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# Novel endodontic sealer with dual strategies of dimethylaminohexadecyl methacrylate and nanoparticles of silver to inhibit root canal biofilms

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## ABSTRACT

**Objective.** Endodontic treatment failures and recontamination remain a major challenge. The objectives of this study were to: (1) develop a new root canal sealer with potent and long-lasting antibiofilm effects using dimethylaminohexadecyl methacrylate (DMAHDM) and nanoparticles of silver (NAg); (2) determine the effects of incorporating DMAHDM and NAg each alone versus in combination on biofilm-inhibition efficacy; and (3) determine the effects on sealer paste flow, film thickness and sealing ability, compared to a commercial control sealer.

**Methods.** Dual-cure endodontic sealers were formulated using DMAHDM mass fractions of 0%, 2.5% and 5%, and NAg mass fractions of 0.05%, 0.1% and 0.15%. The sealing ability of the sealers was measured using linear dye penetration method. Colony-forming units (CFU), live/dead assay, and polysaccharide production of biofilms grown on sealers were determined.

**Results.** The sealer with 5% DMAHDM and 0.15% NAg yielded a flow of  $(22.18 \pm 0.58)$  which was within the range of ISO recommendations and not statistically different from AH Plus control  $(23.3 \pm 0.84)$  ( $p > 0.05$ ). Incorporating DMAHDM and NAg did not negatively affect the film thickness and sealing properties ( $p > 0.05$ ). The sealer with 5% DMAHDM and 0.15% NAg greatly reduced polysaccharide production by the biofilms, and decreased the biofilm CFU by nearly 6 orders of magnitude, compared to AH Plus and experimental controls ( $p < 0.05$ ).

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**Significance.** A therapeutic root canal sealer was developed using 5% DMAHDM with biofilm-inhibition through contact-mediated mechanisms, plus 0.15% of NAg to release silver ions into the complex and difficult-to-reach root canal environment. The novel root canal sealer exerted potent antibiofilm effects and reduced biofilm CFU by 6 orders of magnitude without compromising sealer flow, film thickness and sealing ability. This method provided a promising approach to inhibit endodontic biofilms and prevent recurrent endodontic infections.

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## 1. Introduction

One of the major challenges of endodontic treatment is to eliminate or reduce root canal bacterial biofilm to levels compatible with the host's ability to heal and regenerate the damaged tissues [1,2]. Despite the efforts to disinfect the root canal system through chemo-mechanical debridement and application of highly efficient intracanal medicaments, failure and recontamination often occur, mainly due to the complexity of the root canal system and the resistant nature of root canal biofilms [3–6].

Previous studies have shown the ability of bacteria to infiltrate and penetrate the dentinal tubules to a depth of 200–1500  $\mu\text{m}$ , and therefore are difficult to eradicate through traditional instrumentation and irrigation regimens [7,8]. According to previous studies, 79% of root canals where bacteria were found to remain showed evidence of impaired healing in periapical lesions [1]. Studies investigating the microbial populations of failed root canal treatment cases have shown the dominance of gram-positive bacteria [9]. The role of *Enterococcus faecalis* (*E. faecalis*) in nonhealing root canal cases has been extensively studied. *E. faecalis* has been previously detected in 23–77% of cases with failed endodontic treatment [9–11]. Previous studies have shown the ability of *E. faecalis* to invade the dentinal tubules, can withstand long periods inside the root canal in the absence of nutrients, and is therefore hard to eliminate [12–14].

Root canal sealers applied during endodontic treatment have the ability to fill irregularities present between the core material and the canal wall, adhere to the canal wall, and infiltrate deep into the dentinal tubules [8]. Most root canal sealers possess some antimicrobial properties [15]. However, these properties usually diminish after setting [15,16]. Therefore, efforts have been made to add antibacterial agents to root canal materials to enhance and prolong their antibacterial activity [17–19]. However, the majority of these materials depend on the release of these agents into the surrounding environment, which become depleted over time.

A promising approach for solving this problem is through the application of dual antibacterial agents: one to be released into the more complex anatomy of the root canal and provide a killing effect away from the sealer, and one to provide long-term antibacterial properties through contact-killing mechanism on the sealer surface. Silver (Ag) salts are one of the most common antimicrobial agents that have been incorporated into composites, bonding agents, and root

canal materials [20–22]. The mechanism of action of silver ions works on multiple targets, such as interacting with the sulfhydryl group in proteins, preventing DNA replication, and interfering with cell-wall synthesis and cell division [22]. Recent studies developed nanoparticles of silver (NAg) and chemically incorporated them into dental resins to avoid agglomeration [23,24]. The NAg had a particle size of approximately 2.7 nm with high surface area, which resulted in strong antibacterial properties in low concentrations and without jeopardizing the color or mechanical properties of the material [23,24].

In addition, quaternary ammonium methacrylates (QAMs) were incorporated into dental resins and showed potent and long-lasting antibacterial properties [25–29]. The mechanism of action of QAMs is identified as contact-inhibition. The electrical imbalance that occurs as a result of the contact between the negatively charged bacterial cell wall and the positively charged ( $\text{N}^+$ ) sites of a QAM resin disturbs the bacterial membrane and as a result, the bacterium bursts leaking its cellular contents. Kitagawa et al. incorporated 12-methacryloyloxydodecylpyridinium bromide (MDPB) into a resin-based root canal filling system, which showed potent antibacterial effects against *E. faecalis* [30]. Recently, QAMs with increased alkyl chain length from 5 to 16 were developed [27]. Dental composites and bonding agents containing the new monomer dimethylaminohexadecyl methacrylates (DMAHDM) with alkyl chain length 16 showed the strongest antibacterial activity among the groups tested [27].

Previous studies combined NAg with MDPB in a dental primer and adhesive which showed stronger antibacterial activity than using either agent alone [31,32]. Another study combined NAg and a quaternary ammonium dimethacrylate (QADM) in a primer that significantly reduced the bacterial counts when tested on dentin blocks [33]. Recently, DMAHDM and NAg were incorporated into AH Plus commercial sealer that showed significant reduction of bacterial CFU counts when compared to the unmodified control sealer [34]. However, in the previous study, incorporating high concentrations of DMAHDM into the epoxy-resin-based AH Plus sealer jeopardized its physical properties [34]. Previous studies have shown that the antibacterial effects of both DMAHDM and NAg were concentration-dependent and their addition in higher concentrations improved their antibacterial capabilities [35]. In addition, the previous study tested the modified AH Plus sealer against planktonic *E. faecalis* bacteria, without testing biofilms [34]. Biofilms are much more resistant to antibacterial agents than their planktonic counterparts and therefore, testing on biofilms are required to be clinically relevant [1]. Further-

more, the previous study did not investigate the effects of adding DMAHDM and NAg on the sealing properties, which is considered one of the most important properties of root canal sealers [34]. In the present study, higher concentrations of DMAHDM and NAg were used to formulate a novel antibacterial root canal sealer, and their effects on the physical properties and sealing ability were investigated. In addition, the antibacterial effects of the DMAHDM and NAg containing sealer were tested against *E. faecalis* biofilms, not planktonic bacteria.

Therefore, the objectives of this study were to: (1) Utilize DMAHDM and NAg to develop a root canal sealer with potent and long-lasting antibiofilm properties; (2) investigate the effects of DMAHDM and NAg on sealer flow, film thickness and sealing properties; and (3) investigate the effects of incorporating DMAHDM and NAg into the root canal sealer on biofilm-inhibition. The following hypotheses were tested: (1) incorporating DMAHDM and NAg into the root canal sealer would not compromise the physical properties of the sealer, compared to commercial control; (2) the new root canal sealer would substantially reduce biofilm growth and viability; (3) incorporating dual agents of DMAHDM and NAg together into the sealer would produce much stronger antibiofilm potency than using each agent alone in the sealer.

## 2. Materials and methods

### 2.1. Formulation of endodontic sealer containing DMAHDM and NAg

A resin-based, dual-cured, two-part sealer system was developed. Both parts consisted of bisphenol A glycidyl dimethacrylate (BisGMA, Esstech, Essington, PA, USA) and triethylene glycol dimethacrylate (TEGDMA, Esstech) mixed at 1:1 ratio. 2-hydroxyethyl methacrylate (HEMA, Esstech), and methacryloyloxy ethyl phthalate (MEP, Esstech) were added at 24% and 3% (all mass), respectively. To render the experimental sealer dual curable, 2% benzoyl peroxide (BPO, MilliporeSigma, St. Louis, MO, USA), 1% N,N-dihydroxyethyl-p-toluidine (DHEPT, Sigma-Aldrich) and 1% bisacylphosphine oxides (BAPO, MilliporeSigma) were used as chemical-cure and photo-cure initiators, respectively. Because BisGMA, TEGDMA and HEMA were the major components of this experimental sealer resin, it is referred to as “BTH”.

A modified Menshutkin reaction was used to synthesize DMAHDM, in which a tertiary amine group was reacted with an organo-halide [36]. Briefly, 10 mmol of 2-(dimethylamino) ethyl methacrylate (DMAEMA, MilliporeSigma), 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 [27].

For the synthesis of NAg, 0.12 g of silver 2-ethylhexanoate (Strem, Newburyport, MA, USA) was dissolved into 0.88 g of 2-(tert-butylamino) ethyl methacrylate (TBAEMA, Millipore, Sigma). TBAEMA facilitated the Ag-salt dissolution in the resin likely due to the formation of Ag–N coordination bonds. It

also contained a reactive methacrylate group and therefore could be chemically incorporated into the resin matrix upon polymerization [37,38]. This method allowed for the in situ synthesis of NAg in the polymer matrix and reduced the chance of nanoparticle aggregation [37,38].

Experimental root canal sealers contained 4 levels of NAg (0%, 0.05%, 0.10%, and 0.15%), and 3 levels of DMAHDM (0%, 2.5%, and 5.0%). The purpose was to determine the highest concentrations of DMAHDM and NAg, in order to obtain the strongest antibacterial activities, without compromising the sealer's physical properties. To mechanically reinforce the sealer, silanized barium borosilicate glass particles with a median size of 1.4 μm (Caulk/Dentsply, Milford, DE, USA) were used as fillers. The glass fillers were incorporated into the sealer at mass fraction of 73%; the filler level was selected based on the results of preliminary studies that yielded a paste with physical properties within the International Standards Organization (ISO) recommendations for endodontic sealers [39].

AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany) was tested as a commercial 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.

The following root canal sealers were tested for their flow and film thickness properties:

- (1) Commercial control: AH Plus endodontic sealer control.
- (2) Experimental control BTH sealer: 27% BTH + 73% glass (referred to as “BTH + 73glass control”).
- (3) BTH + 2.5% DMAHDM sealer: 24.5% BTH + 2.5% DMAHDM + 73% glass (BTH + 73glass + 2.5DMAHDM).
- (4) BTH + 5% DMAHDM sealer: 22% BTH + 5% DMAHDM + 73% glass (BTH + 73glass + 5DMAHDM).
- (5) BTH + 0.05% NAg sealer: 26.95% BTH + 0.05% NAg + 73% glass (BTH + 73glass + 0.05NAg).
- (6) BTH + 0.1% NAg sealer: 26.9% BTH + 0.1% NAg + 73% glass (BTH + 73glass + 0.1NAg).
- (7) BTH + 0.15% NAg sealer: 26.85% BTH + 0.15% NAg + 73% glass (BTH + 73glass + 0.15NAg).
- (8) BTH + 2.5% DMAHDM + 0.05% NAg: 24.45% BTH + 2.5% DMAHDM + 0.05% NAg + 73% glass (BTH + 73glass + 2.5DMAHDM + 0.05NAg).
- (9) BTH + 2.5% DMAHDM + 0.1% NAg: 24.4% BTH + 2.5% DMAHDM + 0.1% NAg + 73% glass (BTH + 73glass + 2.5DMAHDM + 0.1NAg).
- (10) BTH + 2.5% DMAHDM + 0.15% NAg: 24.35% BTH + 2.5% DMAHDM + 0.15% NAg + 73% glass (BTH + 73glass + 2.5DMAHDM + 0.15NAg).
- (11) BTH + 5% DMAHDM + 0.05% NAg: 21.95% BTH + 5% DMAHDM + 0.05% NAg + 73% glass (BTH + 73glass + 5DMAHDM + 0.05NAg).
- (12) BTH + 5% DMAHDM + 0.1% NAg: 21.9% BTH + 5% DMAHDM + 0.1% NAg + 73% glass (BTH + 73glass + 5DMAHDM + 0.1NAg).
- (13) BTH + 5% DMAHDM + 0.15% NAg: 21.85% BTH + 5% DMAHDM + 0.15% NAg + 73% glass (BTH + 73glass + 5DMAHDM + 0.15NAg).

## 2.2. Testing of sealer paste flow property

Flow was tested because the root canal bacteria can invade and penetrate the dentinal tubules and are thus difficult to eradicate. Hence, the ability of root canal sealers to flow and penetrate the complex structures of the root canal can aid in the elimination of root canal biofilm, in addition to providing an adequate seal at the sealer-dentin interface [7,8]. Using a graduated 1-mL syringe, 0.05 mL of the sealer paste was placed on a glass slab (40 × 40 × 5 mm). Another glass slab weighing 20 g was placed on top of the sealer paste, followed by a weight of 100 g to make a total mass of 120 ± 2 g [39]. After 10 min, the weight was removed and the major and minor diameters of the compressed disk of sealer were measured by a digital caliper (Mitutoyo, Tokyo, Japan) [39]. Three repeats were carried out (n = 3) and expressed to the nearest millimeter, and the average diameter was calculated to represent the flow of the paste [39].

## 2.3. Sealer film thickness

Endodontic sealers are considered the weakest component in root canal filling systems. They are more susceptible to degradation than core materials and are therefore recommended to be placed in thin films [40]. Incorporating fillers and antibacterial agents could influence the sealer film thickness. Therefore, investigating the effects of adding our bioactive agents on sealer film thickness is particularly important. The combined thickness of two glass plates was measured. A portion of the sealer paste was placed onto the center of one of the plates, and the other glass plate was placed on the top. A load of 150 N was applied on the top glass plate [39]. Ten minutes after the commencement of mixing, the thickness of the two glass plates and the interposed film of sealer were measured using a micrometer (iGaging, Los Angeles, CA, USA) [39]. Three determinations were carried out (n = 3) and the average was calculated [39].

## 2.4. Dye penetration sealing ability testing

The highest concentrations of DMAHDM and NAG that did not compromise the flow and film thickness properties of the sealer were chosen. Based on the results of Sections 2.2 and 2.3, the following groups were selected for the sealing ability and biofilm experiments: AH Plus control; BTH + 73glass control; BTH + 73glass + 5DMAHDM; BTH + 73glass + 0.15NAG; BTH + 73glass + 5DMAHDM + 0.15NAG.

To investigate the sealing ability of the root canal sealers, a dye penetration method was used. Teeth collection was approved by the University of Maryland Baltimore Institutional Review Board. Fifty extracted human teeth with single canals were collected (n = 10). Teeth were kept in 5.25% sodium hypochlorite (NaOCl) for two hours to remove all organic debris and then stored in normal saline until further use. The crowns were removed with a water-cooled diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) and adjusted to a standard length of 14 mm. The canal patency was determined with a 10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) and the working length was adjusted by 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 manually prepared using a step-back technique up to size 60 K-file (Dentsply Maillefer). During instrumentation, the canals were irrigated with 1.0 mL 5.25% NaOCl between each file and then flushed thoroughly 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 and dried. Teeth were obturated with one of the sealers, gutta-percha points matching the size of the master apical file, and accessory cones using the lateral compaction technique. The gutta-percha and accessory cones were removed at the orifice with the application of a heated spreader. The softened gutta-percha was then vertically compacted with a plugger using hand pressure. The canal orifice was then sealed with Cavit (3M ESPE, Seefeld, Germany) [41]. All roots were placed in 100% humidity for 1 week to ensure complete setting of the respective sealer.

All roots were allowed to dry for 2 min and then 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 scraped off and the roots were sectioned longitudinally to determine the apical linear dye penetration under a stereomicroscope [41].

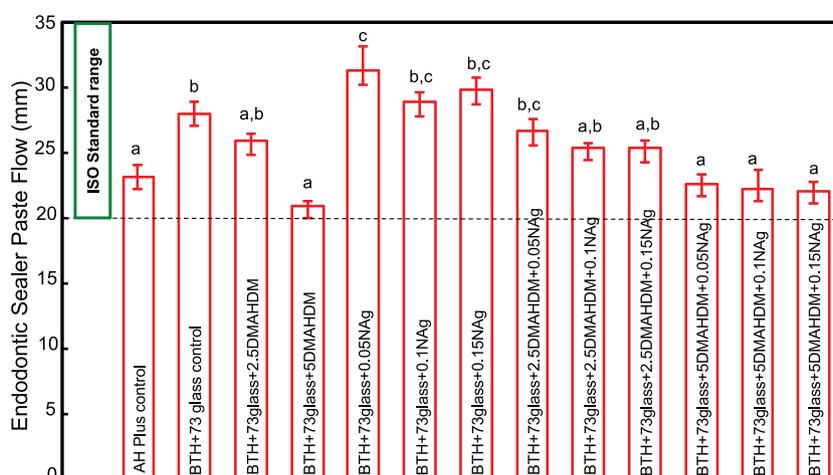
## 2.5. Sealer specimen fabrication for biofilm experiments

A 96-well plate was used as molds to create sealer disks following a previous study [35]. Sealer disks of approximately 8 mm in diameter and 0.5 mm in thickness were obtained by placing 20 µL quantity of sealer into the dent which were then light cured for 30 s (Kerr, Demetron Optilux, VCL 401) [35]. The cured disks were then removed and immersed in water and agitated with a magnetic bar at 100 rpm for 1 h to remove any uncured monomers, following a previous study [42]. The disks were then sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC, USA) and de-gassed for 7 days.

The use of *E. faecalis* was approved by the University of Maryland Baltimore Institutional Review Board (HP-00052180). *E. faecalis* (ATCC29212) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). *E. faecalis* is one of the most commonly retrieved organisms from canals of failed endodontic treatment cases [12]. In addition to the ability of *E. faecalis* to penetrate deep into the dentinal tubules, it can withstand several intracanal disinfectants and medications including calcium hydroxide and survive nutritional deprivation in filled root canals [43]. *E. faecalis* was grown in brain-heart infusion broth (BHI, MilliporeSigma) at 37 °C aerobically (95% air, 5% CO<sub>2</sub>), following ATCC's instructions. The inoculum was adjusted to 10<sup>7</sup> colony-forming unit counts (CFU/mL) for biofilm experiments, based on the standard curve of OD<sub>600</sub> nm versus the CFU/mL [44].

## 2.6. Biofilm formation on sealer disks

The human saliva collection was approved by the University of Maryland Baltimore Institutional Review Board (HP-00050407). Saliva was collected from ten healthy individuals who have not used antibiotics in the past three months. Donors were asked not to brush their teeth for 24 h and abstain from food



**Fig. 1 – Endodontic sealer paste flow properties (mean  $\pm$  sd; n = 3). Adding 5% DMAHDM and 0.15% NAg did not negatively affect the flow properties, compared to AH Plus ( $p > 0.05$ ). All groups had flow values that were in compliance with the ISO requirements of flow of no less than 20 mm.**

and drink intake for 2 h prior to saliva donation. Mixed saliva stock was created by combining an equal volume of saliva from each donor. Saliva was centrifuged at 3000 rpm for 20 min and the supernatant was filter-sterilized through 0.22  $\mu$ m filter (VWR International, Radnor, PA, USA). The sterile saliva contained no bacteria and was used to create a salivary protein pellicle on resin disks. This pellicle is used to promote bacterial adhesion on the resin disks and maintain the complexity of the biofilm structure [44,45].

Each sealer disk was immersed in the sterile saliva in a 24-well plate for 2 h at 37 °C [44]. *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 of a 24-well plate where the saliva-coated disks were transferred. After 24 h, the disks with adherent biofilms were transferred to new 24-well plate with fresh medium, and incubated for additional 24 h. This totaled 48 h of culture. Based on previous studies, 48 h was sufficient to create a relatively mature biofilm on resin [44–46].

### 2.7. Live/dead biofilm staining

Disks with 2-day biofilms were rinsed with phosphate buffered saline (PBS) to remove planktonic bacteria. The BacLight live/dead staining kit (Molecular Probes, Eugene, OR, USA) was used following the manufacturer's instructions. Live bacteria were stained with SYTO9 to produce green fluorescence and dead bacteria with compromised membranes were stained with propidium iodide to produce red fluorescence. Images of each sealer disk were captured using an inverted epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY, USA) [44,46].

### 2.8. Polysaccharide production by biofilms

The water-insoluble polysaccharide in the extracellular polymeric substance (EPS) of biofilms was determined using a phenol-sulfuric acid method [44,46]. Disks with 2-day biofilm were suspended in 2 mL of PBS, and the biofilm was collected

by sonication/vortexing. Biofilm suspensions were centrifuged to yield 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  $\mu$ L of the solution was transferred into a 96-well plate. The amount of polysaccharide in biofilms was determined by measuring the absorbance at OD<sub>490</sub> nm with a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). Standard glucose concentrations were used to convert the OD readings to polysaccharide concentrations, following previous studies [44,46].

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

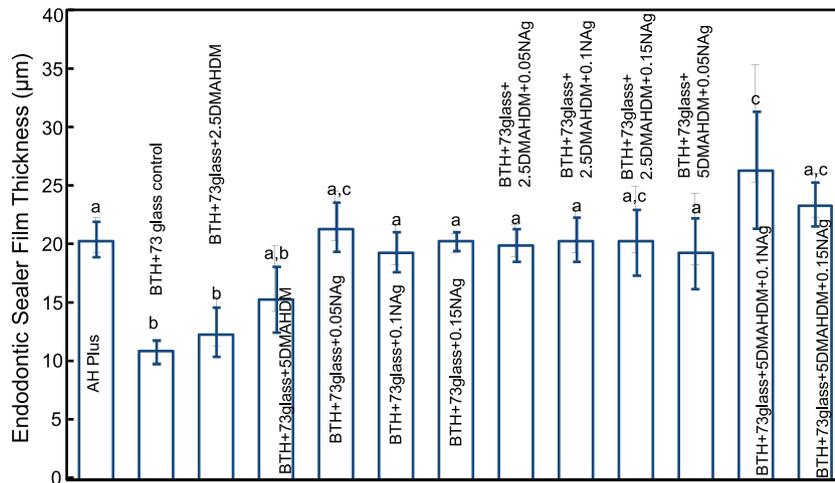
Sealer disks with 2-day biofilms were transferred into vials with 2 mL of PBS, and the biofilms were harvested by scraping and sonication/vortexing (Thermo Fisher Scientific, Pittsburg, PA, USA). BHI agar was used to measure the CFU counts. The bacterial suspensions were serially diluted, spread onto agar plates and incubated at 37 °C aerobically for 48 h. 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 [44,46].

### 2.10. Statistical analyses

One-way analysis of variance (ANOVA) was 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 sealer paste flow properties are plotted in Fig. 1 (mean  $\pm$  sd; n = 3). All the sealers had flow values within the ISO recommended range for endodontic sealers. Therefore, 5% of DMAHDM and 0.15% of NAg could be incorporated into



**Fig. 2 – Endodontic sealer paste film thickness (mean ± sd; n = 3). Adding 5% DMAHDM and 0.15% NAg did not negatively affect the film thickness, compared to AH Plus ( $p > 0.05$ ). All groups had film thickness values that were in compliance with the ISO requirements of no more than 50  $\mu\text{m}$ .**

BTH for the paste to still have sufficient flow as an endodontic sealer.

The sealer paste film thicknesses are plotted in Fig. 2 (mean ± sd; n = 3). Incorporating NAg at all mass fractions (0.05%, 0.1%, and 0.15%) did not significantly influence the film thickness of the sealer when compared to both experimental and AH Plus sealers ( $p > 0.05$ ). Adding 2.5% and 5% DMAHDM did not significantly influence the film thickness of the sealer when compared to both experimental and AH Plus control ( $p > 0.05$ ). When 5% DMAHDM was combined with 0.1% and 0.15% NAg, the film thickness was significantly increased when compared to experimental control ( $p < 0.05$ ), but was similar to that of AH Plus ( $p > 0.05$ ).

The dye penetration results are shown in Fig. 3. Representative stereomicroscopic images are shown in (A–E), with the quantification of dye penetration (mean ± sd; n = 10) in (F). All sealers demonstrated apical dye penetration values that were not significantly different from each other ( $p > 0.05$ ). Incorporating 5% DMAHDM and 0.15% NAg did not adversely affect the sealing properties when compared to both experimental and AH Plus controls ( $p > 0.05$ ).

Fig. 4 shows representative live/dead biofilm images on sealers. Live bacteria were stained green and dead bacteria were stained red. A yellow/orange color represented live and dead bacteria on top of each other. AH Plus and BTH had the most live bacteria. Sealers with 0.15% NAg or 5% DMAHDM showed increasing amounts of dead bacteria. When 5% DMAHDM and 0.15% NAg were combined, bacteria covering the sealer disks were mostly dead.

Polysaccharide production of 2-day biofilms on sealers is plotted in Fig. 5 (mean ± sd; n = 6). BTH+NAg and BTH+DMAHDM sealers significantly reduced the polysaccharide production when compared to experimental and commercial controls ( $p < 0.05$ ). Combining DMAHDM and NAg resulted in a greater reduction of polysaccharide production by biofilms ( $p < 0.05$ ).

CFU counts of 2-day biofilms on sealer disks are plotted in Fig. 6 (mean ± sd; n = 6). CFU counts on sealer disks con-

taining DMAHDM or NAg alone decreased the biofilm CFU, compared to both controls ( $p < 0.05$ ). Combining DMAHDM and NAg resulted in biofilm CFU reduction of 6 logs, which was much more than that achieved by either agent alone ( $p < 0.05$ ).

#### 4. Discussion

In the present study, a novel antibacterial root canal sealer with potent antibacterial properties was developed by combining NAg for its release of silver ions and DMAHDM for its long-term contact killing capability. The dual incorporation of a releasing antibacterial agent (NAg) and a non-releasing agent (DMAHDM) significantly improved biofilm inhibition. The BTH + DMAHDM + NAg sealer greatly reduced CFU counts and polysaccharide production of *E. faecalis* biofilms, without jeopardizing the physical properties and sealing ability of the endodontic sealer. Combining NAg+DMAHDM reduced the endodontic biofilm CFU by nearly 6 orders of magnitude, which was higher than that achieved by using either agent alone.

Endodontic infections are caused by bacterial biofilms harbored in dentin surfaces and extending deep into the dentinal tubules [1]. These biofilms sometimes communicate beyond the apical foreman, resulting in periapical lesions [1]. The root canal biofilm is characterized by a complex microbial community embedded in an extracellular polymeric substance (EPS) that provides mechanical stability, mediates the attachment of biofilms to dentin surfaces and provides several mechanisms of resistance against external stimulus [47]. Some of these mechanisms include: water retention, nutritional storage, exchange of genetic information, and resistance against host cells and antimicrobial agents [47]. In addition to the complexity of the root canal biofilm that makes it difficult to eliminate, the morphology of the root canal system is also complex in nature with accessory canals and apical deltas that have been associated with endodontic infections [48]. Due to the variations and irregularities in the root canal morphology, several walls may be left untouched with conventional instrumentation and irrigation protocols [49]. Therefore, developing

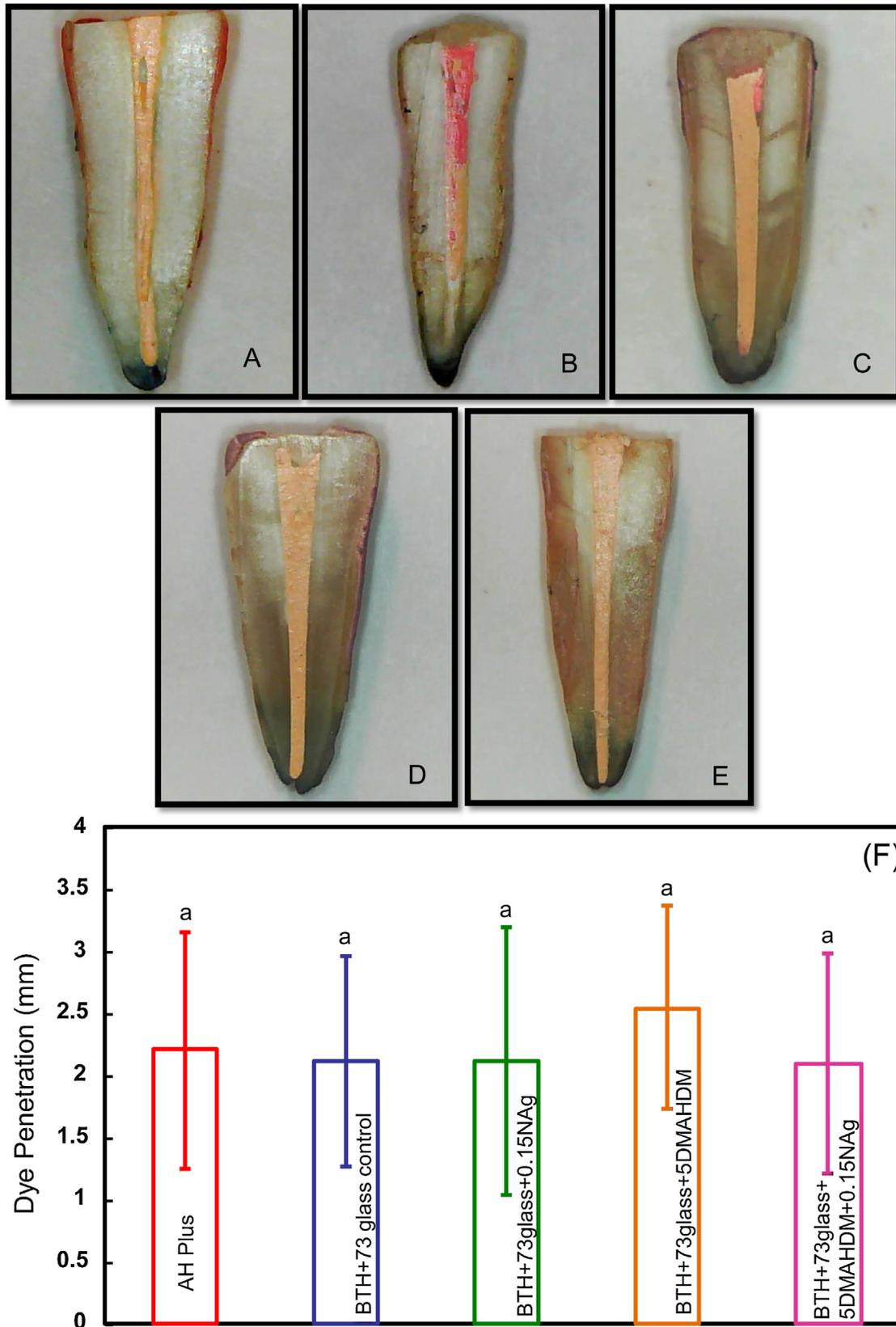
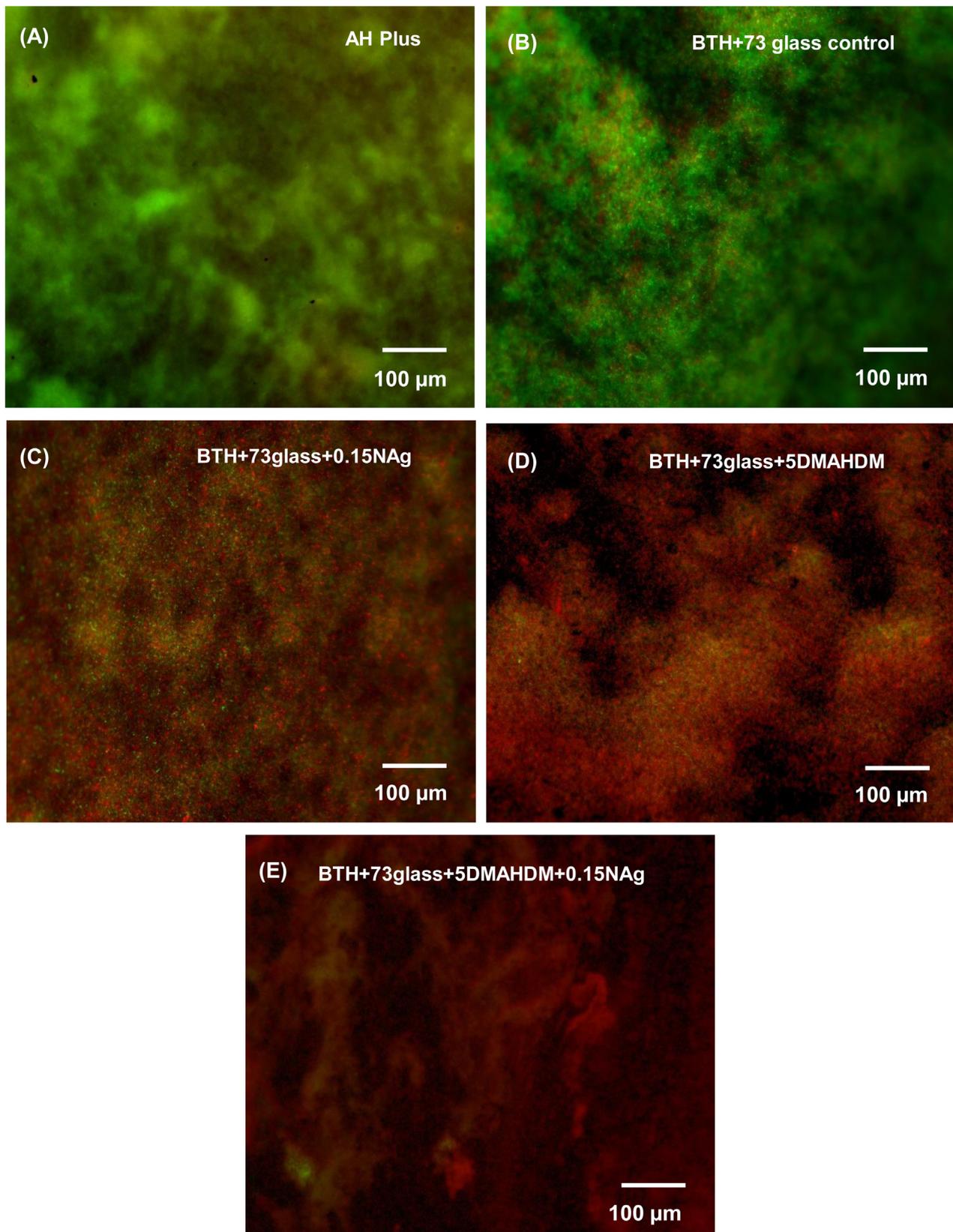
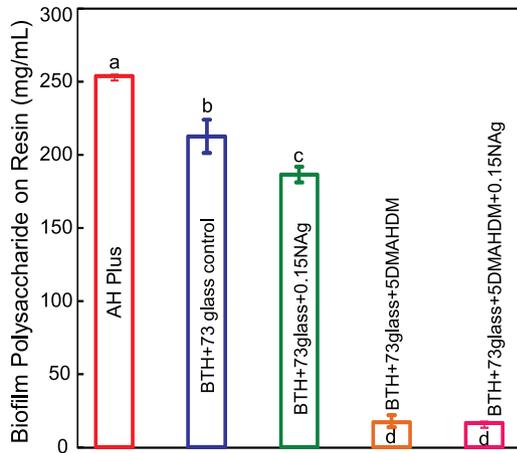


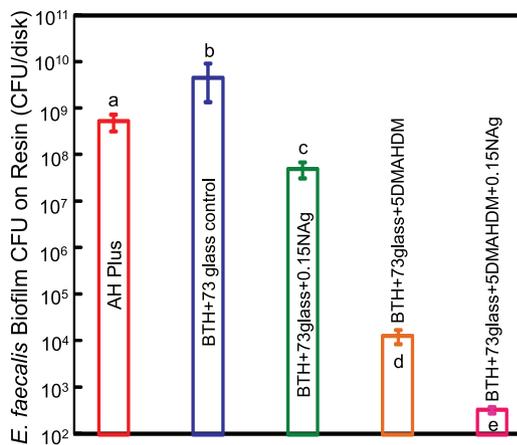
Fig. 3 – Stereomicroscopic images showing apical dye penetration: (A) AH Plus; (B) BTH + 73 glass control; (C) BTH + 73glass + 0.15NAg (D) BTH + 73glass + 5DMAHDM; (E) BTH + 73glass + 5DMAHDM + 0.15NAg. (F) Quantification of dye penetration (mean ± sd; n = 10). Incorporating 0.15% NAg and 5% DMAHDM did not significantly affect the sealing ability, compared to the sealer without NAg and DMAHDM ( $p > 0.05$ ). All experimental sealers showed sealing properties similar to that of AH Plus ( $p > 0.05$ ).



**Fig. 4** – Live/dead images of 2-day biofilms adherent on resins. Live bacteria were stained green. Bacteria with compromised membranes were stained red. Yellow/orange colors represent live and dead bacteria on top of each other. (A) AH Plus and (B) BTH + 73 glass control were primarily covered with live bacteria. (C) BTH + 73glass + 0.15NAg and (D) BTH + 73glass + 5DMAHDM showed less live bacteria and more dead bacteria. (E) BTH+73glass+5DMAHDM+0.15NAg were primarily covered with dead bacteria (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).



**Fig. 5 – Polysaccharide production by biofilms adherent on the disks (mean  $\pm$  sd; n = 6). Disks with NA<sub>g</sub> or DMAHDM alone significantly reduced polysaccharide production by *E. faecalis* when compared to AH Plus ( $p < 0.05$ ). Combining 0.15% NA<sub>g</sub> + 5% DMAHDM resulted in the least polysaccharide production by *E. faecalis*. Dissimilar letters indicate that values are significantly different from each other ( $p < 0.05$ ).**



**Fig. 6 – Colony-forming unit (CFU) counts of 2-day biofilms adherent on the disks (mean  $\pm$  sd; n = 6). Biofilm CFU on disks with NA<sub>g</sub> were more than 1 order of magnitude less than those on AH Plus ( $p < 0.05$ ). Biofilm CFU on disks with 5% DMAHDM were more than 4 orders of magnitude less than those on AH Plus ( $p < 0.05$ ). Combining both 0.15% NA<sub>g</sub> + 5% DMAHDM reduced biofilm CFU by 6 orders of magnitude ( $p < 0.05$ ).**

a bioactive root canal sealer with dual antibacterial agents that complement each other is highly desirable: (1) NA<sub>g</sub> can be released deep into the dentinal tubules to eliminate persisting microbes, and (2) DMAHDM can prevent bacterial ingress in case of leakage through contact-inhibition. This novel NA<sub>g</sub> + DMAHDM dual effect has the potential to play a greater role in preventing treatment failure and secondary endodontic infections beyond what was achieved by previous methods.

In order for the root canal sealer to exert its antimicrobial properties, it should be able to flow and diffuse into the complex anatomy of the root canal that are otherwise difficult to access and disinfect, without being overly flowable to prevent extrusion into the periapical tissues [50]. According to the ISO 6876/2012 standard, root canal sealers should have a minimum flow of 20 mm [39]. Incorporating NA<sub>g</sub> improved the flow when compared to AH Plus control. Using TBAEMA to dissolve silver salts could be the reason for the improved flow, as it can improve the solubility of silver salts in the resin by forming Ag–N bonds [37]. Although incorporating 5% DMAHDM significantly reduced the flow when compared to experimental control, it was still similar to that of AH Plus control and within the range of ISO recommendations.

Another important property for root canal sealers is film thickness. Root canal sealers are meant to fill and seal irregularities present between the core material and the dentinal wall [40]. However, according to previous studies, leakage usually occurs at the sealer-dentin interface, sealer-gutta-percha interface, or within the sealer itself [40,51]. Hence, it is recommended to occupy most of the canal space with the core material and minimize the volume of sealer being placed [40,52]. This is because areas occupied by the sealer are usually more vulnerable and susceptible to degradation [52]. Consequently, endodontic sealers usually provide better sealing properties when they are used in thin films [52]. In this study, combining DMAHDM with NA<sub>g</sub> did not significantly influence the sealer film thickness when compared to AH Plus control, and was still in compliance with the ISO requirements of sealers having a film thickness of no more than 50  $\mu$ m [39]. In addition to the eradication of root canal microbiota, establishing a hermetic seal of the root canal system is another goal of endodontic treatment [53]. Any leakage, whether coronal or apical, can result in bacterial ingress and treatment failure [53]. In addition, any gaps present at the sealer-dentin interface would allow water and fluid uptake by the resinous material, resulting in degradation of its mechanical properties [53]. In this study, a methacrylate resin-based sealer was formulated to allow the antibacterial agents to be chemically bonded to the resin matrix, and not just mechanically interlocked. This would allow for the addition of higher mass fractions of DMAHDM and NA<sub>g</sub> without sacrificing the physical and mechanical properties of the material. Methacrylate resin-based root canal sealers were developed to bond to root dentin and create a mono-block to prevent bacterial proliferation due to microleakage [54]. However, microleakage can still occur due to the forces of polymerization shrinkage and the unfavorable root canal configuration (c-factor) [54]. In addition, leaching of some uncured methacrylate monomers may hinder the healing process and regeneration of the damaged periapical tissues [55]. Although most cytotoxic reactions of polymeric root canal sealers decrease over time, future studies should include cytotoxicity assays and analysis of degree of conversion of our experimental resin to better predict the degree of elution of any uncured monomers [55].

Previously, bonding agents composed of similar resins to the one used in this study were able to copiously infiltrate the dentinal tubules and form numerous resin tags [24]. Previous studies incorporating DMAHDM or NA<sub>g</sub> into methacrylate bonding agents did not adversely alter their bonding prop-

erties [44,56]. In this study, the sealing ability of the root canal sealer was tested instead of its bond strength. This is because materials with good bond strength can still result in microleakage, and therefore investigating the microleakage of endodontic materials is of a greater importance in clinical applications [53]. In this study, incorporating DMAHDM and NAg into the novel sealer did not negatively influence its sealing properties. In addition to the well-established antibacterial properties of QAMs, previous studies showed the ability of QAMs to inhibit dentin matrix metalloproteinases (MMPs) that play a crucial role in the biological degradation of the resin–dentin bond [57,58]. Previous studies tested the inhibitory effects of MDPB on soluble and matrix-bound MMPs and their effects on the durability of the resin–dentin bond. The authors reported significant inhibition of MMPs and improvement in the bond durability via MDPB [57,58]. Another study tested dimethylaminododecyl methacrylate (DMADDM) as an inhibitor for MMPs [59]. The results of that study showed significant reduction in MMPs that appeared to be concentration dependent. Ninety percent inhibition of MMPs was achieved with DMADDM at a concentration of 5% [59]. In this study, 5% DMAHDM was incorporated into the root canal sealer. Due to the similarity in chemical structure and properties between DMADDM and DMAHDM, a similar trend in MMPs inhibition is expected, which would greatly help protect the collagen in the hybrid layer of the bonded interface. However, further studies are required to show the ability of DMAHDM to act as an anti-MMP agent that could improve the durability of the endodontic sealer–dentin bond.

In a previous study, mass fractions of 2.5%, 5%, 7.5% and 10% of DMAHDM were incorporated into a bonding agent to test the live and dead biofilm volumes and live bacteria percentage along the entire biofilm thickness vs. the distance away from the resin surface [60]. Incorporating 10% DMAHDM resulted in dead bacteria along the entire thickness of the biofilm, which was speculated to be the result of a stress condition in bacteria that triggered a built-in programmed cell death (PCD) in the surrounding bacteria. At 2.5% and 5% DMAHDM, bacterial reduction was mainly on or near the resin surface, and the bacteria away from the resin surface were mostly alive [60]. In our present study, greater concentrations of DMAHDM were not included, based on the results of previous studies that showed compromised mechanical and bonding properties of the material at higher DMAHDM mass fractions [44,56].

Advanced disinfection strategies have been developed to enhance elimination of endodontic biofilms. Silver salt has been widely used as antibacterial, antifungal, and antiviral agent [61]. With its wide spectrum antibacterial activity, it could inactivate some of the most vital enzymes necessary for DNA replication and cell wall synthesis [61]. With the advancements in nanotechnology, NAg have been developed and incorporated into dental resins, producing potent anti-microbial effects without compromising the physical or mechanical properties [37,62]. Due to their increased surface area, low concentrations of NAg were needed to produce potent antibacterial properties, without compromising the color of the material [63].

A previous study showed the ability of a NAg-containing bonding agent to inhibit bacteria both on the surface of the resin disks and the bacteria away from the surface, in the cul-

ture medium through the release of silver ions [64]. However, materials releasing silver ions tend to lose their antibacterial activity over time due to the rapid ion release and therefore become depleted over time. Complementing NAg with antibacterial agents that provide long-lasting contact-killing ability is highly desirable. QAMs have been developed and immobilized in a resin matrix so that it does not leach out [63,65]. Increasing the alkyl chain length (CL) of QAMs can improve their antibacterial properties through the penetration of the long CL into bacterial membranes, which results in discontinuity of the cell wall and subsequent release of cellular components [27]. DMAHDM with chain length 16 was developed and incorporated into composites and adhesives and showed enhanced potency in anti-microbial activity [66,67].

In spite of DMAHDM's ability to produce long-term killing, the microbial killing usually occurs at the surface of the resin and not away from the surface, and therefore bacteria residing within the depths of dentinal tubules can still grow and proliferate. However, silver ions released from the NAg containing resin can kill remote bacteria from the resin surface. Therefore, DMAHDM and NAg can work synergistically to eliminate root canal biofilms. In a previous study, NAg and QADM were mutually incorporated into a bonding agent. The results showed the ability of QADM to greatly reduce bacteria coming in contact with the resin surface while having little effect on bacteria in the culture medium. However, NAg was able to inhibit both bacteria in contact or distant from the resin surface [68]. A previous study investigated the effects of NAg and DMAHDM when incorporated into an orthodontic cement [69]. The results showed that DMAHDM or NAg alone greatly reduced the CFU counts, lactic acid production and metabolic activity. However, when the two agents were combined together, an additional reduction was observed. This was accomplished without adversely affecting the shear bond strength even after water-aging for 30 days [69]. Recently, 2.5% DMAHDM and 0.15% NAg were incorporated into a commercial epoxy-resin sealer (AH Plus) [34]. The authors assessed the physical and antibacterial properties over 14 days utilizing the direct contact test (DCT). After 1 day, AH Plus + 0.15% NAg had a significant CFU reduction of one log, but showed no reduction after 7 and 14 days [34]. This was unlike groups with DMAHDM that showed significant CFU reductions at all-time points. Their results showed a 3 log reduction when NAg and DMAHDM were combined, against planktonic *E. faecalis*, without testing biofilms [34].

The present study achieved biofilm CFU reduction of 6 logs, which was 1000 times more potent than that of the previous study [34]. This likely was due to two reasons. First, DMAHDM is a methacrylate-based antibacterial agent and NAg is a mixture of silver salts dissolved in a methacrylate-based monomer. Therefore, mixing these agents into an epoxy resin-based sealer would allow them to be mechanically-interlocked only, without forming any chemical bonds. When the authors of the previous study added high concentrations of DMAHDM, the physical properties were compromised, and therefore, only low concentrations were used [34]. In contrast, in the present study, relatively high mass fractions of DMAHDM and NAg were added without jeopardizing the physical and sealing properties, because these agents were chemically bonded into the polymer matrix and not just mechanically mixed in. Sec-

ond, the antibacterial effects of the modified AH Plus sealer in the previous study were expected to be of a short duration [34]. This was due to the fact that DMAHDM and NAg were not chemically bonded in the polymer matrix but were simply mechanically mixed and hence could be lost over time. Although it is expected that silver ions released from the NAg-containing resin could become depleted over time and thus could lose the antibacterial effects, previous studies showed no change in the antibacterial properties of NAg-containing methacrylate-based bonding agent after being water-aged for 6 months [70]. However, when NAg was incorporated into AH Plus sealer, the antibacterial effects were lost in 7 days, which was likely due to the incompatible mixing of these agents into an epoxy-based sealer accelerating the depletion of silver ions [34]. Therefore, the present study was able to incorporate higher concentrations of NAg and DMAHDM. This was accomplished by formulating a methacrylate-based sealer that allowed the addition of these agents in higher concentrations without jeopardizing the sealer properties. In addition, the antibacterial effects of NAg and DMAHDM were investigated against *E. faecalis* biofilm, which gives a better representation of the effects of these agents if applied inside the complex root canal.

One limitation of this study was that the biofilm tests were performed on resin disks. Therefore, an additional pilot experiment was performed using a root canal biofilm model. This was because dentin can inactivate or reduce the effects of antibacterial agents, and endodontic bacteria can reside in dentinal tubules to escape the antibacterial agents [1]. We performed a pilot test where *E. faecalis* bacteria were impregnated into the root dentin utilizing the power of centrifugation to facilitate the bacteria movement into dentinal tubules, as described by previous studies [71,72]. The *E. faecalis*-impregnated root dentin was then treated with the new sealer containing 5% DMAHDM and 0.15% NAg. Root dentin sections treated with distilled water served as control. The sealers were applied and allowed to set. After 7 days at 37 °C and 100% relative humidity, the bacteria were harvested using a sonication/vortexing method [73]. The harvested bacteria were serially diluted, plated on BHI agar, and incubated for 48 h at 37 °C with 5% CO<sub>2</sub> to count the CFU (n = 4). The results showed that the control group had much higher CFU counts than the group treated with the sealer containing DMAHDM and NAg, at (10<sup>7.4</sup> ± 0.16) vs. (10<sup>4.7</sup> ± 0.28) CFU/mL (p < 0.05), representing a nearly 3 log reduction. This pilot experiment using a root canal biofilm model confirmed the results of the antibiofilm experiments performed on resin disks. A future study using a multi-species root canal biofilm model needs to be performed. In addition, studies investigating the long-term antibacterial effects of DMAHDM and NAg are also needed on endodontic biofilms.

## 5. Conclusions

A novel bioactive endodontic sealer was developed containing dual antibacterial agents of DMAHDM and NAg with potent antibiofilm capabilities, without compromising the physical and sealing properties of the sealer. A commercial endodontic sealer showed no antibacterial properties after setting, while

combining DMAHDM and NAg into the novel sealer greatly reduced biofilm formation and viability. The biofilm CFU was reduced by six orders of magnitude when the two agents were both incorporated compared to the commercial control sealer. The new sealer could provide two main benefits: (1) enhance microbial elimination as an adjunct method to conventional endodontic therapy; and (2) prevent secondary endodontic infections and future recontamination of the canal system. The use of dual agents of DMAHDM and NAg is promising for applications to a wide range of dental resins to achieve potent anti-biofilm functions to combat oral pathogens.

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