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Self-healing adhesive with antibacterial activity in water-aging for 12 months

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ABSTRACT

Objective. Secondary caries and micro-cracks are the main limiting factors for dentin bond durability. The objectives of this study were to develop a self-healing adhesive containing dimethylaminohexadecyl methacrylate (DMAHDM) and nanoparticles of amorphous calcium phosphate (NACP), and investigate the effects of water-aging for 12 months on self-healing, dentin bonding, and antibacterial properties for the first time.

Methods. Microcapsules were synthesized with poly (urea-formaldehyde) (PUF) shells containing triethylene glycol dimethacrylate (TEGDMA) and *N,N*-dihydroxyethyl-*p*-toluidine (DHEPT). The adhesive contained 7.5% microcapsules, 10% DMAHDM, and 20% NACP (all mass). Specimens were water-aged at 37 °C for 1 day to 12 months. Dentin bond strength was measured using extracted human teeth. A single-edge-V-notched-beam (SEVNB) method was used to measure fracture toughness K_{IC} and self-healing efficiency. A dental plaque microcosm biofilm model was used with human saliva as inoculum.

Results. The microcapsules + DMAHDM + NACP group showed no decline in dentin bond strength after water-aging for 12 months, which was significantly higher than that of other groups without DMAHDM ($p < 0.05$). A self-healing efficiency of 67% recovery in K_{IC} was

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obtained even after 12 months of water immersion, indicating that the self-healing ability was not lost in water-aging ($p > 0.1$). The bacteria-killing ability of this adhesive did not decline from 1 day to 12 months ($p > 0.1$), with biofilm CFU reduction by 3–4 orders of magnitude after the resin was water-aged for 12 months, compared to control resin.

Significance. This novel adhesive with triple merits of self-healing, antibacterial and remineralization functions showed an excellent long-term durability in water-aging for 12 months. This multifunctional adhesive has the potential for dental applications to heal cracks, inhibit bacteria, provide ions for remineralization, and increase the restoration longevity.

1. Introduction

Dental adhesive plays an important role in modern dentistry [1]. The adhesive technology has undergone substantial improvements [2,3]. However, the bonded interface is still the weak link of the restoration [4]. Resin-based tooth restorations placed in non-load bearing cavities had a survival rate of only 43% after 10 years in UK [5]. The replacement of failed restorations costs $5 billion in US annually [6]. The most common reason for replacements was secondary caries at the margins due to biofilm acids [7].

Adhesives bond composite restorations to the tooth substrates. Therefore, it is beneficial for the adhesive to be antibacterial to reduce biofilm acids and caries at the margins [8]. Quaternary ammonium methacrylates (QAMs) were synthesized and incorporated into dental resins. 12-methacryloyloxydodecyl-pyridinium bromide (MDPB) [9], methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB) [10], poly(quaternary ammonium salt)-containing polyacid [11], quaternary ammonium polyethylenimine nanoparticles [12] and quaternary ammonium dimethacrylate (QADM) [13] were synthesized and incorporated into dental composites, primer and adhesive. Recently, a new dimethylaminohexadecyl methacrylate (DMAHDM) with an alkyl chain length of 16, was incorporated into dental bonding agents [14,15].

In addition, fabrication of adhesive containing nanoparticles of amorphous calcium phosphate (NACP) with remineralization capability is another effective way to deter secondary caries [16,17]. NACP adhesive was "smart" and dramatically increased the ion release at a low pH, when these ions would be most-needed to inhibit caries [18]. A previous study incorporated DMAHDM and NACP together in the adhesive to achieve antibacterial and ion-releasing effects [19]. The micro-cracks or micro-gaps induced by polymerization shrinkage, cyclic loading, thermal and mechanical fatigue at the bonding interface [20] damage the bonding stability and durability. Accordingly, efforts were made to prolong the durability of resin-dentin bonds. The proposed strategies included utilizing the inhibitor of matrix metalloproteinases (MMPs) [21], attempting new bonding techniques [22], and enhancing the adhesive's mechanical properties [23]. While DMAHDM could inhibit the MMPs, NACP could help remineralize and strengthen the tooth structures [16–19]. However, there has been little research on finding a new approach to inhibit the micro-crack propagation and repair them in the adhesive layer to protect the bond integrity. Micro-crack propagation may lead to micro-leakage, bacteria invasion, restoration dislodgment, and catastrophic failure [24]. Hence, it is highly desirable to be able to self-heal these micro-cracks. Therefore, developing a dental adhesive with self-healing ability would be of high importance [25].

Self-healing polymers have the ability to recover their load-bearing capability after cracking [26]. One method uses self-healing microcapsules [27–30]. The healing agent is stored in the microcapsules. When microcapsules in the polymer matrix are ruptured by damage, the self-healing can be triggered by the polymerization of the released healing liquid with the catalyst in the matrix. In a previous study [27], healing agent dicyclopentadiene (DCPD) was encapsulated in poly(urea-formaldehyde) (PUF) shell, which was added into dental resin composites with Grubb's catalyst. The virgin fracture toughness ($K_{IC\text{-}virgin}$) and the healed fracture toughness ($K_{IC\text{-}healed}$) of the composites were calculated to assess the self-healing efficiency (η): $\eta = K_{IC\text{-}healed}/K_{IC\text{-}virgin}$. A η of 57% was achieved [27]. However, the DCPD toxicity [31], Grubb's catalyst toxicity, its availability and high cost [32], have hindered its further development.

Recently, novel PUF microcapsules containing polymerizable triethylene glycol dimethacrylate (TEGDMA) and *N,N*-dihydroxyethyl-*p*-toluidine (DHEPT) as healing liquid were synthesized with low cytotoxicity [33]. These microcapsules were incorporated into a resin of bisphenol A glycerolate dimethacrylate (BisGMA) and TEGDMA at different mass fractions. Adding microcapsules into the resin increased the virgin fracture toughness, which was about 40% higher at 15% microcapsules than that at 0% microcapsules. A self-healing efficiency of about 65% in K_{IC} recovery was obtained when the microcapsule concentration in the resin was 10% [33]. Next, these microcapsules, together with DMAHDM and NACP, were added into a resin composite. Successful self-healing was obtained, with K_{IC} recovery of 65–81% to regain the mechanical properties. This self-healing DMAHDM-NACP composite displayed a strong antibacterial potency, reducing colony-forming units by 3–4 orders of magnitude, compared to control composite without DMAHDM [34]. In another study, the effect of water-aging on the self-healing properties was investigated [35]. A self-healing efficiency of 64% was achieved even after water-aging for 6 months, and self-healing was able to occur in water [35]. In addition, a self-healing luting cement containing an acidic functional adhesive monomer 4-methacryloyloxyethyl trimellitic anhydride (4-META) was

developed, achieving a self-healing efficiency of 65% after 6 months of water immersion [36]. However, a literature search revealed that to date, there has been no report on dental adhesives with self-healing ability in water-aging. In addition, there has been no report on any self-healing material with water-aging for 12 months.

The objectives of this study were to develop a self-healing dental adhesive with antibacterial and remineralizing properties, and investigate the effects of long-term water-aging for 12 months. The following hypotheses were tested: (1) The new adhesive containing self-healing microcapsules, DMADDM and NACP would not show a decrease in dentin bond strength after water-aging for 12 months; (2) The self-healing efficiency of this adhesive would not decrease during 12 months of water-aging; (3) The novel adhesive would display a strong antibacterial property against dental plaque microcosm biofilm even after 12 months of water-aging.

2. Materials and methods

2.1. Synthesis of microcapsules

Microcapsules were prepared by in situ polymerization of formaldehyde and urea, following previous studies [36,37]. DHEPT (Sigma-Aldrich, St. Louis, MO) at 1% mass fraction was added to TEGDMA monomer (Esstech, Essington, PA). At room temperature, 50 mL of distilled water and 13 mL of a 2.5% aqueous solution of ethylene-maleic anhydride (EMA) copolymer (Sigma-Aldrich) were mixed in a 250 mL round-bottom glass flask. The flask was suspended in a water bath on a hotplate (Isotemp, Fisher Scientific, Pittsburg, PA). The EMA solution was used as a surfactant to form an "oil-in-water" emulsion ("oil" being TEGDMA-DHEPT). Under agitation by a magnetic stir bar (diameter = 7.8 mm, length = 50 mm, Fisher Scientific) at 300 rpm, the shell-forming material urea (1.25 g), ammonium chloride (0.125 g) and resorcinol (0.125 g) (Sigma-Aldrich) were added into the solution. The resorcinol was added in the reaction of shell formation to enhance the rigidity of the shells [37]. The pH was adjusted to 3.5 via drop-wise addition of 1 M sodium hydroxide solution. Then, the agitation rate was increased to 900 rpm [36], and 30 mL of the TEGDMA-DHEPT liquid was added into the flask. A stabilized emulsion of fine TEGDMA-DHEPT droplets was formed after 10 min of agitation. Then, 3.15 g of a 37% aqueous solution of formaldehyde (Sigma-Aldrich) was added, and the flask was sealed with aluminum foil to prevent evaporation. The temperature of the water bath was raised to 55 °C and the shell material was isothermally polymerized for 4 h under continuous agitation. In this process, ammonium chloride catalyzed the reaction of urea with formaldehyde to form PUF at the oil–water interface to develop the shell [37]. These microcapsules were rinsed with water and acetone, vacuum-filtered, and air-dried for 24 h in a hood. The microcapsules were examined with scanning electronic microscopy (SEM, Quanta 200, FEI, Hillsboro, OR). In addition, the microcapsules were examined by optical microscope (TE2000-S, Nikon, Japan), and their sizes were measured with an image analysis software (Nis-Elements BR2.30, Nikon).

2.2. Preparation of DMAHDM and NACP

DMAHDM was synthesized according to a previous report [14]. Briefly, 10 mmol of 2-(dimethy-lamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich) and 10 mmol of 1-bromohexadecane (BHD, TCI America, Port-land, OR) were dissolved in 3 g of ethanol and allowed to react at 70 °C for 24 h under continuous agitation. The solvent was then removed via evaporation, yielding DMAHDM as a clear, colorless, and viscous liquid. NACP were prepared by a spray-drying technique [38]. Briefly, calcium carbonate ($CaCO_3$, Fisher, Fair Lawn, NJ) and dicalcium phosphate anhydrous ($CaHPO_4$, Baker Chemical, Phillipsburg, NJ) were dissolved into an acetic acid solution to obtain Ca and P ionic concentrations of 8 mmol/L and 5.333 mmol/L, respectively, yielding a Ca/P molar ratio of 1.5. Then the solution was sprayed into a heated chamber, and an electrostatic precipitator (AirQuality, Minneapolis, MN) was used to collect the dried particles, producing NACP with a mean particle dimension of 116 nm [39].

2.3. Antibacterial adhesive containing microcapsules

The experimental primer contained pyromellitic glycerol dimethacrylate (PMGDM) (Hampford, Stratford, CT) and 2-hydroxyethyl methacrylate (HEMA) (Esstech) at a mass ratio of 3.3:1, with 50% acetone solvent (all mass fractions). The experimental adhesive consisted of Bis-GMA(Esstech) and TEGDMA at 7:3 mass ratio. The photo-initiator for adhesive was 1% phenyl-bis (2,4,6-trimethylbenzoyl) phosphine oxide (BAPO, Sigma-Aldrich). Benzoyl peroxide (BPO, Sigma-Aldrich) (0.5% of the adhesive) was dissolved into adhesive as an initiator to react with the DHEPT in the released healing agent [33]. Following a previous study [19], mass fractions of 7.5% microcapsules, 10% DMAHDM and 20% NACP were incorporated into the adhesive [19]. Similarly, 10% of DMAHDM was incorporated into the experimental primer, following a previous study [19]. In addition, Scotchbond Multi-Purpose Adhesive and Primer (referred as "SBMP") (3M, St. Paul, MN) were used as a commercial control. According to the manufacturer, SBMP adhesive contained 60–70% of BisGMA and 30–40% of 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. Therefore, six groups were tested:

1) **Commercial SBMP control.** SBMP primer and adhesive.
2) **Experimental bonding agent control.** Experimental primer and adhesive as described above. This experimental bonding agent control is referred to as "Exp bond control".
3) **Self-healing group.** Primer: Unmodified experimental primer. Adhesive: Experimental adhesive + 7.5% microcapsules. This group is referred to as "Exp bond + microcapsules".
4) **Antibacterial group.** Primer + 10% DMAHDM. Adhesive + 10% DMAHDM (referred to as "Exp bond + DMAHDM").
5) **Remineralizing group.** Primer + 10% DMAHDM. Adhesive + 20% NACP (referred to as "Exp bond + NACP").
6) **Self-healing, antibacterial and remineralizing group.** Primer + 10% DMAHDM. Adhesive + 7.5% micro-

capsules + 10%DMAHDM + 20%NACP (referred to as "microcapsules + DMAHDM + NACP").

2.4. Dentin shear bond strength testing

Extracted caries-free human third molars were collected from dental school clinics and stored in 0.01% thymol solution. The protocol was approved by the University of Maryland Baltimore Institutional Review Board. Flat mid-coronal dentin surfaces were prepared by cutting off the tips of the molar crowns with a 150 μm-thick diamond saw (Isomet, Buehler, Lake Bluff, IL). Each tooth was embedded in a poly-carbonate holder (Bosworth, Skokie, IL) and ground perpendicularly to the longitudinal axis on 320-grit silicon carbide paper until the occlusal enamel was completely removed. Then, the dentin surface was etched with 37% phosphoric acid gel for 15 s and rinsed with water [17]. A primer was applied with a brush-tipped applicator and rubbed in for 15 s. The solvent was removed with a stream of air for 5 s. Then, a single coat of an adhesive (which was approximately 8 μL) was applied with a plastic tip. The adhesive was gently blown with a stream of air to spread the adhesive out into a relatively uniform and bright-surfaced film, which was then light-cured for 10 s (Triad 2000, Dentsply, York, PA). A stainless-steel iris with a central opening (diameter = 4 mm, thickness = 1.5 mm) was held against the adhesive-treated dentin surface. The opening was filled with a composite (TPH, Dentsply), and light-cured for 60 s. To measure the dentin shear bond strength, a chisel connected with a computer-controlled Universal Testing Machine (MTS, Eden Prairie, MN) was aligned to be parallel to the composite-dentin interface. A load was applied at a rate of 0.5 mm/min until the bond failed. Dentin shear bond strength = $4P/(\pi d^2)$, where P is the failure load, and d is the diameter of the composite [17]. Ten teeth were tested for each group.

2.5. Measurement of fracture toughness KIC and self-healing efficiency η

A single edge V-notched beam (SEVNB) method was used to measure K_{IC} [40]. For each group, the adhesive was placed into a mold of 2 × 2 × 25 mm and covered with a Mylar strip. The specimens were photo-cured for 1 min on each side and demolded. A notch with a depth of 500 μm was machined into a specimen using a thin diamond blade with a 150 μm thickness (Buehler), following previous studies [40,41]. Then, a diamond paste of 3 μm particle size was placed into the notch tip, and a new razor blade was used to cut the notch further to a total depth of about 700–800 μm. This method produced a relatively sharp notch tip [40]. The notch length was measured using an optical microscope (TE2000-S, Nikon) on both sides of the specimen, and then the notch length was averaged. A Universal Testing Machine (5500R, MTS) was used to fracture the SEVNB specimens in three-point flexure with a span of 10 mm and a crosshead speed of 0.5 mm/min with the notch on the tensile side. K_{IC} was calculated following an established SEVNB method [41]. This yielded the original virgin K_{IC} of the specimen: $K_{IC\text{-virgin}}$. To test the crack-healing efficacy, immediately following specimen fracture, the two halves of the specimen were placed back into the mold to ensure a good contact of the two fractured planes. This would be similar to the case of a tooth cavity restoration, where the cracked adhesive resin would stay in the tooth cavity to allow the released healing liquid to heal the crack. The healing of a completely cracked specimen would be a more rigorous test than the healing of a partial or small crack in the specimen. Because the fracture ruptured the microcapsules in the resin, the released TEGDMA-DHEPT from the microcapsules reacted with the BPO in the resin matrix. This caused the polymerization of the released liquid to heal and bond the two cracked planes into one cohesive specimen. Specimens were incubated in a humidor at 37 °C for 24 h. The notch length of the healed specimen was again measured and made sure that it was the same as the virgin notch length of the same specimen. Then the healed bar was tested again using the same flexural method. This yielded the healed K_{IC} value: $K_{IC\text{-healed}}$. Self-healing efficiency (η) was assessed [19]: $\eta = K_{IC\text{-healed}}/K_{IC\text{-virgin}}$. Six samples were tested for each group. Selected fracture surfaces were sputter-coated with gold and examined in SEM (Quanta 200).

2.6. Live/dead staining of biofilms

The cover of a sterile 96-well plate was used as molds for specimen preparation following a previous study [42]. Ten microliter of adhesive was placed in the bottom of the dent of the cover. After photo-cured for 20 s, a composite (TPH, Dentsply) was placed and photo-cured for another 1 min. This yielded an adhesive/composite disk of approximately 8 mm in diameter and 1 mm in thickness. In clinic, a primer was first applied in restoring a tooth and then an adhesive was placed. The reason no primer was placed on the disks was to directly assess the antibacterial ability of the adhesive [42]. All the cured disks were immersed in distilled water at 37 °C for 1 day [34]. The disks were sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC) [42] and de-gassed following the manufacturer's instructions.

The dental plaque microcosm biofilm model was approved by the University of Maryland Institutional Review Board. Saliva was used as an inoculum to provide biofilms. To represent the diverse bacterial populations, saliva from ten healthy individuals was collected and combined, following a previous study [43]. Saliva was collected from adult donors who had natural dentition without active caries or periopathology, and without the use of antibiotics within the last 3 months. The donors did not brush teeth for 24 h and abstained from food/drink intake for 2 h prior to donating saliva. Stimulated saliva was collected and was kept on ice. The saliva was combined and diluted in sterile glycerol to a concentration of 70%, then stored at −80 °C for subsequent use [44]. The saliva-glycerol stock with 1:50 final dilution was added into a growth medium as inoculum. The components of the McBain artificial saliva growth medium were as follows: mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L; KCl, 0.2 g/L; CaCl2, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; hemin, 0.001 g/L; vitamin K1, 0.0002 g/L, at pH7 [45]. A sterilized adhesive disk was placed into each well of 24-well plate filled with 1.5 mL of inoculum and incubated in 5% CO_2 at 37 °C for 8 h. Then, the disks were transferred to new 24-well plate filled with fresh medium and incubated. After 16 h, the disks were moved to another new 24-well plate with fresh medium and incu-

bated for 24 h. This totaled 48 h of incubation, which was verified to be sufficient to form plaque microcosm biofilms on resins [42]. Specimens with 48 h biofilms were rinsed with phosphate-buffered saline and then stained by a live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained to produce a green fluorescence. Bacteria with compromised membranes were stained to yield a red fluorescence [46]. Specimens were examined using an inverted epifluorescence microscope (Eclipse TE2000-S, Nikon, Japan) [46].

2.7. MTT metabolic assay of biofilms

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay that measures the enzymatic reduction of MTT [46]. Resin specimens with 48 h biofilms were moved to a new 24-well plate, then 1 mL of MTT dye (0.5 mg/mL MTT in phosphate-buffered saline) was added and incubated at 37 °C in 5% CO_2 for 1 h. The metabolically active bacteria reduced the MTT, a yellow tetrazole, to purple formazan [46]. After 1 h, the disks were transferred to another 24-well plate, 1 mL of dimethyl sulphoxide (DMSO) was added to dissolve the formazan crystals, and the plate was incubated for 20 min with gentle mixing in the dark. Then, 200 mL of the DMSO solution from each well was moved to a 96-well plate, and the absorbance at 540 nm was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) [46]. A higher absorbance indicates a higher formazan concentration, which means a higher metabolic activity in the biofilm on the disk [46].

2.8. Lactic acid production by biofilms

Biofilms growing on disks for 2 days were rinsed in cysteine peptone water (CPW) to remove the loosely-attached bacteria. Each disk was placed in a new 24-well plate filled with 1.5 mL of buffered peptone water (BPW) plus 0.2% sucrose. Disks with biofilms were incubated at 5% CO_2 and 37 °C for 3 h to allow the biofilms to produce acid. Then, the BPW solutions were stored for lactate analysis. Lactate concentrations in the BPW solutions were measured using an enzymatic (lactate dehydrogenase) method [46]. The microplate reader was used to measure the absorbance at 340 nm (OD_{340}) for the collected BPW solutions. Standard curves were prepared using a lactic acid standard (Supelco Analytical, Bellefonte, PA) [46].

2.9. Colony-forming unit (CFU) counts of biofilms

Resin disks with 48 h biofilms were transferred into tubes with 2 mL CPW, and the biofilms were harvested by sonication and vortexing via a vortex mixer (Fisher) [46]. Three types of agar plates were prepared to determine the microorganism viability after serial dilution in CPW [46]: tryptic soy blood agar culture plates were used to determine the total microorganisms; mitis salivarius agar culture plates containing 15% sucrose were used to determine the total streptococci; and mitis salivarius agar culture plates plus 0.2 units of bacitracin per mL were used to determine the mutans streptococci [46].

2.10. Water-aging process

Samples for dentin bond strength, antibacterial property and self-healing efficiency test were submerged in distilled water at 37 °C for 1 day, 1 month, 3 months, 6 months and 12 months. Each group was placed in 200 mL of water in a sealed polyethylene container, following previous experiments [35]. Every week, the water was changed once. At the end of each time period, samples were measured by the aforementioned methods.

2.11. Statistical analysis

All data were first checked for normal distribution with the Kolmogorov–Smirnov test and tested for homogeneity using Levene's test. For dentin bond strength, self-healing efficiency, MTT metabolic assay and acid production assay, inter-group differences were estimated using analysis of variance (ANOVA) for factorial models, and individual groups were compared using Fisher's protected least-significant difference test. For CFU, the values were first transformed by log10 to normalize the data distribution, and then ANOVA and Fisher's protected least-significant difference test were performed. Statistical analyses were performed by SPSS 13.0 software (SPSS Inc., Chicago, IL) at a significance level of $p < 0.05$.

3. Results

The microcapsule diameter was measured (mean ± sd; $n = 200$) to be 24 ± 11 μm. A typical SEM image of the microcapsules is shown in Fig. 1(A). The dentin shear bond strengths vs. water aging time from 1 day to 12 months are plotted in Fig. 1(B) (mean ± sd; $n = 10$). The bond strength of commercial SBMP control, Exp bond control and Exp bond + microcapsules groups significantly had about 30% decrease at 1 month ($p < 0.05$). There was no significant bond strength loss thereafter ($p > 0.1$). For Exp bond + DMAHDM, Exp bond + NACP and microcapsules + DMAHDM+NACP, although there was a decreasing trend with increasing time, the differences were not significant ($p > 0.1$). These three groups had higher bond strength than the control after water-ageing ($p < 0.05$).

The specimens were immersed in water for up to 12 months and then tested for self-healing. Fig. 2 plots the virgin and healed K_{IC}. Water-aging decreased the $K_{IC\text{-virgin}}$ from 1 day to 1 month ($p < 0.05$). There was no further decline from 1 to 12 months ($p > 0.1$). For $K_{IC\text{-healed}}$, groups without microcapsules had no healing. Fig. 3 shows the self-healing efficiency vs. water-aging time (mean ± sd; $n = 6$). The η of Exp bond + microcapsules and microcapsules + DMAHDM + NACP varied from 60% to 70%. There was no significant loss in η from 1 day to 12 months ($p > 0.1$). At 12 months, η of Exp bond + microcapsules and microcapsules + DMAHDM + NACP was 68% and 67%, respectively, indicating a good self-healing durability. All groups without microcapsules had no self-healing.

Typical SEM images of fracture surfaces of the microcapsules + DMAHDM + NACP group after water-aged for 12 months are shown in Fig. 4(A) virgin fracture surface, and (B) fractured, healed and re-fractured surface. The virgin fracture

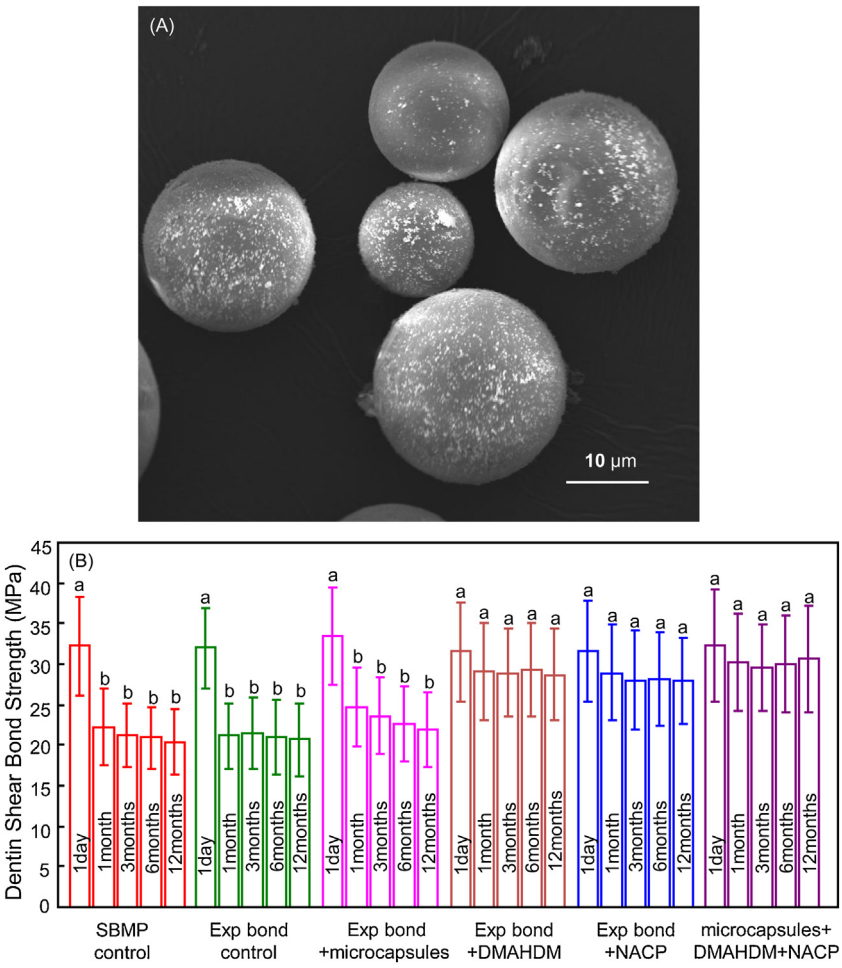

Fig. 1 – Dentin shear bond strength results (mean ± sd; $n = 10$). The bond strengths of groups without antibacterial properties were significantly decreased during 12 months of water-aging ($p < 0.05$). However, there was no significant bond strength loss for bonding agents containing DMAHDM ($p > 0.1$). Primer and adhesive were used together to test the bond strength. Bars with different letters indicate values that are significantly different from each other ($p < 0.05$).

surface indicates the fractured microcapsules. The healed and re-fracture surface contained polymer films (arrows), demonstrating that the healing liquid was released on the fracture surface and then successfully polymerized, consistent with the high self-healing efficiency.

Representative live/dead images are displayed in Fig. 5. Live bacteria were stained green and dead bacteria were stained red. At 1 day, red staining was found on all specimens containing DMAHDM (Exp bond + DMAHDM and microcapsules + DMAHDM + NACP). In contrast, the adhesives without DMAHDM had mostly green biofilm coverage. After 12 months water-aging, the biofilm live/dead images were similar to those at 1 day.

The biofilm viability results are shown in Fig. 6(A) MTT assay, and (B) lactic acid production (mean ± sd; $n = 6$). At 1 day, microcapsules + DMAHDM + NACP and Exp bond + DMAHDM reduced the biofilm metabolic activity by 95%, compared to those without DMAHDM ($p < 0.05$). Lactic acid production showed a similar decrease. Water-aging for 12 months did not reduce the antibacterial efficacy, compared to 1 day ($p > 0.1$). These results indicate that the antibacterial activity of the adhesives containing DMADDM was retained in the 12 months of water-aging.

The CFU of biofilms on adhesives are plotted in Fig. 7 for (a) total microorganisms, (b) total streptococci, and (c) mutans streptococcus (mean ± sd; $n = 6$). At 12 months, Exp bond + DMAHDM and microcapsules + DMAHDM + NACP reduced the biofilm CFU by 4 orders of magnitude over those without DMAHDM ($p < 0.05$). These results demonstrate the potent long-lasting antibacterial activity of DMAHDM groups.

4. Discussion

The present study represents the first report on a self-healing adhesive with antibacterial and remineralization properties in water-aging for 12 months. The hypotheses were proven that the adhesive with microcapsules, DMADDM and NACP did not show a significant decrease in dentin bond strength after water-aging; that this adhesive achieved a relatively high (67%) self-healing efficiency, before and after water-aging; and that it had a strong and long-lasting antibacterial property, reducing

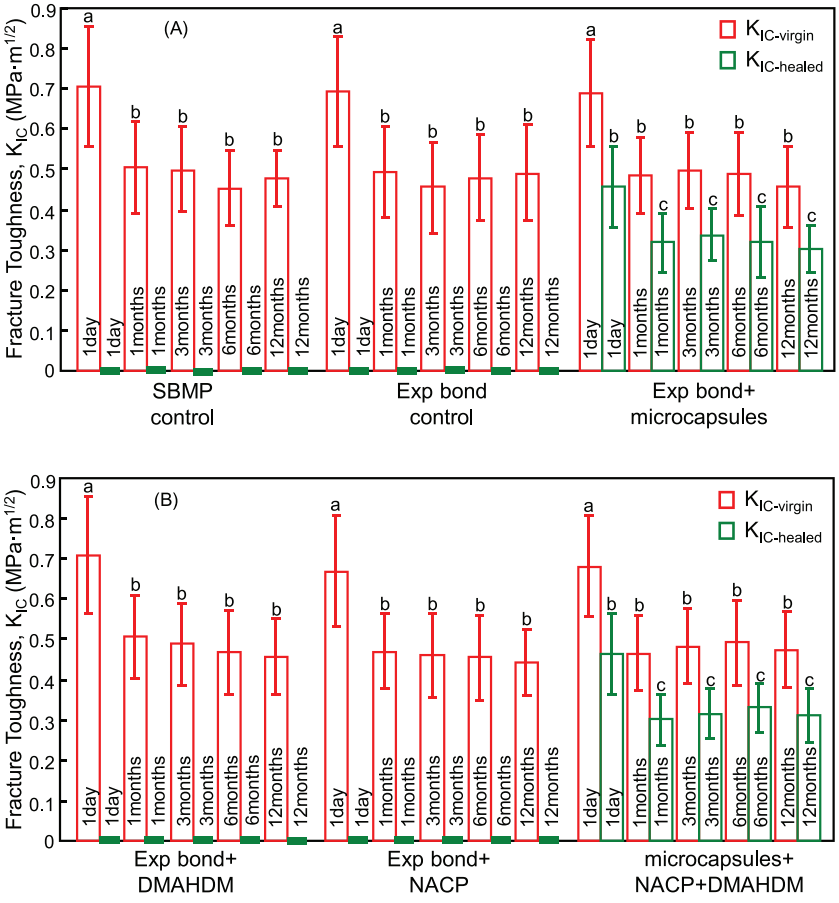

Fig. 2 – Fracture toughness of adhesive resins (mean ± sd; $n = 6$). Water-aging for 1 month decreased $K_{IC\text{-virgin}}$ by 30% for all groups. Groups without the microcapsules had no self-healing. In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

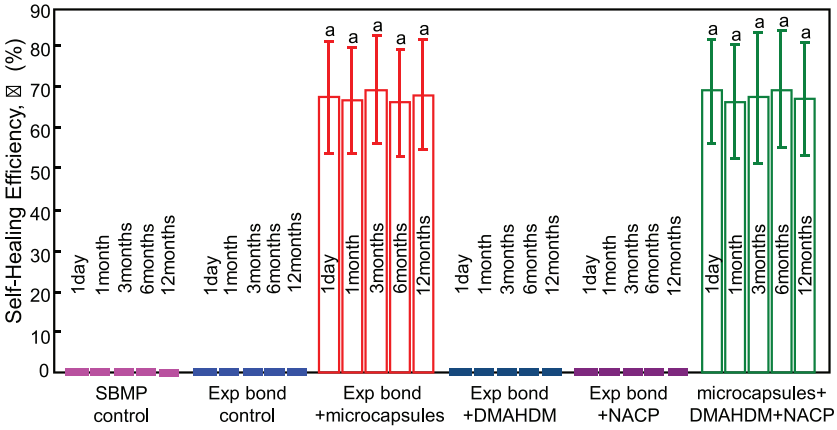

Fig. 3 – Self-healing of efficiency of adhesive resins (mean ± sd; $n = 6$). Healing efficiency of 67% was obtained for microcapsules + DMAHDM + NACP at 12 months, showing a long-lasting healing ability. All groups without microcapsules had no self-healing. In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

the CFU by 4 log for dental plaque microcosm biofilms, even after the resin samples were aged in water for12 months.

QAMs have an aliphatic vinyl group which allows for copolymerization with the resin. The immobilized antibacterial WAM provides antibacterial function that will not diminish over time. Moreover, previous studies suggested that DMAHDM with an alkyl chain length of 16 had stronger antibacterial ability than other QAMs tested [14,15]. The antibacterial mechanism of DMAHDM is that when the negatively charged bacterial cell contacts the positively charged sites of the DMAHDM, the electric balance of cell membrane could be disturbed, and the bacterium would explode under

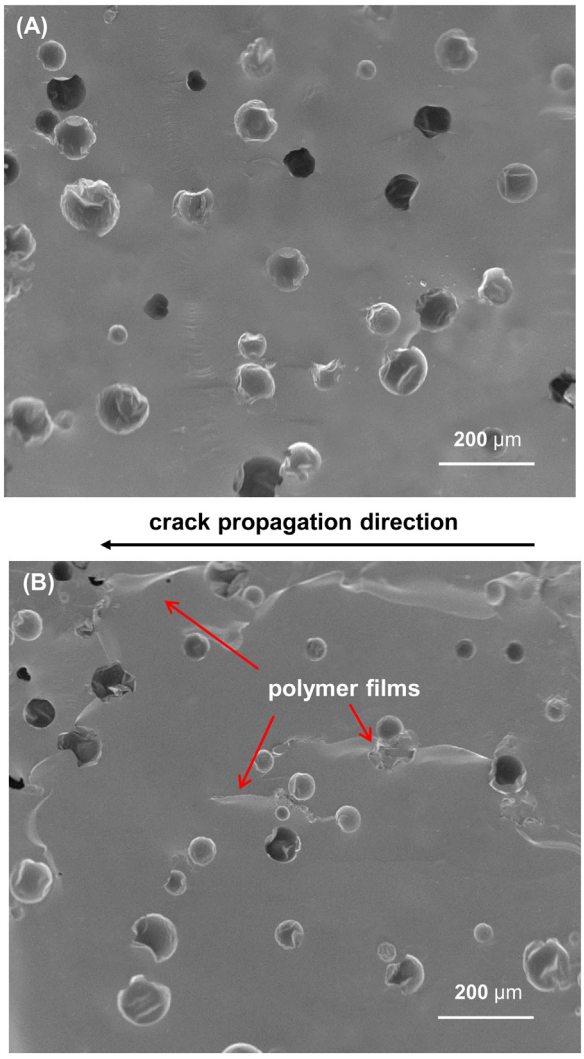

Fig. 4 – Typical fracture surfaces of the microcapsules + DMAHDM + NACP adhesive resin after 12 months of water immersion. (A) Virgin fracture surface, and (B) the healed and re-fractured surface. Arrows represent examples of polymer films from the released healing liquid that was successfully polymerized to heal the crack.

its own osmotic pressure [12]. In addition, the long-chained quaternary ammonium compounds have additional antimicrobial activity by penetrating into bacterial membrane like a needle bursting a balloon, resulting in physical damage [47]. In the present study, the metabolic activity, lactic acid and CFU of biofilms inoculated on a self-healing DMAHDM adhesive were greatly reduced, even after 12 months of water-aging. These results indicated the long-lasting antibacterial property of this self-healing adhesive. This is consistent with previous studies testing 6 months of water-aging for non-self-healing bonding agents containing dimethylaminododecyl methacrylate (DMADDM) [48] or DMAHDM [49]. The copolymerization with adhesive resin and immobilization of DMAHDM endowed a durable antimicrobial effect which was not released or lost over time. Therefore, this novel self-healing antibacterial adhesive has the potential to inhibit the invading bacteria at the bonded interface to combat bacteria and offer protection against secondary caries.

The resin-tooth bonded interface is the weak link in the restoration [50], and aging could further deteriorate the resin-dentin bond strength [51]. The degradation of the hybrid layer at the dentin-adhesive interface was believed to be the primary reason impairing the dentin bond longevity [50]. One major negative factor is that, bacterial enzymes and host-derived MMPs could lead to dissolution of the exposed collagen fibrils in the hybrid layer [52]. The MMP-induced breakdown of collagen could increase the water content, causing further collagen degradation and decrease the durability of dentin bonding [50]. QAMs have promising MMP-inhibitory and anti-enzyme properties. QAMs have cationic quaternary ammonium groups which can change the configuration of the catalytic sites of the MMP via electrostatic binding to the negative charges of glutamic acid residue. This would cause the MMP to lose the ability to hydrolyze and damage the specific peptide bond of the collagen [53]. As a result, the collagen in the hybrid layer was preserved and the dentin bond interface was protected [54]. In the current study, the groups without DMAHDM lost about 30% of the dentin bond strength at 1 month, consistent with previous results [49]. In contrast, the Exp bond + DMAHDM, Exp bond + NACP (which contained DMAHDM in the primer), and microcapsules + DMAHDM + NACP groups displayed no significant reduction in bond strength during water-aging. This was because the immobilized DMAHDM was not leached out over time, thereby exerting a long-lasting anti-MMP effect [48,49]. Some anti-MMP agents, such as chlorhexidine (CHX), were used to inhibit MMPs [53]. However, CHX is easy to be dissolved in water and leach out after being added into the adhesive in a wet environment; hence, CHX does not have a long-term anti-MMP effect [53]. In the present study, DMAHDM was copolymerized in the adhesive resin and not lost in 12 months of water-aging, which provided a durable and long-term MMP-inhibition.

Micro-cracks usually emerge at the bonding interface after repeated cycles of chewing and sliding wear in a fluid oral environment [20,51]. A previous study verified that self-healing could still happen in water [35]. Therefore, the novel self-healing adhesive was expected to be able to automatically repair the micro-cracks in the fluid oral environment to protect the bond strength. Besides the self-healing factor, a recent research confirmed that adding NACP into dental bonding agents had Ca and P ion release to inhabit caries as well as to benefit the bonding strength [18]. The NACP in the adhesive could provide remineralizing ions and acid-neutralization ability to provide further protection to the restoration margins. Previous studies showed that NACP-based nanocomposites achieved enamel lesion remineralization [55] and dentin lesion remineralization [56], and inhibited secondary caries formation at the composite-tooth margins in a human in situ model [57]. Therefore, this new adhesive had triple merits of self-healing, antibacterial and remineralization capabilities.

The self-healing efficiency of a self-repair polymer represents the recovery of the original mechanical properties. Novel dental self-healing resins, composites, cements and adhesives were developed in our previous studies [19,33–36]. The healing liquid of TEGDMA-DHEPT was encapsulated inside the

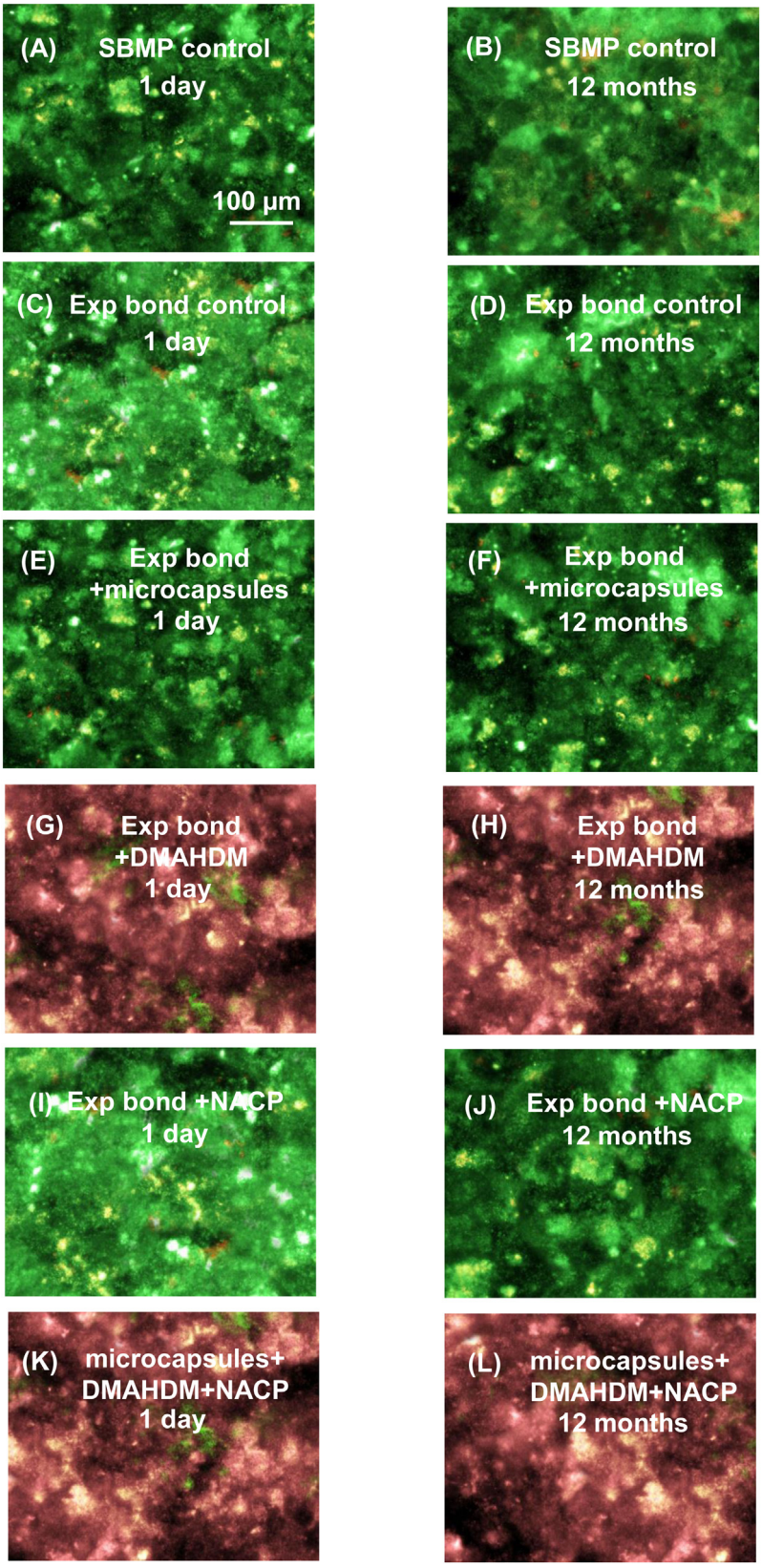

Fig. 5 – Typical live/dead images of dental plaque microcosm biofilms grown on adhesive resins. The resins were water-aged for 1 day and 12 months and then inoculated with bacteria. (A, B) Commercial SBMP control, (C, D) Exp bond control, (E, F) Exp bond + microcapsules, (G, H)Exp bond + DMAHDM, (I, J) Exp bond + NACP, (K, L) microcapsules + DMAHDM + NACP. Live bacteria were stained green, and dead bacteria were stained red. Live and dead bacteria that were close to each other produced yellow/orange colors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

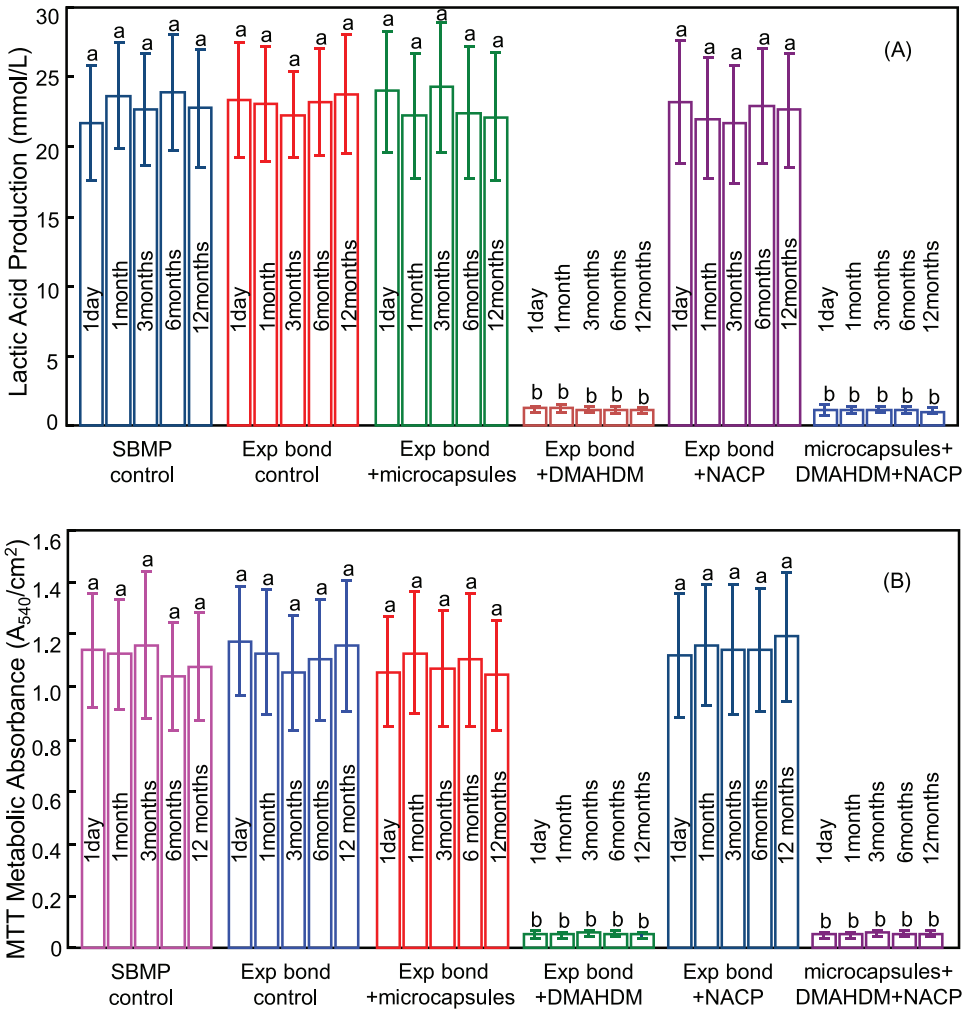

Fig. 6 – Dental plaque microcosm biofilm viability grown for 2 days on adhesive resins: (A) MTT assay, and (B) lactic acid production (mean ± sd; $n = 6$). The resins were first water-aged for 1 day to 12 months, and then inoculated with bacteria. In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

PUF shell, and these microcapsules were incorporated into the resin matrix. These embedded microcapsules were punctured by the approaching crack, releasing healing agents into the crack plane which were polymerized by contacting with the catalyst. The 60–70% self-healing efficiency demonstrated a substantial load-bearing recovery. Although these biomaterials displayed ideal initial self-healing effect [19,27,33,34], the BPO and microcapsulated healing agent in resin matrix still could lose the long-term healing stability in the complicated oral environment. It was documented that the healing efficiency of a solvent-based healing system decreased quickly after one month aging and decreased to zero after eight months due to the lack of residual functionality groups [58,59]. Another investigation of long-term stability of self-healing thermoset demonstrated that up to 68% healing efficiency was obtained after aging for six months under ambient conditions, without water-aging [60]. Our recent study investigated the water-aging effect on self-healing of a dental composite for the first time [35]. Water-aging for 6 months did not decrease the self-healing efficiency when compared to that at 1 day, indicating that water-aging did not degrade the microcapsules and the healing reaction any more than the moderate degradation of the composite matrix itself in water-aging. The present study pro-longed the water-aging time, and represented the first report on aging for 12 months. The novel adhesive having triple benefits showed no significant decrease in its healing efficiency after 12 months of submersion in water. Poly (TEGDMA) films on the fracture surface of the healed samples which had been aged for 12 months (Fig. 4B) proved that this self-healing adhesive had an endurable self-healing behavior. This was likely because the embedded microcapsules maintained an effective rupture during cracking to release the healing liquid, and the initiator BPO in the resin matrix did not leach out or diminish, and was still able to trigger the polymerization of the released healing agent [35].

Three points should be noted. First, the biocompatibility of the microcapsules with formaldehyde content needs to be investigated. As an initial and preliminary effort to investigate the biocompatibility of the self-healing microcapsules, we performed an in vitro cytotoxicity test in a previous study [33]. The results showed that the addition of microcapsules into the resin did not negatively affect the cell viability. That study

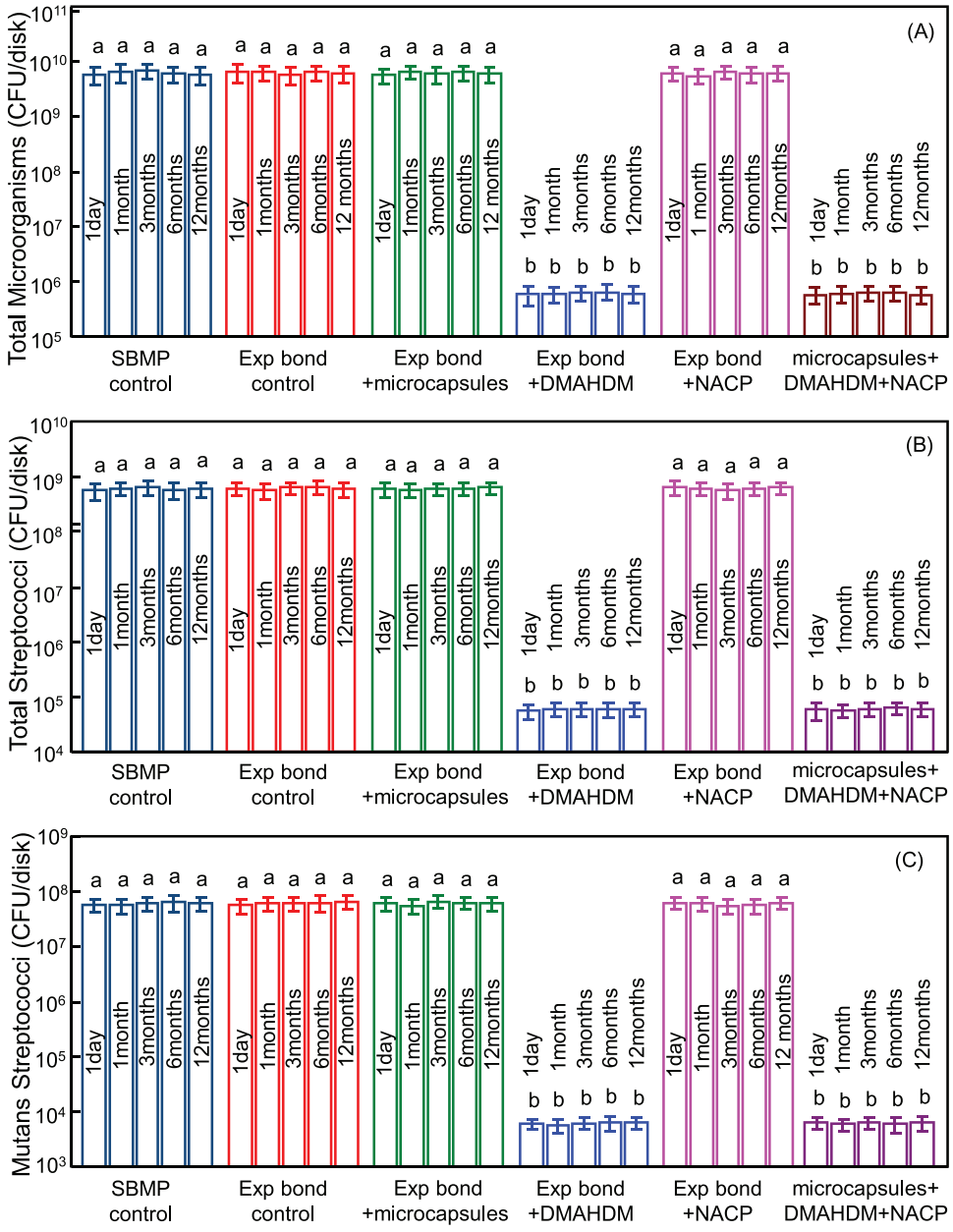

Fig. 7 – Dental plaque microcosm biofilm colony-forming units (CFU) on adhesive resins. (A) Total microorganisms, (B) total streptococci, and (C) mutans streptococci (mean ± sd; $n = 6$). The resins were first water-aged for 1 day to 12 months, and then inoculated with bacteria. For adhesives containing DMAHDM, there was no significant difference in CFU from 1 day to 12 months of water-aging ($p > 0.1$).

also showed that a microcapsule filler mass fraction of 10% or 15% was sufficient to heal the cracks. It was likely that this relatively small amount microcapsules was below the toxicity threshold. However, further studies are needed to develop new self-healing microcapsules without the use of formaldehyde to improve the biocompatibility. Second, once the microcapsule is ruptured to heal the crack, a void is left in the resin matrix which would weaken the mechanical properties. Since the void size that is left in the resin matrix is proportional to the microcapsule diameter, further efforts should develop finer microcapsules, and with silanized microcapsule surfaces to minimize the weakening of the healed resin. Third, further studies are also needed to develop packing techniques to prevent the microcapsules from being ruptured before curing the resin. In addition, the microcapsule stability and shelf-life remain an untested issue. The reactive liquid inside the microcapsules must not diffuse out of the shell wall during shelf storage. In our previous study [33], the microcapsules were exposed to air at the ambient temperature for 1 month, and the results suggested that the microcapsules were stable, with negligible permeability and negligible loss of the liquid TEGDMA through the PUF shells. Furthermore, high temperature testing of TEGDMA polymerization and PUF shell wall fragmentation using differential scanning calorimetry (DSC)

analysis showed that the PUF shell-based microcapsules had durable and stable thermal properties for up to 150 °C [33]. While these results indicate that the microcapsules likely possessed good shelf-life and thermal stability, further longer-term investigations lasting for several years are still needed.

5. Conclusions

This study developed a novel adhesive having triple advantages of self-healing, antibacterial and remineralization properties, and proved its durability in water-aging for 12 months for the first time. Water-aging for 12 months did not decrease the dentin bond strength, compared to those without self-healing and antibacterial properties. The biofilm metabolic activity and lactic acid production were substantially reduced, and biofilm CFU was decreased by 3–4 orders of magnitude, by this new adhesive even after being water-aged for 12 months. In addition, this adhesive had a relatively high self-healing efficiency of 67%, which was maintained without any significant decrease from 1 day to 12 months in water-aging. The new self-healing adhesive containing DMAHDMA and NACP with long-term crack-healing, antibacterial and anti-caries properties is promising for applications in a wide range of dental restorations.

Acknowledgments

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