Novel self-etching and antibacterial orthodontic adhesive containing dimethylaminohexadecyl methacrylate to inhibit enamel demineralization

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Enamel demineralization is one of the most undesired side effects of fixed orthodontic treatment, which will lead to white spot lesions (WSLs) on tooth surfaces. The development of WSLs is due to prolonged accumulation of bacterial plaque and associated acid production. Self-etching adhesives have been used in orthodontic treatments with several advantages over the more traditional acid-etch method. However, current self-etching adhesives in orthodontic treatments have no antibacterial activity. The objectives of this study were to develop a self-etching and antibacterial orthodontic adhesive, and to investigate its enamel bond strength and antibacterial properties. A novel quaternary ammonium monomer dimethylaminohexadecyl methacrylate (DMAHDM) was incorporated into a commercial self-etching adhesive (Adper Easy One, 3M). It showed that the 5% DMAHDM appeared to be optimal in obtaining the strongest antibacterial function without compromising the enamel bond strength both at 15 min and after 30 days of immersion plus thermal cycling.

Keywords: Self-etching adhesive, Antibacterial property, Human saliva microcosm biofilm, Dimethylaminohexadecyl methacrylate, Enamel demineralization
and diminished over time, thereby providing a durable antibacterial ability\(^{15}\). This property is important for orthodontic adhesives due to the long duration (2–3 years) of orthodontic treatments.

Recently, a new QAM, dimethylaminohex-adecyl methacrylate (DMAHDM), was synthesized and incorporated into dental resins showing a strong antibacterial activity\(^{16}\). However, to date, there has been no report of a self-etching and antibacterial adhesive for orthodontic treatments, and there has been no report of incorporating DMAHDM into a self-etching adhesive. Therefore, the objectives of the present study were to develop the first self-etching and antibacterial adhesive for orthodontic treatments, and to investigate the antibacterial properties and enamel-bracket bond strength. For this purpose, the DMAHDM mass fraction on enamel bond strength and antibacterial property was selected to enable an investigation of the effect of (AEO+DMAHDM) mass fractions of 0, 1.5, 3, 5 and 7.5%.

DMAHDM was added into AEO at DMAHDM/ethanol at about 20 mL:10 mmol of 1-bromohexadecane (BHD, TCI America, Portland, OR, USA) in a 20 mL scintillation vial. The vial was stirred at 70°C for 24 h to complete the reaction. Then the solvent was evaporated, and DMAHDM was obtained as a clear, colorless and viscous liquid\(^{17}\). A benefit of this reaction is that the reaction products are generated at virtually quantitative amounts and require minimal purification. Briefly, 3 g of ethanol was combined with 10 mmol of 2-(dimethylamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich, St. Louis, MO, USA) and 10 mmol of 1-bromohexadecane (BHD, TCI America, Portland, OR, USA) in a 20 mL scintillation vial. The vial was stirred at 70°C for 24 h to complete the reaction. Then the solvent was evaporated, and DMAHDM was obtained as a clear, colorless and viscous liquid\(^{17}\).

**Synthesis of DMAHDM**

DMAHDM (Fig. 1) was recently synthesized via a modified Menschutkin reaction in which a tertiary amine was reacted with an organo-halide\(^{17}\). A benefit of this reaction is that the reaction products are generated at virtually quantitative amounts and require minimal purification. Briefly, 3 g of ethanol was combined with 10 mmol of 2-(dimethylamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich, St. Louis, MO, USA) and 10 mmol of 1-bromohexadecane (BHD, TCI America, Portland, OR, USA) in a 20 mL scintillation vial. The vial was stirred at 70°C for 24 h to complete the reaction. Then the solvent was evaporated, and DMAHDM was obtained as a clear, colorless and viscous liquid\(^{17}\).

**Preparation of antibacterial orthodontic adhesives**

A commercial self-etch adhesive (Adper Easy One, 3M, St. Paul, MN, USA) was used as the parent system. AEO is a three-in-one system with etchant+primer+adhesive. According to the manufacturer, AEO contains 2-hydroxyethyl methacrylate (HEMA), Bis-GMA, methacrylated phosphoric esters, 1,6 hexanediol dimethacrylate, methacrylate functionalized polyalkenoic acid, ethanol and water. DMAHDM was incorporated into AEO to provide antibacterial activity. While the present study used AEO as an example, the method of DMAHDM incorporation is applicable to other adhesive systems. DMAHDM was added into AEO at DMAHDM/(AEO+DMAHDM) mass fractions of 0, 1.5, 3, 5 and 7.5%, respectively, to enable an investigation of the effect of DMAHDM mass fraction on enamel bond strength and antibacterial properties. These values were selected following preliminary experiments. The following six groups were tested:

1. Acid-etch control, with 37% phosphoric acid;
2. Self-etch control, AEO adhesive (referred to as AEO+0% DMAHDM);
3. Self-etch AEO adhesive+1.5% DMAHDM (referred to as AEO+1.5% DMAHDM);
4. Self-etch AEO adhesive+3% DMAHDM (referred to as AEO+3% DMAHDM);
5. Self-etch AEO adhesive+5% DMAHDM (referred to as AEO+5% DMAHDM);
6. Self-etch AEO adhesive+7.5% DMAHDM (referred to as AEO+7.5% DMAHDM).

**Orthodontic bracket shear bond testing**

One hundred and twenty extracted human premolars with undamaged buccal surfaces were randomly divided into 6 groups of 20 teeth in each group. The tooth was vertically placed in acrylic resin (New Century Dental Materials, Shanghai, China) so that the buccal surface of the tooth was parallel to the applied force during the subsequent shear bond test. Oil-free pumice and rubber cups were used to polish the coronal portion at a low speed for 10 s, then the samples were washed and dried for 15 s.

The teeth of group 1 were etched with phosphoric acid at 37% (Scotchbond, 3M ESPE, St. Paul, MN, USA) for 60 s, then rinsed for 15 s. The enamel was gently dried with a stream of air until it became whitish\(^{19}\). The teeth of groups 2 to 6 were etched with AEO including 0, 1.5, 3, 5 and 7.5% DMAHDM, respectively. Each paste was added to enamel surface to form a thin, uniform coating by smearing for 20 s and drying for 5 s. Orthodontic brackets (OPK-A, TOMY, Fukushima, Japan) were used and the average base surface areas of the brackets were provided by the manufacturer. Adhesive paste (GC Ortho LC, Fuji, Aichi, Japan) was applied to the bracket base and pushed against the etched enamel of groups 1 to 6. After removing the excess adhesive around the bracket base by a clinical probe, each side (mesial and distal) of the bracket was polymerized for 20 s, for a total of 40 s, using a light-curing unit (Elipar S10 LED, 3M Unitek, Saint Paul, MN, USA)\(^{19,20}\).

Each group was randomly divided into two subgroups of 10 specimens each. One subgroup was applied to shear bond testing at 15 min after being bonded, in order to simulate the clinical situation where force is applied at about 15 min after bonding. In order to test the bond strength durability and simulate the oral environment, the other subgroup was immersed in artificial saliva at 37°C for 30 days.

![Fig. 1 The chemical structure of DMAHDM.](image)
These samples were then subjected to thermal cycling in alternate baths of 5±1 and 55±1°C with a 30 s immersion time in each bath (1 cycle). One thousand such cycles were performed. The specimens were then tested for shear bond strength (SBS). These steps are shown schematically in Fig. 2. A chisel was connected to a Universal Testing Machine (AG-IS 500N, SHIMADZU, Kyoto, Japan) and the chisel tip was placed on the upper part of the bracket. An occluso-gingival force was applied to the bracket at a displacement rate of 0.5 mm/min, producing a shear load at the bracket-tooth interface until the bond failed. The enamel SBS was calculated as the force at bond failure divided by the bracket surface area, as in previous studies.

Saliva collection for biofilm inoculum

Using human saliva for growing dental plaque microcosms is meritorious in keeping the complexity and heterogeneity of the dental plaque. Saliva was collected from ten healthy adult donors having natural dentition without active caries or periopathology, and not having used antibiotics within the preceding 3 months according to a previous study. The donors were told not to brush teeth for 24 h and abstained from food and drink intake for at least 2 h, prior to donating saliva. The saliva was added, with 1:50 final dilution, to the MBain artificial saliva medium as inoculum. The 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; hemin, 0.001 g/L; vitamin K1, 0.0002 g/L, at pH 7.21.

The enamel SBS results showed that group 6 had a significantly lower SBS than the other groups. Therefore, only groups 2–5 were tested for bacterial experiments. Samples were made by placing each adhesive paste into disk molds of 8 mm in diameter and 0.5 mm in thickness. Each disk was light-polymerized for 40 s on each open side of the mold. The cured disks were immersed in sterile water and stirred with a magnetic bar for 1 h to remove any uncured monomers, following previous studies. The samples were then sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC, USA) and de-gassed for 3 days.

Each disk was placed in a well of 24-well plates, and 1.5 mL inoculum was added to each well. Samples were incubated with 5% CO2 at 37°C for 8 h. Then 1 mL from inoculum was aspirated and 1 mL fresh inoculum was added. After incubating for another 16 h, the same operation was done as mentioned above and all the specimens were incubated for another 24 h. This totaled 2 days of culture which was shown to form mature biofilms on resins in previous studies.

Live/dead staining of biofilms

Disks with 2-day biofilms were gently washed with phosphate buffered saline (PBS) and stained using a live/dead kit (Molecular Probes, Eugene, OR, USA). An inverted epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY, USA) was used to examine the stained disks. 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. Three fields of view were randomly photographed for each disk, which provided a total of 18 images for each orthodontic adhesive. The area fraction of live bacteria/green staining area/total area of the image.

MTT assay of metabolic activity

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. Bacteria with compromised membranes were stained with propidium iodide to produce a red fluorescence. Three fields of view were randomly photographed for each disk, which provided a total of 18 images for each orthodontic adhesive. The area fraction of live bacteria/green staining area/total area of the image.

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Statistical analysis

Statistical evaluations were performed with SPSS 17.0. The normality and homogeneity were checked for each variable. SBSs and quantitative microbiological data were analyzed by the analysis of variance (ANOVA) and the Tukey test. ANOVA and Tukey’s tests were performed at a significance level of p<0.05.
RESULTS

The enamel SBS results for the orthodontic adhesives are plotted in Fig. 3 (mean±SD; n=10). Group 1 had significantly higher SBS. Group 2 to 5 had statistically similar SBS values (p<0.05), indicating that adding DMAHDM for up to 5% in AEO did not compromise the bracket-enamel bond strength. However, when the DMAHDM mass fraction was increased to 7.5%, the SBS decreased significantly. For each adhesive, water-aging for 30 days plus thermocycling caused no significant decrease in SBS, compared to that at 15 min.

The MTT metabolic activity results are plotted in Fig. 4 (mean±SD; n=6). Incorporation of DMAHDM at 1.5 and 3% reduced the metabolic activity (p<0.05). Increasing the DMAHDM mass fraction to 5% resulted in the lowest metabolic activity for the biofilms (p<0.05).

Fig. 3 Enamel shear bond strength (SBS) results (mean±SD; n=10). Incorporating 5% DMAHDM into AEO did not significantly decrease the SBS, compared to the self-etch control (p>0.05). Immersing into artificial saliva for 30 days and thermal cycling did not significantly decrease the SBS, compared to that at 15 min (p>0.05), suggesting that the enamel bond strength is durable. Different small letters indicate statically significant differences (p<0.05).

Fig. 4 Metabolic activity of dental plaque microcosm biofilms on resin specimens of self-etching adhesives. The biofilms on the AEO with 5% DMAHDM had the least metabolic activity among all the tested groups (p<0.05). Different small letters indicate statically significant differences (p<0.05).

Fig. 5 Live/dead staining images of 2-day biofilms on self-etching adhesive resin disks: (A) self-etch control, (B) AEO with 1.5% DMAHDM, (C) AEO with 3% DMAHDM, (D) AEO with 5% DMAHDM, and (E) area fraction of live bacteria (green staining) (mean±SD; n=6). Live bacteria were stained green, and compromised bacteria were stained red. When live and dead bacteria were in close proximity or on the top of each other, the staining had yellow or orange colors. The AEO with 5% DMAHDM consisted of primarily compromised bacteria. Dissimilar letters in (E) indicate values that are significantly different from each other (p<0.05). Different small letters indicate statically significant differences (p<0.05).
The live/dead staining results are shown in Fig. 5. 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 yellow and orange colors. In (A–D), the self-etch control adhesive was covered with a thick layer of primarily live bacteria; in contrast, AEO containing 3% DMAHDM had much more compromised bacteria than that containing 1.5%. The live bacteria area fraction (mean±SD; n=6) is plotted in (E). Increasing the DMAHDM mass fraction significantly decreased the live bacteria area fraction.

DISCUSSION

Several factors are recognized as contributing to enamel demineralization. First, etching enamel using the conventional 37% phosphoric acid can cause the loss of surface enamel and demineralization around the brackets. Second, having orthodontic attachments to the teeth can make it difficult to maintain proper oral hygiene, and can prolong the plaque accumulation on tooth surfaces. In addition, a decrease in local pH was found during the course of the treatment, which was produced by changes in the plaque’s metabolism. In general, enamel demineralization is a dietary carbohydrate-modified bacterial infectious disease caused by acid production by biofilms. Hence, it is highly desirable to develop an antibacterial self-etching orthodontic adhesive.

The present study developed an antibacterial orthodontic self-etching adhesive through the incorporation of a new quaternary ammonium monomer DMAHDM and investigated its effects on bracket bond strength and anti-biofilm property for the first time. The hypotheses were accepted that adding DMAHDM into the self-etching adhesive did not compromise the enamel bond strength; adding DMAHDM into the self-etching adhesive yielded potent anti-biofilm activity; and that the antibacterial efficacy was directly proportional to the DMAHDM content in the self-etching adhesive.

The mechanism of the antibacterial effect of QAMs is due to the cationic binding to cell wall components which is negatively charged. This causes the membrane function of the bacteria to be disturbed, and the external leakage of the cytoplasmic material will cause lysis of the bacterial cells. In a previous study, hydrophobic, positively charged long polymeric chains was shown to effectively kill bacteria. The long cationic polymers can penetrate bacterial cells similar to the way a needle bursts a balloon. Hence the ability to penetrate the hydrophobic bacterial membrane is directly related to the alkyl chain length (CL). Indeed, increasing the CL from 3 to 16 greatly enhanced the antibacterial activity. Therefore, DMAHDM with CL of 16 had a strong antibacterial function. Previous studies investigated the antimicrobial activities of DMAHDM in dental composites, adhesives, and orthodontic cements. While these studies did not include self-etching adhesives, they showed substantial reductions in biofilm activity. The present study expanded the application of DMAHDM to self-etch adhesives, showing that AEO containing 5% DMAHDM had strong antibacterial properties.

The surface around the bracket margins is recognized as the most common enamel area to be demineralized. In the present study, the adhesive was applied a 1 mm more than the dimension of the bracket base on each side to achieve a sealing effect to protect the enamel. The present study determined the best concentration of DMAHDM in the self-etching adhesive to balance the antibacterial property and the enamel bond strength. Under the conditions of the present study, when the concentration of DMAHDM was 5%, the adhesive produced a similar SBS to the control group, while achieving a strong antibacterial function. Therefore, 5% DMAHDM was considered the best concentration in this self-etching adhesive.

Compared to the phosphoric acid etching method, the self-etching method was shown to produce a milder etching pattern with less enamel loss. This is because in the self-etching AEO, the active ingredient is a methacrylated phosphoric acid ester, in which the phosphoric acid and the methacrylate group are combined into a molecule that etches and primes at the same time. The phosphate group on the methacrylated phosphoric acid ester dissolves the calcium and removes it from the hydroxyapatite in enamel. But rather than being rinsed away, the calcium forms a complex with the phosphate group and becomes incorporated into the network when the primer is photo-polymerized. While preserving more enamel, the self-etching method still produced adequate enamel bond strength. In the present study, the SBS of the phosphoric acid etch control group was 12 MPa; that of the self-etching control was 11 MPa. The SBSs of group 2–5 ranged from 10 to 12 MPa. Previous studies suggested that the bond strength of stainless steel brackets should be at least 8 to 9 MPa to avoid the braces from being prematurely debonded. Therefore, the novel self-etching and antibacterial group with 5% DMAHDM in AEO would permit adequate bracket-enamel adhesion.

Regarding the long-term durability of the antibacterial properties, the advantage of QAMs is that they can be copolymerized with the resin by forming a covalent bond with the polymer network. Therefore, the QAM is immobilized in the resin and not released or lost over time. This provides a durable antibacterial ability. This is indirectly shown in the enamel SBS after 30 days of immersion and 1,000 thermal cycles, which exhibited no significantly decrease compared to that at 15 min. Had there been release and lost of DMAHDM during the 30 days of immersion plus 1,000 thermal cycles, the resin structure would have been weakened and the bond strength likely would have decreased over time. Further study is needed to investigate the long-term properties of the DMAHDM-containing self-etching adhesive during immersion in artificial saliva for 1–2 years, to ensure that the bond strength and the
antibacterial activity are maintained over the entire orthodontic treatment period. Further studies are also needed to investigate the enamel protection and inhibition of WSLs via the novel DMAHDM-containing self-etching adhesive under in vivo conditions.

CONCLUSION

The present study developed a novel bioactive self-etching adhesive for orthodontic applications to combat enamel demineralization and WSLs. The self-etching adhesive with DMAHDM possessed a strong antimicrobial activity, which increased with increasing the mass fraction of DMAHDM in AEO. The 5% DMAHDM appeared to be optimal in obtaining the strongest antibacterial function without compromising the enamel bond strength both at 15 min and after 30 days of immersion plus thermal cycling. The novel self-etching DMAHDM adhesive has the following advantages: (1) it has adequate enamel bond strength; (2) its antibacterial function can greatly reduce oral biofilms and plaque formation. Furthermore, we can deduce that there are another two advantages of this novel self-etching DMAHDM adhesive: (1) It can result in a smaller extent of enamel demineralization; (2) it can form a protective layer on the surface of the enamel, which could be proved by further study. Therefore, the novel self-etching DMAHDM adhesive is promising to minimize enamel demineralization during orthodontic treatment.

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