Published in final edited form as:

Dent Mater. 2012 August ; 28(8): 842–852. doi:10.1016/j.dental.2012.04.027.

Effect of quaternary ammonium and silver nanoparticle-containing adhesives on dentin bond strength and dental plaque microcosm biofilms

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Abstract

Objectives—Antibacterial bonding agents are promising to hinder the residual and invading bacteria at the tooth-restoration interfaces. The objectives of this study were to develop an antibacterial bonding agent by incorporation of quaternary ammonium dimethacrylate (QADM) and nanoparticles of silver (NAg), and to investigate the effect of QADM-NAg adhesive and primer on dentin bond strength and plaque microcosm biofilm response for the first time.

Methods—Scotchbond Multi-Purpose adhesive and primer were used as control. Experimental adhesive and primer were made by adding QADM and NAg into control adhesive and primer. Human dentin shear bond strengths were measured ($n = 10$). A dental plaque microcosm biofilm model with human saliva as inoculum was used to investigate biofilm metabolic activity, colony-forming unit (CFU) counts, lactic acid production, and live/dead staining assay ($n = 6$).

Results—Adding QADM and NAg into adhesive and primer did not compromise the dentin shear bond strength which ranged from 30 to 35 MPa ($p > 0.1$). Scanning electron microscopy (SEM) examinations revealed numerous resin tags, which were similar for the control and the QADM and NAg groups. Adding QADM or NAg markedly reduced the biofilm viability, compared to adhesive control. QADM and NAg together in the adhesive had a much stronger

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antibacterial effect than using each agent alone ($p < 0.05$). Adding QADM and NAg in both adhesive and primer had the strongest antibacterial activity, reducing metabolic activity, CFU, and lactic acid by an order of magnitude, compared to control.

Significance—Without compromising dentin bond strength and resin tag formation, the QADM and NAg containing adhesive and primer achieved strong antibacterial effects against microcosm biofilms for the first time. QADM-NAg adhesive and primer are promising to combat residual bacteria in tooth cavity and invading bacteria at the margins, thereby to inhibit secondary caries. QADM and NAg incorporation may have a wide applicability to other dental bonding systems.

Keywords

Antibacterial dental adhesive; dentin bond strength; silver nanoparticles; quaternary ammonium dimethacrylate; human saliva microcosm biofilm; caries inhibition

1. Introduction

Of the total 166 million restorations placed in 2005 in the United States, 52.5 million (31.6%) were amalgams, 77.3 million (46.6%) were composites, and 36.2 million (21.8%) were crowns [1]. Resin composites are increasingly used because of their esthetics and improved performance [2-6]. Advances in filler compositions and polymer matrix have enhanced the composite properties [7-12]. One main drawback is that composites tend to accumulate more biofilms/plaques *in vivo* than other restorative materials [13]. Replacement of failed restorations accounts for 50-70% of all restorative dentistry [14], and replacement dentistry costs \$5 billion per year in the U.S. [15]. The main reason for restoration failure is secondary caries at the restoration margins, caused by acid production by biofilms [14-18]. Therefore, antibacterial composites were developed to inhibit biofilms and caries [19-23]. Quaternary ammonium salt (QAS) monomers such as 12-methacryloyloxydodecylpyridinium bromide (MDPB) were copolymerized in resins to yield antibacterial activities [19,24,25]. Recently, a quaternary ammonium dimethacrylate (QADM) was synthesized to possess strong antibacterial properties without compromising the mechanical properties of the resin [26,27]. In other studies, antibacterial nanocomposites containing nanoparticles of silver (NAg) were developed [27,28]. However, there has been no report on incorporating QADM and NAg into dental adhesives.

Composite restorations are bonded to tooth structure via adhesives [29-32]. Extensive studies have been performed to improve, characterize and understand enamel and dentin bonding [33-36]. It is desirable for the adhesive to be antibacterial to inhibit recurrent caries at the tooth-composite margins [19,21,37]. Residual bacteria could exist in the prepared tooth cavity, and microleakage could allow bacteria to invade the tooth-restoration interface. Adhesives that are antibacterial in the cured state could help inhibit the growth of residual and invading bacteria [38,39]. Indeed, MDPB-containing adhesives markedly inhibited the *Streptococcus mutans* (*S. mutans*) growth [19,38]. Another study developed an antibacterial adhesive containing methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB) [37]. Cetylpyridinium chloride (CPC) was also incorporated into a resin with bacteriostatic activity [21]. Besides the adhesive resin, it is also beneficial for the dentin primer to be antibacterial because it directly contacts the tooth structure [24,25,40]. A primer containing MDPB achieved potent antibacterial effects [24,25]. Another primer contained chlorhexidine with antimicrobial activities [40]. There have been only a few reports on the development of antibacterial adhesives and primers. There has been no report on antibacterial adhesive and primer that incorporate QADM and NAg.

The objectives of this study were to develop an antibacterial bonding agent by incorporation of QADM and NAg, and to investigate the effect of QADM-NAg adhesive and primer on dentin bond strength and plaque microcosm biofilm response for the first time. A human saliva microcosm biofilm model was used to evaluate the antibacterial properties. It was hypothesized that: (1) QADM and NAg incorporation would not decrease the bond strength to human dentin, compared to control without QADM and NAg; (2) The adhesive with QADM and NAg would greatly decrease the biofilm viability, metabolic activity and lactic acid production; (3) Combining QADM and NAg together in the adhesive would achieve a stronger antibacterial capability than using each agent alone, and adding QADM and NAg into both adhesive and primer would further increase the antibacterial potency.

2. Materials and methods

2.1. QADM incorporation

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. The purpose was to investigate a model system, and then the method of QADM and NAg incorporation could be applied to other adhesive systems. According to the manufacturer, SBMP etchant contains 37% phosphoric acid. SBMP primer single bottle contains 35-45% 2-Hydroxyethylmethacrylate (HEMA), 10-20% copolymer of acrylic and itaconic acids, and 40-50% water. SBMP adhesive contains 60-70% BisGMA and 30-40% HEMA.

Bis(2-methacryloyloxyethyl) dimethylammonium bromide was a quaternary ammonium dimethacrylate (QADM), and was recently synthesized and incorporated into dental composites [26,27]. The synthesis of QADM was performed using a modified Menshutkin 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 virtually quantitative amounts and require minimal purification [26]. 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. 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 adhesive or primer at a QADM mass fraction of 10%. QADM mass fractions of 20% or higher were not used due to a decrease in dentin bond strength in preliminary study.

2.2. NAg incorporation

Silver 2-ethylhexanoate powder (Strem, Newburyport, MA) was dissolved in 2-(tertbutylamino)ethyl methacrylate (TBAEMA, Sigma) at 0.08 g of silver salt per 1 g of TBAEMA, following previous studies [27,28]. 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 was selected since it contains reactive methacrylate groups and therefore can be chemically incorporated into a dental resin upon photopolymerization [27,28]. This method produced NAg with a mean particle size of 2.7 nm that were well dispersed in the resin matrix [27]. The Ag solution was mixed with SBMP adhesive at silver 2-ethylhexanoate mass fractions of 0.05% and 0.1%. Ag mass fractions of 0.15% or higher were not used due to a decrease in dentin bond strength.

2.3. Dentin Shear bond testing and SEM examination

As listed in Table 1, six groups were used for dentin shear bond strength testing. The purpose of groups 1-3 was to investigate the effects of QADM or NAg individually. The purpose of 3 and 4 was to examine the effect of NAg mass fraction. The purpose of

comparing 2, 3 and 5 was to examine the effect of combining QADM and NAg together in the same adhesive. The purpose of comparing 5 with 6 was to investigate the effects of adding QADM and NAg into both the adhesive and the primer on dentin bond strength and biofilm response.

Extracted caries-free human third molars were cleaned and stored in 0.01% thymol solution. Flat mid-coronal dentin surfaces were prepared by cutting off the tips of molar crowns with a diamond saw (Isomet, Buehler, Lake Bluff, IL). Each tooth was embedded in a polycarbonate holder (Bosworth, Skokie, IL) and ground perpendicular to the longitudinal axis on 320-grit silicon carbide paper until the occlusal enamel was completely removed. As shown schematically in Fig. 1A, the dentin surface was etched with 37% phosphoric acid gel for 15 s and rinsed with distilled water for 15 s, following a previous study [41]. The 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 adhesive was applied and light-cured for 10 s (Optilux VCL 401, Demetron Kerr, Danbury, CT). A stainless-steel iris, having a central opening with a diameter of 4 mm and a thickness of 1.5 mm, was held against the adhesive-treated dentin surface. The central 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 as shown schematically in Fig. 1B [41]. The chisel was connected with a computer-controlled 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 was calculated as: $SD = 4P/(\pi d^2)$, where P is the load at failure, and d is the diameter of the composite. Ten teeth were tested for each group ($n = 10$).

The bonded tooth was cut through the center in the longitudinal direction via the diamond saw (Isomet) with copious water. Three specimens were prepared for each group. The sectioned surface was polished with increasingly finer SiC paper up to 4000 grit. Following a previous study [39], the polished surface was treated with 50% phosphoric acid for 30 s, then with 10% NaOCl for 2 min. After being thoroughly rinsed with water for 10 min, the specimens were air dried and then sputter-coated with gold. The dentin-adhesive bonded interfaces were then examined via scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, OR).

2.4. Saliva collection for plaque microcosm model

The dental plaque microcosm model was approved by the University of Maryland. Human saliva was shown to be ideal for growing plaque microcosm biofilms *in vitro*, with the advantage of maintaining much of the complexity and heterogeneity of the dental plaque *in vivo* [42]. The saliva for biofilm inoculums was collected from a healthy adult donor having natural dentition without active caries or periopathology, and without the use of antibiotics within the last 3 months, following a previous study [43]. The donor did not brush teeth for 24 h and abstained from food/drink intake for at least 2 h prior to donating saliva. Stimulated saliva was collected during parafilm chewing and kept on ice. Saliva was diluted in sterile glycerol to a concentration of 70 %, and stored at -80 °C [43].

2.5. Specimen fabrication for biofilm experiments

Layered disk specimens for biofilm experiments were fabricated following previous studies [24,37]. A polyethylene disk mold (inner diameter = 9 mm, thickness = 2 mm) was situated on a glass slide. For groups 1-5, each adhesive was applied into the mold to cover the glass slide. Then, a composite (TPH) was placed onto the adhesive to fill the disk mold and light-

cured for 1 min. For group 6, the primer was first applied into the mold to cover the glass slide. After drying with a stream of air, the adhesive was applied and cured for 20 s with Optilux. Then, a composite (TPH) was placed on the adhesive to fill the disk mold and light-cured for 1 min. The disks were immersed in sterile water and agitated for 1 h to remove any uncured monomer, following a previous study [24]. The disks were then dried and sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC).

Six groups were tested in biofilm experiments. Groups 1-5 had specimens with adhesives types 1-5 covering the top surface of the composite disk, without primer, in order to test the antibacterial properties of the adhesives, as shown schematically in Fig. 3A. Group 6 had the QADM-NAg primer covering the adhesive on the composite disk in order to test the antibacterial properties of the primer/adhesive combination, as shown schematically in Fig. 3B.

2.6. MTT assay of metabolic activity

The saliva-glycerol stock was added, with 1:50 final dilution, to a growth medium as inoculum. The growth medium contained mucin (type II, porcine, gastric) at a concentration of 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L; KCl, 0.2 g/L; CaCl₂, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K1, 0.0002 g/L, at pH 7 [44]. The inoculum was cultured at 37 °C in an incubator containing 5% CO₂ for 24 h. Each disk specimen was placed into a well of 24-well plates, with the antibacterial surface on the top. 1.5 mL of inoculum was added to each well, and incubated in 5% CO₂ at 37 °C for 8 h. The disks were then transferred to new 24-well plates with fresh medium and incubated. After 16 h, the disks were transferred to new 24-well plates with fresh medium and incubated for 24 h. This 2-day (d) incubation formed plaque microcosm biofilms as shown previously [43].

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay that measures the enzymatic reduction of MTT, a yellow tetrazole, to formazan [26,27]. Each disk with the 2-d biofilm was transferred to a new 24-well plate, then 1 mL of MTT dye (0.5 mg/mL MTT in PBS) was added to each well and incubated at 37 °C in 5% CO₂ for 1 h. During this process, metabolically active bacteria reduced the MTT to purple formazan. After 1 h, the disks were transferred to a new 24-well plate, 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals, and the plate was incubated for 20 min with gentle mixing at room temperature in the dark. After mixing via pipette of the DMSO solution from each well was transferred to a 96-well plate, and the absorbance at 540 nm (optical density OD₅₄₀) was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). A higher absorbance is related to a higher formazan concentration, which indicates a higher metabolic activity in the biofilm on the disk.

2.7. Live/dead staining of biofilms

Microcosm biofilms were grown on the disks for 2 d as described in section 2.6. The biofilms on the disks were gently washed three times with phosphate buffered saline (PBS), and then stained using a live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce a green fluorescence, and bacteria with compromised membranes were stained with propidium iodide to produce a red fluorescence. The stained disks were examined using an epifluorescence microscope (TE2000-S, Nikon, Melville, NY) [27].

2.8. Lactic acid production and colony-forming unit (CFU) counts

Each disk with the 2-d biofilm was rinsed with cysteine peptone water (CPW) to remove loose bacteria. The disks were transferred to 24-well plates containing buffered peptone water (BPW) plus 0.2% sucrose. The samples were incubated in 5% CO₂ at 37 °C for 3 h to allow the biofilms to produce acid. The BPW solutions were then stored for lactate analysis.

Disks with biofilms were transferred into tubes with 2 mL CPW, and the biofilms were harvested by sonication and vortexing via a vortex mixer (Fisher, Pittsburgh, PA). Three types of agar plates were used. First, tryptic soy blood agar culture plates were used to determine total microorganisms [43]. Second, mitis salivarius agar (MSA) culture plates, containing 15% sucrose, were used to determine total streptococci [45]. This is because MSA contains selective agents crystal violet, potassium tellurite and trypan blue, which inhibit most gram-negative bacilli and most gram-positive bacteria except streptococci, thus enabling streptococci to grow [45]. Third, cariogenic mutans streptococci are known to be resistant to bacitracin, and this property is often used to isolate mutans streptococci from the highly heterogeneous oral microflora. Hence, MSA agar culture plates plus 0.2 units of bacitracin per mL was used to determine mutans streptococci [46].

Lactate concentrations in the BPW solutions were determined using an enzymatic (lactate dehydrogenase) method, following a previous study [43]. The microplate reader was used to measure the absorbance at 340 nm (optical density OD₃₄₀) for the collected BPW solutions. Standard curves were prepared using a lactic acid standard (Supelco, Bellefonte, PA).

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 schematics of the dentin shear bond test and the strength results: (A) Schematic of specimen preparation, (B) schematic of shear bond testing, (C) dentin shear bond strength results. In (C), each value is mean $\pm$ sd (n = 10). The six groups had shear bond strengths that were not significantly different ($p > 0.1$), indicating that adding QADM and NAg to adhesive and primer did not compromise the dentin shear bond strength.

The dentin-adhesive interfaces were examined via SEM, and representative images are shown in Fig. 2: (A) SBMP control, and (B) A&P+10QADM+0.05NAg. "HL" refers to the hybrid layer between the adhesive and the underlying mineralized dentin. "T" indicates the resin tags formed by the adhesive resin filling into the dentinal tubules. Numerous resin tags were found in samples of all six groups. The short arrow in (A) indicates a short resin tag. The long arrow indicates an example of a long resin tag. Some tags were shorter because, during sample preparation, the sectioning surface was not exactly parallel to the long axis of dentinal tubules. Some tubules were intersected by the cutting and thus shortened. A mixture of long and short tags was observed in all samples. There was no noticeable difference between the six groups.

Fig. 3 shows the setup for biofilm experiment and the metabolic activity: (A) Schematic of biofilm on the adhesive surface covering the composite, (B) biofilm on the primer covering the adhesive and composite, and (C) MTT metabolic activity. In (C), groups 1-5 followed schematic A. Group 6 used the QADM-NAg primer and followed schematic B. Biofilms on the as-received commercial adhesive had a high metabolic activity. Incorporation of QADM and NAg each markedly reduced the metabolic activity ($p < 0.05$). Adding QADM and NAg together in the adhesive resulted in a much lower metabolic activity than using QADM or

NAg alone ($p < 0.05$). Adding QADM and NAg both in the primer and in the adhesive yielded the lowest biofilm metabolic activity ($p < 0.05$). The metabolic activity of biofilms on A&P+10QADM+0.05NAg was nearly an order of magnitude less than that on adhesive control.

Fig. 4 shows the live/dead staining photos of the biofilms. Live bacteria were stained green, and dead bacteria were stained red. Live/dead bacteria that were close to, or on the top of, each other produced orange or yellow colors. Adhesive control was covered with primarily live bacteria. In contrast, adhesives containing QADM and NAg had much more staining of compromised bacteria. Specimens with primer and adhesive both containing 10% QADM and 0.05% NAg had mostly red and orange staining. Six disks were observed for each group, and the results show a strong antibacterial activity for groups containing NAg and QADM.

Fig. 5 plots CFU counts for: (A) Total microorganisms, (B) total streptococci, and (C) mutans streptococci. Adding QADM or NAg each decreased the CFU, compared to the as-received commercial adhesive control ($p < 0.01$). Increasing the NAg mass fraction from 0.05% to 0.1% decreased the CFU ($p < 0.05$). The combination of QADM and NAg in the adhesive had a much stronger antibacterial effect than using QADM or NAg alone ($p < 0.05$). Incorporation of QADM and NAg together, in both the primer and the adhesive, had the greatest reductions in CFU ($p < 0.05$). All three CFU counts for biofilms on A&P +10QADM+0.05NAg were reduced by an order of magnitude, compared to those on the as-received commercial adhesive.

Fig. 6 plots the lactic acid production by biofilms. Biofilms on adhesive control produced the most acid. Adding QADM or NAg each decreased the acid production ($p < 0.05$). Adhesive with 0.1% NAg had less acid than that with 0.05% NAg ($p < 0.05$). Adhesive with both 10% QADM and 0.05% NAg had less acid than those using either 10% QADM or 0.05% NAg ($p < 0.05$). When 10% QADM and 0.05% NAg were incorporated into both the adhesive resin and the primer, the lactic acid production was further reduced ($p < 0.05$).

4. Discussion

The present study developed antibacterial adhesives and investigated the effects of QADM and NAg incorporation into adhesive and primer on dentin bond strength and microcosm biofilm response for the first time. Previous studies showed that more than half of tooth cavity restorations placed by the dentists are replacements, with secondary caries at the tooth-restoration margins as the main reason for failure [14,15,18]. Caries is a dietary carbohydrate-modified bacterial infectious disease caused by acid production by biofilms [16,17]. Therefore, it is highly desirable to use antibacterial bonding agents at the tooth-restoration interface. There are often residual bacteria present in the prepared tooth cavity [37,38]. This is especially so with the increased interest in less removal of tooth structure and the minimal intervention dentistry [47], which could leave behind more carious tissues with active bacteria in the tooth cavity. Furthermore, while a complete sealing of the tooth-restoration interface is an important goal, it is difficult to achieve. Indeed, many studies revealed microgaps at the tooth-restoration interfaces, which could allow for bacteria invasion [48-52]. These microgaps could further deteriorate due to fatigue stresses and compromise the durability of the bonded interface. The dual attacks by fatigue damage and biofilm acids at the tooth-restoration margins deserve further investigation. Both the residual bacteria in the tooth cavity, and the invading bacteria along the tooth-restoration margins due to bacterial leakage, could harm the pulp and cause recurrent caries. In the present study, when QADM and NAg were added to the primer and adhesive, the biofilm metabolic activity, CFU and lactic acid production were all reduced by nearly an order of magnitude,

compared to the un-modified commercial adhesive. Therefore, the QADM-NAg adhesive and primer have the potential to combat residual bacteria in the tooth cavity and invading bacteria along the margins.

It is desirable to use an antibacterial adhesive to combat biofilms and secondary caries at the tooth-restoration margins. This is because microgap formation occurred between the adhesive resin and the primed dentin, or between the adhesive resin and the hybrid layer [48-52]. This would suggest that a large portion of the marginal gap is surrounded by the cured adhesive resin, hence the invading bacteria would mostly come into contact with the adhesive surface [38]. Furthermore, dentin primer has direct contact with the tooth structure and the dentinal tubules, hence it would also be beneficial to use an antibacterial primer to kill residual bacteria in the tooth cavity. Therefore, the present study incorporated QADM and NAg into both the adhesive resin and the primer. The results on the adhesive resin showed that adding QADM or NAg each rendered the adhesive antibacterial; however, the incorporation of QADM and NAg together in the adhesive yielded an even more potent antibacterial effect. When biofilms were grown on the QADM-NAg primer on the top of the QADM-NAg adhesive, the strongest antibacterial effect was obtained among all the groups tested. Therefore, it is beneficial to incorporate antibacterial agent into both adhesive and primer.

The present study showed that the commercial adhesive without modification was not antibacterial, consistent with previous results showing that the cured commercial adhesives had normal bacteria growth with no antibacterial activity [37,38,53]. Therefore, antibacterial agents are needed [19,21,37,38]. The antimicrobial mechanism of QAS monomers was suggested to be that, when the negatively-charged bacterial cell contacts the positively-charged sites of QAS resin, the electric balance of cell membrane could be disturbed, and the bacterium could explode under its own osmotic pressure [20,21]. Previous studies incorporated QAS monomethacrylates into dental resins [19,21,37,54]. The QADM of the present study is a dimethacrylate, with reactive groups on both ends of the molecule. This could enable the QADM to be incorporated into the resin with less of a negative impact on the mechanical properties. QADM has a relatively low viscosity and is readily miscible with dental methacrylates, and is expected to have minimal monomer leachability due to reactive groups on both ends of the molecule, compared to monomethacrylates. In addition, the synthesis of this QADM was relatively straightforward and the reaction products were generated at quantitative amounts, requiring no further purification [26,27]. The results of the present study indicate that QADM could be a promising antibacterial agent to be incorporated into a wide range of dental adhesives and primers.

NAg also provided a strong antibacterial activity, and the antibacterial potency increased when the NAg mass fraction was increased from 0.05% to 0.1%. Regarding the antimicrobial mechanism, it was suggested that Ag ions could inactivate the vital enzymes of bacteria, thus causing DNA in the bacteria to lose its replication ability, which leads to cell death [55]. Previous studies showed that Ag-containing composites had long-term antibacterial effects and inhibited *S. mutans* growth for more than 6 months [56]. The NAg of the present study had a small particle size, yielding a high specific surface area. This allowed the use of a low filler level of NAg in the adhesive to achieve antibacterial efficacy. Therefore, a strong antibacterial capability was obtained without compromising the resin color and mechanical properties. Indeed, for the A&P+10QADM+0.05NAg group, the biofilm metabolic activity, CFU and lactic acid were reduced by nearly an order of magnitude, without compromising the dentin bond strength. Even without the use of QADM, the 0.1NAg adhesive reduced the CFU and lactic acid to about 1/3 of those on control adhesive. While the color of the modified adhesives remains to be measured, there was no noticeable difference in color between adhesives with 0%, 0.05% and 0.1% NAg.

However, a yellow/brown color appeared when the NAg mass fraction exceeded 0.2%. Therefore, under the experimental conditions of this study, NAg mass fraction of 0.1% could be selected. In addition, the Ag salt was dissolved in the TBAEMA monomer, which was then mixed with the resin and photopolymerized, thus forming the NAg *in situ*. This yielded well-dispersed NAg in the resin without agglomeration [27,28]. This method avoided the need to mix pre-fabricated nanoparticles with the resin, which could cause agglomeration. Due to their small size of 2.7 nm, NAg could flow with the primer and adhesive into dentinal tubules to kill residual bacterial, without compromising dentin bond strength. Indeed, SEM showed that the bonding agents for all six groups filled and wetted the dentin surfaces, manifested by the formation of numerous resin tags from well-infiltrated dentinal tubules. Previous studies suggested that these features are responsible for a strong and durable bond to dentin [35]. In the present study, these features were rampant in all samples, and no noticeable difference was seen in all six groups. Therefore, incorporating QADM and NAg did not negatively affect dentin bonding as compared to the control, consistent with the shear bond strength measurement.

Previous studies tested antibacterial adhesives and primers using single species bacteria models [21,25,37,38,40]. Dental biofilm models can be divided into three groups: Single species, defined consortium, and microcosm [42]. Microcosms are inoculated using material removed from the environment of interest, which maintain much of the complexity and heterogeneity of the original biological sample [42]. Dental plaque is a complicated ecosystem with about 1,000 different bacterial species [17]. In the present study, a dental plaque microcosm biofilm model was used to evaluate adhesives containing QADM and NAg for the first time. In previous studies, saliva from a single donor [27,44], or mixed saliva from multiple donors [57], have both been used to grow microcosm biofilms. Among the microorganisms in human saliva, streptococci play an important role in the caries process. Oral streptococci contain many groups including the mutans streptococci group. Much research has suggested that mutans streptococci are the major pathogens of dental caries [16,17]. The mutans streptococci group contains *S. mutans* and *S. sobrinus*, both playing a main role in causing caries. The results of the present study showed that the QADM-NAg-containing adhesive/primer were equally potent against total microorganisms, total streptococci, and mutans streptococci, reducing them all by approximately the same percentage, compared to the control adhesive. For example, A&P+10QADM+0.05NAg reduced all three CFU counts by the same extent, which was nearly an order of magnitude, compared to the control adhesive. These results were corroborated by a similar extent of reduction in MTT metabolic activity and lactic acid production. A recent paper showed that QADM and NAg in a nanocomposite inhibited *S. mutans* viability and growth [27]. These results together indicate that QADM and NAg are not only effective in composites against single species biofilms, but are also effective in adhesive and primer when evaluated using a clinically-relevant microcosm biofilm model with human saliva as inoculum.

5. Conclusions

The present study incorporated QADM and NAg into dental adhesive and primer which achieved potent antibacterial effects against dental plaque microcosm biofilms for the first time. The results showed that: (1) QADM or NAg each greatly decreased the biofilm viability, CFU and lactic acid production, compared to commercial adhesive control; (2) higher NAg mass fraction rendered the adhesive more strongly antibacterial; (3) QADM and NAg together in the same adhesive yielded a greater reduction in biofilm viability than using each agent alone; (4) QADM and NAg incorporation into both the adhesive and the primer achieved the strongest antibacterial effect. A&P+10QADM+0.05NAg reduced biofilm metabolic activity, CFU and acid production by nearly an order of magnitude, compared to commercial adhesive control. SEM examination revealed numerous resin tags in all the

samples, hence incorporation of QADM and NAg did not compromise dentin bonding, consistent with the relatively high shear bond strengths for all groups. QADM-NAg-containing antibacterial adhesive and primer are promising to combat residual bacteria in the prepared tooth cavity and invading bacteria along the tooth-restoration interfaces due to bacterial leakage, thereby inhibiting recurrent caries. The QADM and NAg incorporation method may have a wide applicability to other dental bonding agents.

Acknowledgments

We thank Drs. J. M. Antonucci, N. J. Lin and S. Lin-Gibson of the National Institute of Standards and Technology, and Dr. G. E. Schumacher and A. A. Giuseppetti of the Paffenbarger Research Center for discussions. We thank Esstech (Essington, PA) for donating the materials, and the technical support of the Core Imaging Facility of the University of Maryland Baltimore. This study was supported by financial support from the School of Stomatology at the Capital Medical University in China (KZ), scholarship from the Coordination for Improvement of Higher Education Personnel CAPES/Fulbright Doctoral Program BEX 0523/11-9 (MASM), National Natural Science Foundation of China grant 81100745 (LC), NIH R01DE17974 and R01DE14190 (HX), and seed fund (HX) from the University of Maryland School of Dentistry.

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Figure 1.

Human dentin shear bond testing: (A) Schematic of specimen preparation, (B) schematic of shear bond strength testing, (C) shear bond strength data. Ten teeth were used for each group, requiring a total of sixty third-molars. Each value is mean $\pm$ sd ($n = 10$). Horizontal line indicates that all six groups had similar shear bond strengths ($p > 0.1$).

Figure 2.

SEM micrographs of dentin-adhesive interfaces: (A) SBMP control, and (B) A&P +10QADM+0.05NAg. Other groups had similar features and are not included here. “HL” refers to the hybrid layer between the adhesive and the underlying mineralized dentin. The adhesive resin was well-infiltrated into dentinal tubules to form resin tags “T”. In (A), the long arrow indicates a long resin tag. The short arrow points to a short tag, which was shortened due to the sectioning surface not being parallel to the tubules. Numerous resin tags were observed in all samples, without noticeable difference between the six groups, indicating that adding QADM and NAg did not affect dentin bonding.

Figure 3.

Schematic of biofilm experiments and MTT metabolic activity: (A) Schematic of biofilm on the adhesive surface covering the composite, (B) biofilm on the primer covering the adhesive and composite, and (C) MTT metabolic activity. Biofilms were grown for 2 d using a microcosm model. Five adhesive groups were tested following schematic A: Control, A+10QADM, A+0.05NAg, A+0.1NAg, A+10QADM+0.05NAg. One group was tested following schematic B with a primer layer: A&P+10QADM+0.05NAg. Each values is mean $\pm$ sd (n = 6). Values with dissimilar letters are different ($p < 0.05$).

Figure 4.

Live/dead staining of biofilms on the specimens. Live bacteria were stained green, and dead bacteria were stained red. Live and dead bacteria in proximity to each other yielded yellow/orange staining. Incorporation of QADM and NAg into the adhesive provided a significant antibacterial effect. Adding QADM and NAg into both adhesive and primer had a potent antibacterial effect, resulting in mostly dead bacteria on the specimen.

Figure 5.

Microcosm biofilm CFU counts: (A) Total microorganisms, (B) total streptococci, and (C) mutans streptococci. Each values is mean $\pm$ sd (n = 6). Values indicated by dissimilar letters are significantly different (p < 0.05). The results showed that: (1) QADM or NAG each decreased the CFU compared to commercial adhesive control; (2) higher NAG mass fraction further decreased the CFU; (3) QADM and NAG together yielded a greater reduction in CFU than each alone; (4) QADM and NAG in both primer and adhesive had the strongest antibacterial effect.

Figure 6.

Lactic acid production by biofilms adherent on the six different types of disks. Each values is mean $\pm$ sd (n = 6). Values with dissimilar letters are different ($p < 0.05$).

Table 1

Compositions of adhesive and primer for dentin bond strength test *

Group	Adhesive resin	Dentin primer	Group name
1	Control	Control	Control
2	Control + 10% QADM	Control	A+10QADM
3	Control + 0.05% NAg	Control	A+0.05NAg
4	Control + 0.1% NAg	Control	A+0.1NAg
5	Control + 10% QADM + 0.05% NAg	Control	A+10QADM+0.05NAg
6	Control + 10% QADM + 0.05% NAg	Control + 10% QADM + 0.05% NAg	A&P+10QADM+0.05NAg

* The control adhesive was the SBMP adhesive without modification. The control primer was the SBMP primer without modification. QADM = quaternary ammonium dimethacrylate. NAg = nanoparticles of silver. In the "Group name" column, A = adhesive resin. P = primer.