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Effects of antibacterial primers with quaternary ammonium and nano-silver on *S. mutans* impregnated in human dentin blocks

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Abstract

Objectives—Recent studies developed antibacterial bonding agents and composites containing a quaternary ammonium dimethacrylate (QADM) and nanoparticles of silver (NAg). The objectives of this study were to investigate: (1) the effect of antibacterial primers containing QADM and NAg on the inhibition of *Streptococcus mutans* (*S. mutans*) impregnated into dentin blocks for the first time, and (2) the effect of QADM or NAg alone or in combination, and the effect of NAg mass fraction, on *S. mutans* viability in dentin.

Methods—Scotchbond Multi-Purpose (SBMP) bonding agent was used. QADM and NAg were incorporated into SBMP primer. Six primers were tested: SBMP primer control, control + 10% QADM (mass %), control + 0.05% NAg, control + 10% QADM + 0.05% NAg, control + 0.1% NAg, and control + 10% QADM + 0.1% NAg. *S. mutans* were impregnated into dentin blocks, then a primer was applied. The viable colony-forming units (CFU) were then measured by harvesting the bacteria in dentin using a sonication method.

Results—Control + 10% QADM + 0.1% NAg had bacteria inhibition zone 8-fold that of control ($p < 0.05$). The sonication method successfully harvested bacteria from dentin blocks. Control + 10% QADM + 0.1% NAg inhibited *S. mutans* in dentin blocks, reducing the viable CFU in dentin by three orders of magnitude, compared to control dentin without primer. Using QADM+NAg was more effective than QADM alone. Higher NAg content increased the potency. Dentin shear bond strength was similar for all groups ($p > 0.1$).

Significance—Antibacterial primer with QADM and NAg were shown to inhibit the *S. mutans* impregnated into dentin blocks for the first time. Bonding agent containing QADM and NAg is

promising to eradicate bacteria in tooth cavity and inhibit caries. The QADM and NAg may have applicability to other adhesives, cements, sealants and composites.

Keywords

Antibacterial dental primer; bacteria impregnation in dentin; quaternary ammonium dimethacrylate; silver nanoparticles; caries inhibition

1. Introduction

Dental caries is a multifactorial disease in which susceptible dental hard tissues are destructed by acidic by-products from bacterial fermentation of dietary carbohydrates [1,2]. Though the disease process and the potential to prevent and control the disease are now much better understood, caries is still prevalent and a heavy financial burden around the world [2,3]. Resin composites are popular filling materials [4,5]. The chemical and physical properties, polymerization characteristics, and placement techniques of composites have been significantly improved [6–13]. Nearly 200 million dental restorations are placed annually in the U.S. [14]. However, half of all restorations fail within 10 years, with recurrent (secondary) caries as the main reason for failure [15,16]. As a result, 50–70% of all restorations were replacements of failed restorations [17,18]. Replacement dentistry is a major economic burden, considering that the annual cost for tooth cavity restorations in the U.S. was approximately \$46 billion in 2005 alone [19].

With the development of new composites and bonding agents, the concept of complete removal of carious dentin during cavity preparation is no longer supported [20,21]. This is partly because that the complete caries removal approach could not really yield caries-free cavities, with often residual infection and bacteria remaining in the cavities [20,22]. On the other hand, minimally invasive techniques are recommended for the treatment of deep caries lesions in order to protect pulp vitality and preserve more tooth tissues [21,23,24]. Modern minimally invasive techniques require removing the infected-dentin to achieve its objective of least-invasive surgical approach, leaving behind the affected-dentin in the cavity. Various methods were developed to help remove caries-infected tissue as selectively as possible, while being minimally invasive through maximum preservation of caries-affected tissue [21]. With the preservation of more affected tissue, the cavity likely will contain more residual bacteria [25]. Therefore, it would be highly beneficial to develop antibacterial adhesives to eradicate or greatly reduce the residual bacteria in the affected-dentin and eliminate the harmful effects caused by bacteria.

Adhesives bond composite restorations to tooth structures, and extensive studies have been performed to enhance the adhesive compositions, bond strength, and durability [26–32]. Antibacterial adhesives containing 12-methacryloyloxydodecylpyridinium bromide (MDPB) were developed, which effectively reduced the growth of oral bacteria such as *Streptococcus mutans* (*S. mutans*) [33,34]. Other studies developed several different types of novel antibacterial dental materials [34–42]. A literature search revealed only a few reports on the development of antibacterial primers: a primer containing MDPB [22,43], a primer containing chlorhexidine [44], and a primer containing quaternary ammonium dimethacrylate (QADM) and nanoparticles of silver (NAg) [45]. The primer containing QADM and NAg showed potent antibacterial effects against dental biofilms with bacteria inoculated on the surface of the cured primer and adhesive resins [40,45]. However, there has been no report on the effect of the QADM-NAg primer against bacteria impregnated into dentin blocks.

Therefore, the objectives of this study were to investigate the antibacterial effects of primers containing QADM and NAg against *S. mutans* impregnated into human dentin blocks for the first time, and to determine the effects of different primer compositions. It was hypothesized that: (1) QADM- and NAg-containing primers would be able to inhibit *S. mutans* inside dentin blocks; (2) Combining two antibacterial agents QADM and NAg in primer would be more effective than QADM alone, and increasing the NAg mass fraction would increase the antibacterial potency against *S. mutans* inside dentin; (3) Incorporating QADM and NAg into primer would not compromise the dentin shear bond strength.

2. Materials and methods

2.1. QADM and NAg incorporation into primer

Scotchbond Multi-Purpose (3M, St. Paul, MN) was used as the parent bonding system and referred as “SBMP”. The SBMP etchant contained 37% phosphoric acid. The SBMP primer contained 35–45% of 2-hydroxyethylmethacrylate (HEMA), 10–20% of a copolymer of acrylic and itaconic acids, and 40–50% of water, according to the manufacturer. The SBMP adhesive contained 60–70% of bisphenol A diglycidyl methacrylate (BisGMA), 30–40% of HEMA, tertiary amines and photo-initiator. In the present study, the primer was modified with antibacterial agents, and the adhesive was used without modification.

Bis(2-methacryloyloxyethyl) dimethylammonium bromide, a quaternary ammonium dimethacrylate (QADM), was synthesized following previous studies [38,39]. The synthesis employed a modified Menshutkin reaction, in which a tertiary amine group was reacted with an organo-halide. A benefit of this reaction is that the reaction products are generated at virtually quantitative amounts and require minimal purification [38]. 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 Labs, Trevose, PA) were combined with 3 g of ethanol in a 20 mL scintillation vial. A magnetic stir bar was added, and the vial was stirred at 60 °C for 24 h. The solvent was then removed, yielding QADM as a clear, colorless, and viscous liquid. The QADM was mixed with the SBMP primer at a QADM mass fraction of 10%, following a previous study [45].

To develop primers containing NAg, silver 2-ethylhexanoate powder (Strem, Newburyport, MA) was dissolved in 2-(tert-butylamino)ethyl methacrylate (TBAEMA, Sigma) at 0.1 g of silver salt per 0.9 g of TBAEMA, following previous studies [39,46]. TBAEMA was used because it improves the solubility by forming Ag-N coordination bonds with Ag ions, thereby facilitating the Ag salt to dissolve in the resin solution. TBAEMA contains reactive methacrylate groups and therefore can be chemically incorporated into a dental resin [39,46]. When the Ag solution was mixed with a resin, upon photopolymerization which was accompanied with a simultaneous reduction of the silver salt, NAg were formed in situ in the resin. Previous studies showed well-dispersed NAg with a mean particle size of 2.7 nm in the cured resin [39,46]. In this study, the Ag solution was mixed with SBMP primer at two different mass fractions of silver 2-ethylhexanoate of 0.05% and 0.1%, respectively, following previous studies [40,45]. Six primers were tested as listed in Table I.

2.2. Agar disk diffusion test (ADT)

The use of *S. mutans* (ATCC 700610, UA159, American Type Culture, Manassas, VA) was approved by the University of Maryland. Fifteen µL of stock bacteria was added to 15 mL of growth medium consisting of brain heart infusion (BHI) broth (BD, Franklin Lakes, NJ) supplemented with 0.2% sucrose. The samples were incubated at 37 °C with 5% CO₂ for 24 h, then 0.4 mL of the bacteria suspension was swabbed uniformly across an agar plate with a diameter of 90 mm and a thickness of 4 mm. For each of the six primers in Table I, 20 µL of primer was impregnated into a sterile paper disk with a diameter of 6 mm and a thickness of

1.5 mm, following previous studies [22,45]. The primer-impregnated disks were placed on agar plates which had been inoculated with the *S. mutans* bacterial suspension. The plates were incubated in 5% CO₂ for 48 h at 37 °C. The size of the inhibition zones for each material was calculated from the diameter of the halo of the bacterial inhibition: Inhibition zone size = (diameter of halo - diameter of paper disk)/2 [22,45].

2.3. SEM examination of bacteria impregnation into dentin block

Extracted molars were obtained from patients under informed consent, and dentin blocks of approximately 4 × 4 × 0.5 mm were cut from the crown [47]. The dentin block was cut so that one dentin surface of 4 × 4 mm faced the occlusal enamel, while the other 4 × 4 mm surface faced the pulp. The dentin surface that faced the pulp was cut at a distance of approximately 1 mm away from the pulp. In subsequent bacterial impregnation, the dentin surface that faced the occlusal enamel was used for impregnation of the bacterial suspension. The thickness of the dentin blocks was adjusted to approximately 4 mm × 4 mm × 200 μm using 1500-grit silicon carbide abrasive paper [47]. The dentin blocks were treated with 37% phosphoric acid for 3 min to remove the smear layer and partially demineralize the surface of the dentin, which were then thoroughly rinsed with distilled water for 60 s [48]. The dentin blocks were sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC) for 12 h according to the manufacturer's specifications. The disks were then de-gassed for 7 days to remove the ethylene oxide gas.

A 2-μL aliquot of *S. mutans* suspension prepared to 10¹⁰ CFU/mL in BHI was placed on the surface of the dentin block. The suspension was left to infiltrate into the dentin for 10 min, to simulate bacterial colonization of carious dentin, following a previous study [22]. For examination with scanning electron microscopy (SEM), the dentin blocks with the impregnated bacteria were immersed in 1% glutaraldehyde in PBS for 4 h at 4 °C. The dentin was then subjected to graded ethanol dehydrations, and rinsed twice with 100% hexamethyldisilazane. The dentin blocks were then sputter-coated with gold and examined via SEM (Quanta 200, FEI, Hillsboro, OR). Dentin blocks without bacteria were also included for examination. In addition, to examine bacteria impregnation into the interior of the dentinal tubules, dentin blocks after bacteria impregnation were opened and the cross-sections were examined in SEM.

2.4. Harvesting the bacteria in dentin blocks impregnated with *S. mutans*

A 2-μL aliquot of *S. mutans* suspension at 10¹⁰ CFU/mL in BHI was impregnated into dentin as described above. Each dentin block was then placed into a tube with 2 mL of CPW, and the bacteria were harvested by sonication (3510R-MTH, Branson Ultrasonics, Danbury, CT) at a frequency of 40 kHz for 5 minutes, and by vortexing at the maximum speed for 20 s using a vortex mixer (Fisher, Pittsburgh, PA). The bacterial suspension thus collected was serially diluted in CPW and plated on BHI agar plates. The BHI agar plates were incubated at 5% CO₂ and 37 °C for 3 d, and the number of colonies was counted on the agar plates. This yielded the CFU harvested from the dentin block by the sonication method.

To examine whether the sonication method successfully harvested all the bacteria in the dentin block, the dentin after sonication was cut into small pieces and homogenized in 500 μL of CPW, following a previous study [22]. The cutting and homogenizing method was previously shown to successfully harvest the bacteria in dentin blocks [22]. The solution with small dentin pieces were vortexed at the maximum speed for 20 s using the vortex mixer. The purpose of this was to harvest any residual bacteria remaining in the dentin after being harvested by the sonication method. The homogenized solution was plated on BHI agar plates and incubated for 3 d, and the number of colonies was counted. This yielded the residual bacteria CFU in the dentin block after being harvested by the sonication method.

The rationale for this experiment was that the sonication method for harvesting the bacteria in dentin was a simpler method. If the sonication method could effectively harvest the bacteria from the dentin block, then the step of cutting and homogenizing the dentin block could be avoided.

2.5. Live/dead bacterial staining assay

Two μL of *S. mutans* suspension at 10^{10} CFU/mL was placed on the dentin block to simulate bacteria colonization of dentin, as described above. Then each of the six primers in Table I was applied to the dentin and left in place for 20 s, with 2 μL of primer per dentin block, and six dentin blocks for each primer. The dentin blocks were then stained using a live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce green fluorescence, and bacteria with compromised membranes were stained with propidium iodide to produce red fluorescence. The stained samples were imaged using an epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY) [40,45].

2.6. Effects of primers on the inhibition of *S. mutans* impregnated in dentin

Two μL of *S. mutans* at 10^{10} CFU/mL was placed on the dentin block, which was shown by the results of Section 2.3 to successfully impregnate the bacteria into the interior dentinal tubules. Then each of the six primers in Table I was applied. The dentin blocks were then used for CFU measurements. The results of Section 2.4 showed that the sonication method successfully harvested 99.99% of the total amount of bacteria in the dentin blocks. Hence, the sonication method was used here to measure the viable CFU in dentin blocks after placement of different types of antibacterial primers. Dentin blocks with *S. mutans* impregnation and primer application were placed into tubes with 2 mL of CPW, and the bacteria were harvested by sonication (Branson Ultrasonics) and vortexing at the maximum speed for 20 seconds using a vortex mixer (Fisher). The bacterial suspensions were serially diluted in CPW and plated on BHI agar plates. After incubating the agar plate at 5% CO_2 and 37 °C for 3 d, the number of colonies was counted [40,45].

2.7. Dentin shear bond testing

The protocol was approved by the University of Maryland. Extracted caries-free human third molars were cleaned and stored in 0.01% thymol solution. Sixty teeth (10 teeth per primer group) were prepared following previous studies [40,45]. Briefly, the tips of the molar crowns were cut off with a diamond saw (Isomet, Buehler, Lake Bluff, IL) to prepare the flat mid-coronal dentin surfaces. Each tooth was embedded in a poly-carbonate holder (Bosworth, Skokie, IL), and the occlusal enamel was completely removed by polishing with 320-grit silicon carbide paper. The dentin surface was etched with 37% phosphoric acid gel for 15 s and rinsed with distilled water for 15 s [45,49]. A primer was applied with a brush-tipped applicator and rubbed in for 15 s. The solvent was removed with a stream of air for 5 s. Then the unmodified SBMP adhesive was applied and light-cured for 10 s (Optilux VCL 401, Demetron Kerr, Danbury, CT). A stainless-steel iris with a central opening (diameter = 4 mm, thickness = 1.5 mm) was held against the adhesive-treated dentin surface. The opening was filled with a composite (TPH, Caulk/Dentsply, Milford, DE), and light-cured for 60 s. The bonded specimens were stored in distilled water at 37 °C for 24 h. The dentin shear bond strength, S_D , was measured. A chisel was connected with a Universal Testing Machine (MTS, Eden Prairie, MN) and held parallel to the composite-dentin interface. Load was applied at a rate of 0.5 mm/min until the bond failed. $S_D = 4P/(\pi d^2)$, where P is the load at failure, and d is the diameter of the composite [40,45,49].

For SEM examination, the bonded tooth was cut through the center in the longitudinal direction via the diamond saw (Isomet). The sectioned surface was polished with

increasingly finer SiC paper up to 4000 grit. Following a previous study [47], the polished surface was treated with 50% phosphoric acid for 30 s, then with 10% NaOCl for 2 min. The specimens were sputter-coated with gold and the dentin-adhesive bonded interfaces were examined via SEM (Quanta 200) [40,45].

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was performed to detect the significant effects of the variables. Tukey's multiple comparison was used to compare the data at a p value of 0.05.

3. Results

Fig. 1 shows the ADT results of the antibacterial activity of uncured primers (mean $\pm$ sd; $n = 6$). The control primer had minimal inhibition zone sizes. The antibacterial primers 10QADM, 0.05NAG, 10QADM+0.05 NAG and 0.1 NAG had much larger inhibition zones than the control ($p < 0.05$). The inhibition zone size for 10QADM+0.1NAG was the largest, which was about 8-fold that of the control primer ($p < 0.05$).

Typical SEM micrographs of dentin and *S. mutans* impregnation are shown in Fig. 2. Examples of dentinal tubules "T" before *S. mutans* impregnation are shown in (A). In (B), the external surface of dentin is shown, in which *S. mutans* were impregnated into dentinal tubules. In (C), *S. mutans* in dentinal tubules on the cross-section of dentin is shown when the dentin was opened after bacteria impregnation, showing successful bacteria impregnation into the interior of dentin. A higher magnification of a dentin cross-section is shown in (D) with *S. mutans* in the dentinal tubule. These examinations demonstrated that the *S. mutans* were successfully impregnated into the interior of the dentin blocks.

Fig. 3 plots the results of the bacteria harvest method after the dentin was impregnated with *S. mutans* (mean $\pm$ sd; $n = 6$). The sonication method successfully harvested nearly all the *S. mutans* in dentin blocks. After sonication, the residual bacteria in dentin harvested by cutting the dentin into small pieces and homogenization was 4 orders of magnitude lower than that harvested by the sonication method. The total CFU in the dentin block was estimated as the the CFU harvested by sonication plus the residual bacteria harvested by cutting the dentin into small pieces and homogenization. The CFU harvested by sonication was equal to approximately 99.99% of the total CFU in the dentin block.

Bacteria-impregnated dentin blocks were live/dead stained, and representative images were shown in Fig. 4(A–F) after placement of the six primers. The staining images showed bacteria impregnation in tubules in dentin surfaces similar to that in Fig. 2B. Dentin with SBMP control primer had much more live bacteria than the other primers. Adding QADM into the primer resulted in a noticeable increase in red staining of compromised bacteria. Adding NAG to the primer yielded even more dead bacteria staining. The use of both QADM and NAG in primer appeared to produce the most amount of dead bacteria.

These qualitative observations were corroborated with quantification of *S. mutans* CFU in dentin blocks in Fig. 5 (mean $\pm$ sd; $n = 6$). The CFU was the highest in the control dentin blocks without primer. The antibacterial primers with QADM and NAG greatly lowered the CFU of *S. mutans* in dentin blocks, compared to SBMP control primer ($p < 0.05$). The CFU from dentin treated with the 10QADM+0.1NAG primer was about 5% of the CFU of SBMP control primer. Dentin treated with the 10QADM+0.1NAG primer reduced the CFU by three orders of magnitude, compared to the CFU in control dentin without primer. These results demonstrated that the antibacterial primers were able to inhibit the bacteria residing in the dentinal tubules of the dentin blocks.

Fig. 6 shows (A) the shear bond strength (mean $\pm$ sd; $n = 10$), and (B) SEM micrograph of the dentin-adhesive interface. The shear bond strengths were not significantly different from each other ($p > 0.1$). All six groups had similar dentin-adhesive interfacial features in SEM, with a hybrid layer filled with primer and adhesive resin and the formation of numerous resin tags. These results indicate that incorporating QADM and NAg into the primer did not adversely affect the dentin bonding properties.

4. Discussion

This study showed that antibacterial primers containing QADM and NAg were effective in inhibiting the *S. mutans* impregnated into dentin blocks for the first time. *S. mutans* were successfully impregnated into the interior of the dentinal tubules of the dentin blocks. In addition, the sonication method was effective in harvesting the bacteria from the dentin blocks. The novel antibacterial primers were able to inhibit the bacteria inside dentin blocks, by greatly reducing the CFU of *S. mutans* harvested from the dentin. Cavity disinfectants such as chlorhexidine or peroxide are routinely used by many dental clinicians in the treatment of caries, because it is difficult to determine whether the lesion has been completely removed from the prepared cavity [22]. However, two problems exist regarding the use of cavity disinfectants. First, there is concern on whether the application of cavity disinfectants would reduce the bond strength of dentin adhesive systems. Several previous studies indicated that pretreatment with traditional cavity disinfectants had a negative effect on the dentin bond strength of bonding systems [50,51]. Second, traditional cavity disinfectants could not offer long-term antibacterial effects, and the use of cavity disinfectants did not completely eliminate the viable microorganisms in the prepared tooth cavity [52].

While residual bacteria in the tooth cavity can lead to caries and pulp damage, dentin primer has direct contact with the tooth structure and the dentinal tubules, and therefore is a promising vehicle for the delivery of antimicrobial agents. The present study showed that the uncured primer containing QADM and NAg reduced the bacterial CFU in dentin blocks by three orders of magnitude, compared to control dentin without primer. In addition, previous studies showed that bonding agents containing QADM and NAg continued to have a strong antibacterial activity after being photo-cured, thereby exerting a long-lasting effect against residual bacteria in the dentinal tubules as well as new invading bacteria along the margins due to microleakage [40,45,53]. Therefore, the novel antibacterial primer has two advantages. First, when the primer was applied after tooth cavity preparation, it could act as both a primer and a cavity disinfectant without significantly influencing the shear bonding strength. Second, long-term antibacterial effect in the cured state could provide lasting protection for the restoration.

In the agar disk diffusion test, the 10QADM+0.1NAg primer increased the *S. mutans* inhibition zone by 8-fold, compared to the commercial control primer. This demonstrated the potent antibacterial functions of the new primer. When the antibacterial primer was placed on the dentin block which had been impregnated with *S. mutans*, the primer readily flowed into the tubules to kill the bacteria inside the dentin. Three points should be noted here. First, the dentin block was etched for 3 min with 37% phosphoric acid to remove the smear layer and partially demineralize the surface of the dentin; it did not demineralize the entire dentin block. The purpose was to open the dentinal tubules to facilitate bacterial impregnation. The incubation of bacterial suspension on the dentin block for 10 min was successful in bacterial infiltration into the dentin block (Fig. 2); however, these were planktonic bacteria and not biofilms. Second, the 2 μ L of bacterial suspension contained 2×10^7 CFU of *S. mutans*, but the total *S. mutans* harvested from the dentin block without primer were only 2.51×10^6 CFU per dentin block. The suspension at the dentinal tubule

openings could readily flow into the tubules, because the tubule openings had diameters of about 3–4 μm , while the diameters of the *S. mutans* were only about 0.6–1.0 μm . However, when the 2 μL of bacterial suspension was placed on the small dentin block, some solution flowed into the dentin while the other portion of the solution flowed into culture well in which the dentin block was situated. The results indicated that $(2.51 \times 10^6)/(2 \times 10^7) = 12.6\%$ of the *S. mutans* were impregnated into the dentin, and 87.4% of the *S. mutans* flowed into the culture well. Third, the bacteria in dentin were harvested by cutting the dentin block into small pieces and homogenizing in PBS in a previous study [22]. The sonication method of the present study, without cutting the dentin into small pieces and homogenization, successfully harvested nearly all the bacteria in the dentin block. Therefore, the sonication method was adequate in harvesting the bacteria in dentin blocks.

Quaternary ammonium methacrylates such as MDPB were incorporated into dental resins for antibacterial functions [33,36–41]. Previous studies showed that the uncured primer with MDPB was antibacterial [54], and its antibacterial effect was maintained after curing [43]. Resins containing other antibacterial monomers, such as methacryloxylethyl cetyl dimethyl ammonium chloride and cetylpyridinium chloride, were also developed [36,37,55]. The antimicrobial mechanism of quaternary ammonium materials is that when the negatively-charged bacterial cell contacts the positively-charged sites of the monomer, the electric balance of cell membrane could be disturbed, and the bacterium could explode under its own osmotic pressure [55,56]. The QADM of the present study is unique because the reaction products were generated at quantitative amounts and required no further purification, so its synthesis method was straightforward [38]. Additionally, while monomers such as MDPB were monomethacrylates, QADM is a dimethacrylate with reactive groups on both ends of the molecule. Therefore, it could be incorporated into the resin with less of a negative impact on the mechanical properties. QADM has a low viscosity and is miscible with dental dimethacrylates, and is expected to have minimal monomer leach-out due to reactive groups on both ends of the molecule, compared to monomethacrylates [38,39].

Ag is a widely-used antibacterial agent [46,57–59]. The advantages of Ag include that it has a low toxicity and good biocompatibility with human cells [58], a long-term antibacterial effect due to sustained Ag ion release [60], and a lower level of bacterial resistance than antibiotics [61]. The antimicrobial mechanism was suggested to be that the Ag ions could inactivate the vital enzymes of bacteria, causing the DNA to lose its replication ability, which leads to cell death [62]. In the present study, the Ag salt was reduced to NAg in the resin in situ, avoiding the need to mix pre-fabricated nanoparticles with the resin, which could cause agglomeration. The NAg had a high surface area, which provided potent antibacterial effects at a low filler level of 0.05% and 0.1% to avoid negative influence on color and mechanical properties. A recent study [63] tested five different NAg mass fractions in a resin: 0%, 0.08%, 0.12%, 0.25%, and 0.50%. The resin containing 0.12% of NAg had no noticeable change in color, compared to that with 0%. However, when the NAg mass fraction was increased to 0.25%, the resin started to have a yellow/brown color. When the NAg was further increased to 0.5%, the resin had a darker brown color, accompanied by a precipitous decrease in the mechanical strength [63]. Therefore, the present study used 0.05% and 0.1% of NAg in the resin. Strong antibacterial effects were achieved via NAg incorporation, and increasing the NAg mass fraction from 0.05% to 0.1% increased the antibacterial potency. The CFU of bacteria in dentin blocks was significantly lower with the primer containing 0.1% NAg, compared to that using 0.05% NAg.

In recent investigations [39,40,45], combining QADM and NAg together in the resin yielded stronger antibacterial properties than using QADM or NAg alone. A primer containing QADM and NAg in the cured state exhibited a stronger inhibition on the growth and metabolism of dental biofilms than using QADM or NAg alone [45]. However, in the

present study on inhibition of *S. mutans* in dentin blocks, while the primer containing both QADM and NAg had stronger antibacterial effect than that using QADM alone, it was not significantly different from that using NAg alone ($p > 0.1$). This may be because that the NAg primer already reduced the CFU in dentin by three orders of magnitude compared to dentin without primer, hence the primer with NAg plus QADM did not further reduce the bacterial CFU. However, the use of QADM is still expected to be beneficial, because QADM is copolymerized with the resin after curing and provides a long-term antibacterial effect. A recent study showed that the anti-biofilm effect of a QADM-containing composite after 6 months of water-aging was the same as that at 1 day [64]. Further study is needed to investigate the effectiveness of the antibacterial primer containing QADM and NAg in inhibiting bacteria in dentinal tubules in the prepared tooth cavity in vivo.

5. Conclusions

The present study showed effective cavity disinfecting effects of QADM and NAg containing primers in bacteria-impregnated dentin for the first time. The results demonstrated that: (1) Antibacterial primer containing QADM+NAg had much larger bacteria inhibition zones than a commercial control primer; (2) antibacterial primer containing QADM+NAg inhibited *S. mutans* that had been impregnated into dentin blocks, reducing the CFU by three orders of magnitude, compared to control dentin without primer; (3) a simple sonication method successfully harvested the *S. mutans* in dentin blocks; (4) QADM- and NAg-containing primer did not decrease the dentin bond strength compared to control primer. Therefore, the bonding agent containing QADM and NAg is promising to eradicate bacteria in the tooth cavity and help inhibit recurrent caries. QADM and NAg may be useful for incorporation into other dental adhesives, cements, sealants and composites to achieve potent antibacterial functions.

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Fig. 1. Agar disk diffusion results of uncured primers against *S. mutans*: (A) Representative examples of SBMP control primer, and primer with 10QADM+0.1NAG; (B) *S. mutans* inhibition zone sizes (mean $\pm$ sd; n = 6). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

Fig. 2.

Representative SEM micrographs of dentin and *S. mutans* impregnation: (A) Dentinal tubules before *S. mutans* impregnation, (B) *S. mutans* impregnation into tubules on the external surface of dentin with the tubules perpendicular to the surface, (C) *S. mutans* in tubules parallel to the surface on the cross-section of dentin by opening the dentin block after bacteria impregnation, (D) higher magnification of the cross-section as in (C). T: dentinal tubules. Arrows indicate *S. mutans* in tubules. Hence, the *S. mutans* were successfully impregnated into the interior of the dentin blocks.

Fig. 3.

Harvesting the bacteria that had been impregnated in dentin blocks. The y-axis is a log scale. The sonication method was able to harvest nearly all the *S. mutans* in dentin blocks (mean $\pm$ sd; n = 6). After sonication, the residual bacteria in dentin were harvested by cutting the dentin into small pieces and homogenization. The CFU harvested by sonication + the residual CFU in dentin = the total CFU in dentin. The CFU harvested by sonication was 99.99% of the total CFU. Therefore, the simple sonication method can be used to harvest the *S. mutans* in the dentin blocks.

Fig. 4. Representative live/dead bacterial staining images of dentin that had been impregnated with *S. mutans* and then treated with primer: (A) SBMP primer control, (B) 10QADM, (C) 0.05NAg, (D) 10QADM+0.05NAg, (E) 0.1NAg, and (F) 10QADM+0.1NAg. Live bacteria were stained green, and dead bacteria were stained red. The incorporation of QADM and NAg greatly increased the staining amounts of dead bacteria and decreased the staining amounts of live bacteria.

Fig. 5.

S. mutans CFU in dentin blocks harvested by sonication (mean $\pm$ sd; n = 6). The CFU of the bacteria-impregnated dentin without primer was 2.51×10^6 (CFU/dentin block). The other groups had the units of 10^4 CFU/dentin block. The CFU of dentin treated with primer 10QADM+0.1NAG was three orders of magnitude less than the CFU of control dentin without primer. Therefore, the novel antibacterial primer effectively inhibited the bacteria in the dentinal tubules of the dentin blocks.

Fig. 6. Dentin bonding using extracted human molars: (A) shear bond strength (mean $\pm$ sd; n = 10), and (B) typical SEM micrograph of the dentin-adhesive interface. Horizontal line indicates values that are not significantly different from each other ($p > 0.1$). All six groups had similar dentin-adhesive interfacial features in SEM examination revealed well-infiltrated hybrid layer and numerous resin tags.

Table I

Compositions of dentin primers

Group	Dentin primer	Group name
1	SBMP primer control	SBMP
2	Control + 10% QADM	10QADM
3	Control + 0.05% NAg	0.05NAg
4	Control + 10% QADM + 0.05% NAg	10QADM+0.05NAg
5	Control + 0.1% NAg	0.1NAg
6	Control + 10% QADM + 0.1% NAg	10QADM+0.1NAg