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Dual antibacterial agents of nano-silver and 12-methacryloyloxydodecylpyridinium bromide in dental adhesive to inhibit caries

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Abstract

Dental resins containing 12-methacryloyloxydodecylpyridinium bromide (MDPB) showed potent antibacterial functions. Recent studies developed antibacterial resins containing nanoparticles of silver (NAg). The objectives of this study were to develop an adhesive containing dual agents of MDPB and NAg for the first time, and to investigate the combined effects of antibacterial adhesive and primer on biofilm viability, metabolic activity, lactic acid, dentin bond strength, and fibroblast cytotoxicity. MDPB and NAg were incorporated into Scotchbond Multi-Purpose (SBMP) adhesive "A" and primer "P". Five systems were tested: SBMP adhesive A; A+MDPB; A+NAg; A+MDPB+NAg; P+MDPB+NAg together with A+MDPB+NAg. Dental plaque microcosm biofilms were cultured using mixed saliva from ten donors. Metabolic activity, colony-forming units, and lactic acid production of biofilms were investigated. Human fibroblast cytotoxicity of bonding agents was determined. MDPB+NAg in adhesive/primer did not compromise dentin bond strength ($p>0.1$). MDPB or NAg alone in adhesive substantially reduced the biofilm activities. Dual agents MDPB+NAg in adhesive greatly reduced the biofilm viability compared to each agent alone ($p<0.05$). The greatest inhibition of biofilms was achieved when both adhesive and primer contained MDPB+NAg. Fibroblast viability of groups with dual antibacterial agents was similar to control using culture medium without resin eluents ($p>0.1$). In conclusion, this study showed for the first time that the antibacterial potency of MDPB adhesive could be substantially enhanced via NAg. Adding MDPB+NAg into both primer and adhesive achieved the strongest anti-biofilm efficacy. The dual agent (MDPB+NAg) method could have

wide applicability to other adhesives, sealants, cements and composites to inhibit biofilms and caries.

Keywords

Antibacterial adhesive; dentin bond strength; silver nanoparticles; MDPB; dental plaque microcosm biofilm; fibroblast cytotoxicity

INTRODUCTION

Half of all dental restorations fail within 10 years, and secondary caries at the tooth-restoration margins has been cited as the main reason [1,2]. Nearly 70% of all restorations were replacements of failed restorations [3,4]. Replacement dentistry is costly, considering that the annual cost for tooth cavity restorations in the U.S. was \$46 billion in 2005 [5]. Composites have grown popular for tooth cavity restorations due to their esthetics and direct-filling capabilities [6–10]. While the physical and mechanical properties of composites have been significantly improved [2,9,11–13], one remaining drawback is that composites tend to accumulate more biofilms/plaques in vivo than other restorative materials [14,15]. Because biofilms with fermentable carbohydrates are responsible for caries [16], the biofilm accumulation at composite restorations needs to be reduced or inhibited in order to combat secondary caries and increase the longevity of the restorations.

Accordingly, efforts were made to develop antibacterial dental materials [17–24]. Resins with quaternary ammonium methacrylates (QAMs) were synthesized, and 12-methacryloyloxydodecyl-pyridinium bromide (MDPB) was among the first antibacterial monomers [17,18,20]. MDPB has been extensively investigated and shown to possess potent antibacterial activities against various oral bacteria including facultative and obligate anaerobe in coronal lesions, and bacterial species isolated from root caries such as actinomyces and *Candida albicans* [20]. A MDPB-containing primer was applied to cavities in teeth of dogs infected with *S. mutans* and exhibited in vivo antibacterial effects [25]. A composite restoration containing MDPB was shown to inhibit the progression of artificial secondary root caries lesions using extracted human teeth with Class V cavities [26].

Since secondary caries can occur at the restoration-tooth margins, it is important to develop antibacterial adhesives [20,21,27]. Adhesives serve as an important link between the composite and the tooth structure to maintain a bonded interface. Adhesive composition, the effectiveness of bonding, and bond strength durability have been improved through extensive studies [28–32]. After tooth cavity preparation, a primer can be applied, and then an adhesive and a composite are placed to form the restoration. The primer contacts the tooth structure and flows into dentinal tubules. Hence, it is important to develop antibacterial primers [17,18,33]. A MDPB-containing primer was reported as “The world’s first antibacterial adhesive system” [20]. MDPB primer inhibited bacteria growth [17,18] and inactivated residual bacteria in tooth cavities in vitro and in vivo [25,34]. These studies typically used only one antibacterial agent in the primer. A recent study combined two antibacterial agents, namely, MDPB and nanoparticles of silver (NAg), into a primer [35]. The addition of NAg substantially enhanced the antibacterial potency of the MDPB primer [35]. Besides the primer, antibacterial adhesive is also beneficial to inhibit bacteria and offer protection against secondary caries and pulpal damage [20,36]. Due to polymerization shrinkage and cyclic loading and wear, microgaps often form between the adhesive resin and the primed dentin, or between the adhesive resin and the hybrid layer [37,38]. 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

[39]. Therefore, it is also important for the adhesive to be antibacterial. However, there has been no report on the use of dual antibacterial agents MDPB and NAg in the adhesive. Furthermore, the effects of dual agents MDPB + NAg in both the adhesive and the primer on biofilm properties have not been investigated.

Therefore, the objectives of this study were to: (1) develop antibacterial adhesive containing dual agents MDPB and NAg for the first time; (2) investigate the combined effects of antibacterial adhesive and primer on dental plaque microcosm biofilm viability, metabolic activity and lactic acid production, as well as dentin bond strength and fibroblast cytotoxicity. It was hypothesized that: (1) MDPB or NAg alone in adhesive would impart a strong antibacterial function; (2) MDPB + NAg in adhesive would achieve much more potent antibacterial activity than each agent alone; (3) Rendering both adhesive and primer antibacterial via MDPB + NAg would be more effective in inhibiting biofilms than antibacterial adhesive alone; (4) Developing antibacterial adhesive and primer via MDPB + NAg would not compromise dentin bond strength and human fibroblast cytotoxicity.

MATERIALS AND METHODS

Incorporating MDPB and NAg into dental adhesive

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, and 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.

Silver (Ag) is known to have antibacterial, antifungal and antiviral capabilities [40]. Recently, NAg were formed in dental resins in situ without the need of mixing nanoparticles with resin, which showed a strong antibacterial activity [41–43]. Briefly, silver 2-ethylhexanoate (Strem, Newburyport, MA) of 0.1 g was dissolved into 0.9 g of 2-(tert-butylamino)ethyl methacrylate (TBAEMA, Sigma). TBAEMA was used to improve the solubility of the silver salt by forming Ag-N coordination bonds with Ag ions, thereby facilitating the Ag salt to dissolve in the resin solution. TBAEMA was specifically selected since it contains reactive methacrylate groups and therefore can be chemically incorporated into the polymer network. 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 [41,43].

MDPB was provided by Kuraray Medical Inc. (Tokyo, Japan) in the form of a white powder. It is a polymerizable bactericide and synthesized by combining a quaternary ammonium dodecylpyridinium bromide and a methacryloyl group [20]. To investigate the effect of dual agents (MDPB + NAg) on the adhesive properties, four adhesives were tested:

1. SBMP adhesive control (A);
2. SBMP adhesive + 2.5% MDPB (mass fraction) (referred to as “A+MDPB”);
3. SBMP adhesive + 0.1% NAg (mass fraction) (referred to as “A+NAg”);
4. SBMP adhesive + 2.5% MDPB + 0.1% NAg (referred to as “A+MDPB+NAg”).

The mass fraction of 2.5% MDPB in adhesive followed previous studies [39,36]. Mass fraction of 0.1% NAg followed a previous study, which showed that 0.1% NAg in the adhesive had a potent antibacterial effect without compromising dentin bond strength [44].

In addition, a 5th bonding system was formulated with MDPB and NAg in both primer and adhesive, to investigate the effects of combining the MDPB+NAg primer with the MDPB +NAg adhesive. The adhesive was the same as A+MDPB+NAg. The primer contained 5% MDPB and 0.05% NAg. This 5th system is referred to as “P+MDPB+NAg, A+MDPB +NAg”. The 5% MDPB in primer followed previous studies [39,36]. The 0.05% NAg in primer followed a previous study which greatly inhibited microcosm biofilm growth without adversely affecting dentin bond strength [45]. However, these previous studies did not include the systems with dual MDPB and NAg antibacterial agents.

Dentin shear bond testing

Caries-free human molars were used for dentin bond test, and the protocol was approved by the University of Maryland. After removing the tooth crown by sawing (Isomet, Buehler, Lake Bluff, IL), the tooth was ground perpendicularly to its longitudinal axis on 320 grit SiC paper until occlusal enamel was completely removed. The dentin surface was etched for 15 seconds (s) and rinsed with water [46]. A primer was applied, and the solvent was evaporated with an air stream. Then, an adhesive was applied and light-cured for 10 s (Optilux VCL401, Demetron, Danbury, CT). For adhesives (1) to (4) described above, the unmodified SBMP primer was used, to examine the effect of MDPB and NAg in adhesive on dentin bond strength. 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, Dentsply, Milford, DE) and light-cured for 60 s. The bonded specimens were stored in water at 37 °C for 24 h. The dentin shear bond strength, S_D , was measured as previously described [46]. A chisel was held parallel to the composite-dentin interface and loaded via a Universal Testing Machine (MTS, Eden Prairie, MN) at 0.5 mm/min until the composite-dentin bond failed. S_D was calculated as: $S_D = 4P/(d^2)$, where P is the load at failure, and d is the diameter of the composite [46].

Dental plaque microcosm biofilm model

Human saliva was used as an inoculum to grow biofilms consisting of multi-species organisms found in the oral cavity. Saliva is ideal for growing biofilms to maintain much of the complexity and heterogeneity in vivo [47]. To represent the diverse bacterial populations, saliva from ten healthy individuals was combined for the experiments, following a previous study [48]. The donors had natural dentitions without active caries or periodontal pathology, and without using antibiotics within the last 3 months [48,49]. They did not brush teeth for 24 h and abstained from food/drink intake for 2 h prior to donating saliva [44,45]. Stimulated saliva was collected during parafilm chewing and kept on ice. An equal volume of saliva from each of the ten donors was combined, and diluted to 70% saliva and 30% glycerol [48,49]. Aliquots of 1 mL were stored at -80 °C for subsequent use.

Specimen fabrication for biofilm experiments

Layered disks for biofilm tests were fabricated following previous studies [17,44,45]. To test the adhesives (1) to (4), each adhesive was placed into a polyethylene mold (inner diameter = 9 mm, thickness = 2 mm) with a glass slide underneath. Ten μ L of adhesive was applied into the mold on the glass slide and cured for 20 s with Optilux. Then, a composite (TPH) was placed on the adhesive to completely fill the mold and cured for 1 min (Triad 2000 Visible Light, Dentsply, Milford, DE). No primer was used for adhesives (1) to (4), in order to investigate the antibacterial effects of the adhesives directly. It should be noted that in restoring a tooth cavity, a primer was first applied and then an adhesive was placed. The reason no primer was used for adhesives (1) to (4) was to measure the antibacterial activity of the adhesive resin. This is because marginal gaps at the tooth-restoration interface would often expose the cured adhesive resin, with which the invading bacteria would come into contact [37–39]. Therefore, groups (1) to (4) were designed to test the antibacterial activity

of the adhesives, and group (5) was designed to test the antibacterial activity of the primer. This design enabled the antibacterial activity of both the adhesive and the primer to be separately determined.

For the 5th bonding system “P+MDPB+NAg, A+MDPB+NAg”, the primer was first brushed onto the glass in the mold, dried for 30 s with a gentle stream of air, then 10 μ L of the adhesive was applied on top of the primer and cured for 20 s with Optilux. Then, a composite (TPH) was placed on the adhesive to fill the mold and cured for 1 min with Triad. All the cured specimens were agitated in water for 1 h to remove any uncured monomers [17]. The specimens were then dried and sterilized with ethylene oxide sterilizer (Anprolene AN 74i, Andersen, Haw River, NC).

Inoculum and live/dead staining of biofilms

Adhesives (1) to (4) were tested with biofilm inoculation on the adhesive surface, as shown schematically in Fig. 2A; no primer was included. For the 5th system “P+MDPB+NAg, A+MDPB+NAg”, the biofilm was inoculated on primer as shown schematically in Fig. 2B.

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 [50]. Each disk was placed into 24-well plate with 1.5 mL inoculum, and incubated with 5% CO₂ at 37 °C for 8 h. Specimens were then transferred into a new 24-well plate with 1.5 mL fresh medium, and incubated for 16 h. Then the specimens were transferred into a new 24-well plate with 1.5 mL fresh medium and incubated for 24 h. This constituted a total of 48 h of culture, which was shown to form mature microcosm biofilms on resin disks [45].

The 2-day biofilms on disks were rinsed with phosphate-buffered saline (PBS), live/dead stained (Molecular Probes, Eugene, OR), and examined using an epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY). 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. Four representative images were taken for each disk, with three disks yielding 12 images for each material.

MTT assay of metabolic activity

Disks of each group (n = 6) with 2-day biofilms were transferred to a new 24-well plate for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT is a colorimetric assay that measures the enzymatic reduction of MTT, which is a yellow tetrazole, into formazan [43]. One 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 metabolized the MTT and reduced it to purple formazan inside the living cells. After 1 h, the specimens 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. Then, 200 μ L of the DMSO solution from each well was transferred to a 96-well plate, and the absorbance at 540 nm was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) [43].

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

Disks of each group (n = 6) with 2-day biofilms were rinsed with cysteine peptone water (CPW) to remove loose bacteria, then transferred to 24-well plates containing buffered-

peptone water (BPW) plus 0.2% sucrose, and incubated for 3 h to allow biofilms to produce acid. BPW solutions were stored and lactate concentrations were determined using an enzymatic method [45]. The 340-nm absorbance of BPW was measured with the microplate reader. Standard curves were prepared using a standard lactic acid (Supelco Analytical, Bellefonte, PA) [44,45].

For colony-forming unit (CFU) counts, specimens with 2-day biofilms were transferred into tubes with 2 mL CPW. The biofilm on each disk was harvested by sonication and vortexing (Fisher, Pittsburgh, PA). Three types of agar plates were prepared to assess the microorganism viability after serially dilution in CPW. First, tryptic soy agar (TSA) with sheep blood plates were used to determine total microorganisms [45]. Second, mitis salivarius agar (MSA) plus 15% sucrose plates were used to determine total streptococci. 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 [51]. Third, MSA plus 0.2 units of bacitracin/mL plates were used to determine mutans streptococci. This is because cariogenic mutans streptococci are known to be resistant to bacitracin, while other species of oral microflora cannot grow on these plates [44,45,52].

Biocompatibility of bonding agents with human gingival fibroblasts

Eluents from cured antibacterial bonding agents were used to culture fibroblasts to assess the cytotoxicity [53]. To make the specimens, the cover of a sterile 96-well plate having round dents with a diameter of approximately 8 mm was used [21]. For adhesives (1) to (4), 20 μ L of each adhesive was applied into the bottom of the dent, covered with a translucent celluloid strip, and photo-polymerized for 20 s with Optilux. For the 5th system “P+MDPB +NAg, A+MDPB+NAg”, 10 μ L of primer was first applied into the bottom of the dent. After drying with a stream of air, 20 μ L of adhesive was applied on top of the primer and cured for 20 s. The cured disks ($n = 6$) of each group were sterilized with ethylene oxide, and then immersed in 10 mL of fibroblast medium (FM) (ScienCell, San Diego, CA). The samples were agitated with a magnetic stir bar for 24 h at 37 °C to obtain eluents from the disks. Cytotoxicity was then tested using the original extract solution along with a series of dilutions [54]. The original extract solution, which was concentrated with resin eluents, was serially diluted to concentrations relevant to conditions in vivo. Three solutions were thus prepared, at dilutions of 32-fold (1 mL of original extract + 31 mL of fresh FM), 64-fold, and 128-fold, respectively. This is because the amount of saliva flow for an average person is approximately 1000 to 1500 mL per day [55], hence the 128-fold dilution corresponded to a total solution of 1280 mL for the six resin disks. The 32-fold and 64-fold corresponded to a total solution of 320 mL and 640 mL respectively. This corresponded to 1/4 and 1/2 of the normal saliva amount per day, yielding resin eluent concentrations higher than that with normal saliva flow, to take into account of patients with reduced saliva production. The total volume for the six cured resin disks was approximately 150 mm³. Therefore, the resin volume (mm³) divided by the total solution volume (mL) for the three solutions was 0.12, 0.24, and 0.48 mm³/mL, respectively. Fresh FM without any resin eluent (resin/solution volume ratio = 0 mm³/mL) served as control. Hence, four types of FM solutions were prepared for the fibroblast cytotoxicity test, with eluents obtained at resin/solution volume ratios of 0, 0.12, 0.24, and 0.48 mm³/mL, respectively.

Human gingival fibroblasts (HGF, ScienCell) were cultured in FM supplemented with 2% fetal bovine serum, 100 IU/mL penicillin and 100 IU/mL streptomycin. A seeding density of 4,000 cells/well was used in 96-well plates [53]. After 24 h of incubation at 37 °C with 5% CO₂ in air, the medium was removed and replaced with 100 μ L of one of the four types of FM solutions described above. The cells were cultured for another 48 h, then 20 μ L of MTT solution at a concentration of 5 mg/mL was added to each well. After incubating in

darkroom for 4 h, the unreacted dye was removed and 150 μ L/well of DMSO was added [53]. The solution absorbance was measured via the microplate reader at 492 nm [53]. The absorbance for fibroblasts cultured in control FM without resin eluent was set as 100%. Fibroblast viability cultured in medium containing resin eluents = Absorbance of the eluent group/absorbance of control [53].

Statistical analysis

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

RESULTS

Dentin shear bond strength using human extracted teeth are plotted in Fig. 1 (mean $\pm$ sd; n = 10). The horizontal line indicates values that are not significantly different from each other ($p > 0.1$). These results indicate that incorporating MDPB and NAg into the bonding agent did not degrade the dentin bond strength.

Representative live/dead bacterial staining images are shown in Fig. 2(C–G), for the 5 groups, respectively. Groups 1–4 in (C–F) used the experimental setup shown in (A), with biofilm directly on the adhesive without a primer, to investigate the antibacterial activity of the adhesive. Group 5 in (G) used the setup shown in (B). SBMP adhesive control was completely covered by biofilms and the bacteria were primarily alive. Disks with MDPB, on the other hand, had much red staining, which indicated compromised bacteria, and so were disks with NAg. Examination of 12 images for each material indicated that the adhesive A +MDPB+NAg had more red staining and less green staining than those with MDPB or NAg alone. The 5th group with primer and adhesive both containing MDPB+NAg appeared to have the most red staining and least green staining.

Fig. 3 plots the MTT metabolic activity of biofilms (mean $\pm$ sd; n = 6). Biofilms on adhesive control had the highest absorbance. Adhesives with MDPB or NAg alone greatly reduced the MTT absorbance, compared to adhesive control ($p < 0.05$). Dual agents MDPB+NAg in adhesive further substantially reduced the MTT absorbance, compared to MDPB alone or NAg alone ($p < 0.05$). When dual agents MDPB+NAg were incorporated into both primer and adhesive, the lowest metabolic activity was reached ($p < 0.05$).

Fig. 4 plots the CFU counts for biofilms on the resin disks (mean $\pm$ sd; n = 6). Oral streptococci consists of many species including mutans streptococci, and the mutans group includes cariogenic species *S. mutans* and *S. sobrinus*. Therefore, CFU counts for total microorganisms (A), total streptococci (B), and mutans streptococci (C) were all measured. All three counts were greatly reduced by adding MDPB or NAg in the adhesive, indicating the indiscriminating killing of all bacterial species by MDPB and NAg. The CFU was further reduced via dual agents MDPB+NAg ($p < 0.05$), and was still further reduced via both primer and adhesive containing the dual antibacterial agents ($p < 0.05$).

Lactic acid production of biofilms on the disks is plotted in Fig. 5 (mean $\pm$ sd; n = 6). Biofilms on the SBMP adhesive produced the most amount of acid ($p < 0.05$). Adding MDPB or NAg alone in the adhesive reduced the biofilm acid to approximately 1/3 of that of adhesive control. Using dual agents MDPB+NAg in the adhesive further reduced the biofilm acid to about 1/3 of that using MDPB alone. Lastly, with both primer and adhesive containing the dual agents MDPB+NAg, the biofilms produced the least acid ($p < 0.05$).

Fig. 6 plots the human gingival fibroblast cytotoxicity data (mean $\pm$ sd; $n = 6$). The control had fibroblast culture in FM without resin eluents, and its cell viability was set at 100%. For groups with resins, the fibroblasts were cultured in FM containing resin eluents. The fibroblast viability for all the bonding agent groups was nearly 100%. There was generally little difference between MDPB and NAg groups and the non-antibacterial adhesive control, or between bonding agent groups and the FM control without resin eluents ($p > 0.1$).

DISCUSSION

The incorporation of MDPB into dental resins has been extensively investigated, and its potent antibacterial activities have been demonstrated [20]. The first antibacterial bonding system with MDPB has been commercialized [20]. The present study showed for the first time that while the adhesive resin containing MDPB was indeed strongly antibacterial, its potency can be substantially enhanced via a dual agent method (MDPB + NAg). The addition of a small amount of NAg (0.1% by mass) did not visibly change the adhesive color, and did not adversely affect the dentin bond strength and fibroblast cytotoxicity. However, it did substantially enhance the antibacterial potency of the MDPB-containing adhesive. The metabolic activity, CFU and lactic acid of biofilms were all greatly reduced via dual agents of MDPB + NAg, compared to MDPB alone. The reduction in biofilm activity by the dual agents appeared to be synergistic with a multiplying effect. For example, for the MTT metabolic activity, experimental results showed that MDPB alone in the adhesive reduced the MTT activity to about 1/3 of that of the control adhesive. NAg alone in the adhesive reduced the MTT to approximately 1/3 of that of the control adhesive. Therefore, dual agents MDPB+NAg in the adhesive would be expected to further reduce the metabolic activity to approximately 1/3 of that by MDPB alone. The multiplying effect would yield $(1/3) \times (1/3) = 1/9$. Hence, the multiplying effect would predict that the metabolic activity of MDPB+NAg adhesive would be approximately an order of magnitude lower than that of the control adhesive. This prediction approximately agreed with the experimental results in Fig. 3. A similar trend was observed in the CFU and lactic acid results, suggesting that MDPB and NAg as dual antibacterial agents in the adhesive had a multiplying effect in reducing the biofilm activities.

Furthermore, the mode of action for MDPB and NAg could be complimentary to each other in inhibiting bacteria. MDPB is a quaternary ammonium methacrylate and can be immobilized in the resin by co-polymerization [20]. Hence, its mode of action is contact-inhibition, which inhibits bacteria on its surface, but not away from its surface [20]. A recent study [56] investigated the incorporation of another quaternary ammonium methacrylate, QADM, into a bonding agent. The results showed that the QADM bonding agent inhibited biofilm growth and gene expressions of bacteria contacting the resin surface, while having no effect on bacteria in the culture medium away from the resin surface. In dramatic contrast, the bonding agent with NAg inhibited not only the bacteria on the resin surface, but also the remote bacteria suspended in the culture medium away from the resin surface [56]. Therefore, while quaternary ammonium-containing resins have contact-inhibition, NAg-containing resins possess long-distance inhibition to kill bacteria away from its surface. This indicates that the dual agents MDPB+NAg in adhesive could serve as complimentary antibacterial agents to inhibit biofilms both on surface and away from the surface.

In addition, the present study showed that adding the dual agents MDPB+NAg into both the primer and the adhesive reached stronger antibacterial properties than adding the dual agents into the adhesive only. For lactic acid production, for example, the acid amount for “P +MDPB+NAg, A+MDPB+NAg” was reduced to 40% of that of the A+MDPB+NAg group. Similar reductions were achieved in metabolic activity and CFU counts via MDPB+NAg in both primer and adhesive. Oral biofilms produce acids from carbohydrates to cause

secondary caries at the tooth-restoration margins. Caries is a result of demineralization of enamel and dentin via acids generated by bacterial biofilms or dental plaque [16]. Therefore, it is beneficial to develop antibacterial resins [20,21]. While residual bacteria could exist in the prepared tooth cavity, microleakage may occur during service and new bacteria can invade the tooth-restoration margins [17,18,20,57]. Antibacterial adhesives would be important in both of these cases to combat bacteria and offer protection against secondary caries and pulpal damage [20,36]. The results of the present study indicated that it is beneficial to add the dual agents (MDPB+NAg) into both adhesive and primer which greatly enhanced the antibacterial potency.

Ag has been shown to possess antibacterial, antifungal, and antiviral functions [40], and is an effective antibacterial agent against a wide range of micro-organisms [58–60]. It was suggested that Ag ions could inactivate the vital enzymes of bacteria to cause the bacterial DNA to lose its replication ability, leading to cell death [58,60]. Ag has a low toxicity and good biocompatibility with human cells [59], with a long-term antibacterial effect due to sustained silver ion release [61]. It was noted that Ag had less bacterial resistance than antibiotics [62]. Due to the small size of NAg and the high specific surface area, a low NAg filler level could be used in the resin to achieve a potent antibacterial efficacy [41,43]. It is beneficial to use a low NAg filler level in the resin so that the NAg did not adversely affect the resin color and physical/mechanical properties. In the present study, the presence of NAg in adhesive and primer did not compromise the dentin bond strength or cytotoxicity. Preliminary study indicated that adding 0.2% or more of NAg decreased the dentin bond strength and turned the adhesive into a yellowish/brownish color; however, using 0.05% to 0.15% of NAg had no effect on color or dentin bond strength. Therefore, the present study tested conservative amounts of 0.05% and 0.1% NAg in the bonding agent, which indeed did not compromise the bond strength and cytotoxicity.

It is important for the new antibacterial bonding agents to be non-cytotoxic for mammalian cells. The MDPB adhesive, NAg adhesive, MDPB+NAg adhesive, and the primer/adhesive containing dual MDPB+NAg of the present study all showed fibroblast viability similar to that of control fibroblasts cultured in FM without resin eluents. Tests were performed using dilutions of the original extracts at total solution amounts comparable to the daily saliva amount in vivo, as well as at total solution volumes of 1/2 or 1/4 of the daily saliva volume in vivo. The latter two cases yielded resin eluent concentrations that were artificially higher than those under normal saliva flow conditions. Even with the higher eluent concentrations, the MDPB and NAg groups still had nearly 100% of fibroblast viability, similar to the FM group without eluents. Therefore, the MDPB and NAg containing bonding agents could achieve strong antibacterial functions without adversely affecting the cytotoxicity.

The present study used 5% of MDPB in the primer, following previous studies [36,39], and 0.05% NAg in the primer according to a previous report [45]. In the adhesive, the present study used 2.5% of MDPB following previous studies [36,39], and 0.1% NAg in the adhesive [44]. It should be noted that the present study focused on the effects of dual agents (MDPB + NAg) on microcosm biofilm viability, metabolic activity, CFU and lactic acid, as well as dentin bond strength and cytotoxicity, without systematically varying the MDPB and NAg mass fractions in the bonding agent. It remains to be investigated if the MDPB mass fraction in the adhesive could be increased to 4% or 5% or higher, and if the NAg mass fraction in primer and adhesive could also be increased to perhaps 0.15%, without compromising other physical and mechanical properties of the bonding agent. The important conclusion from the present study is that using dual agents (MDPB + NAg) in adhesive as well as in primer achieves substantially stronger antibacterial potency than using MDPB alone, without compromising dentin bonding and cytotoxicity properties. The actual application of this dual agent method and the optimal MDPB and NAg mass fractions

require further investigation, as the optimal mass fraction will likely differ when incorporating into different bonding systems, cements, sealants, and composites.

Summary

The present study incorporated dual antibacterial agents MDPB and NAg into an adhesive for the first time, and showed that the antibacterial potency could be greatly increased via the dual agent method. Dental plaque microcosm biofilm viability, CFU and lactic acid were greatly reduced with MDPB or NAg alone in the adhesive. The use of dual agents MDPB +NAg further substantially reduced the biofilm activities, compared to each agent alone. The extent of reduction in biofilm activity by the dual agents appeared to be having a multiplying effect. While the mode of action of MDPB was known to be contact-inhibition, that of NAg is expected to include long-distance inhibition, hence they were complimentary to each other. Adding MDPB+NAg into both primer and adhesive achieved stronger antibacterial properties than adding into adhesive only. The use of MDPB+NAg in adhesive and primer did not affect dentin bond strength or fibroblast cytotoxicity. Hence, the dual agent MDPB +NAg method may have a wide applicability to various bonding systems, cements, sealants, and composites to obtain potent antibacterial functions to inhibit caries.

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Fig. 1.

Dentin shear bond strength using human extracted teeth (mean $\pm$ sd; n = 10). P = SBMP control primer. A = SBMP control adhesive. NAg = nanoparticles of silver. Horizontal line indicates statistically similar values ($p > 0.1$).

Fig. 2. Biofilm experimental setup and live/dead images. (A) Schematic for groups 1 to 4, with biofilm directly on the adhesive surface without a primer, to investigate the antibacterial activity of the adhesive. (B) Schematic for group 5 with a primer and an adhesive. (C–G) Typical live/dead staining images. Live bacteria were stained green, and dead bacteria were stained red. Live and dead bacteria in close vicinity and on the top of each other appeared yellow or orange.

Fig. 3.

Metabolic activity of dental plaque microcosm biofilms via MTT assay (mean $\pm$ sd; $n = 6$). A higher absorbance indicates a higher formazan concentration, which in turn indicates more metabolic activity in the biofilms. Adding MDPB or NAg alone in the adhesive greatly reduced the metabolic activity. Dual agents MDPB + NAg were substantially more potent than MDPB alone. Both primer and adhesive containing the dual antibacterial agents yielded the strongest effect. Values with dissimilar letters are significantly different from each other ($p < 0.05$).

Fig. 4. Colony-forming unit counts for biofilms on resin disks: (A) Total microorganisms, (B) total streptococci, and (C) mutans streptococci (mean $\pm$ sd; n = 6). Values with dissimilar letters are significantly different from each other (p < 0.05).

Fig. 5.

Lactic acid production by biofilms (mean $\pm$ sd; n = 6). Adding MDPB or NAG alone in the adhesive greatly reduced the biofilm acids. The dual agents MDPB+NAG had a substantially more potent effect than MDPB alone. When both primer and adhesive contained dual agents MDPB+NAG, the biofilms produced the least acid. Values with dissimilar letters are significantly different from each other ($p < 0.05$).

Fig. 6.

Human gingival fibroblast cytotoxicity (mean $\pm$ sd; n = 6). The control fibroblasts were cultured in the fibroblast medium (FM) without any addition of resin eluents, and its cell viability was set at 100%. For the groups with resin, the fibroblasts were cultured in FM containing resin eluents. The resin volume was in unit mm³, and the FM was in unit mL. Values with dissimilar letters are significantly different from each other ($p < 0.05$). All the groups with resin eluent addition in the FM had fibroblast viability that matched that of the control FM without resin eluents ($p > 0.1$).