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Comparison of quaternary ammonium-containing with nano-silver-containing adhesive in antibacterial properties and cytotoxicity

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Abstract

Objective—Antibacterial primer and adhesive are promising to help combat biofilms and recurrent caries. The objectives of this study were to compare novel bonding agent containing quaternary ammonium dimethacrylate (QADM) with bonding agent containing nanoparticles of silver (NAg) in antibacterial activity, contact-inhibition vs. long-distance inhibition, glucosyltransferases (*gtf*) gene expressions, and cytotoxicity for the first time.

Methods—QADM and NAg were incorporated into Scotchbond Multi-Purpose adhesive and primer. Microtensile dentin bond strength was measured. *Streptococcus mutans (S. mutans)* biofilm on resin surface (contact-inhibition) as well as *S. mutans* in culture medium away from the resin surface (long-distance inhibition) were tested for metabolic activity, colony-forming units (CFU), lactic acid production, and *gtf* gene expressions. Eluents from cured primer/adhesive samples were used to examine cytotoxicity against human gingival fibroblasts.

Results—Bonding agent with QADM greatly reduced CFU and lactic acid of biofilms on the resin surface ($p < 0.05$), while having no effect on *S. mutans* in culture medium away from the resin surface. In contrast, bonding agent with NAg inhibited not only *S. mutans* on the resin surface, but also *S. mutans* in culture medium away from the resin surface. Bonding agent with QADM suppressed *gtfB*, *gtfC* and *gtfD* gene expressions of *S. mutans* on its surface, but not away from its surface. Bonding agent with NAg suppressed *S. mutans* gene expressions both on its surface and away from its surface. Bonding agents with QADM and NAg did not adversely affect microtensile bond strength or fibroblast cytotoxicity, compared to control ($p > 0.1$).

Significance—QADM-containing adhesive had contact-inhibition and inhibited bacteria on its surface, but not away from its surface. NAg-containing adhesive had long-distance killing capability and inhibited bacteria on its surface and away from its surface. The novel antibacterial adhesives are promising for caries-inhibition restorations, and QADM and NAg could be complimentary agents in inhibiting bacteria on resin surface as well as away from resin surface.

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Keywords

Antibacterial dental adhesive; quaternary ammonium; silver nanoparticles; *Streptococcus mutans* biofilm; microtensile dentin bond strength; fibroblast cytotoxicity

1. Introduction

Nearly half of all dental restorations fail within 10 years, and replacing them accounts for 50–70% of all restorative dentistry [1–3]. Composites are popular filling materials because of their esthetics and direct-filling capabilities [4–10]. One main problem, however, is that composites tend to accumulate more biofilms than other restorative materials in vivo [11–13]. Biofilms at the restoration margins could produce acids and cause secondary caries, the main reason for restoration failure [14,15]. Acidogenic bacteria such as *Streptococcus mutans* (*S. mutans*) and their biofilms, upon exposure to fermentable carbohydrates, are responsible for dental caries [16–18]. Therefore, efforts have been made to develop antibacterial dental composites. Novel polymers containing quaternary ammonium salts (QAS) were developed [19–28]. Monomers such as 12-methacryloyloxydodecylpyridinium bromide (MDPB) could copolymerize with other dental monomers to form antibacterial polymer matrices that can effectively reduce bacteria growth [19,20].

Bonding agents adhere the composite restoration to the tooth structure to form a functional and durable interface [29–32]. Bonding agent compositions and bond strengths have been improved in previous studies [33–37]. Antibacterial adhesives are promising to combat bacteria and reduce recurrent caries at the tooth-restoration margins [19–21,38,39]. Residual bacteria often exist in the prepared tooth cavity, and microleakage could allow new bacteria to invade the margins. In previous studies, adhesives containing MDPB substantially reduced the growth of *S. mutans* [19,38]. A methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB)-containing adhesive also effectively reduced biofilm growth [21]. MDPB was incorporated into a primer which showed a strong antibacterial activity [40,41]. In addition, chlorhexidine was used in primer to achieve antibacterial effects [42].

Recently, a quaternary ammonium dimethacrylate (QADM) was synthesized and incorporated into resins to inhibit biofilm growth [25,26,43]. In addition, recent studies developed antibacterial nanocomposites containing nanoparticles of silver (NAg) with a potent antibacterial activity [26,44]. QADM and NAg were also incorporated into primer and adhesive which greatly reduced biofilm growth [45,46]. QADM is immobilized in the resin due to the covalent bonding with the polymer network to exert “contact inhibition” [19,20]. Hence, the cured QADM-containing adhesive could inhibit bacteria adherent on its surface, but would have no effect on bacteria in the culture medium away from its surface. In contrast, the resin containing NAg is expected to inhibit not only bacteria on its surface, but also bacteria in the culture medium away from its surface due to Ag ion release. However, there has been no report on the comparison of antibacterial effects of QADM-adhesive and NAg-adhesive side by side, and how they inhibit bacteria on the surface and away from the surface differently.

Therefore, the objective of this study was to investigate the antibacterial differences of a QADM-adhesive and a NAg-adhesive via a side-by-side comparison for the first time, to determine their effects on the surface-adherent bacteria and the bacteria away from the surface in the culture medium. In addition, previous studies on adhesives with QADM and NAg reported dentin shear bond strength, without measuring the microtensile bond strength [45,46]. Hence, the microtensile bond strength of adhesive and primer containing QADM and NAg were measured in this study. Furthermore, the cytotoxicity and *S. mutans* gene

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expressions were determined. It was hypothesized that: (1) QADM-adhesive will inhibit *S. mutans* on its surface, but not *S. mutans* away from its surface in the culture medium; (2) NAg-adhesive will inhibit not only *S. mutans* on its surface, but also *S. mutans* away from its surface in the culture medium; (3) Incorporation of QADM or NAg into primer and adhesive would impart potent antibacterial activity without adversely affecting the microtensile dentin bond strength and fibroblast viability.

2. Materials and methods

2.1. Antibacterial bonding agents containing QADM or NAg

Scotchbond Multi-Purpose bonding system (3M, St. Paul, MN), referred as "SBMP", was used as the parent bonding system to test the effect of incorporation of QADM and NAg. According to the manufacturer, SBMP etchant contains 37% phosphoric acid. SBMP primer contains 35–45% 2-Hydroxyethylmethacrylate (HEMA), 10–20% copolymer of acrylic/itaconic acids, and 40–50% water. SBMP adhesive contains 60–70% BisGMA and 30–40% HEMA.

Bis(2-methacryloyloxyethyl) dimethylammonium bromide, a quaternary ammonium dimethacrylate (QADM), was recently synthesized [25,26]. Its synthesis employed a modified Menschutkin reaction, where a tertiary amine group was reacted with an organo-halide. A benefit of this reaction is that the reaction products are generated at quantitative amounts and require minimal purification [25,26,43]. Briefly, 10 mmol of 2-(N,N-dimethylamino)ethyl methacrylate (DMAEMA, Sigma, St. Louis, MO) and 10 mmol of 2-bromoethyl methacrylate (BEMA, Monomer-Polymer and Dajec, Trevose, PA) were combined with 3 g of ethanol in a 20 mL scintillation vial. The vial was stirred at 60 °C for 24 h. The solvent was then removed, yielding QADM as a clear, colorless, and viscous liquid. QADM was mixed with SBMP adhesive at QADM/(SBMP adhesive + QADM) mass fraction of 10%, following a previous study [45]. The same 10% mass fraction was used in SBMP primer [45].

Silver 2-ethylhexanoate salt (Strem, New Buryport, MA) was dissolved in 2-(tert-butylamino)ethyl methacrylate (TBAEMA, Sigma) at 0.08 g of silver salt per 1 g of TBAEMA [26,44,45]. TBAEMA improved the solubility by forming Ag-N bonds with Ag ions to facilitate Ag salt to dissolve in resin solution. TBAEMA contains reactive methacrylate groups which can be chemically bonded in the resin upon photopolymerization. Ag was incorporated into SBMP primer at a silver 2-ethylhexanoate/(primer + silver 2-ethylhexanoate) mass fraction of 0.05%, following a previous study [45]. The same 0.05% mass fraction was used in SBMP adhesive.

Transmission electron microscopy (TEM) was used to examine the NAg in resin, following a recent method [44]. Briefly, a thin sheet of mica was partially split and the Ag-containing resin was placed in the gap. The resin-mica sandwich was pressed with an applied load of 2.7×10^7 N to form a thin sheet of resin in between the two mica layers [44]. The resin was photo-cured for 1 min. The mica sheet was then split apart after 1 day to expose the polymerized film. An ultrathin layer of carbon was vacuum-evaporated onto the resin (Electron Microscopy Sciences, Hatfield, PA). The carbon-coated sample was then partially submerged in distilled water in order to float the thin film onto the water's surface. A copper grid was then used to retrieve the film. A high-resolution TEM (Tecnai T12, FEI, Hillsboro, OR) was used at an accelerating voltage of 120 kV. Images were collected and the NAg size was measured using AMT V600 image analysis software (Advanced Microscopy Techniques, Woburn, MA) [44].

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2.2. Microtensile dentin bond strength

Three primer/adhesive groups were tested: (1) SBMP primer, SBMP adhesive (termed SBMP control); (2) primer + 10% QADM, adhesive + 10% QADM (termed SBMP +QADM); (3) primer + 0.05% NAg, adhesive + 0.05% NAg (termed SBMP+NAg).

Human third molars were collected with donor consent under a protocol approved by the University of Maryland. The roots of teeth were removed via a water-cooled cutting saw (Isomet, Buehler, Lake Bluff, IL) [45]. The occlusal one-third of the tooth crown was removed to expose midcoronal dentin. The dentin surface was polished with 600-grit SiC paper, etched with 37% phosphoric acid gel for 15 s, and rinsed with distilled water. A primer was applied and the solvent was removed with a stream of air for 5 s. Then the adhesive was applied and light-cured for 10 s (Optilux VCL 401, Demetron Kerr, Danbury, CT). A composite (TPH, Caulk/Dentsply, Milford, DE) was applied and light-cured for 60 s [45–47]. After storage in de-ionized water at 37 °C for 24 h, each bonded tooth was vertically sectioned into slabs with a 0.9 mm thickness [48]. The central slab was used for microscopy. The other slabs were sectioned into 0.9 × 0.9 mm composite-dentin beams [48]. Eight teeth were used for each bonding agent group. Five beams were obtained from each tooth, yielding 40 beams for each group (n = 40). Each beam was stressed to failure under uniaxial tension in a computer-controlled Universal Testing Machine (MTS, Eden Prairie, MN) at a cross-head speed of 1 mm/min [48]. The load-at-failure divided by the cross-sectional area at the site of failure yielded the microtensile dentin bond strength [48].

For scanning electron microscopy (SEM), the composite-dentin slabs were polished, and treated with 37% phosphoric acid gel for 15 s, and then 5.25% NaOCl for 10 min [45,48]. Specimens were dehydrated using increasing ethanol concentrations of 50%, 70%, 85% and 100% [45,48]. Specimens were sputter-coated with gold and examined via SEM (Quanta 200, FEI, Hillsboro, OR). For TEM, thin composite-dentin sections of an approximate thickness of 120 μm were cut and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde, following a previous study [33]. Samples were embedded in epoxy (Spurr's, Electron Microscopy Sciences, PA). Ultra-thin sections (approximate thickness=100 nm) were cut using a diamond knife (Diatome, Bienne, Switzerland) with an ultra-microtome (EM-UC7, Leica, Germany). The non-demineralized sections were examined in TEM (Tecnai-T12, FEI).

2.3. Cytotoxicity of adhesive eluent with human gingival fibroblasts

The cover of a sterile 96-well plate was used for specimen preparation [21]. Ten μL of a primer was placed in the bottom of the dent. After drying with a stream of air, 20 μL of adhesive was applied. A micro applicator with a small brush tip (Benda, Centrix, Shelton, CT) was used to mix the primer and adhesive together, which was then photo-polymerized for 20 s using a Mylar strip covering. This yielded a cured primer/adhesive disk of approximately 8 mm in diameter and 0.5 mm in thickness. The primer/adhesive disk was well cured, and after the cured samples were removed from the 96-well plate, there was no primer left on the well cover as confirmed with optical microscopy. After sterilization with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC), the disk was immersed in 10 mL fibroblast medium (FM, ScienCell, San Diego, CA) and agitated for 24 h at 37 °C to obtain eluent from the disks [49]. Cytotoxicity was then tested using the original extract solution along with a series of dilutions [49]. The original extract was diluted with fresh FM at dilutions of 2-fold (1 part of original extract + 1 part of fresh FM), 4-fold, 8-fold, 16-fold, 32-fold, 64-fold, and 128-fold, which were then used for the fibroblast cytotoxicity test.

Human gingival fibroblasts (HGF, ScienCell, San Diego, USA) were cultured in FM supplemented with 2% fetal bovine serum, 100 IU/mL penicillin and 100 IU/mL

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streptomycin. The protocol of using HGF was approved by the University of Maryland. A seeding density of 4000 cells/well was used in 96-well plates [50]. After 24 h of incubation at 37 °C with 5% CO_2 in air, the culture medium was removed and replaced with 100 μL of the adhesive eluent at one of the aforementioned dilution folds. The cells were cultured for another 48 h, and then 20 μL of sterile filtered MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) solution at a concentration of 5 mg/mL was added to each well. After incubating in a darkroom for 4 h, the unreacted dye was removed and 150 μL/well of dimethylsulfoxide (DMSO, Sigma) was added [50]. The solution absorbance was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnvale, CA) at 492 nm [50]. FM without any resin eluent was used to culture fibroblasts as control, and its absorbance was set as 100%. The fibroblast viability for cells cultured with eluents = Absorbance with eluents/absorbance of control [50].

2.4. Live/dead assay of S. mutans on primer/adhesive/composite tri-layer disks

The cover of a 96-well plate was used for specimen preparation [21]. As in Section 2.3, primer and adhesive were applied and photo-polymerized. Then a composite (TPH) was placed and photo-cured for 1 min to obtain a disk of 8 mm in diameter and 1 mm in thickness. Disks were immersed in water and agitated for 1 h to remove any uncured monomer, following a previous study [40]. The disks were sterilized with ethylene oxide (Anprolene AN 74i).

The use of *S. mutans* (ATCC700610, American Type, Manassas, VA) was approved by the University of Maryland. *S. mutans* is a cariogenic bacterium and is the primary causative agent of caries. *S. mutans* was cultured overnight at 37 °C in Brain Heart Infusion (BHI, Becton, Sparks, MD) in an anaerobic atmosphere. Bacterial suspension obtained was adjusted to an optical density (OD) of 0.5 at 600 nm for further usage [21]. The resin disks were placed, with the primer surface facing up, in a 24-well plate with 2 mL of BHI supplemented with 0.2% sucrose. Bacterial suspension was diluted by 1:100, and then 10 μL of the suspension was inoculated in each well. After 24-h, the biofilm on the disk was used in the following experiments, and the planktonic bacteria in the medium were also collected separately [21].

Disks with 24-h biofilms were washed with phosphate buffered saline (PBS). The bacteria were stained using a live/dead bacterial kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce a green fluorescence. Bacteria with compromised membranes were stained with propidium iodide to produce a red fluorescence. Separately, the planktonic bacteria in the medium were collected, centrifuged at 5 kg for 4 min, and similarly live/dead stained. Each test was done at n = 6. The stained specimens were examined with an epifluorescence microscope (TE2000-S, Nikon, Melville, NY) [45].

2.5. MTT metabolic activity of S. mutans

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a colorimetric assay that measures the enzymatic reduction of MTT, a yellow tetrazole, to formazan. Briefly, disks with 1-d biofilms were transferred to new 24-well plate, with 1 mL of MTT dye in each well [26]. Separately, the collected medium with planktonic bacteria from each well was transferred to a tube containing 100 μL of MTT dye. All specimens were incubated at 37 °C in 5% CO_2 for 1 h. During this process, metabolically active bacteria reduced the MTT to purple formazan. After 1 h, the biofilm specimens were transferred to a new 24-well plate. The planktonic bacteria were collected by centrifugation at 5 kg for 4 min. An aliquot of 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals [26]. After incubation for 20 min in the dark, 200 μL of the DMSO solution was transferred to a 96-well plate, and the absorbance at 540 nm was measured via a microplate reader

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(SpectraMax M5) [26]. A higher absorbance indicates a higher formazan concentration, which in turn indicates more metabolic activity of the bacteria. Six replicates were tested for each group (n = 6).

2.6. Lactic acid production and colony-forming units (CFU)

Disks with 24-h biofilms were rinsed in cysteine peptone water (CPW) to remove loose bacteria and placed in a new 24-well plate. Separately, the planktonic bacteria from each well was transferred to a tube and collected by centrifugation at 5 kg for 4 min. An aliquot of 1.5 mL of buffered peptone water (BPW) supplemented with 0.2% sucrose was added to each well or tube. Samples were incubated at 5% CO_2 and 37 °C for 3 h to allow the bacteria to produce acid. After 3 h, the BPW solutions were stored for lactate analysis. Lactate concentrations were determined using an enzymatic method [26]. The microplate reader was used to measure the absorbance at 340 nm for the collected BPW solutions. Standard curves were prepared using a lactic acid standard (Supelco Analytical, Bellefonte, PA) [26].

Bacteria in biofilms on disks were harvested by sonicating (3510R, Branson, Danbury, CT) and vortexing (Fisher, Pittsburgh, PA). Separately, the CFU counts of the planktonic bacteria from the medium were also measured. The bacterial suspensions were serially diluted, and spread onto BHI agar plates for CFU analysis (n = 6) following previous studies [26,45].

2.7. gtf gene expression of S. mutans

S. mutans can synthesize extracellular glucans by glucosyltransferases (GTFs), which is important for bacterial cell adhesion and biofilm formation [16,51]. The GTF expression was shown to involve three types of genes: *gtfB*, *gtfC*, and *gtfD* [52]. These gene expressions were measured here because it is desirable for the antibacterial primer/adhesive to hinder these gene expressions, thereby suppressing glucans synthesis, biofilm formation, and secondary caries [21]. To collect sufficient amount of bacteria for RNA extraction and gene expression analysis, larger primer/adhesive disks were fabricated. Disks were fabricated in the covers of 12-well plates using 50 μL of primer, 50 μL of adhesive, and a composite (TPH). This yielded a primer/adhesive/composite tri-layer disk of 20 mm in diameter and 1 mm in thickness, while otherwise following the same method described in Section 2.3. Each disk was placed in a 6-well plate with 5 mL of BHI supplemented with 0.2% sucrose. Bacterial suspension was diluted by 1:100, then 50 μL of the suspension was inoculated in each well and incubated for 24 h.

Biofilm on each disk and the corresponding bacteria in medium were used separately for gene expression measurement. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA) was used. Total RNA for *S. mutans* was isolated with a TRIzol Max bacterial RNA isolation kit (Invitrogen, CA) according to the manufacturer's instructions [21]. cDNA was synthesized by mixing total RNA (2 μg) and high-capacity cDNA reverse transcription kit (Applied Biosystems) in a 20 μL reaction volume [21]. RT-PCR was carried out with a Taqman fast universal PCR master mix (Applied Biosystems). Oligonucleotide primers and probes for *gtfB, gtfC, gtfD* and *16S rRNA* (a housekeeping gene as an internal control) were used. The expression levels of all genes were normalized with the amplification of the *16S rRNA* gene of *S. mutans* as an internal standard [21]. Three separate experiments each with duplicates were performed (total n = 6).

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2.8. Statistical analysis

All data were first checked for normal distribution with the Kolmogorov-Smirnov test and for homogeneity with Levene's test. Inter-group differences were estimated by a statistical analysis of variance (ANOVA) for factorial models; individual groups were compared with Fisher's protected least-significant difference test. Statistical analyses were performed by SPSS 13.0 software (SPSS, Chicago, IL) at a significance level of $p < 0.05$.

3. Results

Fig. 1 shows the NAg and the dentin bonding results. In (A), TEM image showed that NAg were well dispersed in the resin. Measurement of 100 particles (mean ± sd) yielded particle size of (2.7 ± 0.6) nm. In (B), microtensile bond strengths (mean ± sd; n = 40) were not significantly different for the three groups ($F = 1.407$; $p = 0.249$). Representative SEM images of the composite-dentin interfaces are shown in (C–E) for the three groups. The adhesive resin was well-infiltrated into dentinal tubules to form resin tags, without noticeable difference among the three groups. Hence, incorporation of QADM or NAg did not compromise dentin bond strength and formation of resin tags. In (F), TEM image of tooth sections for SBMP+NAg showed examples of NAg inside resin tags that flowed with adhesive resin into dentinal tubules.

The cytotoxicity of resin eluents is shown in Fig. 2. The x axis starts with the original extract, which was then serially diluted. The x axis ends with the commercial FM without any resin eluents addition. Cell viability for all three groups was below 20% in the original extract, then gradually increased with greater dilution folds, reaching 100% with 16-fold dilution. SBMP+NAg had slightly lower cell viability at 2–8 dilution folds ($p < 0.05$). Between dilution folds of 16–128, all three groups had cell viability matching that of FM control ($p > 0.1$), where the addition of QADM or NAg into bonding agent did not significantly affect the cytotoxicity, compared to the commercial non-antibacterial bonding agent ($p > 0.1$).

Representative live/dead images are shown in Fig. 3 for biofilms on resin disks (left column), and planktonic bacteria in the medium (right column). The control had primarily live bacteria. In (B) and (C), bonding agents containing QADM or NAg had more compromised bacteria, compared to (A). For bacteria in the culture medium, only (F) had increased amounts of dead bacteria; (D) and (E) both had predominantly live bacteria. These results indicate that the QADM-containing resin inhibited bacteria growth on its surface (B), but the bacteria away from its surface were still primarily alive (E). In sharp contrast, the NAg-containing resin inhibited bacteria both on its surface (C) and in the culture medium away from the resin surface (F).

Quantitative results for *S. mutans* biofilms on resin surfaces are plotted in Fig. 4: (A) metabolic activity, (B) CFU, and (C) lactic acid. In (A), control group yielded the highest MTT absorbance. The incorporation of QADM or NAg into bonding agent decreased the metabolic activity of *S. mutans* biofilms ($p < 0.05$). There was no significant difference between QADM group and NAg group ($p > 0.1$). Similar trends were obtained in (B) and (C).

For the planktonic *S. mutans* in culture medium away from the resin surface, the results are shown in Fig. 5: (A) metabolic activity, (B) CFU, and (C) lactic acid. A striking difference was observed from the results in Fig. 4. In Fig. 5, there was no significant difference between the control and the QADM group ($p > 0.1$), indicating that QADM resin had no effect on bacteria away from its surface in culture medium. However, the NAg group decreased the metabolic activity, CFU, and lactic acid, compared to the control ($p < 0.05$).

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These results indicate that the QADM resin inhibited *S. mutans* only on its surface (Fig. 4), but did not inhibit the *S. mutans* in the culture medium away from its surface (Fig. 5). In contrast, the NAg-containing bonding agent inhibited *S. mutans* both on the surface (Fig. 4) and away from its surface (Fig. 5).

The RT-PCR results are plotted in Fig. 6. For *S. mutans* biofilms on resin surfaces, all three gene expressions were greatly reduced via QADM and NAg, compared to control ($p < 0.05$). The *gtfB* expression was reduced to about 25% of that of control. The *gtfC* expression was reduced to 30–40% of control, and *gtfD* expression was reduced to 20% of control. For *S. mutans* in culture medium away from the resin surface, no significant difference was found in *gtf* genes of control and QADM-incorporating group ($p > 0.1$), indicating that the QADM resin had no inhibitory effect on *S. mutans* away from the resin surface. In contrast, significant decreases in all three *gtf* gene expressions were found in the NAg-containing group ($p < 0.05$), indicating that NAg resin inhibited *S. mutans* in the culture medium away from its surface.

4. Discussion

The present study compared QADM-containing adhesive with NAg-containing adhesive side-by-side for the first time and showed that QADM resin inhibited *S. mutans* on its surface but had no effect on bacteria away from its surface, while NAg-resin inhibited *S. mutans* both on its surface and away from its surface. In addition, this study investigated the effects of incorporating QADM and NAg on microtensile bond strength, fibroblast cytotoxicity, and *S. mutans gtf* gene expressions for the first time. The QADM and NAg bonding agents greatly inhibited *S. mutans* biofilm growth, metabolic activity, CFU counts, and lactic acid production, compared to the commercial bonding agent. Antibacterial activity was obtained without compromising microtensile bond strength, or cytotoxicity against human gingival fibroblasts. Oral bacteria in vivo colonize on the tooth-restoration surfaces to form biofilms, and cariogenic bacteria such as *S. mutans* in the biofilm can metabolize carbohydrates to organic acids. This in turn leads to tooth decay and secondary caries at the tooth-restoration margins. Therefore, the new QADM- and NAg-containing antibacterial bonding agents with substantial reductions in biofilm growth and lactic acid production are promising for caries-inhibition applications.

Previous studies developed novel resins containing QAS monomers with antibacterial properties [19–23,28,53]. QAS materials can cause bacteria lysis by binding to cell membrane and causing cytoplasmic leakage [53]. When the negatively charged bacterial cell contacts the positively charged (N^+) sites of the QAS resin, the electric balance of the cell membrane could be disturbed, and the bacterium could explode under its own osmotic pressure [54]. An advantage of QAS resins is that the antibacterial agent is copolymerized with resin by forming a covalent bonding with the polymer network. Therefore, the antibacterial agent is immobilized and not lost over time. MDPB-containing resin maintained its antibacterial effect after 3 months of water-aging [11]. Glass ionomer cements with QAS bromides and chlorides also possessed a long-lasting antibacterial activity [22]. Recently, a nanocomposite containing QADM after 6 months of water-aging was shown to have potent anti-biofilm properties, which were similar to the 1-day properties [43]. QADM has reactive groups on both ends of the molecule, which could be incorporated into the resin with less of a negative impact on the mechanical properties of the resin. Furthermore, QADM is a low viscosity monomer which is miscible with common dimethacrylates and is expected to have minimal monomer leachability, compared with other quaternary ammonium salts based on monomethacylates [25,26]. The present study showed that QADM in a bonding agent greatly reduced biofilm metabolic activity, CFU, acid production and gene expressions, without adversely affecting microtensile dentin bond

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strength and cytotoxicity. Hence, QADM is promising for use in antibacterial adhesives for contact-inhibition.

Ag is another important antibacterial agent against a wide range of micro-organisms [55,56]. The antimicrobial mode appears to be that Ag ions can inactivate the vital enzymes of bacteria, and cause the DNA in the bacteria to lose its replication ability, leading to cell death [55,57]. Ag has several advantages, including a low toxicity and good biocompatibility with human cells [56], long-term antibacterial activity due to sustained ion release [58], and a low bacterial resistance compared to antibiotics [59]. Ag nanoparticles were shown to possess potent antibacterial properties [55,57]. Ag nanoparticle were recently incorporated into dental resins [26,44,60]. Their small particle size and large surface area could enable them to release more Ag ions at a low filler level, thereby reducing Ag particle concentration necessary for efficacy [26,44,45,60]. This is desirable because low Ag filler levels in the resin would not adversely affect the resin color and mechanical properties. The present study obtained strong antibacterial properties using a NAg filler level of 0.05% in primer and adhesive, which caused no decrease in microtensile dentin bond strength and no noticeable change in the color of primer and adhesive. Ag salt was dissolved in TBAEMA monomer, which was then mixed with primer or adhesive. The Ag ions in the resin agglomerated to form nanoparticles that became part of the resin upon photo-polymerization [26,44,45]. An advantage of this method was that it reduced the Ag salt to Ag nanoparticles in the resin in situ, which avoided the difficulty of mixing pre-formed Ag nanoparticles that could cause agglomeration [26,44,45]. Therefore, the concurrent reduction of Ag ions and polymerization of dimethacrylate-based polymers is a promising method for incorporating NAg into primers and adhesives with potent antibacterial properties.

The present study compared the antibacterial effects of QADM with NAg against the *S. mutans* biofilm on the resin surface and the *S. mutans* away from the resin surface for the first time. The live/dead assay, MTT, CFU, lactic acid, and *gtf* gene expression results all consistently demonstrated that QADM-containing bonding agent had antibacterial activity against bacteria on its surface, but not bacteria away from its surface. This is consistent with the contact-inhibition characteristic of quaternary ammonium methacrylate resins in general [19,20]. In contrast, the NAg-containing bonding agent inhibited not only the *S. mutans* on its surface, but also the *S. mutans* away from its surface suspended in the culture medium. This indicates that NAg-containing resin has a long-distance killing capability, likely due to the release of Ag ions [55]. Previous studies suggested that Ag-containing resin composites had a long-lasting antibacterial activity due to the sustained Ag ion release [58]. An Ag-containing dental composite was shown to inhibit *S. mutans* growth when tested for a duration of 6 months [61]. However, while the present study focused on the QADM-resin and NAg-resin comparisons and properties including gene expressions and cytotoxicity, further study is needed to investigate the Ag ion release and long-term properties of the new NAg-containing adhesive and primer.

Bacteria in biofilms reside in a matrix of extracellular polymeric substances (EPS) [62]. EPS is beneficial for bacteria survival by providing a three dimensional scaffold for mechanical integrity as well as enhanced bacterial adhesion and cell-cell interactions [62]. *S. mutans* can synthesize extracellular glucans by glucosyltransferases (GTFs), which is important for bacterial cell adhesion and biofilm formation [16,51]. The GTF expression was shown to involve three types of genes: *gtfB*, *gtfC*, and *gtfD* [58]. The *gtfB* and *gtfC* genes encode enzymes that produce mainly water-insoluble glucans. The *gtfD* gene encodes an enzyme that catalyzes the synthesis of a water-soluble glucan. Changes in *gtf* gene expressions will change the glucan production which affects the bacterial cell adhesion and biofilm formation. This in turn will affect the occurrence and progression of caries. It would be highly desirable for a restorative to suppress the *gtf* gene expressions to inhibit glucan

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production and biofilm formation. The present study showed that antibacterial bonding agent containing QADM greatly reduced the *gtf* expressions for *S. mutans* in biofilms on resin surfaces, but not for *S. mutans* away from the resin in the culture medium. The NAg-containing bonding agent, on the other hand, greatly reduced the *gtf* gene expressions for both adherent biofilms and those in medium away from the resin surface.

While it is important to inhibit bacterial growth and suppress their gene expressions, it is equally important for the new antibacterial bonding agents to be non-cytotoxic for mammalian cells. The present study showed that, under dilution folds approaching the saliva volume in vivo, QADM- and NAg-containing bonding agents had human fibroblast viability matching that of a commercial non-antibacterial control, and that of FM control without any resin eluent. The NAg-containing bonding agent had fibroblast viability slightly lower than that of the commercial bonding agent at 2–8 folds of dilution of the original extract. However, at 16–128 folds of dilution, both antibacterial bonding agents had fibroblast viability matching that of commercial control and FM control without resin eluents. The original extract was obtained by immersing cured primer/adhesive disks of 8 mm in diameter and 0.5 mm in thickness in 10 mL medium at 37 °C for 24 h. The typical saliva flow for an average person is about 1000 to 1500 mL per day [63]. Hence, the 128-fold dilution of the original extract (which would yield a total of 1280 mL of culture medium) may approximate the amount of saliva in the mouth in 24 h. Furthermore, even at the 16-fold dilution, with a total solution volume about 1/8 of the saliva volume per day in vivo, the QADM and NAg groups still had nearly 100% fibroblast viability. Therefore, the QADM- and NAg-containing bonding agents could achieve a potent antibacterial activity without compromising the fibroblast cytotoxicity.

Antibacterial primers and adhesives are promising to inhibit recurrent caries. There are often residual bacteria present in the prepared tooth cavity [21,38]. With the increased practice of less removal of tooth structure and minimal intervention dentistry [64], more carious tissues could be left in tooth cavity containing active bacteria. Besides residual bacteria, the tooth-restoration margins will experience invading bacteria during service. While a complete sealing of the tooth-restoration interface is ideal, it is often difficult to achieve. This is evident in studies showing microgaps at tooth-restoration interfaces, which could allow for bacteria invasion [65–67]. Therefore, the QADM and NAg primer and adhesive could combat not only the residual bacteria in the tooth cavity, but also the invading bacteria along the tooth-restoration margins due to bacterial leakage, thereby to reduce the occurrence of pulp damage and secondary caries. In addition, QADM and NAg can also be incorporated into pit and fissure sealants, orthodontic bracket cements and inlay and crown cements, to exert a unique combination of contact- inhibition and long-distance killing capability to inhibit biofilm growth and dental caries.

5. Conclusion

The present study compared a QADM-containing adhesive with a NAg-containing adhesive in antibacterial activity, contact-inhibition vs. long-distance inhibition, microtensile bond strength, *S. mutans* gene expressions, and fibroblast cytotoxicity for the first time. The bonding agents inhibited biofilm growth and suppressed *S. mutans* gene expressions without compromising microtensile bond strength and cytotoxicity. Bonding agent with QADM greatly reduced the metabolic activity, CFU and lactic acid of *S. mutans* biofilms on the resin surface, while having no effect on *S. mutans* in culture medium away from resin surface. In contrast, bonding agent with NAg inhibited not only the *S. mutans* on surface, but also the *S. mutans* away from the surface in the culture medium. These results indicate the contact-inhibition mode of QADM resin, and the ion release and long-distance killing ability of NAg resin. The *gtfB*, *gtfC* and *gtfD* gene expression results corroborated with

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biofilm viability and lactic acid data. Bonding agents with QADM and NAg had eluent cytotoxicity against fibroblasts similar to culture medium control without resin. The novel QADM- and NAg-containing adhesives are promising for antibacterial and caries-inhibition restorations, and the QADM and NAg could be complimentary agents in inhibiting both bacteria on restoration surface and away from surface.

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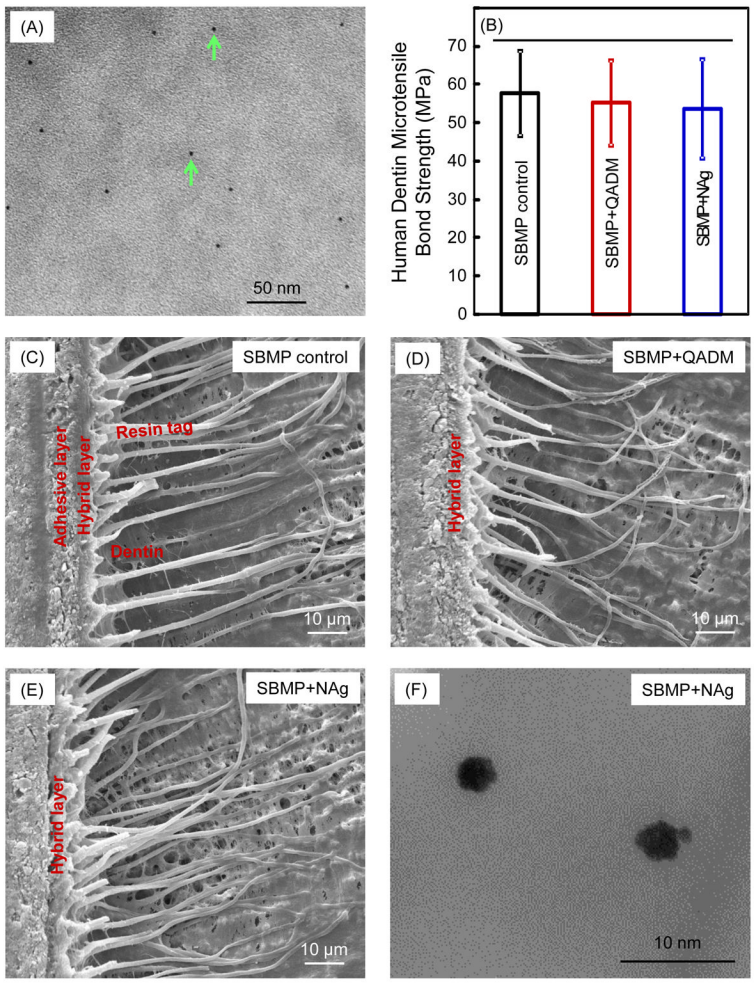

Fig. 1.

Nanoparticles of silver (NAg) and dentin bonding. (A) TEM of NAg in resin. (B) Microtensile bond strength (mean ± sd; n = 40). Horizontal line indicates no significant difference ($p > 0.1$). (C, D, E) Representative SEM images of composite-dentin interfaces for the three groups, respectively. The hybrid layer is between the adhesive layer and the underlying mineralized dentin. Numerous resin tags were observed, similar for all groups. (F) TEM of thin tooth section for SBMP+NAg, showing NAg inside the resin tag. Hence, the NAg flowed with the adhesive resin into the dentinal tubules.

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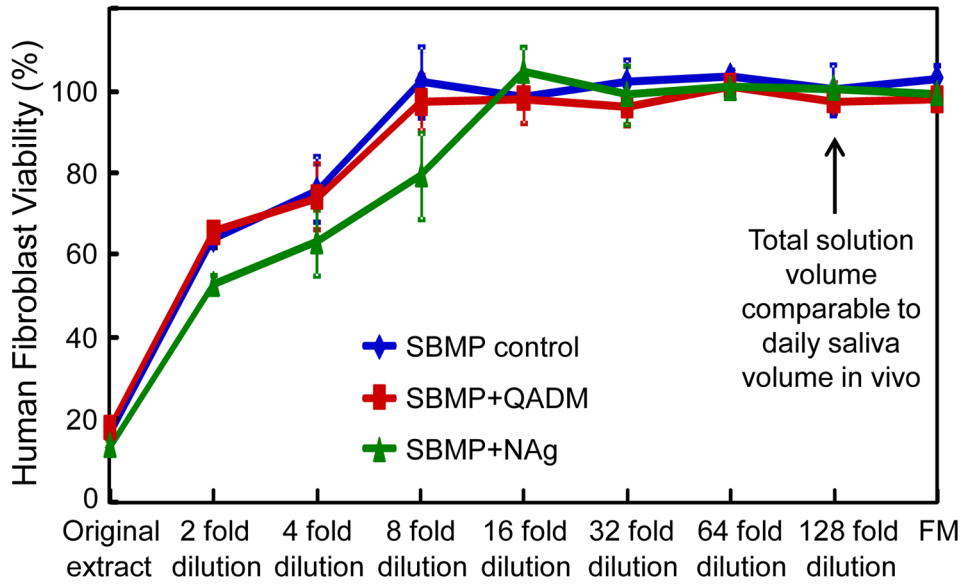

Fig. 2.

Human gingival fibroblast cytotoxicity using eluents from cured primer/adhesive disks. The original 10 mL extract was diluted, and each dilution was used to culture fibroblasts. “FM” refers to cell culture in fibroblast medium without any resin eluents. Fibroblast viability was measured by MTT absorbance and normalized by FM control. Between 16–128 folds, all groups had cell viability matching that of FM ($p > 0.1$). The 128-fold dilution yielded a total solution volume comparable to the daily saliva volume in vivo.

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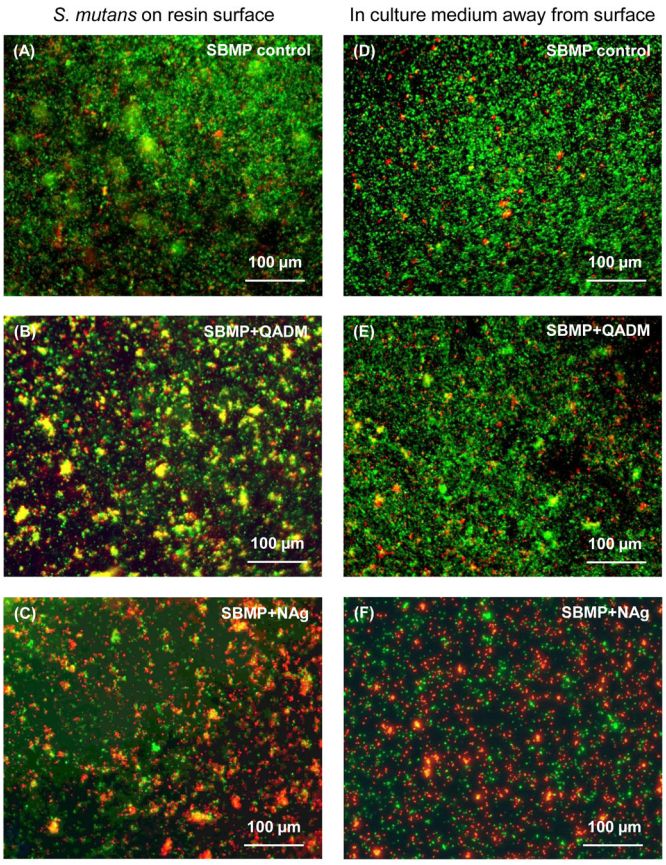

Fig. 3.

S. mutans live/dead assay. The left column shows biofilms on primer/adhesive disks. The right column shows planktonic bacteria from culture medium. Live bacteria were stained green, and dead bacteria were stained red. Live/dead bacteria that were close to each other produced orange/yellow colors. (A and D) Controls had mostly live bacteria. (B and C) Disks with QADM or NAg had more compromised bacteria on their surfaces. (E) Disk with QADM did not kill the planktonic bacteria away from its surface in the medium. (F) Disks with NAg inhibited bacteria away from its surface in the medium.

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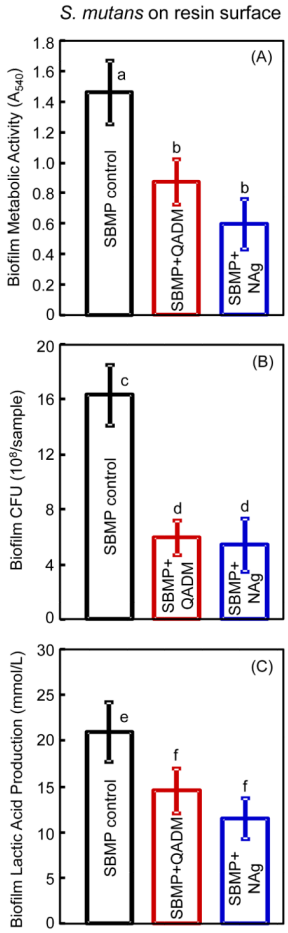

Fig. 4.

Results for *S. mutans* biofilms on primer/adhesive disk surfaces: (A) Metabolic activity, (B) CFU, and (C) lactic acid production (mean ± sd; n = 6). QADM and NAg in bonding agents significantly reduced biofilm activity, compared to control ($p < 0.05$).

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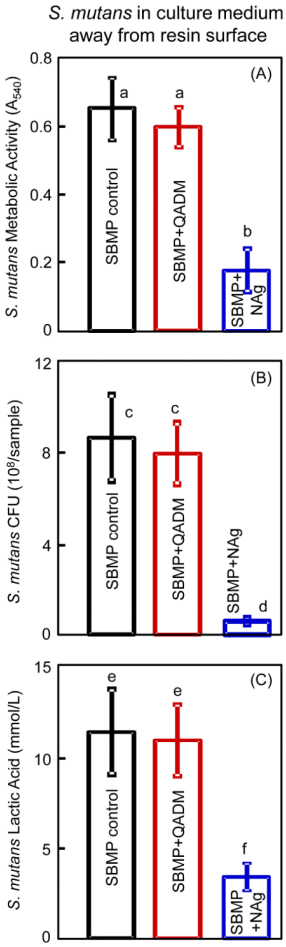

Fig. 5.

S. mutans suspended in culture medium away from the resin surfaces: (A) Metabolic activity, (B) CFU, and (C) lactic acid production (mean ± sd; n = 6). For each property, there is no significant difference between the control and the QADM group ($p > 0.1$). However, NAg significantly reduced bacterial activity, compared to control ($p < 0.05$).

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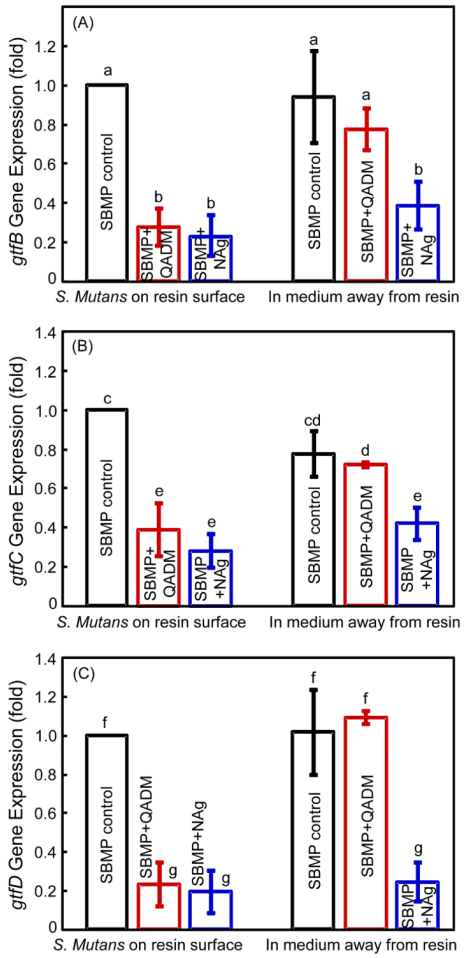

Fig. 6.

RT-PCR of *gtf* gene expressions of *S. mutans* (mean ± sd; n = 6). For *S. mutans* biofilms on the resin surface, all three gene expressions were greatly reduced by QADM and NAg ($p < 0.05$). However, for planktonic *S. mutans* in the culture medium away from the resin surface, the gene expression was substantially reduced by NAg, but not by QADM, compared to control. These data demonstrate that QADM-resin inhibited bacteria on its surface, but not bacteria away from its surface. In contrast, NAg-resin inhibited not only the bacteria on its surface, but also the bacteria in culture medium away from its surface.