN) was used to collect the dried NACP powders. This yielded NACP with a mean particle size of 116 nm [20]. NACP were incorporated into the adhesive at mass fractions of 0%, 10%, 20% and 30%, following previous studies showing high releases of Ca and P ions while having no significant adverse effect on mechanical properties [13,28,29]. Therefore, the following five groups were tested:

(1) Commercial Scotchbond Multi-Purpose Adhesive and Primer (designated “SBMP Control”).
(2) SBMP primer and adhesive each had 5% DMAHDM, no NACP (designated “SBMP + DMAHDM + 0NACP”).
(3) SBMP Primer + 5% DMAHDM. SBMP Adhesive + 5% DMAHDM + 10% NACP (SBMP + DMAHDM + 10NACP).
(4) SBMP Primer + 5% DMAHDM. SBMP Adhesive + 5% DMAHDM + 20% NACP (SBMP + DMAHDM + 20NACP).
(5) SBMP Primer + 5% DMAHDM. SBMP Adhesive + 5% DMAHDM + 30% NACP (SBMP + DMAHDM + 30NACP).

2.3. Dentin microtensile bond strength (μTBS) test

Fifty extracted caries-free human third molars were collected with donor consent under a protocol approved by the Institutional Review Board (IRB) of the University of Maryland, Baltimore. The occlusal enamel of the tooth was removed to expose midcoronal dentin via a water-cooled low-speed saw (Isomet, Buehler, Lake Bluff, IL). Each dentin surface was polished with 600-grit SiC paper under running water for 60 s, etched with 37% phosphoric acid gel for 15 s and rinsed with distilled water [30]. A primer was applied and the solvent was removed with a stream of air for 5 s. Then an adhesive was applied and

light-cured with continuous output using a light intensity of 430 mW/cm^2, with a curing distance of 5 mm and lasting for 10 s (Optilux VCL 401, Demetron Kerr, Danbury, CT). Two 2-mm thick layers of a hybrid composite (Universal, 3 M, St. Paul, MN) were placed over the bonded surface. Each layer was light-cured following the same aforementioned photo-cure method for 40 s. After storing in artificial saliva at 37 °C for 24 h, each bonded tooth was vertically sectioned into beams with 0.9 x 0.9 mm cross-section. Ten beams were obtained from each tooth and divided randomly into two groups: one group for immediate μTBS after 1 day immersion, and the other group for testing after 6 months of storage in artificial saliva. The artificial saliva contained (mmol/L): $CaCl_2$ (0.7), $MgCl_2$ $6H_2O$ (0.2), KH_2PO_4 (4.0), KCl (30), NaN_3 (0.3), and HEPES buffer [31]. The artificial saliva was changed once every week. Fifty beams were tested for each bonding agent at each time period.

For all groups, each beam was tested in uniaxial tension on a computer-controlled Universal Testing Machine (MTS, Eden Prairie, MN) at a cross-head speed of 1 mm/min. The load-at-failure divided by the cross-sectional area at the site of failure yielded the μTBS. Failure modes were examined under a stereoscopic microscope and classified as adhesive failure (failure along the adhesive interface), mixed failure (failures in the adhesive joint together with failure in composite or in dentin), or cohesive failure (failure in the composite or in dentin) [32]. Furthermore, four representative debonded specimens per group that failed in adhesive mode were selected to analyze the micromorphology of the fractured surface with SEM. The specimens were dried overnight, sputter-coated with gold and observed with a scanning electron microscope (Quanta 200, FEI, Hillsboro, OR) at an accelerating voltage of 10 KeV.

2.4. Bacterial experiments

The cover of a sterile 96-well plate (Costar, Corning Inc., Corning, NY) was used as molds to fabricate resin disks following a previous study [15]. Briefly, 10 μL of a primer was placed in the bottom of each dent of the 96-well plate. After drying with a stream of air, 20 μL of adhesive was applied to the dent and photo-polymerized for 60 s (Optilux), using a Mylar strip covering to obtain a disk of 8 mm in diameter and 0.5 mm in thickness. The cured resin disks were immersed in distilled water and magnetically stirred with a bar at a speed of 100 rpm for 1 h to remove any uncured monomers, following a previous study [33]. The disks were then sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC) and de-gassed for 3 days, following the manufacturer's instructions. For the aging process, the specimens of each group were first immersed in artificial saliva at 37 °C for 6 months, and then sterilized and subjected to the following tests. The artificial saliva was changed every week.

The use of *Streptococcus mutans* (*S. mutans*) (ATCC 700610, UA159, American Type Culture, Manassas, VA) was approved by the University of Maryland IRB. *S. mutans* is a cariogenic, aerotolerant anaerobic bacterium and the primary causative agent of dental caries. Brain heart infusion (BHI) broth (BHI, Becton, Sparks, MD) supplemented with 1% sucrose is termed the "growth medium". Ten μL of stock bacteria was added into 10 mL of BHI broth and incubated at 37 °C with 5% CO_2 for 16 h. During this culture, the *S. mutans* bacteria were suspended in the BHI broth. Then, this *S. mutans* culture was diluted by 10 folds in the growth medium to form the inoculation medium.

2.5. Live/dead assay of S. mutans biofilms on resins

Each resin disk was placed in a well of a 24-well plate, and 1.5 mL of the inoculation medium was added to each well. The samples were incubated at 5% CO_2 and 37 °C for 24 h. Then each disk with biofilm was transferred to a new 24-well plate containing 1.5 mL of fresh growth medium, and cultured for another 24 h. Specimens with 2-day biofilms were washed with phosphate buffered saline (PBS). Then the biofilms on the disks were stained using the BacLight live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce green fluorescence. Bacteria with compromised membranes were stained with propidium iodide to produce red fluorescence. An inverted epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY) was used to examine three disks for each group [28]. Six images were collected at random locations on each disk, yielding 18 images per group.

2.6. MTT metabolic activity of S. mutans biofilms

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a colorimetric assay that measures the enzymatic reduction of MTT, a yellow tetrazole, to formazan [34]. Briefly, disks with 2-day biofilms were transferred to new 24-well plate, with 1 mL of 0.5 mg/mL MTT dye in each well. All specimens were incubated at 37 °C in 5% CO_2 for 1 h. During this process, metabolically active bacteria reduced the MTT to purple formazan. After 1 h, the biofilm specimens were transferred to a new 24-well plate. An aliquot of 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. After incubation for 20 min in the dark, 200 μL of the DMSO solution was transferred to a 96-well plate, and the absorbance at 540 nm was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). A higher absorbance indicates a higher formazan concentration, which means more metabolic activity of the bacteria. Six replicates were tested for each group (n = 6).

2.7. Lactic acid production and colony-forming units (CFUs) of biofilms

Disks with 2-day biofilms were rinsed in cysteine peptone water (CPW) to remove loose bacteria and placed in a new 24-well plate. An aliquot of 1.5 mL of buffered peptone water (BPW) supplemented with 0.2% sucrose was added to each well. Samples were incubated at 5% CO_2 and 37 °C for 3 h to allow the bacteria to produce acid. After 3 h, the BPW solutions were stored for lactate analysis. Lactate concentrations were determined using an enzymatic method [35]. The microplate reader was used to measure the absorbance at 340 nm for the collected BPW solutions. Standard curves were prepared using a lactic acid standard (Supelco Analytical, Bellefonte, PA) [36].

Bacteria in 2-day biofilms on the disks were harvested by sonication (3510R-MTH, Branson, Danbury, CT) for 3 min and vortexing (Fisher, Pittsburgh, PA) for 20 s. The bacterial suspensions were serially diluted, and spread onto BHI agar plates for CFU analysis (n = 6) following previous studies [12,28,37].

2.8. Ca and P ion concentrations in biofilm culture medium

The biofilm medium after 48 h of incubation was collected and centrifuged at 12,000 rpm for 5 min (Eppendorf 5415, Brinkmann, Westbury, NY). Then, the supernatant was analyzed for Ca and P ion concentrations via a spectrophotometric method (DMS-80 UV–vis, Varian, Palo Alto, CA) using known standards and calibration curves, following previous studies [13,20].

2.9. The pH of biofilm culture medium

The pH of the biofilm culture medium was measured via a pH meter (Accumet Excel XL25, Fisher, Pittsburgh, PA) at 48 h. The pH was not measured during the first 24 h of culture, because the non-adherent bacteria in the growth medium could contribute to the pH [37]. Since the resin disk with adherent biofilm at 24 h was removed from the well and placed into a new well, only the adherent biofilm on the resin would be growing from 24 h to 48 h. This ensured that the measurement at 48 h yielded the pH that was solely related to the adherent biofilm on the resin disk, avoiding any contribution from planktonic bacteria in the medium.

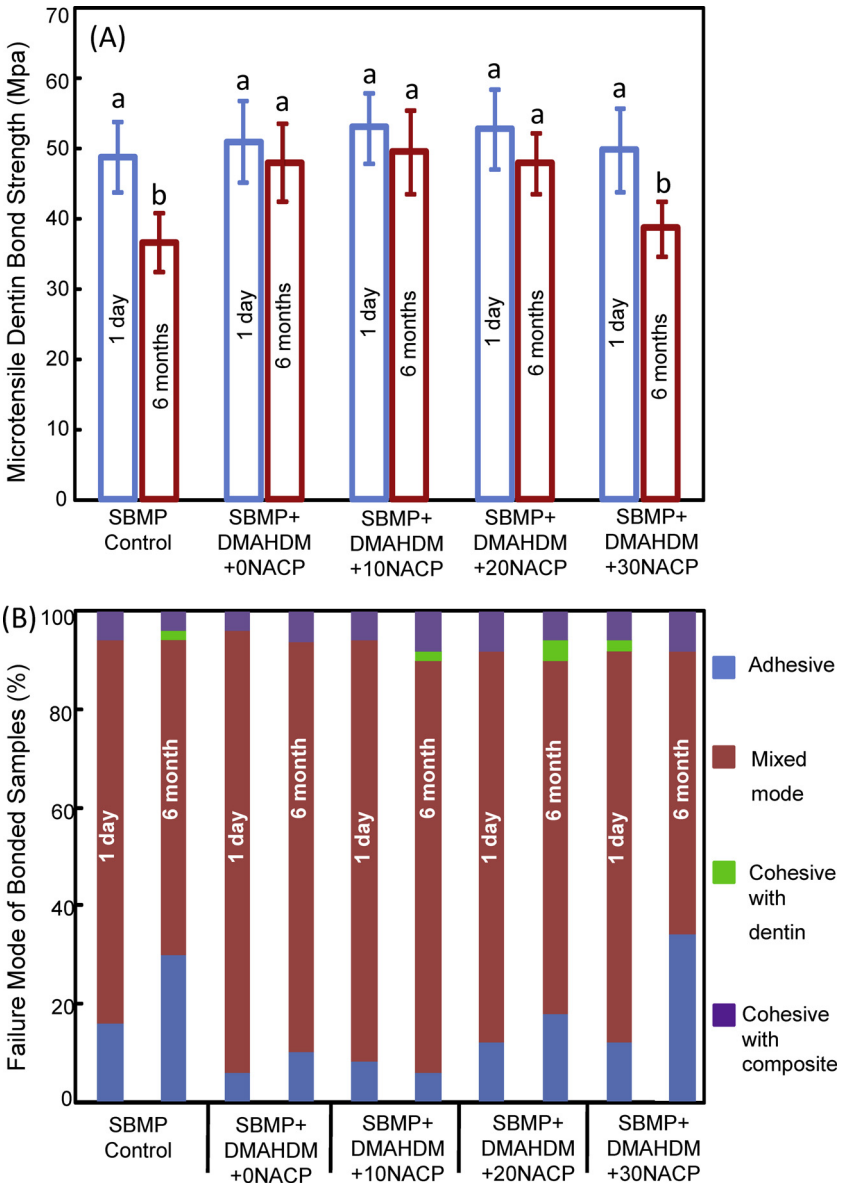

Fig. 1. Dentin bonding. (A) Microtensile bond strength (μTBS) using extracted human teeth, tested after storage in saliva for 1 day and for 6 months aging treatment (mean ± sd; n = 50). Values with dissimilar letters are significantly different from each other ($p < 0.05$). (B) The effect of DMAHDM and NACP on the distribution of μTBS failure modes.

2.10. Statistical analyses

Statistical analysis was conducted with two-way analysis of variance to examine the effects of "different type of adhesives" and "two immersion time periods", and the interaction of these two factors on microtensile bond strength, biofilm experiments and Ca and P ion release. Parametric statistical methods were employed under the premise that the normality and equal variance assumptions of the data sets were not violated. Post-hoc pairwise comparisons were performed using the Tukey's statistics. Statistical significance was set at $\alpha = 0.05$.

3. Results

Dentin μTBS at 1day and 6 months of artificial saliva immersion are plotted in Fig. 1(A) (mean ± sd; n = 50). Two-way ANOVA revealed that the immersion time and the type of adhesive had significant effects on μTBS, with a significant interaction between the two factors ($p < 0.05$). There were no significant differences at 1 day ($p > 0.05$). However, after 6 months, the μTBS of SBMP Control and SBMP + DMAHDM + 30NACP were significantly lower ($p < 0.001$) than other groups. Significant differences in μTBS between 1 day and 6 months were detected for SBMP Control ($p < 0.001$) and SBMP + DMAHDM + 30NACP ($p < 0.001$). No significant reduction was observed in μTBS for the other three groups ($p > 0.05$). Fig. 1B shows the failure mode distribution in the bonded samples. Mixed failure was the predominant mode. After 6 months, the ratio of adhesive failure was increased in SBMP Control by 30%, and in SBMP + DMAHDM + 30NACP group by 34%.

Fig. 2 shows representative SEM images of adhesive fracture surfaces for: (A, B) SBMP Control, (C, D) SBMP + DMAHDM + 20NACP, (E, F) SBMP + DMAHDM + 30NACP, at 1 day and 6 months in artificial saliva. SBMP + DMAHDM + 0NACP and SBMP + DMAHDM + 10NACP had fracture surfaces similar to those of SBMP + DMAHDM + 20NACP, and were not included here to avoid redundancy. For all groups, the predominant failure mode was mixed failure. At 1 day, the mixed failure mainly occurred in the middle or near the top of the hybrid layer, because the demineralized dentin was covered by adhesive, and resin tags were left in dentinal tubules. At 6 months, SBMP Control

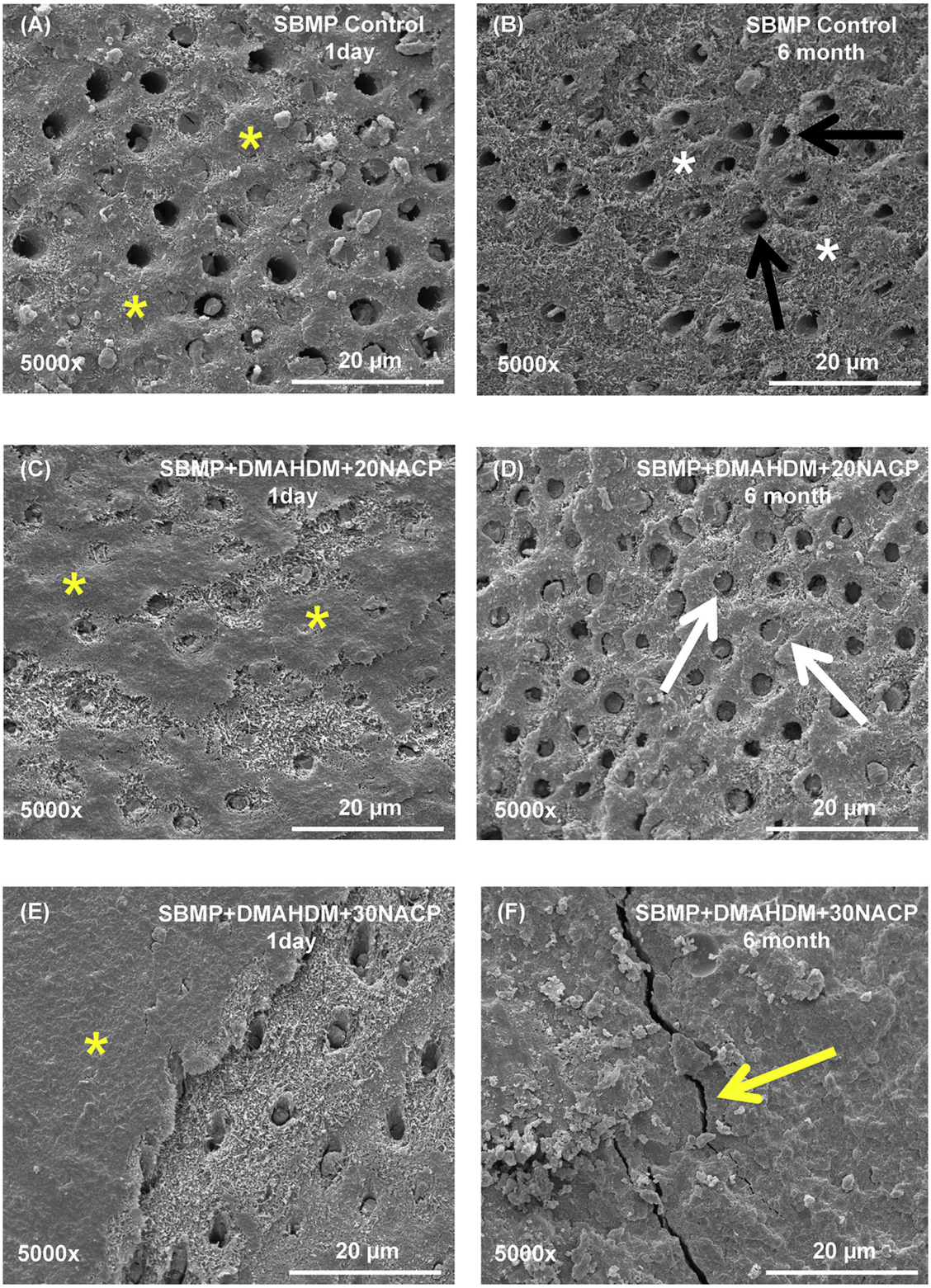

Fig. 2. Typical SEM images of fracture surfaces of: (A, B) SBMP Control, (C, D) SBMP + DMAHDM + 20NACP, (E, F) SBMP + DMAHDM + 30NACP, at storage in saliva for 1 day and for 6 months aging treatment. SBMP + DMAHDM + 0NACP and SBMP + DMAHDM + 10NACP had fracture surfaces similar to SBMP + DMAHDM + 20NACP, and hence were not included here to avoid redundancy. Mixed failure occurred in the hybrid layer for all groups at 1 day (A, C and E). Differences were found between SBMP Control with adhesive fracture at the bottom of the hybrid layer (B), and SBMP + DMAHDM + 30NACP with cohesive fracture in adhesive layer (F). No obvious change was observed in SBMP + DMAHDM + 20NACP after 6 months storage in artificial saliva. Adhesive resin (yellow asterisk); exposed collagen at the bottom of the hybrid layer (white asterisk); exposed dentinal tubules (black arrow); resin tag (white arrow); crack in adhesive resin layer (yellow arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

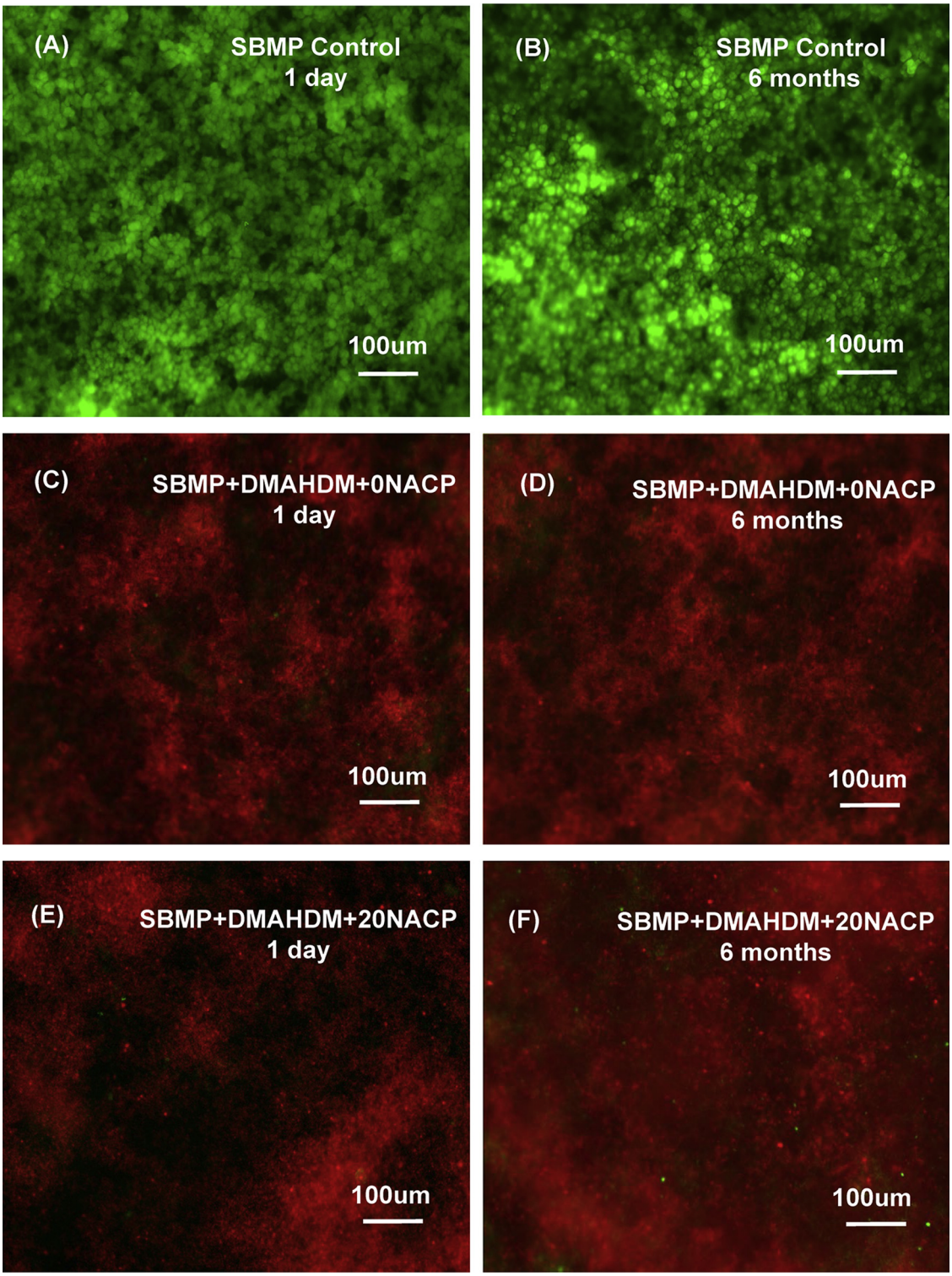

Fig. 3. Representative live/dead images of 2-day biofilms on adhesive resins: (A)-(F) SBMP Control, SBMP + DMAHDM + 0NACP and SBMP + DMAHDM + 20NACP after 1day and 6 months of immersion in artificial saliva. SBMP + DMAHDM + 10NACP and SBMP + DMAHDM + 30NACP (not included to avoid redundancy) had similar images to SBMP + DMAHDM + 20NACP. Live bacteria were stained green, and bacteria with compromised membranes were stained red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

failed at the bottom of the hybrid layer (Fig. 2B); tubules were mostly exposed, with few tubules containing resin tags. Partially-exposed collagen at the bottom of the hybrid layer was also visible. However, for SBMP + DMAHDM + 20NACP, the adhesive fracture was localized in the middle of the hybrid layer, the tubules were filled with resin tags, and intertubular dentin was covered by adhesive (Fig. 2D). In contrast, SBMP + DMAHDM + 30NACP had cohesive fracture that was localized in the adhesive layer (Fig. 2F).

Representative live/dead images of 2-day biofilms on resins are shown in Fig. 3. In (A) and (B), SBMP Control were fully covered by live bacteria. In contrast, images (C)–(F) show biofilms consisting of primarily compromised bacteria. There was no noticeable difference

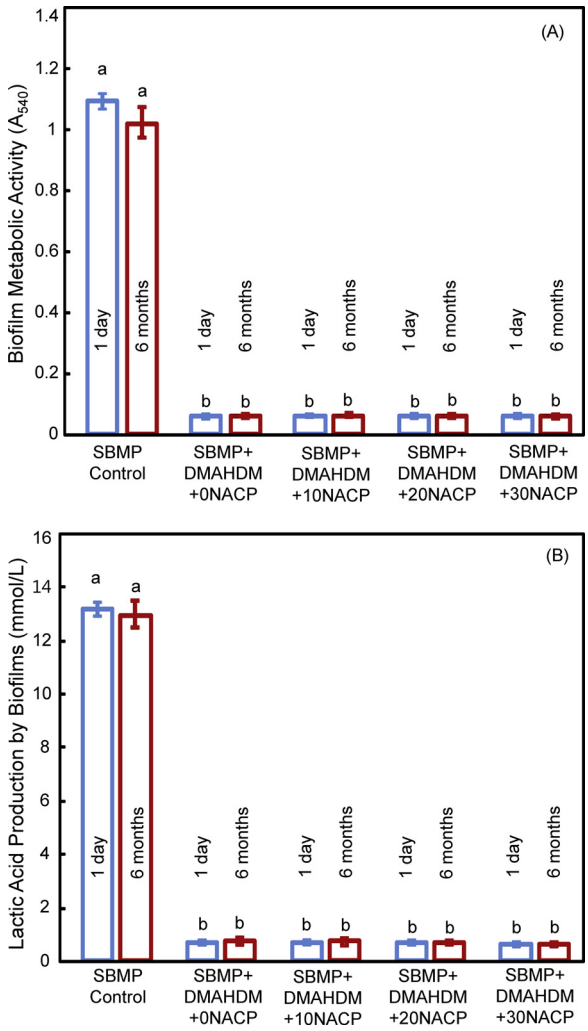

Fig. 4. Biofilm viability on resins: (A) MTT metabolic activity, (B) lactic acid production (mean ± sd; n = 6). The resin disks were first stored in artificial saliva for 1 day or 6 months, and then the resin disks were used to inoculate the bacteria and cultured for 2 days. In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

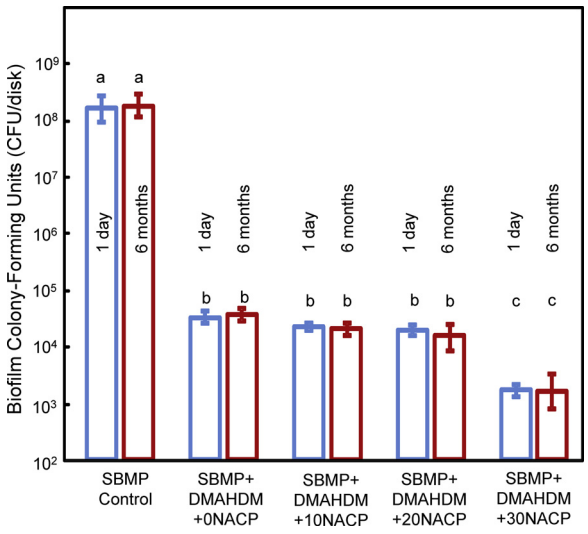

Fig. 5. Colony-forming units (CFU) of 2-day biofilms on resins (mean ± sd; n = 6). The resin disks were first stored in artificial saliva for 1 day or 6 months, and then the resin disks were used to inoculate the bacteria and cultured for 2 days. Values with dissimilar letters are significantly different from each other ($p < 0.05$). Note the log scale in the y-axis.

between 1 day and 6 months of immersion, indicating that the antibacterial activity was not lost in artificial saliva aging. SBMP + DMAHDM + 10NACP and SBMP + DMAHDM + 30NACP were similar to SBMP + DMAHDM + 20NACP, and hence are not included here to avoid redundancy.

Metabolic activity of 2-day biofilms on resins is plotted in Fig. 4(A) (mean ± sd; n = 6). The adhesive types had a significant effect on the metabolic activity ($p < 0.001$), but the immersion time had an insignificant effect ($p > 0.05$). No significant difference between 1 day and 6 months was detected for all groups ($p > 0.05$). In Fig. 4(B), biofilms on SBMP Control produced the most acid. Incorporation of DMAHDM dramatically decreased the acid production, to less than 1/22 that of SBMP Control ($p < 0.001$). These results demonstrate a long-lasting antibacterial activity of bonding agents containing DMAHDM and NACP that was not lost in artificial saliva aging.

Fig. 5 plots the CFU of 2-day biofilms on resins (mean ± sd; n = 6). Groups containing DMAHDM reduced the biofilm CFU counts, which were almost 4 orders of magnitude less than those of control ($p < 0.001$). The antibacterial potency did not decrease with from 1 day to 6 months ($p > 0.05$).

Ca and P ion concentrations in biofilm culture medium are plotted in Fig. 6 (mean ± sd; n = 6). The BHI medium itself contained Ca and P ions, manifested by the measured Ca and P ion concentrations in the culture medium for SBMP Control and SBMP + DMAHDM + 0NACP groups. However, with increasing NACP mass fraction, the Ca and P ion concentrations increased significantly ($p < 0.05$). Even after 6 months of immersion in artificial saliva and with the change of fresh medium every week, the Ca and P ion concentrations in the biofilm culture medium of SBMP + DMAHDM + 20NACP and SBMP + DMAHDM + 30NACP were still significantly higher than those of SBMP control ($p < 0.05$). These results demonstrate that the NACP-containing resins could provide a long-term reservoir of Ca and P ions.

The pH of the 2-day biofilm medium is plotted in Fig. 7 (mean ± sd; n = 6). The pH for SBMP Control stayed at 4.5, which could cause demineralization to tooth structures. In sharp contrast, the pH for all DMAHDM + NACP groups was in a safe region of above 6.5. For each group, there was no difference between 1 day and 6 months ($p > 0.05$). For all DMAHDM + NACP groups, their ability to raise the biofilm pH was not lost in the 6 months of artificial saliva aging, compared to 1 day ($p > 0.05$).

4. Discussion

The present study determined the effects of immersion time in artificial saliva for 6 months on bonding durability, antibacterial activity and remineralization ability of bioactive bonding agents containing DMAHDM and NACP for the first time. The hypotheses were proven that SBMP + DMAHDM + 10NACP and SBMP + DMAHDM + 20NACP exhibited no decrease in dentin bond strength after 6 months storage in artificial saliva, while SBMP Control and SBMP + DMAHDM + 30NACP had 25% and 21% of loss in bond strength, respectively. The antibacterial activity of all DMAHDM + NACP bonding agents had no decrease from 1 day to 6 months of immersion in artificial saliva, which reduced biofilm CFU by four orders of magnitude, and possessed long-term potent anti-biofilm functions. Furthermore, SBMP + DMAHDM + 20NACP and SBMP + DMAHDM + 30NACP maintained their remineralization ability, with ion concentrations after 6 months of immersion still being much higher than control, and with biofilm pH in a safe region of above 6.8, while commercial control had a cariogenic biofilm pH of 4.5. Based on these results, The optimal composition appeared to be SBMP + DMAHDM + 20NACP, which successfully maintained the dentin bond strength during 6 months of aging in saliva, and which had much higher Ca/P ion concentration than

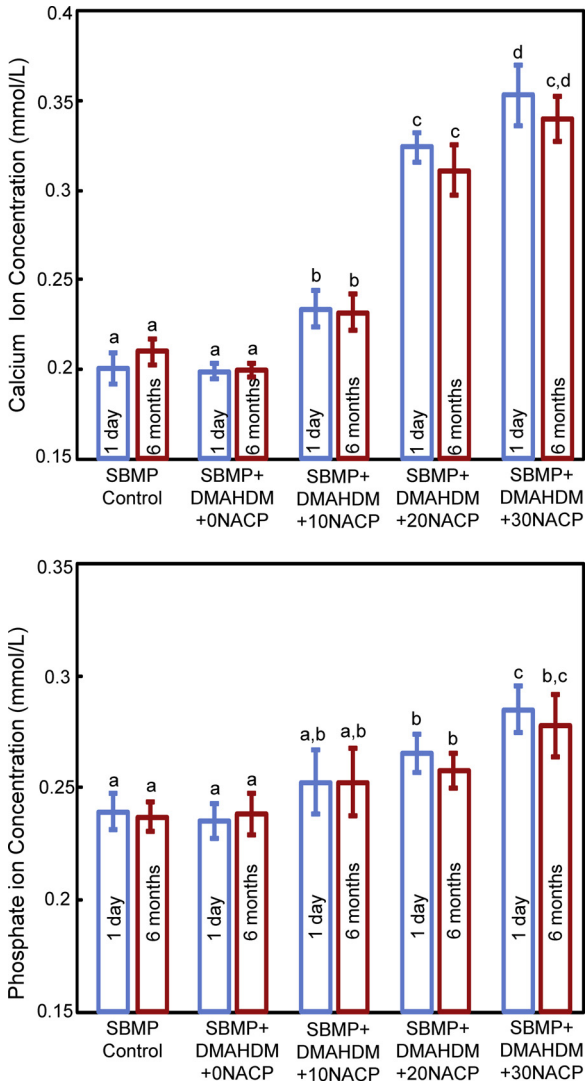

Fig. 6. Calcium (Ca) and phosphate (P) ion concentrations in 2-day biofilm medium (mean ± sd; n = 6). The resin disks were first stored in artificial saliva for 1 day or 6 months, and then the resin disks were used to inoculate the bacteria and cultured for 2 days. Then the ion concentrations in the medium were measured: (A) Ca ions, (B) P ions. In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

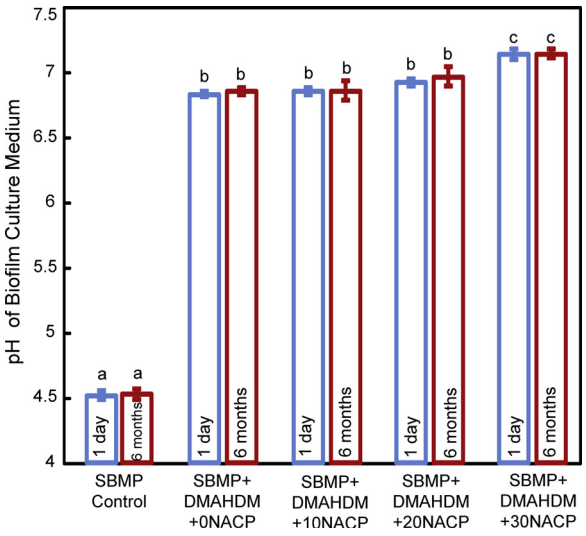

Fig. 7. The pH of 2-day biofilm culture medium where the biofilms grew on the resin disks (mean ± sd; n = 6). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

SBMP + DMAHDM + 10NACP.

Although the immediate bond strengths of contemporary commercial adhesives are encouragingly satisfactory, substantial decreases in resin-dentin bond strength occur after aging [38], with continuous loss of bonded restorations over time for both etch-and-rinse and self-etch adhesive systems [39]. The stability of the resin-dentin bonded interfaces still remains a major challenge [40,41]. In the oral environment, progressive degradation of the hybrid layer at the resin-dentin interface can weaken the adhesion and lead to gaps between the teeth and restoratives, which can be caused by hydrolytic and enzymatic degradation of the resin, collagenolysis by bacterial enzymes, as well as endogenous matrix metalloproteinases (MMPs) and cysteine cathepsins [31,42–46]. In the present study, SBMP + DMAHDM + 10NACP and SBMP + DMAHDM + 20NACP showed no significant decrease after 6 months of storage in artificial saliva, while SBMP + DMAHDM + 30NACP and SBMP control has significant losses in bond strength. Like chlorhexidine, QAMs are cationic compounds, can inhibit soluble MMP-9, and can almost completely inhibit the demineralized dentin collagen degradation [47]. But unlike chlorhexidine, QAMs are copolymerized with and immobilized in the adhesive monomers, and hence are not leached out and lost over time. Indeed, in vitro experiments showed that MDPB, DMAE-CB and DMADDM inhibited the host-derived collagenolytic enzymes from demineralizing the dentin matrix [48,49]. The covalently-bonded QAM with long-lasting anti-MMP effects may explain the μTBS for the DMAHDM-containing adhesives showing no loss during 6 months of artificial saliva aging. Further studies are needed to validate the anti-MMP effect of DMAHDM on inhibiting collagenolytic enzymes in the hybrid layer.

Further addition of 10% and 20% of NACP into the DMAHDM adhesive did not negatively affect the immediate μTBS and long-term μTBS after 6 months of aging, compared to the DMAHDM adhesive without NACP. However, at 30% NACP, the μTBS after 6 months of aging was lower than that at 1 day. Such a decrease was similar to SBMP Control. During aging, the hydrophilic monomer HEMA is vulnerable to hydrolysis, due to the presence of ester linkages [50]. Previous studies have correlated the hydrophilic monomers in adhesives with decreased longevity of the resin-dentin bonds [51]. This is because adhesives with high concentrations of hydrophilic monomers exhibit greater water sorption and are more susceptible to hydrolytic degradation [52]. In the present study, there was an increased percentage of adhesive failure mode in SBMP Control (30%) and SBMP + DMAHDM + 30NACP (34%) after 6 months of aging. The main adhesive failure mode in SBMP + DMAHDM + 30NACP group was cohesive failure in the adhesive layer. This means that the mechanical properties of SBMP + DMAHDM + 30NACP adhesive deteriorated in 6 months of saliva aging. This was likely because at 30% NACP, there were more interfaces between NACP particles and resin matrix causing more water diffusion, which increased the hydrolytic degradation of the resin. However, at 10% and 20% NACP, there was no increase in cohesive failure mode in the adhesive layer, consist with their excellent μTBS showing no decrease after 6 months of aging. Therefore, it appears that 20% NACP caused no significant increase in resin hydrolysis than SBMP control, while the DMAHDM exerted anti-MMP effects, yielding much greater μTBS after 6 months of aging than SBMP control.

Furthermore, excessive mass fractions of NACP addition into the adhesive may decrease the flowability of the adhesive and increase the thickness of adhesive layer. A previous study found that adding 30% NACP into an adhesive increased the average thickness of the adhesive layer to 70 μm, while the adhesive without NACP had a layer thickness of only 20 μm [28]. A thicker adhesive with a relatively high concentration of HEMA could be more prone to fracture duo to hydrolytic degradation. Therefore, it is important to optimize the NACP mass

fraction in the particular system of application. The NACP mass fraction of 20% appeared to be optimal in obtaining high levels of Ca and P ions and high pH, while simultaneously achieving a strong and durable dentin bond, under the conditions of the present study.

The antibacterial monomer DMAHDM was incorporated into the dental adhesive to inhibit bacterial aggregation and reduce acid production. The excellent antibacterial properties of DMAHDM were previously investigated using different concentrations in various resins [28,53,54]. However, the warm and moist oral environment can accelerate the hydrolysis of the polymerized resin materials and cause deterioration of the mechanical and bioactive prosperities [55]. In the present study, after 6 months of aging, the SBMP control resin was completely covered by live *S. mutans* biofilm, while the biofilm on SBMP + DMAHDM consisted of primarily dead bacteria. This was due to the co-polymerization of DMAHDM in the resin matrix without being leached out or lost over time, thereby providing a lasting anti-biofilm effect. Regarding biocompatibility, a previous study tested uncured monomers and cured resin eluents, showing that DMAHDM had acceptable cytotoxicity similar to that of several common clinically-used dental monomers [15]. DMAHDM had fibroblast and odontoblast cell viability similar to that of commercial monomers HEMA and triethylene glycol dimethacrylate (TEGDMA); they all had less cytotoxicity than that of BisGMA [15]. This was likely because that DMAHDM was co-polymerized by forming covalent bonds with the polymer network, with no significant DMAHDM leach-out thus minimizing the cytotoxicity [15].

Bioactive materials with the ability of releasing and delivering high levels of Ca and P ions are advantageous since they can deliver these ions to infiltrate the carious subsurface to prevent demineralization and promote remineralization. With the increasing popularity of minimal intervention dentistry, more carious tissues and residual bacteria would be left in the prepared tooth cavity [56–58]. NACP-containing resin materials have ion release and acid-neutralization capability, which can increase the Ca and P ion concentrations of the local environment and facilitate dental tissue remineralization [59]. Providing Ca and P ions can help reduce demineralization and tilt the balance toward remineralization [60]. Indeed, our previous studies showed that NACP-containing resins were effective in remineralizing enamel and dentin lesions in vitro, achieving an enamel remineralization efficacy that was 4-fold that of a fluoride-releasing commercial composite control [61]. NACP-containing resin achieved dentin remineralization that was 9.6-fold that of a commercial control [21]. NACP also helped reduce enamel demineralization at the enamel-composite margins to 1/3 the demineralization of a control composite under oral biofilms in a human in situ study [62]. Besides the ion release from NACP, the calcium and phosphate ions from artificial saliva and BHI culture medium also impacted the absolute values of the measured ion concentrations. That was why the ion concentrations in Fig. 6 for SBMP control were greater than 0. However, this did not affect the relative ranking, or the difference, between the experimental group and the control group. This was because the contribution from artificial saliva and BHI culture medium would be the same among all the tested groups. Therefore, ion release from NACP increased the ion concentrations, tilted the balance toward remineralization, and caused a net beneficial effect. Increasing the NACP mass fraction in the resin significantly increased the Ca and P ion concentrations. This is beneficial because the ultimate goal of dental adhesives is to provide durable adhesion to dentin and to protect the demineralized dentin matrix from degradation. Re-incorporation of minerals into the demineralized dentin matrix is important because the precipitated minerals could help repair the nanometer-sized voids such as nanoleakage in the hybrid layer. The minerals could also block the MMPs from penetrating and degrading the collagen matrix [63]. Indeed, with the help of NACP, the hardness of an acid-etched demineralized dentin was increased by almost 7-fold in a lactic acid solution, and by 1.6-fold in a cyclic artificial saliva/acid treatment, compared with control [64,65]. The remineralized dentin is more resistant to degradation in the oral environment and can resist and neutralize biofilm acids [66]. Besides remineralizing and protecting tooth structures, the Ca and P ion supplement strategy could potentially also be useful for bone tissue engineering applications [67–69].

In the present study, the acid-neutralization and ion release capability of DMAHDM + NACP adhesive was maintained in a *S. mutans* biofilm model even after 6 months of aging in artificial saliva. SBMP + DMAHDM + 20NACP had a biofilm pH of 6.8, while SBMP Control had a biofilm pH of 4.5. By inhibiting the growth of biofilms which produce acids and enzymes, DMAHDM + NACP bonding agent could help combat secondary caries and improve the bonding durability. DMAHDM could help kill the bacteria, while NACP could deliver Ca and P ions to remineralize the carious tissues and induce alkaline stresses to reduce the growth of bacteria [70]. Protecting the demineralized dentin matrix and promoting the formation of the newly remineralized tissues are beneficial to resisting the degradation at the bonded interface [4]. Further study is needed to investigate how the pH difference would affect caries prevention and tooth protection. Further studies are also needed to investigate the DMAHDM + NACP efficacy and mechanisms on the remineralization of the collagen in the acid-treated dentin bonded interfacial region using an in vivo model.

5. Conclusion

This study investigated the effects of immersion in artificial saliva on the bonding durabiliy, antibacterial activity, ions liberation and pH measurement of bonding agents containing DMAHDM and NACP for the first time. The commercial bonding agent lost 25% of its dentin bond strength in 6 months; however, the bonding agents with 5% DMAHDM and 20% NACP showed no loss in dentin bond strength. DMAHDM + NACP substantially reduced the biofilm metabolic activity and lactic acid, reducing biofilm CFU by four orders of magnitude. The novel adhesive increased Ca and P ion concentrations, neutralized biofilm acids and raised the biofilm pH to 6.8, while biofilms of commercial adhesive had a cariogenic pH of 4.5. There was no decrease in the antibacterial potency, remineralization and acid neutralization capability after artificial saliva-aging for 6 months. Therefore, the novel DMAHDM + NACP adhesive provided three benefits: (1) Protecting dentin bonded interface from degradation; (2) long-lasting antibacterial activity; and (3) Ca and P ion reservoir and high pH to promote remineralization.

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References

[1] J.L. Ferracane, Resin composite—state of the art, Dent. Mater. 27 (1) (2011) 29–38.
[2] C.D. Lynch, N.J. Opdam, R. Hickel, P.A. Brunton, S. Gurgan, A. Kakaboura, A.C. Shearer, G. Vanherle, N.H. Wilson, Guidance on posterior resin composites: academy of operative dentistry-European section, J. Dent. 42 (4) (2014) 377–383.
[3] D. Watts, A. Marouf, A. Al-Hindi, Photo-polymerization shrinkage-stress kinetics in resin-composites: methods development, Dent. Mater. 19 (1) (2003) 1–11.
[4] Y. Liu, L. Tjäderhane, L. Breschi, A. Mazzoni, N. Li, J. Mao, D.H. Pashley, F. Tay, Limitations in bonding to dentin and experimental strategies to prevent bond degradation, J. Dent. Res. 90 (8) (2011) 953–968.
[5] M. Bernardo, H. Luis, M.D. Martin, B.G. Leroux, T. Rue, J. Leitão, T.A. DeRouen, Survival and reasons for failure of amalgam versus composite posterior restorations placed in a randomized clinical trial, J. Am. Dent. Assoc. 138 (6) (2007) 775–783.
[6] D. Eltahlah, C.D. Lynch, B.L. Chadwick, I.R. Blum, N.H. Wilson, An update on the reasons for placement and replacement of direct restorations, J. Dent. 72 (5)

(2018) 1–7.
[7] S. Imazato, Antibacterial properties of resin composites and dentin bonding systems, Dent. Mater. 19 (6) (2003) 449–457.
[8] S. Imazato, Bio-active restorative materials with antibacterial effects: new dimension of innovation in restorative dentistry, Dent. Mater. J. 28 (1) (2009) 11–19.
[9] J.M. Antonucci, D.N. Zeiger, K. Tang, S. Lin-Gibson, B.O. Fowler, N.J. Lin, Synthesis and characterization of dimethacrylates containing quaternary ammonium functionalities for dental applications, Dent. Mater. 28 (2) (2012) 219–228.
[10] F. Li, Z. Chai, M. Sun, F. Wang, S. Ma, L. Zhang, M. Fang, J. Chen, Anti-biofilm effect of dental adhesive with cationic monomer, J. Dent. Res. 88 (4) (2009) 372–376.
[11] L. Huang, F. Yu, X. Sun, Y. Dong, P.-t. Lin, H.-h. Yu, Y.-h. Xiao, Z.-g. Chai, X.-d. Xing, J.-h. Chen, Antibacterial activity of a modified unfilled resin containing a novel polymerizable quaternary ammonium salt MAE-HB, Sci. Rep. 23 (6) (2016) 33858.
[12] K. Zhang, L. Cheng, E.J. Wu, M.D. Weir, Y. Bai, H.H. Xu, Effect of water-ageing on dentine bond strength and anti-biofilm activity of bonding agent containing new monomer dimethylaminododecyl methacrylate, J. Dent. 41 (6) (2013) 504–513.
[13] C. Chen, M.D. Weir, L. Cheng, N.J. Lin, S. Lin-Gibson, L.C. Chow, X. Zhou, H.H. Xu, Antibacterial activity and ion release of bonding agent containing amorphous calcium phosphate nanoparticles, Dent. Mater. 30 (8) (2014) 891–901.
[14] Y.-p. Gou, J.-y. Li, M.M. Meghil, C.W. Cutler, H.H. Xu, F.R. Tay, L.-n. Niu, Quaternary ammonium silane-based antibacterial and anti-proteolytic cavity cleanser, Dent. Mater. 34 (12) (2018) 1814–1827.
[15] F. Li, M.D. Weir, H.H. Xu, Effects of quaternary ammonium chain length on antibacterial bonding agents, J. Dent. Res. 92 (10) (2013) 932–938.
[16] H. Zhou, M.D. Weir, J.M. Antonucci, G.E. Schumacher, X.-D. Zhou, H.H. Xu, Evaluation of three-dimensional biofilms on antibacterial bonding agents containing novel quaternary ammonium methacrylates, Int. J. Oral Sci. 6 (2) (2014) 77.
[17] S. Wang, H. Wang, B. Ren, H. Li, M.D. Weir, X. Zhou, T.W. Oates, L. Cheng, H.H. Xu, Do quaternary ammonium monomers induce drug resistance in cariogenic, endodontic and periodontal bacterial species? Dent. Mater. 33 (10) (2017) 1127–1138.
[18] S. Imazato, S. Ma, J.-h. Chen, H.H. Xu, Therapeutic polymers for dental adhesives: loading resins with bio-active components, Dent. Mater. 30 (1) (2014) 97–104.
[19] L. Tjäderhane, Dentin bonding: can we make it last? Oper. Dent. 40 (1) (2015) 4–18.
[20] H.H. Xu, J.L. Moreau, L. Sun, L.C. Chow, Nanocomposite containing amorphous calcium phosphate nanoparticles for caries inhibition, Dent. Mater. 27 (8) (2011) 762–769.
[21] M.D. Weir, J. Ruan, N. Zhang, L.C. Chow, K. Zhang, X. Chang, Y. Bai, H.H.K. Xu, Effect of calcium phosphate nanocomposite on in vitro remineralization of human dentin lesions, Dent. Mater. 33 (9) (2017) 1033–1044.
[22] S. Imazato, M. Torii, Y. Tsuchitani, J. McCabe, R. Russell, Incorporation of bacterial inhibitor into resin composite, J. Dent. Res. 73 (8) (1994) 1437–1443.
[23] F. Yu, Y. Dong, H.-h. Yu, P.-t. Lin, L. Zhang, X. Sun, Y. Liu, Y.-n. Xia, L. Huang, J.-h. Chen, Antibacterial activity and bonding ability of an orthodontic adhesive containing the antibacterial monomer 2-methacryloxylethyl hexadecyl methyl ammonium bromide, Sci. Rep. 7 (2017) 41787.
[24] L. Cheng, K. Zhang, C.-C. Zhou, M.D. Weir, X.-D. Zhou, H.H. Xu, One-year water-ageing of calcium phosphate composite containing nano-silver and quaternary ammonium to inhibit biofilms, Int. J. Oral Sci. 8 (3) (2016) 172.
[25] W.L.d.O. da Rosa, E. Piva, A.F. da Silva, Bond strength of universal adhesives: a systematic review and meta-analysis, J. Dent. 43 (7) (2015) 765–776.
[26] C.B. André, B.P.F.A. Gomes, T.M. Duque, R.N. Stipp, D.C.N. Chan, G.M.B. Ambrosano, M. Giannini, Dentine bond strength and antimicrobial activity evaluation of adhesive systems, J. Dent. 43 (4) (2015) 466–475.
[27] M.A. Melo, S. Orrego, M.D. Weir, H.H. Xu, D.D. Arola, Designing multiagent dental materials for enhanced resistance to biofilm damage at the bonded interface, ACS Appl. Mater. Interfaces 8 (18) (2016) 11779–11787.
[28] N. Zhang, M.A. Melo, C. Chen, J. Liu, M.D. Weir, Y. Bai, H.H. Xu, Development of a multifunctional adhesive system for prevention of root caries and secondary caries, Dent. Mater. 31 (9) (2015) 1119–11131.
[29] L. Zhang, M.D. Weir, G. Hack, A.F. Fouad, H.H. Xu, Rechargeable dental adhesive with calcium phosphate nanoparticles for long-term ion release, J. Dent. 43 (12) (2015) 1587–1595.
[30] R.M. Carvalho, J. Mendonca, S. Santiago, R. Silveira, F. Garcia, F. Tay, D.H. Pashley, Effects of HEMA/solvent combinations on bond strength to dentin, J. Dent. Res. 82 (8) (2003) 597–601.
[31] D.H. Pashley, F. Tay, C. Yiu, M. Hashimoto, L. Breschi, R. Carvalho, S. Ito, Collagen degradation by host-derived enzymes during aging, J. Dent. Res. 83 (3) (2004) 216–221.
[32] F.-c. Tian, X.-y. Wang, Q. Huang, L.-n. Niu, J. Mitchell, Z.-y. Zhang, C. Prananik, L. Zhang, J.-h. Chen, L. Breshi, Effect of nanolayering of calcium salts of phosphoric acid ester monomers on the durability of resin-dentin bonds, Acta Biomater. 38 (2016) 190–200.
[33] S. Imazato, T. Imai, R. Russell, M. Torii, S. Ebisu, Antibacterial activity of cured dental resin incorporating the antibacterial monomer MDPB and an adhesion-promoting monomer, J. Biomed. Mater. Res. 39 (4) (1998) 511–515.
[34] F.G. Basso, C.F. Oliveira, A. Fontana, C. Kurachi, V.S. Bagnato, D.M. Spolidório, J. Hebling, C.A. Costa, In vitro effect of low-level laser therapy on typical oral microbial biofilms, Braz. Dent. J. 22 (6) (2011) 502–510.
[35] L. Cheng, K. Zhang, M.A. Melo, M. Weir, X. Zhou, H. Xu, Anti-biofilm dentin primer with quaternary ammonium and silver nanoparticles, J. Dent. Res. 91 (6) (2012) 598–604.
[36] C. Van Loveren, J. Buijs, J. Ten Cate, The effect of triclosan toothpaste on enamel demineralization in a bacterial demineralization model, J. Antimicrob. Chemother. 45 (2) (2000) 153–158.
[37] L. Cheng, M.D. Weir, H.H. Xu, A.M. Kraigsley, N.J. Lin, S. Lin-Gibson, X. Zhou, Antibacterial and physical properties of calcium–phosphate and calcium–fluoride nanocomposites with chlorhexidine, Dent. Mater. 28 (5) (2012) 573–583.
[38] T. Vaidyanathan, J. Vaidyanathan, Recent advances in the theory and mechanism of adhesive resin bonding to dentin: a critical review, J. Biomed. Mater. Res. B: Appl. Biomater. 88 (2) (2009) 558–578.
[39] K.L. Van Landuyt, J. Snauwaert, J. De Munck, M. Peumans, Y. Yoshida, A. Poitevin, E. Coutinho, K. Suzuki, P. Lambrechts, B. Van Meerbeek, Systematic review of the chemical composition of contemporary dental adhesives, Biomaterials 28 (26) (2007) 3757–3785.
[40] L. Breschi, A. Mazzoni, A. Ruggeri, M. Cadenaro, R. Di Lenarda, E.D.S. Dorigo, Dental adhesion review: aging and stability of the bonded interface, Dent. Mater. 24 (1) (2008) 90–101.
[41] M. Cardoso, A. de Almeida Neves, A. Mine, E. Coutinho, K. Van Landuyt, J. De Munck, B. Van Meerbeek, Current aspects on bonding effectiveness and stability in adhesive dentistry, Aust. Dent. J. 56 (s1) (2011) 31–44.
[42] M. Carrilho, R.M. Carvalho, F.R. Tay, C. Yiu, D.H. Pashley, Durability of resin-dentin bonds related to water and oil storage, Am. J. Dent. 18 (6) (2005) 315–319.
[43] B. Shokati, L.E. Tam, J.P. Santerre, Y. Finer, Effect of salivary esterase on the integrity and fracture toughness of the dentin-resin interface, J. Biomed. Mater. Res. B Appl. Biomater. 94 (1) (2010) 230–237.
[44] A. Mazzoni, D.H. Pashley, Y. Nishitani, L. Breschi, F. Mannello, L. Tjäderhane, M. Toledano, E.L. Pashley, F.R. Tay, Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives, Biomaterials 27 (25) (2006) 4470–4476.
[45] J. De Munck, P. Van den Steen, A. Mine, K. Van Landuyt, A. Poitevin, G. Opdenakker, B. Van Meerbeek, Inhibition of enzymatic degradation of adhesive-dentin interfaces, J. Dent. Res. 88 (12) (2009) 1101–1106.
[46] I.L. Tersariol, S. Geraldeli, C.L. Minciotti, F.D. Nascimento, V. Pääkkönen, M.T. Martins, M.R. Carrilho, D.H. Pashley, F.R. Tay, T. Salo, Cysteine cathepsins in human dentin-pulp complex, J. Endod. 36 (3) (2010) 475–481.
[47] A. Tezvergil-Mutluay, K. Agee, T. Uchiyama, S. Imazato, M. Mutluay, M. Cadenaro, L. Breschi, Y. Nishitani, F. Tay, D.H. Pashley, The inhibitory effects of quaternary ammonium methacrylates on soluble and matrix-bound MMPs, J. Dent. Res. 90 (4) (2011) 535–540.
[48] F. Li, H. Majd, M.D. Weir, D.D. Arola, H.H. Xu, Inhibition of matrix metalloproteinase activity in human dentin via novel antibacterial monomer, Dent. Mater. 31 (3) (2015) 284–292.
[49] N. Liu, F. Li, Y.-j. Chen, L. Zhang, S. Lu, J.-J. Kang, J.-h. Chen, The inhibitory effect of a polymerisable cationic monomer on functional matrix metalloproteinases, J. Dent. 41 (11) (2013) 1101–1108.
[50] J.L. Ferracane, Hygroscopic and hydrolytic effects in dental polymer networks, Dent. Mater. 22 (3) (2006) 211–222.
[51] M. Peumans, P. Kanumilli, J. De Munck, K. Van Landuyt, P. Lambrechts, B. Van Meerbeek, Clinical effectiveness of contemporary adhesives: a systematic review of current clinical trials, Dent. Mater. 21 (9) (2005) 864–881.
[52] J. Malacarne, R.M. Carvalho, F. Mario, N. Svizero, D.H. Pashley, F.R. Tay, C.K. Yiu, M.R. de Oliveira Carrilho, Water sorption/solubility of dental adhesive resins, Dent. Mater. 22 (10) (2006) 973–980.
[53] L. Wang, X. Xie, M.D. Weir, A.F. Fouad, L. Zhao, H.H. Xu, Effect of bioactive dental adhesive on periodontal and endodontic pathogens, J. Mater. Sci. Mater. Med. 27 (11) (2016) 168.
[54] L. Cheng, K. Zhang, N. Zhang, M. Melo, M. Weir, X. Zhou, Y. Bai, M. Reynolds, H. Xu, Developing a new generation of antimicrobial and bioactive dental resins, J. Dent. Res. 96 (8) (2017) 855–863.
[55] J. Santerre, L. Shajii, B. Leung, Relation of dental composite formulations to their degradation and the release of hydrolyzed polymeric-resin-derived products, Crit. Rev. Oral Biol. Med. 12 (2) (2001) 136–151.
[56] M. Jingarwar, N. Bajwa, A. Pathak, Minimal intervention dentistry–a new frontier in clinical dentistry, J. Clin. Diagn. Res. JCDR 8 (7) (2014) ZE04.
[57] C.D. Lynch, K.B. Frazier, R.J. McConnell, I.R. Blum, N.H. Wilson, Minimally invasive management of dental caries: contemporary teaching of posterior resin-based composite placement in US and Canadian dental schools, J. Am. Dent. Assoc. 142 (6) (2011) 612–620.
[58] M. Hayashi, T. Yamada, C.D. Lynch, N.H. Wilson, Teaching of posterior composites in dental schools in Japan–30 years and beyond, J. Dent. 76 (9) (2018) 19–23.
[59] M.A. Melo, M.D. Weir, V.F. Passos, M. Powers, H.H. Xu, Ph-activated nano-amorphous calcium phosphate-based cement to reduce dental enamel demineralization, Artif. Cells Nanomed. Biotechnol. 45 (8) (2017) 1778–1785.
[60] A. Hara, R. Karlinsey, D. Zero, Dentine remineralisation by simulated saliva formulations with different Ca and Pi contents, Caries Res. 42 (1) (2008) 51–56.
[61] M. Weir, L. Chow, H. Xu, Remineralization of demineralized enamel via calcium phosphate nanocomposite, J. Dent. Res. 91 (10) (2012) 979–984.
[62] M.A.S. Melo, M.D. Weir, L.K. Rodrigues, H.H. Xu, Novel calcium phosphate nanocomposite with caries-inhibition in a human in situ model, Dent. Mater. 29 (2) (2013) 231–240.
[63] Y.K. Kim, S. Mai, A. Mazzoni, Y. Liu, A. Tezvergil-Mutluay, K. Takahashi, K. Zhang, D.H. Pashley, F.R. Tay, Biomimetic remineralization as a progressive dehydration mechanism of collagen matrices–implications in the aging of resin–dentin bonds, Acta Biomater. 6 (9) (2010) 3729–3739.
[64] K. Liang, M.D. Weir, M.A. Reynolds, X. Zhou, J. Li, H.H. Xu, Poly (amido amine) and nano-calcium phosphate bonding agent to remineralize tooth dentin in cyclic artificial saliva/lactic acid, Mater. Sci. Eng. C Mater. Biol. Appl. 72 (2017) 7–17.
[65] K. Liang, S. Xiao, M.D. Weir, C. Bao, H. Liu, L. Cheng, X. Zhou, J. Li, H.H. Xu, Poly (amido amine) dendrimer and dental adhesive with calcium phosphate

nanoparticles remineralized dentin in lactic acid, J. Biomed. Mater. Res. B Appl. Biomater. 106 (6) (2018) 2414–2424.
[66] R. Osorio, E. Osorio, A. Medina-Castillo, M. Toledano, Polymer nanocarriers for dentin adhesion, J. Dent. Res. 93 (12) (2014) 1258–1263.
[67] R.C. de Morais, R.E. Silveira, M. Chinelatti, S. Geraldeli, Fd.C.P. Pires-de, Bond strength of adhesive systems to sound and demineralized dentin treated with bioactive glass ceramic suspension, Clin. Oral Investig. 22 (5) (2018) 1923–1931.
[68] D. Fernando, N. Attik, N. Pradelle-Plasse, P. Jackson, B. Grosgogeat, P. Colon, Bioactive glass for dentin remineralization: a systematic review, Mater. Sci. Eng. C Mater. Biol. Appl. 76 (6) (2017) 1369–1377.
[69] M.A.S. Melo, L. Cheng, K. Zhang, M.D. Weir, X. Zhou, Y. Bai, L.K. Rodrigues, H.H. Xu, Novel nanostructured bioactive restorative materials for dental applications, in: Polina Prokopovich (Ed.), Biological and Pharmaceutical Applications of Nanomaterials, CRC Press, New York, 2015, pp. 151–165.
[70] X. Huang, R. Exterkate, J. Ten Cate, Factors associated with alkali production from arginine in dental biofilms, J. Dent. Res. 91 (12) (2012) 1130–1134.