In Vitro Evaluation of Antibacterial Effect of AH Plus Incorporated with Quaternary Ammonium Epoxy Silicate against *Enterococcus faecalis*

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Abstract

Introduction: The purpose of this study was to evaluate the *in vitro* antibacterial effect of AH Plus (Dentsply, DeTrey, Konstanz, Germany) incorporated with quaternary ammonium epoxy silicate (QAES) against *Enterococcus faecalis*. **Methods:** QAES particles were synthesized by the cocondensation of tetraethoxysilane with 2 trialkoxysilanes (3-[trimethoxysilyl]propyldimethyloctadecyl ammonium chloride and 3-glycidyloxypropyltrimethoxysilane) through a 1-pot sol-gel route. Dried QAES particles were then characterized by attenuated total reflection Fourier transform infrared spectroscopy and scanning electron microscopy. AH Plus sealers incorporated with 0–8 wt% QAES were tested after 4 weeks of water aging to assess the *in vitro* antibacterial activity against *E. faecalis* by the direct contact test (DCT) and 3-dimensional image analysis of live/dead-stained *E. faecalis* biofilms using confocal laser scanning microscopy. **Results:** The Fourier transform infrared spectroscopy spectrum of QAES particles revealed the coexistence of the characteristic absorbance band of the siloxane backbone (Si-O-Si) from 1,000–1,100 cm^{-1}, epoxide band peaking at ~916 cm^{-1}, and C-N stretching vibration peaking at 1,373 cm^{-1}. The scanning electron microscopic image showed the spherical morphology of QAES particles with ~120 nm in diameter and a rough surface. DCT results revealed that AH Plus alone (0 wt% QAES) after 4 weeks of water aging had no inhibitory effect on *E. faecalis* growth ($P = .569$). AH Plus incorporated with QAES (2–8 wt%) showed antibacterial activity against *E. faecalis* as shown in DCT and biofilm viability results ($P < .001$). **Conclusions:** The incorporation of QAES into epoxy resin–based AH Plus may be a promising approach for controlling endodontic infection at the time of canal filling and preventing subsequent reinfection. *(J Endod 2014;■:1–5)*

Key Words

AH Plus, antibacterial, biofilm, *Enterococcus faecalis*, ORMOSIL, quaternary ammonium

The ultimate goal of endodontic treatment is to eliminate microorganisms localized in root canal systems and to prevent reinfection through completely filling the canals with stable materials. However, current strategies can hardly achieve complete elimination of biofilms from endodontic sites, leaving 40%–60% of the root canals still positive for bacterial presence (1). The bacteria that survive in the root canals at the time of filling have been shown to increase the possibility of post-treatment apical periodontitis (2, 3). *Enterococcus faecalis* is the most frequently isolated species; it is recovered in over one third of the canals of root-filled teeth with persisting periapical infection (4, 5). When growing as a biofilm in the root canal system, *E. faecalis* can endure nutritional deprivation and becomes highly resistant to irrigants and medicaments, including sodium hypochlorite and calcium hydroxide (6, 7).

By penetrating into dentinal tubules and contacting survived bacteria after chemomechanical preparation, endodontic sealer with antimicrobial activity can kill bacteria on contact and thus holds promise for potential applications in controlling microbial infection. Literature abounds on antimicrobial sealers that are based on the sustained release of antimicrobial agents, such as amoxicillin (8), chlorhexidine, and cetrimide (9). Despite their proven antibacterial effect against *E. faecalis*, their activities are expected to disappear when these agents are depleted. In addition, the released antimicrobial agents under minimum inhibitory concentration during the tail-release phase may contribute to the possible development of microbial resistance (10). Moreover, the release of antimicrobial compounds may compromise the integration of sealer with core or dentin, which creates the risks for bacterial recolonization (11). In this sense, sealers with inherent antibacterial activity that have minimal leaching are still in great need.

Polymerizable antibacterial macromolecules bearing quaternary ammonium have been triumphantly introduced into dental resinous materials, yielding nonleaching antimicrobial activities (12). Although the exact antimicrobial mechanism of quaternary ammonium has yet to be determined, it is suggested that it causes lysis of bacteria by binding to and puncturing their cell wall components. This leads to leakage of the cytoplasmic components that ultimately results in bacterial death (13). One excellent example of this concept is 12-methacryloyloxydodecylpyridinium bromide, which has been successfully incorporated into methacrylate-based materials, such as dentin primer (14), adhesive (15), and resin composite (16). However, there is no report on quaternary ammonium–functionalized antimicrobials that can be copolymerized with epoxy resin–based endodontic sealers, such as AH Plus (Dentsply, DeTrey, Konstanz, Germany). In the present study, a technique for synthesizing quaternary ammonium epoxy silicate (QAES) particles with dual functionalities by cocondensation of tetraethoxysilane (TEOS) with 2 trialkoxysilanes, 3-(trimethoxysilyl)propyldimethyloc-

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tadecyl ammonium chloride (SiQAC), and 3-glycidyloxypropyltrimethoxysilane (3-GPTS) was established. The quaternary ammonium from SiQAC is responsible for the antimicrobial potential of QAES (17), whereas the epoxy group from 3-GPTS provides the feasibility to copolymerize with the polymer network of epoxy resin–based materials. The *in vitro* antibacterial activity of AH Plus incorporated with QAES that had been water aged for 4 weeks was assessed by the direct contact test (DCT) and 3-dimensional (3D) image analysis of live/dead-stained biofilms that were grown on sealer-coated surface using confocal laser scanning microscopy (CLSM).

Materials and Methods

Unless stated otherwise, all chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO) without further purification.

Synthesis and Characterization of QAES Particles

QAES particles were synthesized by a silane-based sol-gel reaction. Briefly, 20 mL ethanol, 100 mL MilliQ water (EMD Millipore Corp, Billerica, MA), and 0.7 mL hydrochloric acid (2 mol/L) were mixed and stirred at 1,200 rpm. To this clear solution, 5 mL mixture of TEOS, 3-GPTS, and SiQAC (9:1:1 molar ratio) that were predissolved in 20 mL ethanol was rapidly added. After 1 minute, 1.4 mL sodium hydroxide (2 mol/L) was added, and the stirring speed was reduced to 350 rpm. After 2 hours of reaction at room temperature, QAES particles were harvested through centrifuging the homogeneous solution at 7,500 rpm and then washed by a copious amount of ethanol. Synthesized QAES particles were vacuum dried and stored in tightly sealed vial until use.

Dried QAES particles were characterized by attenuated total reflection Fourier transform infrared spectroscopy and scanning electron microscopy. Fourier transform infrared spectrum was recorded between 4,000 and 400 cm^{-1} using a Fourier transform infrared spectrometer (Nicolet 6700; Thermo Scientific, Waltham, MA) with an attenuated total reflection setup at a resolution of 4 cm^{-1}. The morphology of QAES particles was observed under a field emission scanning electron microscope (Quanta 450 FEG; FEI, Eindhoven, The Netherlands) operating at 15 kV.

Experimental QAES Containing Sealers

Epoxy resin–based endodontic sealer AH Plus sealers were prepared according to manufacturers' recommendations (ie, mixing mass ratio is 1 g paste A [epoxy resin matrix] to 1.18 g paste B [amine catalyst]). QAES particles were added at 0, 2, 4, or 8 wt% concentration.

Bacteria Preparation

E. faecalis ATCC 29212 (American Type Culture Collection, Manassas, VA) was used in this study. The subcultured bacterial strain was grown in brain-heart infusion (BHI) broth (Difco, Becton-Dickinson and Co, Sparks, MD) overnight in an anaerobic atmosphere at 37°C. Bacterial pellets were harvested by centrifugation at 2,500 rpm for 5 minutes at 4°C. Bacterial suspension was adjusted to 1×10^8 colony-forming units/mL by resuspending the cell pellet with the appropriate amount of sterile growth medium according to a regression line derived from McFarland turbidity standards (Pro-Lab Diagnostics, Richmond Hill, ON, Canada).

DCT

First introduced by Weiss et al in 1996 (18), DCT has been proven to be a reliable, quantitative, and reproducible method that allows the detection of an antimicrobial effect of insoluble materials based on turbidimetric determination of bacterial growth in wells of microtiter plates coated with test materials (19–21). Sidewalls of 96-well plates (Corning Costar, Tewksbury, MA) were coated with equal amounts of freshly mixed sealers containing varied concentrations of QAES (ie, 0–8 wt%). Sealers from each group were applied in 6 replica wells ($N = 6$). After 8 hours of setting, cured sealers were subjected to water aging by placing 300 μL distilled water in each well at 37°C for 4 weeks. The coated plates were disinfected with ultraviolet light for 2 hours before inoculation of bacterial suspension; 10-μL *E. faecalis* bacterial suspension (approximately 10^6 bacteria) was applied on the test material. Bacterial suspension placed on uncoated wells was used as the control. After incubation in a humid atmosphere at 37°C for 1 hour, evaporation of liquid was evident to ensure direct contact between bacteria and material surface and 200 μL sterile BHI broth was then added into each well of the 5 groups (ie, uncoated wells and wells coated with sealers containing 0, 2, 4, 8 or wt% QAES). After 5 hours of incubation, the 96-well plate was placed in a microplate spectrophotometer (Bio-Rad, Hercules, CA) to determine turbidimetric readings at 630 nm as instructed. The DCT tests were conducted twice independently. The results were analyzed by 1-way analysis of variance followed by Tukey multiple comparison at $\alpha = .05$.

Biofilm Viability

Freshly mixed sealer pastes were spread in a thin layer on round disks (diameter = 10 mm) cut from a Teflon (DuPont, Wilmington, DE) sheet. After setting, sealer-coated surfaces were polished using silicon carbide papers from 500- to 1,500-grit sheets to achieve planar substrates for biofilm formation. Cured sealer coatings were then water aged at 37°C for 4 weeks. Ultraviolet light–sterilized sealer-coated disks were placed into wells of 12-well plates containing 2.7 mL BHI broth. Each well was inoculated with 0.3 mL aforementioned *E. faecalis* suspension. The disks were incubated under anaerobic conditions at 37°C for 1 week. Subsequently, the disks were rinsed with 0.85% sodium chloride saline to remove the unattached bacteria and BHI broth.

The *E. faecalis* biofilms grown on the sealer-coated surfaces from each of the 4 groups (surfaces coated with sealers containing 0, 2, 4, or 8 wt% QAES) were stained using a LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Eugene, OR) as instructed and then imaged using CLSM (OLYMPUS FV500, Tokyo, Japan) at excitation wavelengths of 488 nm (SYTO 9) and 568 nm (propidium iodide), respectively. For each of the 6 sealer-coated disks in a particular group, an image stack (Z-stack) was obtained at a location that was characteristic of each biofilm on that disk. Images (field size = 212 × 215 μm) were acquired at a 2-μm z-step (the distance between 2 adjacent images of a stack) beginning from the bottom of the biofilm that was in contact with the sealer surface to the top of the biofilm ($N = 6$). Three-dimensional (3D) image analysis was performed for each stack using image analysis software (BioImageL v2.1; Faculty of Odontology, Malmö University, Malmö, Sweden) (22). Two measurement parameters were used: biovolume of interest and contact surface biomass. The biovolume of interest, representing the first 12 μm of each Z-stack (ie, 1st–7th images), was analyzed for the percentage distribution of live and dead bacteria. The contact surface biomass (biomass along the basal layer of each biofilm, ie, the first image of a Z-stack), representing direct contact of the microbes with the sealer-coated surfaces, was also analyzed to identify the effect of contact killing by the immobilized QAES. For each parameter, the data were analyzed by 1-way analysis of variance followed by Tukey multiple comparison at $\alpha = .05$.

Results

As shown in Figure 1, the Fourier transform infrared spectrum of QAES revealed the characteristic absorbance band of siloxane

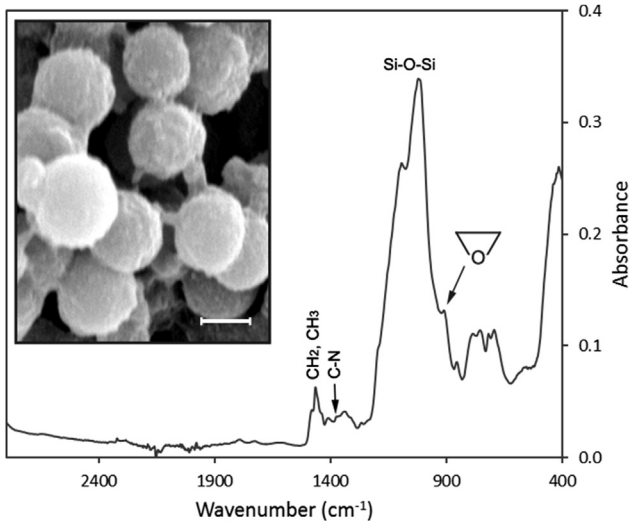

Figure 1. The FTIR spectrum of QAES particles displays the presence of the characteristic absorbance band of the siloxane backbone (Si-O-Si) from 1,000–1,100 cm^{-1} as well as epoxide band peaking at ~916 cm^{-1} and C-N stretching vibration peaking at 1,373 cm^{-1}. The scanning electron microscopic image (*inset*) shows the spherical morphology of QAES with ~120 nm in diameter. Scale bar = 100 nm.

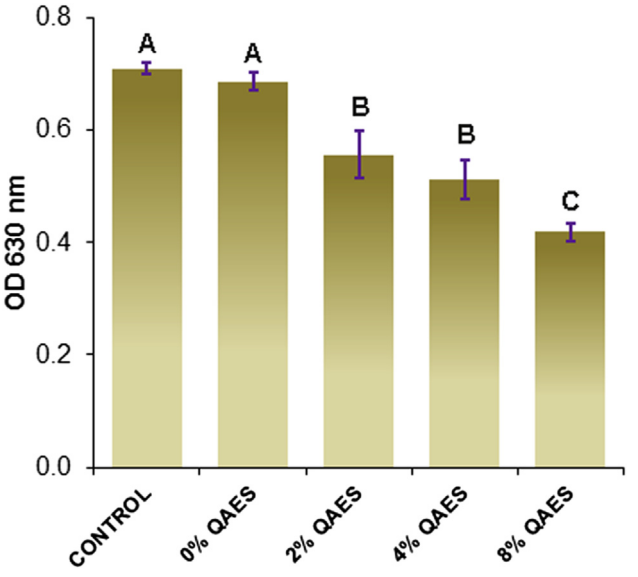

Figure 2. Growth of *E. faecalis* after being in contact with sealers containing varied concentrations of QAES (0–8 wt%) for 5 hours. Bacterial suspension placed on uncoated wells was used as the control. The growth is assessed as the average absorbance measurements at 630 nm in 6 replica wells ($N = 6$). Groups with the same upper case letters are not statistically significant ($P > .05$).

backbone (Si-O-Si) from 1,000–1,100 cm^{-1} that is assigned to asymmetric stretching vibration of Si-O-Si groups. Two separate peaks were identified, indicating 2 components corresponding to Si-O-Si groups in cyclic (~1,080 cm^{-1}) and linear (~1,040 cm^{-1}) configurations (23). Epoxide band peaking at ~916 cm^{-1} and C-N stretching vibration peaking at 1,373 cm^{-1} implied the presence of the epoxy group and alkylammonium chain from SiQAC that are covalently linked to siloxane backbone. The inserted scanning electron microscopic image showed the spherical morphology of QAES particles with ~120 nm in diameter and a rough surface.

The growth of *E. faecalis* was reflected in absorbance measurements at 630 nm of bacterial suspension. The mean growth after 5 hours was presented in Figure 2. Compared with the control group, AH Plus alone (0 wt% QAES) after 4 weeks of water aging showed no inhibition of bacterial growth ($P = .569$). In sharp contrast, the incorporation of QAES at a concentration as low as 0.4 wt% resulted in the reduction of bacterial growth with statistical significance ($P < .001$). The viability of *E. faecalis* biofilms grown on sealer-coated surfaces was shown in Figure 3 and Supplemental Figure S1 (Supplemental Figure S1 is available online at www.jendodon.com). For both parameters (ie, biovolume of interest and contact surface biomass), there were significant reductions of live bacteria within the biofilms that were dependent on the concentration of QAES incorporated into AH Plus sealers ($P < .001$). A representative distribution of live/dead bacteria within biofilms grown on surfaces coated by sealer with different concentrations of QAES was shown in Figure 3; the distributions of live (green) and dead (red) bacteria within the biovolume of interest of those biofilms are depicted as 3D plots in the left column, whereas the percentage distributions of live and dead bacteria at each level of a Z-stack were summarized in the line plots in the right column.

Discussion

To evaluate the antibacterial effect of QAES containing sealers on sessile *E. faecalis*, we used 2 methods: DCT and viability analysis of biofilms. Compared with the traditional agar diffusion test, DCT is more suitable for water-insoluble materials with nonleaching antibacterial properties (24). However, 1 drawback of DCT is that the results are based on the use of bacterial suspension (ie, planktonic cells). There is evidence that sessile microorganisms in the biofilms are up to 1,000 times more resistant to antibacterial agents than the same bacteria in planktonic status (25). Importantly, the current concept in endodontic microbiology considers endodontic disease as a biofilm-induced infection, emphasizing the importance of biofilm formation as an adaptive mechanism on how bacteria survive in persisting endodontic infection (26). In current study, 3D imaging of live/dead-stained *E. faecalis* biofilms characterized live and dead bacterial distributions within biofilms, which allowed us to investigate the antibacterial effects of QAES containing sealers on sessile *E. faecalis*.

AH Plus is an epoxy resin–based root canal sealer that has been commonly used in endodontic practice because of its excellent sealing ability (27), cytocompatibility (28), and tissue tolerance (29). As a polymeric material, unpolymerized residues (ie, epoxide and amine) may be released into the surrounding milieu during the setting process, resulting in provisional antibacterial activity, as suggested by Kayaoglu et al (11). However, Pizzo et al (30) reported that AH Plus sealer has no inhibitory effect after 24 hours, which is consistent with our current findings. The incorporation of insoluble particles may render endodontic sealers with antibacterial properties without a discernable decrease over a certain period of aging time (24). This enhanced antibacterial property is desirable in endodontic sealers because the sealers can achieve good control of microbial infection by killing microorganisms in the dentinal tubules that come in contact and avoiding reinfection. Indeed, our current *in vitro* study revealed that cured AH Plus sealers incorporated with QAES particles can remain antibacterial activities even after 4 weeks of water aging.

Theoretically, permanent antibacterial activity can only be achieved on nonleaching antimicrobial surfaces that kill bacteria on contact (31). The rationale of QAES synthesis is based on a sol-gel route that uses TEOS as an anchoring unit for SiQAC and 3-GPTS, producing organically modified silicate (ORMOSIL) bearing 2 covalently bound organic functionalities: the antimicrobial alkylammonium chain and the epoxy group. The

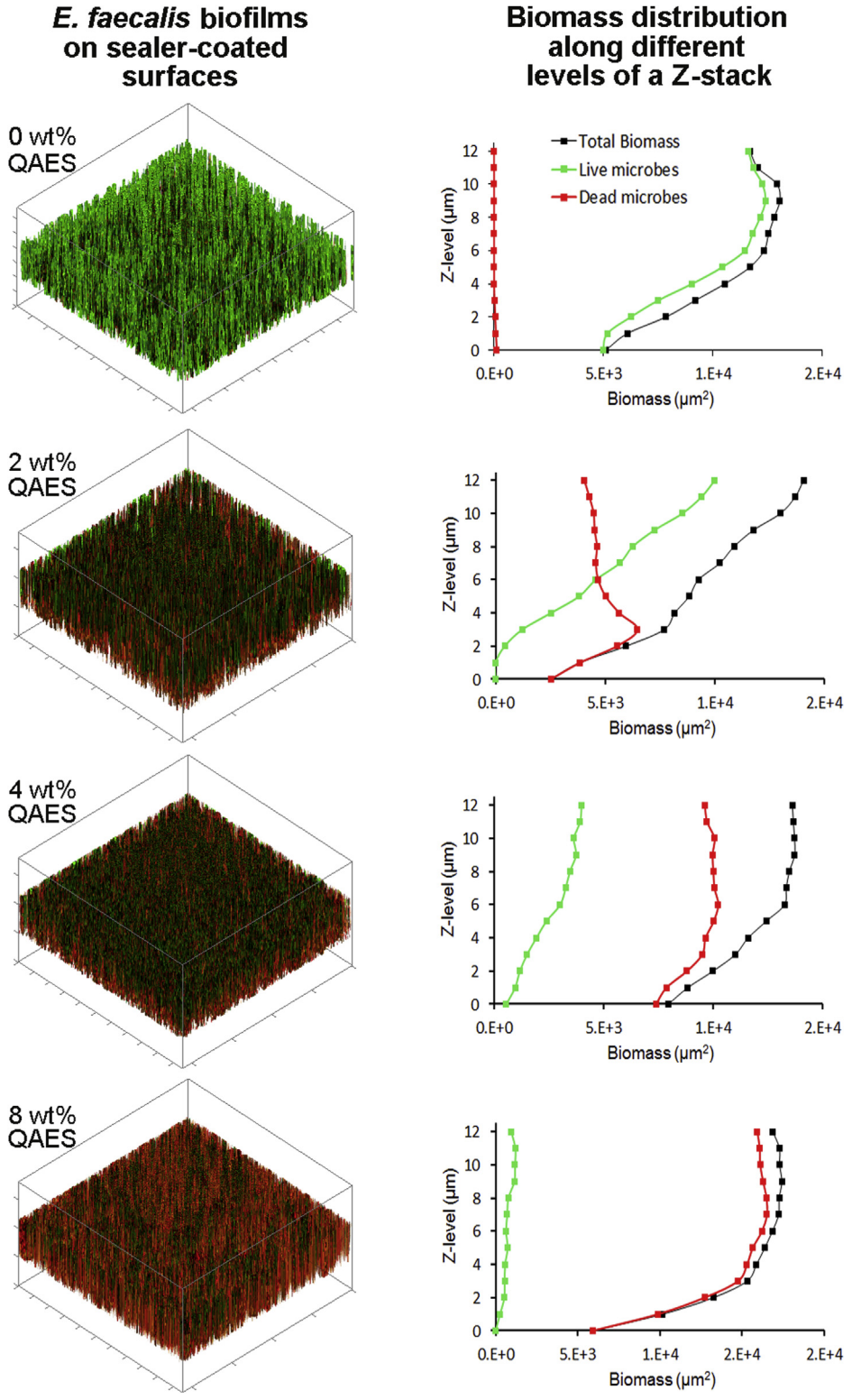

Figure 3. Representative live/dead-stained *E. faecalis* biofilms that grown on sealer-coated surfaces. *Green:* live bacteria (bacteria with intact cell membranes); *red:* dead bacteria (bacteria with compromised cell membranes). *(Left column)* 3D plots of the 12-μm-thick biovolume of interest from each biofilm. *(Right column)* Biomass of live and dead bacteria as a function of biofilm level (z-step = 2 μm).

latter enables the copolymerization of QAES with epoxy resin–based AH Plus, the curing mechanism of which relies on the polyaddition reaction of epoxide and amine. Hence, the immobilized antimicrobial alkylammonium chain that does not leach from the cured AH Plus is proposed to contribute to the antibacterial activity of 4-week water-aged sealer against *E. faecalis* in our study. It is worthy to note that dead bacteria are not only confined to the substrate surface in the current study (Fig. 3). However, one cannot draw the conclusion that the diffusion of noncovalently bound

QAES from the sealer is responsible for the killing of bacteria within the bulk of a biofilm given that toxic substances released by dead bacteria that are in contact with the substrate surface may also have resulted in a reduction of the viable biovolume (32). A previous study has shown successful immobilization of antimicrobial methacrylate–functionalized silicates into polymethyl methacrylate resin by forming covalent bonds, which allows negligible leaching of antimicrobials over 3 months of water aging (33). Further study would be necessary to validate the minimal release of QAES after long-term water aging.

Although antibacterial endodontic sealers incorporating quaternary ammonium particles have been reported recently (34, 35), to the best of our knowledge, this is the first report about quaternary ammonium–functionalized antimicrobials that can be copolymerized with epoxy resin–based AH Plus. With respect to the complexity of the root canal system in which QAES containing AH Plus will function as a commercial product, the following considerations have to be taken into account: the impact of QAES on the physicochemical and mechanical properties (ie, setting time, adhesion to dentin, flow behavior, and so on) of AH Plus, the penetrating ability of sealers into dentinal tubules, the localization of QAES in the tubules, and the antibacterial activity of QAES containing sealer in the root canal infection model. Furthermore, the cytotoxicity of the sealer in relationship to human cells should be evaluated, in particular, when the sealer is extruded during obturation. The aforementioned also designate what is required to be accomplished in further investigations before clinical testing.

In conclusion, this study showed the feasibility of synthesizing an antibacterial nanoparticle that can be copolymerized with epoxy resin–based sealer. AH Plus containing QAES possessed antibacterial activity against *E. faecalis* both in planktonic and sessile status after 4 weeks of water aging as revealed by DCT and confocal laser scanning microscopic analysis of biofilm viability *in vitro*. This pilot study laid the foundation for further study of using AH Plus sealer containing QAES in root canal infection models.

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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2014.03.010).

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