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Microbial biofilm proliferation within sealer-root dentin interfaces is affected by sealer type and aging period

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Abstract

Background—Root canal fillings are intended to prevent microbial proliferation over time in the canal after treatment.

Objective—To assess biofilm proliferation within the sealer-dentin interfaces of two methacrylate resin-based systems, self-etch (SE) and total-etch (TE), and an epoxy resin-based sealer (EP), aged for up to 6 months.

Methods—Standardized specimens (n=45) comprising the coronal 5 mm of human roots were filled with the test materials and gutta-percha. Specimens were either not pre-incubated (control; n=9), or incubated in sterile saline for 1 week, 1 month, 3 months or 6 months (n=3/group). Monospecies biofilms of *Enterococcus faecalis* were grown on the specimens for 7 days in a chemostat-based biofilm fermentor mimicking pathogenic oral conditions. The extent of *E. faecalis* proliferation within the sealer-dentin interface for each material and incubation period group was assessed using fluorescence microscopy of dihydroethidium-stained specimens.

Results—TE had less biofilm proliferation than both EP and SE (p<0.01). Deeper biofilm proliferation was detected in SE and EP specimens aged for 1 and 3 months than those aged for 1 week or 6 months (p<0.05). Maximum depth of biofilm penetration was recorded for SE at 1 month (p<0.05).

Conclusion—Within the test model used, the self-etch and epoxy resin-based sealers were more susceptible to interfacial biofilm proliferation than the total-etch restorative material. This susceptibility diminished after aging the materials' interfaces for 6 months.

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Keywords

resin-dentin interface; endodontic sealer; resin-composite; biofilm; *E faecalis*; fluorescence microscopy

Introduction

Root canal fillings, comprising a core and a flowable sealer, should prevent bacterial ingress into the canal after treatment (1). Sealers that adhere or bond to root dentin (2) are expected to resist bacterial proliferation within the sealer-dentin interface (3). Epoxy resin (ER)-based sealers adhere to dentin and are considered the “gold standard” (4). Methacrylate resin (MR)-based sealers bond to conditioned dentin (5) by penetrating the tubules (6) and interlocking with dentin collagen forming a hybrid layer (2, 7, 8). Two main MR-based systems are currently available: i) “Total-etch” systems, requiring acid-etching, priming and bonding to form a hybrid layer (8). Although they are the benchmark for bonded restorative systems (7), they are not available as commercial endodontic sealers. ii) “Self-etch” systems employ one-step etching, priming and bonding, incorporating the smear layer into the hybrid layer (9). They are available as endodontic sealers, but concerns have recently emerged about inadequacy of their bond (9, 10).

Bonding MR-based sealers to root dentin is challenging (2, 10, 11). Resin polymerization is inhibited by dentin exposure to sodium hypochlorite (12), shrinkage-related debonding occurs due to unfavorable cavity configuration (13), and interfacial degradation over time, allowing salivary and tissue fluid movement between the hybrid layer and dentin (14), may lead to bacterial proliferation and re-infection of the tooth (15).

Sealer-dentin interfaces have been studied using *in-vitro* models measuring penetration of dyes (16), endotoxins (17), inoculated bacteria (18), or saliva (19), and by assessment of their mechanical (10) and physicochemical (20) properties, with questionable clinical relevance (10, 20, 21). Our group has introduced the use of the chemostat-based biofilm fermentor (CBBF) for assessment of interfacial bacterial biofilm proliferation of the cariogenic biofilm organism *Streptococcus mutans* after aging of MR-dentin specimens (14). The purpose of the present study was to assess biofilm proliferation within the sealer-dentin interface of two MR-based systems, self-etch and total-etch, and an ER-based sealer, using the CBBF model.

Material and Methods

Specimen preparation and aging

Intact, human teeth with single canals (University of Toronto Human Ethics Protocol #24315) were sterilized by gamma-irradiation (4080Gy) (22) and decoronated at the cemento-enamel junction. Canals were negotiated to the apical foramen with K-files (Lexicon Flex SSK, Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) and cleaned and shaped with ProTaper rotary instruments (Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) up to a size F4 at the foramen, with intermittent 5.25% sodium-hypochlorite irrigation. The last rinse with 5 mL of 5.25% NaOCl was activated with the EndoActivator (Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) to sonically agitate the irrigation solution. Smear layer was removed with 5 mL of 17% EDTA solution (2) (Vista Dental, Racine, WI, USA), followed with 5 mL of 5.25% NaOCl and a final flush with 10mL distilled water. Canals were then dried with paper points. . Roots were randomly divided into three sealer type groups (n=18/group): EP, an ER-based sealer (AH Plus Dentsply, Konstanz, Switzerland); SE, a MR-based self-etch sealer (RealSeal, SybronEndo, Orange,

CA); TE, a MR-based total-etch restorative material (Adper Scotchbond multi-purpose, 3M, St Paul, MN and Bisfil 2B self-cured resin, Bisco, Schaumburg, IL).

In EP and SE, sealers were applied with a Lentulo (Dentsply), canals filled with injectable gutta-percha (Elements, SybronEndo) compacted with Schilder pluggers (Dentsply), and the coronal end light-cured (SE only). Canals in TE were etched with 37% phosphoric acid (Bisco) for 15 sec, rinsed with sterile water for 15 sec, lightly air-dried, treated with Scotchbond primer and adhesive, light-cured, coated with Bisfil 2B, and filled with RealSeal SE gutta-percha points (SybronEndo) using passive lateral compaction.

Filled roots were stored for 72hrs at 37°C and 100% humidity (Hera Cell 150, Heraeus, Newton, CT), and sectioned horizontally 5mm from the coronal end with a slow-speed water-cooled rotary diamond disc (Brasseler, Savannah, GA) under sterile conditions, obtaining standardized 5mm-long specimens. Peripheral cementum, apical surfaces and exposed coronal dentin adjacent to root fillings were coated with nail varnish to prevent bacterial access to the sealer-dentin interface through cut dentinal tubules. Specimens were subjected to aging in vials with sterile phosphate-buffered saline (PBS), incubated (37°C, pH 7.0) for 1 week, 1, 3 or 6 months (n=3/material group/time).

Biofilm cultivation

Aged specimens were suspended in CBBF (37°C) to cultivate monospecies biofilms of *Enterococcus faecalis* (ATCC 47077) over interfacial margins (14), under continuous flow of fresh Tryptic Soy Broth (BD Bioscience, Sparks, MD) with 0.25% (wt/vol) glucose, at 0.72L/d (23) and dilution rate $D=0.075/\text{hr}$ mimicking the resting salivary flow rate (15).

Specimens were aseptically removed after 7 days and gently rinsed with sterile PBS. To assess bacterial viability, a 10 mL sample was collected from each vial, serially-diluted, and spot-plated in triplicate onto Brain Heart Infusion agar for bacteria colony forming unit (CFU) counting after 24hr of incubation at 37°C. Bacterial counts in the order of 10^9 CFU/mL were obtained for all tested samples.

Outcome assessment

Specimens were stained with dihydroethidium (Invitrogen, Molecular Probes, Carlsbad, CA) and examined with an epifluorescence microscope (DMIRE2, Leica Microsystems; Wetzlar, Germany). Red fluorescence was visualized with TX2 filter cube (excitation BP560/40, dichroic 595nm, emission BP645/75).

Biofilm proliferation and individual bacterial cell penetration were analyzed at 4 cardinal points (100X magnification). Captured images (CCD; Hamamatsu, Shizuoka, Japan) were processed (Openlab 4.0.2, Improvion, Waltham, MA) and Z-stack series established in a coronal-apical direction for each specimen in 5µm increments up to 500µm. Three-dimensional images of biofilm formation were reconstructed from Z-stack series by Image J software (NIH)(14).

Analysis

We performed one-way ANOVA and LSD post-hoc analysis ($p<0.05$) to determine the effects of material and aging period on biofilm proliferation and bacterial cell penetration along the sealer-dentin interfaces.

Results

Representative three-dimensional image reconstructions from Z-stack series captured from 1 month-aged specimens revealed different patterns of interfacial *E. faecalis* biofilm formation for EP, SE and TE (Fig. 1, top). Z-stack images depict individual bacterial cells at different depths within the interfaces of all test materials (Fig.1, bottom).

Mean interfacial biofilm proliferation depth ranged across test materials and aging periods (Fig. 2). Lumping aging periods together, biofilm proliferation depth differed significantly ($p<0.005$) among test materials; TE had significantly less biofilm proliferation than EP and SE ($p<0.01$), which did not differ significantly from each other.

SE and EP revealed biofilm proliferation for all aging periods with increased levels for specimens aged for 1 and 3 months than those aged for 1 week or 6 months ($p<0.05$). Maximum depth of biofilm proliferation was recorded for SE at 1 month ($p<0.05$). No biofilms were detected for TE at 3 and 6 months periods.

Individual bacterial cells within the sealer-dentin interfaces were detected consistently deeper than biofilm aggregates for all test materials at all aging periods (results not shown). Their mean depth ranged from $198\pm66\mu\text{m}$ for 6 month-aged TE specimens, to $431\pm21\mu\text{m}$ for 3 month-aged EP specimens. TE showed less individual bacterial cell penetration depth than SE and EP ($p<0.05$).

Discussion

Novel endodontic sealers require assessment with physiologically relevant models. Our previously used experimental model (14) was adapted for assessment of root canal sealers' interface with root dentin. Interfaces were post-aged in CBBF to simulate pathogenic intraoral conditions, and subsequently challenged by *E. faecalis*, an endogenous oral bacterial species frequently isolated in root canal infections (24). *E. faecalis* forms monospecies biofilms over root dentin, survives disinfection regimens and nutritional deprivation, and invades filled canals and dentinal tubules (25). Coronal-third root dentin was used, since it is the first challenged by bacteria invading through the pulp chamber (15). Its structure differs from that of apical root dentin where abundant branching and irregularities (26) preclude standardization of specimens.

Sealers that adhere or bond to dentin, a desirable property (2, 10,27) expected to curtail interfacial bacterial ingress and proliferation, were tested. ER-based AH Plus is widely used and frequently tested against novel sealers (4, 10, 20). Scotchbond and Bisfil 2B are typical components of MR-based total-etch systems, producing durable dentin bonds (7). This design allowed comparison of RealSeal SE, representing the new direction of MR-based self-etch sealers, to the ER-based sealer and MR-based total-etch restorative, both extensively tested and used clinically for many years.

Interfacial bacterial ingress occurred consistently, with less biofilm proliferation than individual bacterial cell penetration. Biofilm proliferation overall was deeper for SE than for TE, peaking in specimens aged for 1 month when it was also deeper than for EP. The susceptibility of SE to biofilm proliferation suggested that it did not fully satisfy the requirements for root filling materials (27). After aging for 6 months, biofilm proliferation declined for SE and EP and was undetected for TE, suggesting that prolonged aging of the materials' interfaces might have changed the ecological milieu in a manner that curtailed biofilm proliferation despite invasion of individual bacterial cells. This change may be related to altered sealing capacity of the materials (14, 28) and to the materials' degradation by-products released into the interfacial margins (14, 29). Previous study showed that free-

floating planktonic cells of *E. faecalis* readily invaded all interfaces within 7 days of inoculation prior to formation of biofilms (30). This observation supported the aforementioned hypothesis that the extent of biofilm proliferation could be influenced by the ecological milieu within the interfaces, which in turn might be affected by aging-related degradation.

Despite the ability of AH Plus to adhere to root dentin, its ability to resist bacterial invasion has been disputed (31, 32). Adhesive and mixed failures of its bond with root dentin (33) might lead to gaps where bacteria can invade and proliferate. Indeed, our results indicated that AH Plus was susceptible to interfacial bacterial invasion, even though biofilm proliferation appeared to diminish with prolonged aging of the sealer-dentin interface.

MR-based sealers are expected to form a micromechanical bond with root dentin (2, 10), but undergo biodegradation and dissolution over time (14, 20, 34) allowing bacterial invasion and biofilm formation (14, 18). Interfacial areas where the resin does not completely infiltrate the dentin substrate, or where it does not polymerize within the hybrid layer, are especially susceptible (35). RealSeal SE was previously reported to be inferior to AH Plus (36). Our results indicated that after aging for 1 month, RealSeal SE was more susceptible to interfacial biofilm proliferation to a greater extent than the total-etch and AH Plus systems. This phenomenon could be attributed to the greater hydrophilicity of the self-etch MR-based system that promotes water sorption and hydrolytic breakdown of the interface (37), and to its delayed degree of vinyl group conversion (28).

MR-based total-etch systems are widely used in restorative dentistry as they produce the most durable bond (7). Acid etching enhanced bonding to cervical root canal dentin up to 200% (38). Our results indicated that the total-etch restorative material was only minimally susceptible to interfacial biofilm proliferation, and significantly less than the self-etch sealer. However, no total-etch endodontic sealers are commercially available, possibly because of application challenges within the root canal configuration. Thus, even though the self-etch sealers represent an innovation (39), developing a sealer that would be impervious to interfacial biofilm proliferation remains an elusive goal.

Conclusions

This study presented a non-invasive assessment of interfacial biofilm proliferation between sealers and root dentin simulating pathogenic oral conditions. The self-etch and epoxy-resin based endodontic sealers were more susceptible at early stages to interfacial biofilm proliferation than the total-etch restorative material, but this susceptibility diminished after aging of the materials' interfaces for 6 months. Given the importance of biofilm forming processes in protecting bacteria, and their relevance to disease pathogenesis, the experimental model presented herein could be instrumental for testing novel strategies aimed at improving the resistance of the sealer-dentin interface to bacterial invasion and biofilm proliferation.

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Figure 1.

Top images- Representative three-dimensional reconstruction of select Z-stack series of *E. faecalis* biofilms captured from sealer-dentin interfaces of epoxy resin-based (EP), self-etch (SE) and total-etch (TE) sealers, aged for 1 month. Specimens were dihydroethidium-stained and examined using epifluorescence microscopy (excitation in the range of 560/40, dichroic 595 nm and emission of 645/75 nm, 100X magnification). Three-dimensional Images were reconstructed using Image J software (National Institutes of Health, Bethesda, MD). Levels of luminescence demonstrate different patterns of biofilm formation for the different materials.

Bottom images- Representative Z-stack images of *E. faecalis* captured from the sealer-dentin interface of specimens aged for 1 month. Images show differences in trends of bacterial cell presence within the interface (μm from the specimen's surface in an apical direction) for each material group.

Figure 2.

E. faecalis biofilm formation along the sealer-dentin interfaces of epoxy resin-based (EP), self-etch (SE) and total-etch (TE) methacrylate-based sealers. Specimens were aged in PBS (37°C, pH=7.0) for 1 week, 1, 3 and 6 months, then incubated with *E. faecalis* in biofilm forming constant media conditions for 7 days. Mean $\pm$ SE values with same superscripts denote statistically not-significant differences between groups ($p > 0.05$, one-way ANOVA, LSD post-hoc test). Mean biofilms depths ranged from 0 to 355 ± 21 μm from the specimen's surface. TE showed lower biofilm proliferation than EP and SE ($p < 0.01$). No biofilm were detected for TE specimens pre-incubated for 3 and 6 months. Maximum depth of biofilm proliferation was recorded for SE at 1 month ($p < 0.05$).