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Novel bioactive root canal sealer with antibiofilm and remineralization properties

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ABSTRACT

Objectives: (1) To develop a novel bioactive root canal sealer with antibiofilm and remineralization properties using dimethylaminohexadecyl methacrylate (DMAHDM) and nanoparticles of amorphous calcium phosphate (NACP); (2) investigate the effects on *E. faecalis* biofilm inhibition, sealer flow and sealing ability, compared with an epoxy-resin-based sealer AH Plus; and (3) investigate the calcium (Ca) and phosphate (P) ion release from the sealers.

Methods: A series of dual-cure endodontic sealers were formulated with DMAHDM and NACP at 5% and 20% by mass, respectively. Flow properties and sealing ability of the sealers were measured. Colony-forming units (CFU), live/dead assay, and polysaccharide production of biofilms on sealers were determined. Ca and P ion releases from the sealers were measured.

Results: The new sealer containing 20% NACP and 5% DMAHDM yielded a paste flow of (28.99 ± 0.69) mm, within the range of ISO recommendations. The sealing properties of the sealer with 5% DMAHDM and 20% NACP were similar to a commercial control ($p > 0.05$). The sealer with DMAHDM decreased *E. faecalis* biofilm CFU by more than 4 orders of magnitude, compared to AH plus and experimental controls. The sealer with 20% NACP and 5% DMAHDM had relatively high levels of Ca and P ion release necessary for remineralization.

Conclusions: A new bioactive endodontic sealer was developed with strong antibiofilm activity against *E. faecalis* biofilms and high levels of Ca and P ion release for remineralization, without compromising the paste flow and sealing properties.

Clinical significance: The bioactive antibacterial and remineralizing root canal sealer is promising to inhibit *E. faecalis* biofilms to prevent endodontic treatment failure and secondary endodontic infections, while releasing high levels of Ca and P ions that could remineralize and strengthen the tooth structures and potentially prevent future root fractures and teeth extractions.

1. Introduction

The main objective of endodontic therapy is to eradicate root canal microorganisms and achieve a fluid-tight apical seal and coronal seal to prevent the persistence of microbes in the root canal, thereby reducing or eliminating the chance of future reinfection [1–3]. The presence of microorganisms and their by-products in the root canal inhibit the hosts' ability to heal and regenerate the damaged tissues [1–3]. In endodontic therapy, the elimination of microorganisms is accomplished through adequate chemo-mechanical instrumentation, intra-canal medication, and root canal filling material that acts as a barrier against microbial ingress from the oral cavity or from the periapical tissues [4,5].

However, histological evaluations of failed root canal-treated teeth

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indicate that the main cause of failure of endodontic therapy is the persistence of microorganisms within the root canal system after treatment [3,6]. This is mainly due to the complex anatomy of the root canal system and the resistance of biofilms to disinfecting agents [6,7]. Endodontic treatment failures are dominated by anaerobic facultative gram-positive cocci and rods [4,8]. The role of *Entercoccus faecalis* (*E. faecalis*) in failed endodontic treatment has been extensively studied. Using polymerase chain reaction (PCR) detection methods, *E. faecalis* was detected in 67–77% of teeth with failed endodontic treatment [8,9]. This gram-positive coccus is able to invade dentinal tubules, adhere to collagen, and withstand long periods of starvation, which could contribute to its ability of surviving chemo-mechanical instrumentation and intra-canal medication [8,10,11].

One of the highly-desired properties of root canal sealers is a high antibacterial efficacy, which can help eliminate residual microorganisms and prevent re-contamination if micro-leakage occurs. Previous studies reported that most endodontic sealers have some antibacterial activity [12,13]. However, most sealers show some antibacterial activity for only a few days due to the initial diffusion of some of the freshly mixed sealer components into dentinal fluids, like eugenol, zinc oxide, thymol, and paraformaldehyde [12,13]. Therefore, it would be highly desirable to develop a root canal sealer with potent and long-lasting antibacterial properties that can potentially increase the success rate of endodontic therapy and prevent future tooth loss.

Methacrylate resin-based root canal sealers have been developed to adhere to root dentin and create an impervious seal to prevent microbial ingress from saliva and impede their proliferation within the sealer-dentin interface [14]. However, due to unfavorable cavity configuration (C-factor) of the root canal, the lack of relief of the shrinkage stresses from rapid polymerization of methacrylate resin-based sealers may create gaps along the sealer-dentin interface [14]. These gaps may cause micro-leakage, bacterial penetration and treatment failure [14,15]. To inhibit the bacteria, quaternary ammonium methacrylates (QAMs) have been developed and immobilized in dental resins so that the QAM does not leach out, thereby providing long-term antibacterial effects [16–20].

Various compositions of QAMs have been incorporated into resins, including 12-methacryloyloxydodecylpyridinium bromide (MDPB), quaternary ammonium dimethacrylate (QADM), quaternary ammonium polyethylenimine (QPEI), and quaternary ammonium methacryloxy silicate (QAMS) [17–20]. Only a few reports exist on the use of QAMs in endodontic applications [21,22]. MDPB was incorporated into a methacrylate resin-based root canal filling system and was able to produce strong antibacterial activity against endodontic pathogens when compared to a commercial methacrylate-based sealer [21]. In another study, QPEI was also mixed into two commercial sealers which showed potent antibacterial effects against *E. faecalis* biofilms [22].

Recent studies have shown that increasing the alkyl chain length (CL) of QAMs can improve their antibacterial potency. Long CLs can penetrate the bacterial membranes which can disrupt the cell membrane [18]. Dimethylaminohexadecyl methacrylates (DMAHDM) with CL 16 was developed and incorporated into composites and adhesives, showing strong antimicrobial activities [18,23,24]. Recently, DMAHDM was incorporated into a commercial sealer which significantly reduced bacterial CFU counts when compared to the unmodified control sealer [25]. In addition, it would be highly desirable for root canal sealers to be able to remineralize root dentin through the release of calcium (Ca) and phosphate (P) ions. In previous studies, nanoparticles of amorphous calcium phosphate (NACP) were incorporated into composites and adhesives, which showed continuous Ca and P ion release [26,27]. Hence, the incorporation of NACP into a sealer could help strengthen the root structures through remineralization and help prevent micro-leakage through the release of Ca and P ions into areas with incomplete resin infiltration to regenerate minerals to block movement of fluids [26–28]. However, to date, there has been no report on the incorporation of DMAHDM and NACP into a root canal sealer to achieve potent, long-lasting antibacterial and Ca and P ion release capabilities.

Therefore, the objectives of this study were to: (1) Develop a novel root canal sealer with antibacterial and remineralization capability through the incorporation of DMAHDM and NACP, without adversely affecting the other properties of the sealer; and (2) investigate the antibiofilm properties on *E. faecalis* biofilms and evaluate the Ca and P ion release of the antibacterial root canal sealer for the first time. Four hypotheses were tested: (1) Incorporating DMAHDM and NACP into the sealer would yield physical properties matching those of commercial control; (2) Incorporating DMAHDM and NACP into the sealer would yield sealing properties matching those of commercial control; (3) The novel root canal sealer would greatly reduce *E. faecalis* biofilm growth and viability; (4) The sealer would release high levels of Ca and P ions.

2. Materials and methods

2.1. Fabrication of endodontic sealer containing DMAHDM and NACP

The experimental sealer is a resin-based, dual-cured, two-part sealer system. Both parts consisted of 35.475% (all mass) bisphenol A glycidyl dimethacrylate (BisGMA, Esstech, Essington, PA, USA), 35.475% triethylene glycol dimethacrylate (TEGDMA, Esstech), 24% 2-hydroxyethyl methacrylate (HEMA, Esstech), and 3% methacryloyloxy ethyl phthalate (MEP, Esstech). In addition, 2% benzoyl peroxide (BPO, Sigma-Aldrich, St. Louis, MO, USA), 1% N,N-dihydroxyethyl-p-toluidine (DHEPT, Sigma-Aldrich) and 1% bisacylphosphine oxides (BAPO, Sigma-Aldrich) were used as chemical-cure and photo-cure initiators, respectively. The root canal sealer was dual-cured. The light-cure provided an immediate coronal seal to prevent leakage from the oral cavity into the root canal system. The chemical-cure component enabled the sealer to cure in the deeper portions of the root canal, as well as allowing the sealer to flow along the canal wall and relieve some of the shrinkage stresses [14]. This resin is referred to as “BTH”.

NACP was synthesized using spray-drying technique [26]. Briefly, calcium carbonate and dicalcium phosphate anhydrous were dissolved in acetic acid to produce Ca and P ion concentrations of 8 and 5.333 mmol/L, respectively. This yielded a Ca/P molar ratio of 1.5, the same as amorphous calcium phosphate (ACP) [$Ca_3(PO_4)_2$]. The solution was sprayed into a heated chamber and an electrostatic precipitator was used to collect the dried particles. This produced NACP with a mean particle size of 116 nm [26]. NACP were mixed into BTH at a filler mass fraction of 20% of the final sealer. The 20% was selected following preliminary experiments to maintain a good flow for the paste. The 20% NACP was also shown to enable the release of high levels of Ca and P ions for remineralization in previous studies [26–30].

In addition, silanized barium boroaluminosilicate glass particles with a median size of 1.4 μm (Caulk/Dentsply, Milford, DE, USA) were used as co-filler to provide mechanical reinforcement to the sealer. The glass fillers were incorporated into the sealer at mass fractions of 40%, 45%, and 50%, respectively. These filler levels were selected based on preliminary studies to obtain the paste flow properties within the ISO-recommended flow for endodontic sealers.

DMAHDM was synthesized via modified Menschutkin reaction where a tertiary amine group was reacted with an organo-halide [31]. Briefly, 10 mmol of 2-(dimethylamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich), and 10 mmol of 1-bromohexadecane (BHD; TCI America, Portland, OR, USA) were combined with 3 g of ethanol in a 20 mL scintillation vial. The vial was stirred at 70 °C for 24 h. After that, the solvent was removed via evaporation, yielding DMAHDM as a clear and viscous liquid [18]. DMAHDM was mixed into the BTH resin at 5% mass fraction of the final sealer. The 5% DMAHDM was selected following a previous study that showed strong antibacterial activity without adversely affecting the physical properties of the resin [32].

A commercial endodontic sealer (AH Plus, Dentsply DeTrey, Konstanz, Germany) was used as comparative control. According to the

manufacturer, AH Plus contained a total filler mass fraction of 76% of finely ground calcium tungstate with an average particle size of 8 μm, and finely ground zirconium oxide with an average particle size of 1.5 μm.

2.2. Test of paste flow property

One of the most important properties of root canal sealers is their ability to flow and penetrate into the complex anatomy of the root canal, such as lateral canals, fins and ramifications, to provide an adequate seal and exert their antimicrobial properties [33]. The flow test was conducted according to the International Standards Organization (ISO) 6876/2012 standard for root canal sealing materials [34]. Briefly, a volume of 0.1 mL of the mixed sealer was placed on a glass slab (40 × 40 × 5 mm) using a graduated 1-mL syringe. A second glass slab weighing approximately 20 g was then placed on top of the sealer paste, followed by weight of approximately 100 g to make a total mass of 120 ± 2 g. After 10 min, the weight was removed and the maximum and minimum diameters of the compressed disk of sealer were measured by a digital caliper (Mitutoyo, Tokyo, Japan). If the maximum and minimum diameters agreed with each other to within 1 mm, the mean of the two diameters was recorded as the flow rate of the sample. If the two diameters differed by more than 1 mm, then the test was repeated. Three determinations were carried out (n = 3) which were expressed to the nearest millimeter, and the average was calculated [34]. With the aforementioned justifications for selecting the mass fractions of the components, the following ten root canal sealers were tested for their flow properties:

(1) **Control:** commercial AH Plus endodontic sealer control.
(2) **Glass-filled sealer:** 60% BTH + 40% glass.
(3) **Glass-filled sealer:** 55% BTH + 45% glass.
(4) **Glass-filled sealer:** 50% BTH + 50% glass.
(5) **Remineralizing sealer:** 40% BTH + 20% NACP + 40% glass.
(6) **Remineralizing sealer:** 35% BTH + 20% NACP + 45% glass.
(7) **Remineralizing sealer:** 30% BTH + 20% NACP + 50% glass.
(8) **Antibacterial and remineralizing sealer:** 35% BTH + 20% NACP + 5% DMAHDM + 40% glass.
(9) **Antibacterial and remineralizing sealer:** 30% BTH + 20% NACP + 5% DMAHDM + 45% glass.
(10) **Antibacterial and remineralizing sealer:** 25% BTH + 20% NACP + 5% DMAHDM + 50% glass.

2.3. Sealing ability test

The flow test results showed that the experimental sealers with 40% glass and 20% NACP had a flow value within that recommended by the ISO standard. Therefore, the following four groups were selected for the subsequent experiments:

Control: commercial AH Plus endodontic sealer control.

Glass-filled sealer: 60% BTH + 40% glass.

Remineralizing sealer: 40% BTH + 20% NACP + 40% glass (referred to as NACP sealer);

Antibacterial and remineralizing sealer: 35% BTH + 20% NACP + 5% DMAHDM + 40% glass (referred to as NACP + DMAHDM sealer).

The dye penetration method was used to evaluate the sealing ability of the root canal sealers [35]. Teeth collection was approved by the University of Maryland Institutional Review Board. Forty extracted single-rooted teeth were collected. All teeth were stored in 5.25% sodium hypochlorite (NaOCl) for two hours to remove all organic debris and stored in normal saline until further use. The crown of all the teeth were removed with a water-cooled diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) to obtain a standard length of 14 mm. The working length was determined using a 10 K-file to determine canal patency (Dentsply Maillefer, Ballaigues, Switzerland) and then subtracting 1 mm from the apical foreman. The apical part of the canal was prepared up to size 35 K-file as the master apical file (Dentsply Maillefer). The canals were then prepared using a step-back technique up to size 60 K-file (Dentsply Maillefer). At each change of file, canals were irrigated with 1.0 mL 5.25% NaOCl and received a final flush with 3.0 mL of 17% ethylenediaminetetraacetic acid (EDTA) which was allowed to remain in the canal for 5 min. Finally, the canals were flushed with 5 mL of distilled water.

A primer containing pyromellitic dianhydride glycerol dimethacrylate (PMGDM, Esstech) and 2-hydroxyethyl methacrylate (HEMA, Esstech) at a 10:3 mass ratio, with 50% acetone solvent (all by mass), was first applied to the root canal wall. Paper points were inserted inside the canal to remove any excess. The canals were further dried by applying a gentle burst of air. Teeth were then obturated with one of the sealers, gutta-percha points, and accessory cones using the lateral compaction technique. The gutta-percha and accessory cones were removed at the orifice and vertical compaction of the gutta-percha was performed. All samples were light-cured for 60 s at the canal orifice to create an immediate coronal seal. The access cavity was then sealed with Cavit (3 M ESPE, Germany) [35]. All roots were placed in 100% humidity for 1 week to ensure complete setting.

To prepare the specimens for stereomicroscopic analysis of dye penetration, roots were dried for two minutes and covered with two layers of nail varnish except for the apical 2 mm. The samples were then immersed in 1% methylene blue dye for 72 h. The nail varnish was then removed and roots were sectioned longitudinally to determine the vertical dye penetration under a stereomicroscope, all measurements were scored in millimeters [35].

After dye penetration, teeth sections filled with the antibacterial and remineralizing sealer were washed with distilled water to remove any debris and treated with 50% phosphoric acids and 10% sodium hypochlorite. The surfaces were then exposed to graded ethanol dehydration, dried, mounted, and sputter-coated with gold to be examined by scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, Oreg) to view the sealer-dentin interface.

2.4. Specimen fabrication for E. faecalis biofilm experiments

Sealer disks were made using the cover of a 96-well plate as molds following a previous study [34]. Twenty μL of sealer was placed into the dent and photo-polymerized for 30 s (Demetron VCL401), using a mylar strip covering to obtain a disk of approximately 8 mm in diameter and 0.5 mm in thickness [36]. The cured disks were immersed in water and stirred with a magnetic bar at 100 rpm for 1 h to remove any uncured monomers, following a previous study [37]. The disks were sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC, USA) and de-gassed for 7 days.

The use of bacterial species in this study was approved by the University of Maryland Institutional Review Board (HP-00052180). *E. faecalis* (ATCC29212) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). *E. faecalis* was selected as it is one of the most commonly-detected organisms in persistent endodontic infections. *E. faecalis* possesses several virulence factors and is able to withstand various intracanal medicaments including calcium hydroxide [8]. *E. faecalis* was grown in brain-heart infusion broth (BHI, Sigma-Aldrich) at 37 °C aerobically (95% air, 5% CO_2), following ATCC's instructions. The inoculum was adjusted to 10^7 colony-forming unit counts CFU/mL for biofilm experiments, based on the standard curve of OD_{600} nm versus the CFU/mL [24].

2.5. Biofilm formation on sealer resin disks

The human saliva collection was approved by the University of Maryland Institutional Review Board (HP-00050407). Saliva was collected from ten healthy donors without caries, periopathology and endodontic infections, and without the use of antibiotics in the past three

months. Prior to saliva donation, the donors were asked not to brush their teeth for 24 h and to abstain from food and drink intake for 2 h. An equal volume of saliva from each of the donors was mixed together. Saliva was centrifuged at 3000 rpm for 20 min to remove bacterial cellular debris, and the supernatant was filter-sterilized through 0.22 μm filter (VWR International, Radnor, PA, USA). The resulting saliva without bacteria was used to create a salivary protein pellicle layer on the resin disks to enhance the subsequent bacterial attachment and biofilm formation tests [38,39]. Sealer disks were coated with sterile saliva by immersing each disk in sterile saliva in a 24-well plate for 2 h at 37 °C [24]. The saliva-coated disks were transferred to a new 24-well plate where *E. faecalis* was inoculated at a concentration of 10^7 CFU/mL in 1.5 mL of brain heart infusion (BHI) medium in each well. After 24 h, the disks with adherent biofilms were transferred to new 24-well plate filled with fresh medium, and incubated for additional 24 h. This totaled 48 h of culture, which was previously shown to be sufficient to form relatively mature biofilms on resins [24,38,39].

2.6. Live/dead staining of biofilms

Sealer disks with 2-day biofilms were washed with phosphate buffered saline (PBS) to remove planktonic bacteria. Disks were stained with the BacLight live/dead kit (Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. A mixture of 2.5 μM SYTO 9 and 2.5 μM propidium iodide was used to stain each sample for 15 min. Live bacteria with intact membranes were stained with SYTO9 to emit a green fluorescence. Bacteria with disrupted membrane integrity were stained with propidium iodide to emit a red fluorescence. An inverted epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY, USA) was used to examine the stained biofilm disks [24,38].

2.7. Measurement of polysaccharide production by biofilms on resins

The water-insoluble polysaccharide in the extracellular polymeric substance (EPS) of biofilms was determined using a phenol-sulfuric acid method [24,38]. Each disk with 2-day biofilm was immersed in 2 mL of PBS, and the biofilm was collected by sonication/vortexing. Centrifugation was performed which yielded a precipitate that was rinsed with PBS and resuspended in 1 mL of de-ionized water. Then, 1 mL of 6% phenol solution was added, followed by 5 mL of 95–97% sulfuric acid. After 30 min of incubation, 100 μL of the solution was transferred into a 96-well plate. The amount of polysaccharide in biofilms was determined by measuring the absorbance at OD_{490} nm with a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). The OD readings were converted to polysaccharide concentrations based on standards with five glucose concentrations of 0, 5, 10, 20, 50 and 100 mg/mL, following previous studies [24,38].

2.8. Colony-forming unit (CFU) counts of biofilms on resins

Sealer disks with 2-day biofilms were transferred into vials with 2 mL of PBS, and the biofilms were harvested by scraping and sonication/vortexing (Fisher, Pittsburgh, PA, USA). BHI agar was used to measure the CFU counts. Biofilm suspensions were serially diluted, spread onto agar plates and incubated at 37 °C aerobically for 48 h. Then, the number of colonies was counted by a colony counter (Reichert, NY, USA) and used, along with the dilution factor, to calculate the CFU counts [24,38].

2.9. Measurement of Ca and P ion release

The ion release from the NACP sealer and NACP + DMAHDM sealer were measured to investigate the effects of adding DMAHDM on the ion release. A sodium chloride (NaCl) solution (133 mmol/L) was buffered to three different pH values: pH 4 with 50 mmol/L lactic acid, pH 5.5 with 50 mmol/L acetic acid, and pH 7 with 50 mmol/L HEPES

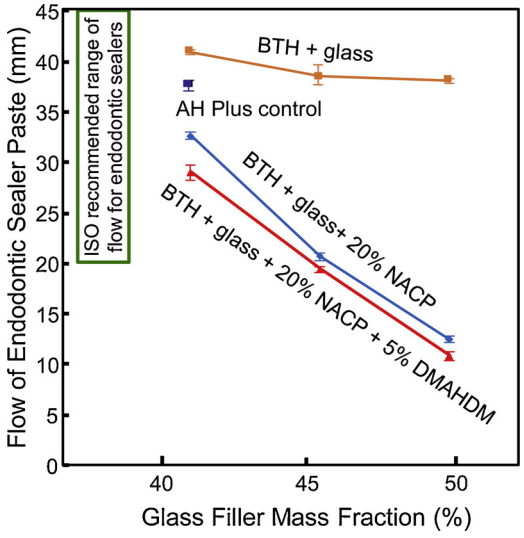

Fig. 1. Mean flow rate measured in mm (mean ± sd; n = 3). Increasing the glass filler mass fraction significantly decreased the flow rate when compared to commercial control. Group with NACP + DMAHDM significantly decreased flow rate when compared to commercial control.

[26,40,41]. Three specimens of approximately 2 × 2 × 12 mm were immersed in 50 mL of solution to yield a specimen volume/solution of 2.9 mm^3/mL, similar to a specimen volume per solution of about 3.0 mm^3/mL following previous studies [40,41]. For each solution, the concentrations of Ca and P ions released from the specimens were measured at 1, 3, 5, 7, 14, 21, and 28 days [26,40]. At each time, aliquots of 0.5 mL were removed and replaced by fresh solution. The aliquots were analyzed for Ca and P ions via a spectrophotometric method (SpectraMax M5) using known standards and calibration curves following previous studies [26,40].

2.10. Statistical analyses

Independent-samples *t*-test and one-way ANOVA were performed to detect significant effects of the variables. Tukey's multiple comparison test was used to compare the data. Statistical analyses were performed by SPSS 19.0 (SPSS, Chicago, IL, USA) at an alpha of 0.05.

3. Results

The flow values are plotted in Fig. 1 (mean ± sd; n = 3). Increasing the glass filler mass fraction significantly decreased the flow in both experimental groups (40% BTH + 20% NACP + 40% glass and 35% BTH + 20% NACP + 5% DMAHDM + 40% glass) ($p < 0.05$). Both groups with NACP and NACP + DMAHDM significantly decreased the flow when compared to commercial control ($p < 0.05$). However, incorporating 40% glass filler + 20% NACP + 5% DMAHDM still produced flow within the range of ISO requirement (≥ 20 mm).

The dye penetration results are shown in Fig. 2, with representative images in (A–D), and the dye penetration data (mean ± sd; n = 10) in (E). All sealers demonstrated apical leakages that were not significantly different from each other ($p > 0.05$). Incorporating 5% DMAHDM and 20% NACP did not adversely affect the sealing ability compared to the control ($p > 0.05$).

Fig. 3 shows typical SEM images of the sealer-dentin interface. After examination from low to high magnifications, an intimate contact at the interface was evident between dentin and the antibacterial and remineralizing sealer without gaps. Numerous resin tags penetrating the dentinal tubules were prevalent at the bonded interface.

Representative live/dead images of 2-day *E. faecalis* biofilms on sealers are shown in Fig. 4. Live bacteria were stained green. Bacteria

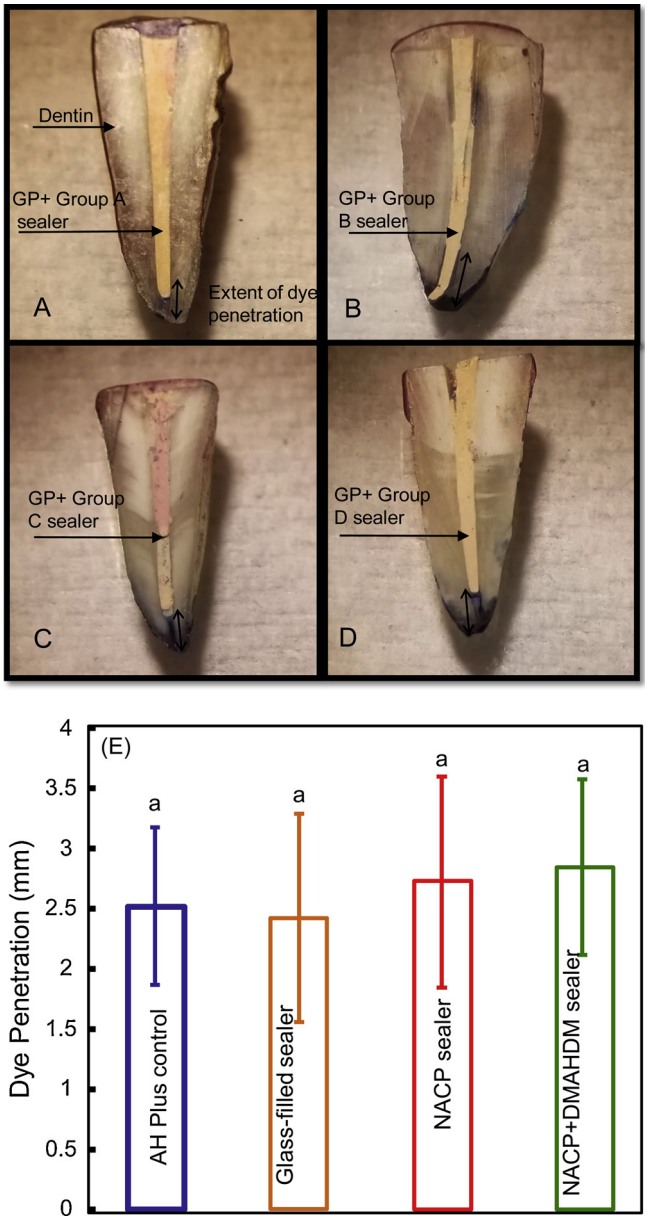

Fig. 2. Stereomicroscopic images of tested groups showing the rate of apical dye penetration: (A) AH Plus control; (B) glass-filled sealer; (C) NACP sealer; (D) NACP + DMAHDM sealer. (E) Quantification of dye penetration (mean ± sd; n = 10). Incorporating 20% NACP and 5% DMAHDM did not significantly affect the sealing ability of the sealer when compared to sealer without NACP and DMAHDM ($p > 0.05$). All experimental sealers showed sealing properties similar to that of AH Plus ($p > 0.05$).

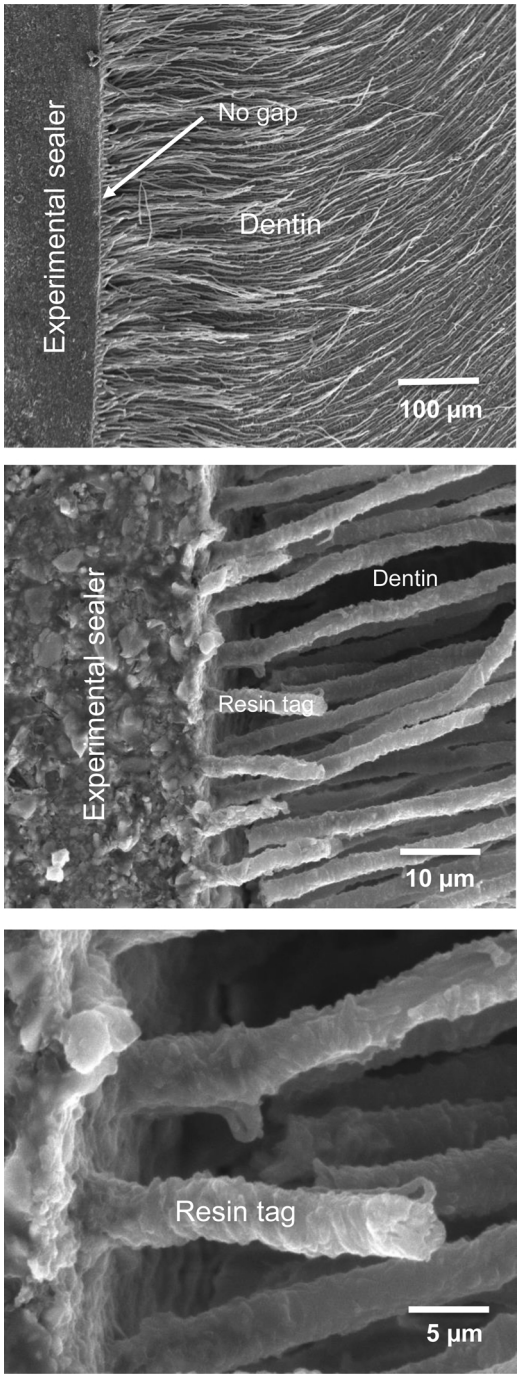

Fig. 3. Representative SEM images at lower and higher magnifications of longitudinal sections of teeth showing the sealer-dentin interface. The canals were filled with the NACP and DMAHDM containing root canal sealer. An intimate sealer-dentin interface was successfully obtained without gaps. The sealer formed numerous resin tags that penetrated the dentinal tubules.

with disrupted membranes were stained red. Live and dead bacteria on the top of each other yielded yellow/orange colors. Commercial sealer control, glass-filled sealer, and NACP sealer were primarily covered by live bacteria. In contrast, NACP + DMAHDM sealer had substantial amounts of compromised bacteria.

Polysaccharide results of 2-day *E. faecalis* biofilms on sealers are plotted in Fig. 5A (mean ± sd; n = 6). Glass-filled sealer, and NACP sealer without DMAHDM had results similar to those of AH Plus ($p > 0.05$). DMAHDM greatly reduced the polysaccharide production of biofilms ($p < 0.05$).

The CFU counts of 2-day *E. faecalis* biofilms on sealers are plotted in Fig. 5B (mean ± sd; n = 6). CFU counts on disks containing DMAHDM had 4–5 log reductions in CFU. Glass-filled sealer, and NACP sealer without DMAHDM had CFU results similar to those of AH Plus ($p > 0.05$).

Ca and P ion releases from endodontic sealers are plotted in Fig. 6 (Ca ions) and Fig. 7 (P ions) (mean ± sd; n = 4). Fig. 5 shows Ca release from (A) NACP sealer, and (B) NACP + DMAHDM sealer. Fig. 6. Shows P ion release from (A) NACP sealer and (B) NACP + DMAHDM sealer. The concentrations of released ions increased with time and decreasing pH. Higher ion concentrations are released when the oral environment is more acidic, and when these ions are most needed to prevent demineralization of tooth structure. At day 28, incorporating DMAHDM into the sealer slightly reduced the Ca and P ion release

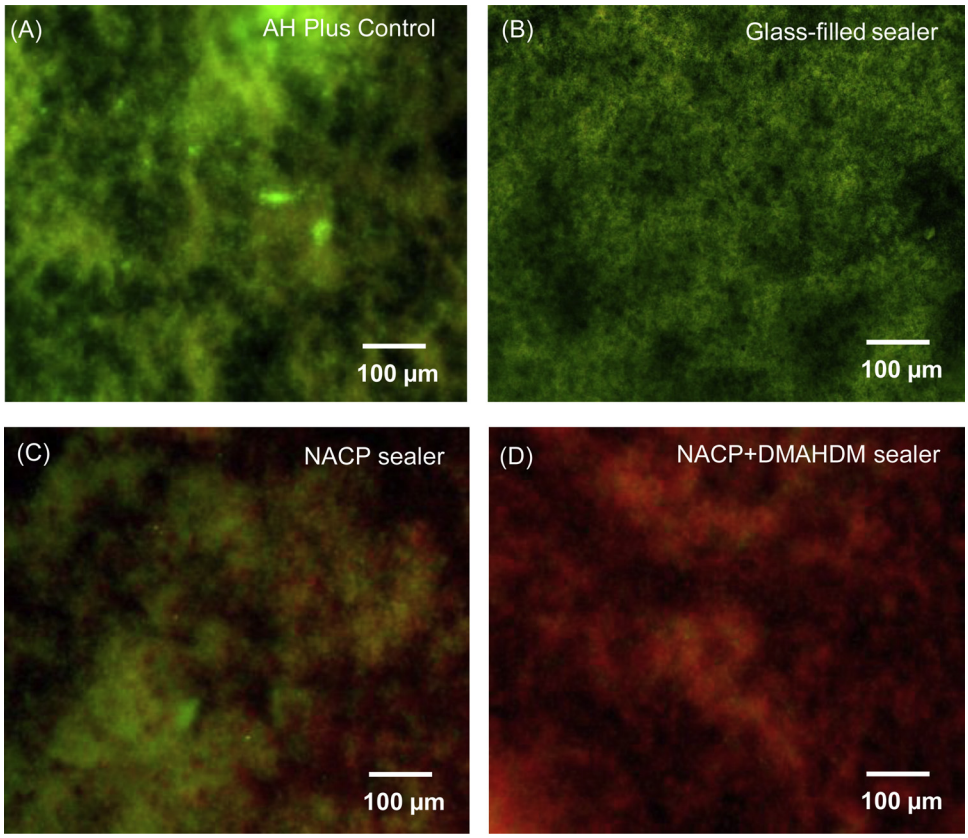

Fig. 4. Live/dead staining images of biofilms adherent on resin disks. Live bacteria were stained green. Bacteria with disrupted membranes were stained red. Live and dead on the top of each other yielded yellow/orange colors. Disks of the AH Plus control (A), glass-filled sealer (B), and NACP sealer (C) were primarily covered with live bacteria. Disks with NACP + DMAHDM (D) were primarily covered with dead bacteria.

compared to group without DMAHDM; however, these reductions were not significant ($p > 0.05$).

4. Discussion

This study developed a novel root canal sealer with antibacterial and Ca and P ion release capabilities. The sealer's ability to inhibit *E. faecalis* biofilms while releasing high levels of Ca and P was achieved. The hypotheses were proven that the incorporation of DMAHDM and NACP into the sealer did not adversely affect the flow and sealing properties, while greatly reducing *E.faecalis* biofilm growth and viability. The *E. faecalis* biofilm CFU was reduced by 4–5 orders of magnitude via the new sealer. In addition, the novel root canal sealer had the ability to release high levels of Ca and P ions to potentially remineralize and strengthen root structures.

The present study focused on the effects of DMAHDM and NACP in endodontic therapy. It should be noted that the important basic requirements for the endodontic sealer includes biocompatibility of the material, dimensional stability during setting, long-term chemical stability, stability with heat so the sealer can be used with warm-vertical compaction technique, etc. These properties should be comprehensively investigated in future studies. Furthermore, the present study used a methacrylate resin as a model system. Further studies are needed to investigate the applicability of the novel methods and relationships established in the present study to various other resin matrix systems, including non-methacrylate matrices and low-shrinkage resins.

In root canal treatments, although predictable microbial debridement can be achieved using traditional instrumentation and irrigation techniques, this is usually achieved in accessible areas where instruments and disinfecting agents are able to access. Inaccessible areas that remain un-instrumented such as fins, ramifications, lateral canals, and dentinal tubules, usually remain contaminated with endodontic bacteria and biofilms [1]. In addition, the nature of root canal biofilms renders bacterial elimination more difficult when compared to the bacteria in their planktonic state. Clinical studies have revealed persistence of bacteria within the root canal system in spite of cleaning, shaping, and applications of highly efficient antimicrobial agents [1,6]. Using antibacterial root canal sealing materials that flow into inaccessible areas of the root canal could aid in the eradication of the remaining root canal microbiota, leading to higher success rates for the endodontic therapy.

An important property of root canal sealers is to obtain a hermetic seal at the sealer-dentin interface and fill the space between the core material and the dentin wall to prevent micro-leakage from saliva or periapical tissues from causing future re-infection [42]. In addition, the presence of voids at the sealer-dentin interface can permit fluid movement and water-uptake by resins which can result in the degradation of their physical and mechanical properties [15]. Various tests have been previously utilized to evaluate the sealing ability of root canal sealers. A common test is the dye penetration test [42–45]. Methylene blue dye was chosen for the present study due to its strong staining potency and low molecular weight similar to that of bacterial metabolites [46]. The novel sealer with 20% NACP and 5% DMAHDM achieved sealing properties similar to those of the commercial control sealer, while obtaining antibacterial and remineralizing properties that the commercial sealer did not have.

With advancements in adhesive dentistry, methacrylate resin-based sealers were developed in an effort to create a bondable root canal filling system to root dentin and to introduce the concept of monoblock [14]. A number of methacrylate resin-based sealers are commercially available and are classified according to their generation. Three generations of root canal sealers have been commercially available. For example, EndoREZ is an example of a second-generation root canal sealer, Epiphany a third-generation root canal sealer, and Meta SEAL is fourth-generation root canal sealer [14].

Several studies have shown favorable results regarding the bondability and sealing ability of these sealers. Recently, Saranga et al. compared the pushout bond strengths of MetaSEAL, and AH Plus. The

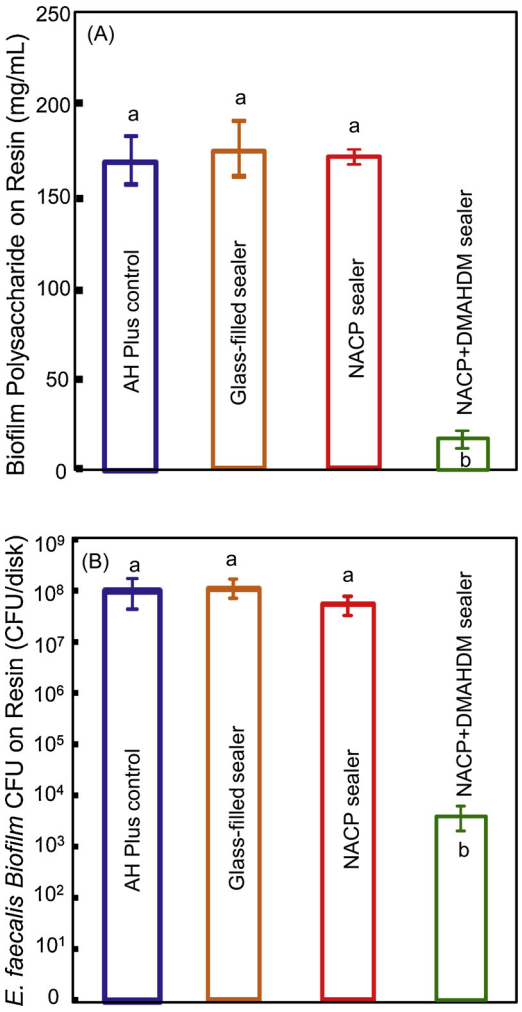

Fig. 5. Polysaccharide production by biofilms adherent on the disks (mean ± sd; n = 6). Glass-filled sealer, and NACP sealer had Polysaccharide production similar to those of AH Plus control. Disks with DMAHDM significantly reduced polysaccharide production by *E. faecalis* ($P < 0.05$). Dissimilar letters indicate that values are significantly different from each other ($P < 0.05$). Colony-forming unit (CFU) counts of biofilms adherent on the disks (mean ± sd; n = 6). Glass-filled sealer, and NACP sealer had CFU results similar to those of AH Plus control. Disks with DMAHDM were more than 4 orders of magnitude less than those without DMAHDM. Dissimilar letters indicate that values are significantly different from each other ($p < 0.05$).

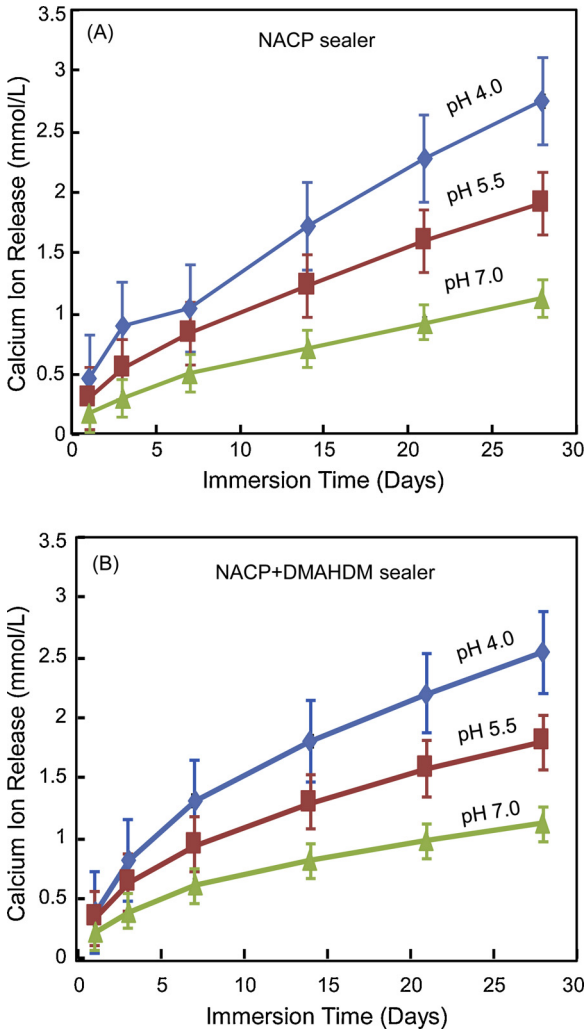

Fig. 6. Calcium (Ca) ion release from endodontic sealers (A) NACP sealer, and (B) NACP + DMAHDM sealer (mean ± sd; n = 4).

authors reported higher bond strength values with MetaSeal (1.49 ± 0.09 MPa) when compared to AH Plus (0.90 ± 0.04 MPa) [47]. Another study compared the sealing ability of Endosequence BC (a bioceramic sealer), AH Plus sealer, and Epiphany system (a methacrylate-based sealer) using the dye penetration test. Although all sealers showed dye leakage to some extent, the Endosequence BC and Epiphany system showed significantly less dye penetration than AH Plus [45]. The mechanism by which the methacrylate-based sealers adhere to root dentin is mainly through resin infiltration into the collagen matrix and dentinal tubules with the formation of a hybrid layer [11]. However, some studies have reported unfavorable results, showing lower push-out strengths in roots obturated with methacrylate resin–based sealers when compared to conventional nonbonding sealers [48,49]. The sub-optimal bonding properties could be attributed to several factors. First, the unfavorable cavity configuration of the root canal could prevent the relief of stresses generated from polymerization shrinkage of the sealer, which could result in gaps at the sealer-dentin interface [14]. However, shrinkage of the resin could possibly be compensated by the fluid uptake and swelling of the hydrophilic monomer system [14]. The second factor was the accelerated setting of the sealer due to heat generation when warm vertical compaction techniques were applied [14]. Therefore, it could be particularly important to incorporate antibacterial agents to the methacrylate resin-based sealers to prevent bacterial proliferation in case of microleakage.

SEM images in previous studies on adhesives showed their ability to infiltrate into the dentinal tubules [50]. Previous studies showed that incorporating similar mass fractions of NACP and DMAHDM into methacrylate-based adhesives did not adversely affect their dentin bond strength [51,52]. For endodontic practices, micro-leakage is a main reason for endodontic failure. The degradation of the sealer-dentin bond can be accelerated in the presence of bacterial enzymes and matrix metalloproteinases (MMPs) produced by both the bacteria and the host [15]. In previous studies, QAMs such as MDPB effectively inhibited soluble MMPs and matrix-bound dentin MMPs [53,54]. Another study tested the inhibitory effects of dimethylaminododecyl methacrylate (DMADDM) against MMPs [55]. The results showed significant inhibition of MMPs, achieving a high level of 90% of inhibition [55]. Further study is needed to investigate the NACP + DMAHDM sealer for its antibacterial and anti-MMP properties to potentially prevent biological degradation of the sealer-dentin bond.

Another important property of root canal sealers is to kill endodontic bacteria and biofilms. Efforts have been made to improve root canal disinfection either via soluble agents or through copolymerization of antibacterial substances to root canal sealers or bonding agents

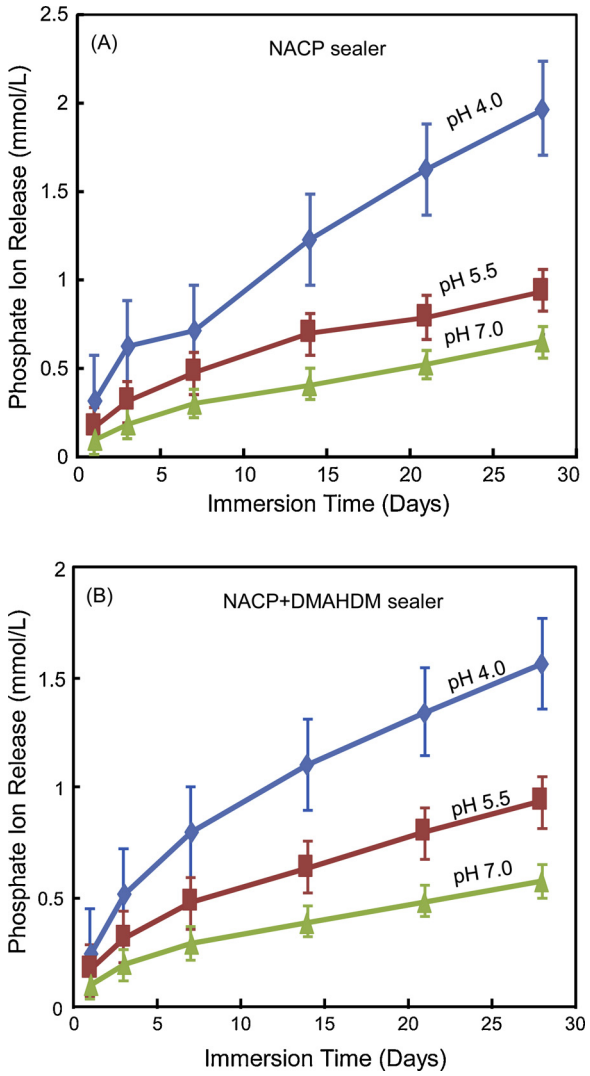

Fig. 7. Phosphate (P) ion release from endodontic sealers (A) NACP sealer, and (B) NACP + DMAHDM sealer (mean ± sd; n = 4).

[16,56]. In previous studies, micro- and nano-sized particles of chitosan, zinc oxide, and silver were incorporated [57,58]. Although promising results were achieved, some of the major draw backs of these materials include their burst release over a short period of time and bacterial selectivity [59,60]. The mechanism of action of QAMs is identified as contact-inhibition. The electrostatic interaction between the negatively-charged bacterial cell and the positively-charged (N^+) sites of a QAM resin causes a disturbance in the electric balance of the cell membrane. As a result, the bacterium bursts under its own osmotic pressure [17,18]. The results of the present study showed that incorporating 5% DMAHDM greatly reduced *E. faecalis* biofilm CFU by more than four orders of magnitude. The polysaccharide produced in the extracellular polymeric substance was greatly reduced in the DMAHDM group compared to groups without DMAHDM and commercial sealer.

In a previous study, DMAHDM was incorporated in bonding agents, showing potent inhibition of *E. faecalis* biofilms and CFU reduction of nearly three orders of magnitude [24]. Another study tested the effects of DMAHDM and NACP against endodontic polymicrobial biofilms [38]. The results also showed substantial biofilm-inhibition and orders of magnitude CFU reduction, without jeopardizing the push-out bond strength of the sealer [38]. Recently, DMAHDM and nanosilver (NAg) were incorporated into AH Plus sealer [25]. AH Plus sealer is an epoxy-resin-based sealer in which the methacrylate-based DMAHDM is mechanically mixed, without chemical bonds. Therefore, only low concentrations of DMAHDM were used, because adding higher concentrations jeopardized the physical properties of the sealer [25]. In addition, the antibacterial effect was expected to be short-term because DMAHDM was not bonded in the polymer structure. In contrast, in the present study, a new methacrylate-based sealer was formulated which allowed the incorporation of higher concentrations of DMAHDM that was covalently bonded in the resin. Hence, it would not leach out and lost over time, thus providing a long-term effect. The present study achieved *E. faecalis* biofilm CFU reductions of 4–5 orders of magnitude, compared to the previous study that achieved only 3 orders of magnitude reduction. In addition, in the present study, incorporating higher concentrations of DMAHDM into the methacrylate-based sealer showed stronger antibacterial effects without jeopardizing the physical and sealing properties. Furthermore, the previous study tested the modified AH Plus sealer against *E. faecalis* bacteria in their planktonic state [25]. In contrast, in the present study, the antibacterial effects of the new DMAHDM sealer were tested against *E. faecalis* biofilms. Biofilms are much more resistant to antibacterial agents than their planktonic counterparts. Therefore, in the present study, the CFU reductions of 4–5 orders of magnitude in biofilms indicate a much more potent antibacterial endodontic sealer than that in the previous study.

E. faecalis was chosen as the test organism for the present study because it plays a major role in the persistence of periradicular lesions following root canal treatment [8]. It is also the most-commonly found organism in failed root canal treatments, due to its ability to withstand various root canal disinfecting agents and intracanal medicaments [8]. In addition, other organisms have been frequently detected in failed endodontic treatments, including, and not limited to, *Fusobacterium nucleatum (F. nucleatum)*, *Prevotella* spp, and *Actinomyces* spp [61]. A previous study tested the antibacterial effects of DMAHDM against an endodontic biofilm model composed *of Actinomyces naeslundii* (*A. naeslundii*), *F. nucleatum* and *E. faecalis* species [38]. The results showed a significant reduction in endodontic biofilm growth and viability [38]. In the present study, the new sealer showed strong antibacterial and Ca and P ion properties, without compromising the physical and sealing properties when compared to AH plus. Although DMAHDM is expected to show variable antibacterial effects against different bacterial species, *E. faecalis* is one of the more difficult species to be killed [8,62,63]. DMAHDM is immobilized in the resin matrix and is expected to kill residual bacteria coming in contact with the sealer. DMAHDM is also expected to prevent bacterial ingress in case of leakage through contact-inhibition and therefore preventing secondary endodontic infections.

Another highly-desirable property for the endodontic sealer would be to remineralize and strengthen the tooth root structures. Endodontic preparation of the root canal involves the use of various irrigant solutions such as NaOCl and chlorhexidine (CHX) that help dissolve organic tissues, kill bacteria, and flush away debris [64]. They could cause dentin demineralization and lower the dentin hardness, potentially predisposing to root fracture [65,66]. These alterations in the chemistry and structure of root dentin could potentially be reversed through the incorporation of NACP into the root canal sealer, which were previously shown to remineralize the demineralized dentin [29,67]. The present study incorporated 20% NACP, which was able to release high levels of Ca and P ions. In a previous study, NACP was incorporated into a composite that was able to achieve an enamel remineralization that was 4 folds that achieved by a fluoride-releasing commercial control [29]. In another study, through the release of Ca and P ions, NACP nanocomposite achieved dentin lesion remineralization that was 48% higher than that achieved by a commercial composite [67].

The present study investigated the release of Ca and P ions in acidic and neutral pH. During certain endodontic infections, the pH of the root canal environment could become acidic and decrease to pH 5 [38,68]. To simulate the effects of metabolic byproducts produced by anaerobic bacteria during endodontic infections, previous studies investigated the ion release at acidic pH, such as pH 5.5 and compared it to neutral pH

[68]. In addition, chelating agents used on root dentin to remove the smear layer, such as citric acid, lactic acid, phosphoric acid and certain antibiotic mixtures, could further contribute to the acidity of the root canal environment to demineralize root dentin. Stresses applied on the root structures during canal preparation and instrumentation could weaken the root structure and reduce its hardness, often resulting in a higher susceptibility to vertical root fractures [69,70]. The new NACP-containing root canal sealer could increase the release of Ca and P ions at lower pH when these ions would be most needed to strengthen the root structures.

In previous studies, bonding agents containing NACP, along with polyamidoamine (PAMAM) dendrimer as nucleation template, were tested for their ability to remineralize the pre-demineralized human dentin in a cyclic artificial saliva/lactic acid challenge environment [71,72]. The results showed the ability to neutralize acid, promote dentin remineralization, and increase the hardness of the pre-demineralized dentin. The NACP and PAMAM method regenerated the dentin minerals and reversed the pre-demineralized dentin hardness to match that of heathy dentin [71,72]. The reduction in dentin hardness can cause crack propagation and reduce fracture resistance [73,74]. Therefore, the incorporation of NACP, previously shown to increase dentin hardness, could be beneficial in roots treated with antibiotic mixtures or calcium hydroxide medicaments which could diminish the root dentin hardness and fracture resistance [74]. The possible strengthening of tooth roots via the NACP sealer requires further study.

Bonding to dentin is highly dependent on the micro-mechanical retention by resin infiltration into the demineralized superficial dentin [15]. Incomplete resin infiltration results in resin sparse zones of demineralized dentin that permits fluid movement and subsequent bond degradation [15]. The release of Ca and P ions into these demineralized zones could potentially improve the sealing ability of the resin-based endodontic sealers and prevent micro-leakage through dentin remineralization. Further studies are needed to investigate the antibacterial effects of the novel root canal sealer on the killing of multi-species endodontic biofilms that are more clinically relevant. In addition, further studies are needed to evaluate the effects of Ca and P ion release from the sealer on root dentin remineralization and fracture resistance of tooth roots.

5. Conclusions

A novel bioactive endodontic sealer was developed containing dual agents of DMAHDM and NACP with strong anti-biofilm activity against *E. faecalis* and high levels of Ca and P ion release for remineralization, without compromising the flow and sealing properties. The new bioactive sealer was able to greatly reduce biofilm formation and viability. Biofilm CFU was reduced by more than four orders of magnitude on the NACP + DMAHDM endodontic sealer, compared to a commercial control sealer. Incorporating DMAHDM into the sealer did not adversely affect the sealer's Ca and P ion release, which could potentially help remineralize the root dentin and strengthen the root structures. The novel NACP + DMAHDM root canal sealer is promising for endodontic applications, as it effectively inhibited *E. faecalis* biofilm, commonly associated with failed endodontically treated teeth. The novel sealer also released high levels of Ca and P ions that could promote remineralization in tooth root structures.

Conflict of interest

The authors do not have any conflict of interest related to this study.

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