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Enzyme-catalyzed hydrolysis of dentin adhesives containing a new urethane-based trimethacrylate monomer

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Abstract

A new trimethacrylate monomer with urethane-linked groups, 1,1,1-tri-[4-(methacryloxyethylamino-carbonyloxy)-phenyl]ethane (MPE), was synthesized, characterized, and used as a co-monomer in dentin adhesives. Dentin adhesives containing 2-hydroxyethyl methacrylate (HEMA, 45% w/w) and 2,2-bis[4(2-hydroxy-3-methacryloyloxy-propyloxy)-phenyl] propane (BisGMA, 30% w/w) in addition to MPE (25% w/w) were formulated with H_2O at 0 (MPE0), 8 (MPE8) and 16 wt % water (MPE16) to simulate the wet demineralized dentin matrix and compared with controls [HEMA/BisGMA, 45/55 w/w, at 0 (C0), 8 (C8) and 16 wt% water (C16)]. The new adhesive showed a degree of double bond conversion and mechanical properties comparable with control, with good penetration into the dentin surface and a uniform adhesive/dentin interface. On exposure to porcine liver esterase, the net cumulative methacrylic acid (MAA) release from the new adhesives was dramatically ($P < 0.05$) decreased relative to the control, suggesting that the new monomer improves esterase resistance.

Keywords

Dentin adhesives; Photopolymerizaiton; Enzymatic degradation; Urethane-based trimethacrylate; Interface

Introduction

The use of restorative composites in dentistry has been primarily driven by the esthetic features of these materials. Interest in dental composites as an alternative to amalgam has been further promoted by the public's concern about mercury release from dental amalgam.[1,2] Despite their extensive use, the short clinical lifetime of composites is a significant limitation.[3] While the clinical lifetime of traditional mercury-containing dental amalgam restorations is generally 10-20 years, the lifetime for methacrylate-based composite restorations is about 8 years in anterior sites and as little as 2-4 years in posterior sites.[4]

The primary factor in the premature failure of composite restorations is recurrent caries at the margins of these restorations.[5] Recurrent decay is most often localized gingivally[5] and is linked to the lack of a consistent seal at the tooth/material interface.[6]

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Water in the mouth is a major interfering factor when bonding adhesives and/or composites to the tooth7. The water content of the dentin surface varies as a function of depth8,9, the nature of the substrate (i.e. caries-affected or healthy dentin)10 and the presence of residual rinse water. Under *in vivo* conditions, there is little control over the amount of water left on the tooth during dentin bonding. In our previous work, we reported the effect of water on photopolymerization behavior, phase separation, enzymatic degradation, and material properties of dentin adhesives.11-16 Water may be trapped within the matrix during photopolymerization or can enter the adhesive matrix by diffusion into the loosely cross-linked or hydrophilic HEMA-rich domains.7 Water also facilitates the degradation of methacrylate adhesives, which have numerous ester groups subject to both chemical and enzymatic hydrolysis, the latter mediated by salivary enzymes.17-19 Studies have shown dental polymer networks to be degraded to produce small molecules such as methacrylic acid and bishydroxypropoxyphenylpropane (bis-HPPP) through passive hydrolysis and enzymatic reaction.7,20-22 The carboxylate and alcohol degradation products of ester hydrolysis are more hydrophilic than the parent ester, further enhancing the local ingress of water which may result in accelerating biodegradation. In addition, methacrylic acid can cause irritation of mucosal membranes and cytotoxicity in the mouth20,21; these factors must be considered when developing biocompatible restorative materials. The extent of hydrolysis of methacrylates appears to be largely dependent on the chemical structure of the monomers, suggesting that degradation can be reduced by changes in monomer structure. For example, Hagio *et al.*23 have demonstrated that dimethacylates containing a urethane linkage show a particular resistance to salivary hydrolysis.

Here, we report the synthesis and characterization of a novel urethane-linked trimethacrylate monomer for use as a co-monomer in dentin adhesives. The study tests the hypothesis that in the presence of moist clinically relevant dentin substrates, dentin adhesives that include the new monomer will provide less methacrylic acid release without sacrificing polymerization conversion, penetration into dentin or mechanical properties, as compared to model adhesives that are representative of state-of-the-art commercial dentin adhesives.

Materials and Methods

Materials

2-Hydroxyethylmethacrylate (HEMA, Acros Organics, NJ) and 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy) phenyl]-propane (BisGMA, Polysciences, Warrington, PA) were used as received without further purification as monomers in dentin adhesives. 1,1,1-tri-[4-(methacryloxyethylaminocarbonyloxy)-phenyl]ethane (MPE) was used as a co-monomer and synthesized in-house (Figure 1). 1,1,1-tris(4-hydroxyphenyl)ethane, dibutyltin dilaurate (DBTL), and 2-isocyantoethyl methacrylate (IEM) were obtained from Aldrich, Milwaukee, WI, USA and used for MPE synthesis. Camphorquinone (CQ) and ethyl-4-(dimethylamino)benzoate (EDMAB) were obtained from Aldrich (Milwaukee, WI, USA) and used as photoinitiators without further purification. Porcine liver esterase (PLE, EC 3.1.1.1) was obtained from Sigma Chemical Co., St. Louis, USA. All other chemicals were reagent grade and used without further purification.

Monomer synthesis (MPE)

The new monomer, MPE, was synthesized following the procedures described by Xie *et al.*, with slight modification24. Briefly, to a three-neck flask containing 1,1,1-tris(4-hydroxyphenyl)ethane (THPE, 8.89g, 0.029 mol), dibutyltin dilaurate (DBTL, 0.03g), and dry tetrahydrofuran (THF, 50 mL) under N_2 atmosphere, 2-isocyantoethyl methacrylate (IEM, 13.73 g, 0.089 mol) was added dropwise with stirring at 0 °C. Following complete addition of IEM, the reaction was allowed to continue at room temperature for another 5 hrs.

The reaction was monitored by thin layer chromatography (mobile phase: $CHCl_3$:MeOH=9:1). After the reaction was completed, the product-containing solution was purified by washing with distilled water and ethyl acetate until the solution was clear. After drying over anhydrous $MgSO_4$, 0.05 wt% of 2,6-di-*tert*-butyl-4-methylphenol (BHT) was added and the solvent removed with a rotary evaporator at 35-40 °C. The yield of this trimethacrylate-based monomer, 1,1,1-tri-[4-(methacryloxyethylamino-carbonyloxy)-phenyl]ethane (MPE), which is a white foamy compound, was in the range of 87-90 %. The scheme for the MPE synthesis is shown in Figure 1.

Preparation of adhesive formulations and their specimens

Control adhesive formulations consisted of HEMA and BisGMA with a mass ratio of 45/55, which is similar to commercial dentin adhesives such as Single Bond (3M ESPE, St. Paul, MN). Control adhesives were formulated with 0 wt% (C0), 8 wt% (C8) and 16 wt% (C16) water to simulate the moist environment of the mouth. The experimental adhesive formulations, HEMA/BisGMA/MPE=45/30/25 w/w ratio, in which MPE was used as a co-monomer, were also formulated with 0 wt% (MPE0), 8 wt% (MPE8) and 16 wt% (MPE16) water. CQ (0.5 wt%) and EDMAB (0.5 wt%) were used as photoinitiator and co-initiator, respectively, with respect to the total amount of monomer. The resin mixtures were shaken on an orbital shaker for 2 days to dissolve the initiators completely and form a homogeneous solution. The resin solution was then placed into an aluminum mold (4 mm diameter and 1 mm thickness) and covered with a plastic film to form disc specimens for biodegradation studies. Rectangular beam specimens ($1\times1\times11$ mm^3) cured in a glass-tubing mold (Fiber Optic Center Inc., Vitrocom hollow square capilliaries, 1.00 mm square I.D., 0.200 mm wall thickness, borosilicate glass) were prepared for the determination of mechanical properties. The adhesives placed in the mold were light-cured for 40 s at room temperature at a distance of 1 mm using a commercial visible-light-curing unit (Spectrum® 800, Dentsply, Milford, DE, USA) at an intensity of 550 mW cm^{-2}, according to techniques published previously.25 The cured specimens were removed from the mold after storage for 24 hrs in a dark room at room temperature.

The degree of conversion of the double bond

The degree of conversion (DC) of the methacrylate double bond of the adhesives was determined using a Perkin-Elmer Spectrum One Fourier transform infrared spectrophotometer (FTIR) with a resolution of 4 cm^{-1}. 25,26 One drop of adhesive solution was placed on the diamond crystal top-plate of an attenuated total reflectance (ATR) accessory (Perkin-Elmer, Waltham, MA, USA), covered with a mylar film to prevent oxygen inhibition of polymerization. A 40 sec-exposure to the commercial visible-light-polymerization unit (described above) was initiated after 50 spectra had been recorded. Real-time IR spectra were continuously recorded for 600 sec after light activation began. The ATR crystal was zinc selenide (ZnSe) with a transmission range between 650 and 4000 cm^{-1}. A time-based spectrum collector (Spectrum TimeBase, Perkin-Elmer) was used for continuous and automatic collection of spectra during polymerization. Three replicates were obtained for each adhesive formulation. The change of the band ratio profile (1637 cm^{-1}(C=C)/1608 cm^{-1}(phenyl)) was monitored and DC was calculated using the following equation based on the decrease in the absorption intensity band ratios before and after light curing. The average of the last 50 of time-based spectra is reported as the DC value.

$$DC=(1-\frac{Absorbance^{sample}_{1637cm^{-1}}/Absorbance^{sample}_{1608\,cm^{-1}}}{Absorbance^{monomer}_{1637cm^{-1}}/Absorbance^{monomer}_{1608\,cm^{-1}}})\times 100\%$$

Determination of mechanical properties

Rectangular beam specimens ($1\times1\times11mm^3$) were used to determine mechanical properties. Ten specimens were prepared for each of the control (C0, C8, and C16) and experimental adhesives (MPE0, MPE8, and MPE16). Tensile properties were determined for all samples after either 24h storage-in-air (n = 5 per sample type) at room temperature or after storage for 24h in distilled deionized water (n = 5 per sample type). Following storage, specimens were attached tightly to the upper and lower grips using cyanoacrylate cement (Zapit, Dental Ventures of America, Corona, CA, USA) and were loaded at a cross-head speed of 0.5 mm/min using an SSTM-5000 mechanical tester (United Calibration Corporation, CA, USA) with a 150 lb load cell. The toughness (T, m MN m^{-3}) of the specimen was calculated as the area under the stress-strain curve. Percent elongation (EL, %) was calculated as the value at the point of failure divided by the original gauge length of the specimen. The ultimate tensile strength (UTS, MPa) is the maximum resistance to fracture, and was measured from the maximum force at the point of failure divided by the specimen cross-sectional area. The elastic modulus (E, GPa) was obtained as the slope of the linear portion of the stress-strain curve between 5% and 15% strain for all specimens. Four to eight specimens in each group were tested.

The results were analyzed statistically using analysis of variance (ANOVA), together with Tukey's test at $\alpha=0.05$ (Microcal Origin Version 6.0, Microcal Software Inc., Northampton, MA).

Dentin-adhesive specimen preparation for SEM and staining light microscopy

Extracted non-carious, unerupted human third molars stored at 4 °C in 0.9% wt/vol NaCl containing 0.002% sodium azide were used to evaluate the ability of the adhesives to bond with dentin. Teeth were collected after the patients' informed consent was obtained. The teeth were collected under a protocol approved by the University of Missouri Adult Health Sciences institutional review board. Specimen preparation has been detailed previously.[27,29,31] In brief, dentin disks were prepared by first cutting the roots at the cementum-enamel junction with a water-cooled low speed diamond saw (Buehler, Lake Bluff, IL). The occlusal one-third of the crown was then removed by means of a second, parallel section. Dentin surface without any enamel remnants or exposure of the pulp chamber was prepared. A uniform smear layer was created by abrading the exposed dentin surface with 600 grit silicon carbide under water. Control and experimental adhesives were applied to the prepared dentin surfaces according to the following protocol.[28] The dentin surfaces were etched with 35% phosphoric acid gel for 15 s and rinsed with distilled water. Excess distilled water was then removed, but the dentin surface was allowed to remain visibly moist. Next, two consecutive coats of the adhesive resin were applied and the surface gently dried using air from an air-water syringe. The adhesive layer was then photo-cured for 40 s by exposure to a visible light source, as described previously. The prepared specimens were stored for 24 h in distilled water at 25 °C before being sectioned. The treated dentin surfaces were sectioned perpendicular and parallel to the bonded surfaces using a water-cooled low-speed diamond saw. The resulting dentin/adhesive specimens were rectangular slabs (~8 mm × ~2 mm × 1.5 mm).

Differential staining microscopy

The specimens were prepared according to the following protocol.[29] The rectangular, 8 mm × 2 mm × 1.5 mm, slabs of dentin/adhesive specimens were mounted on a poly(methyl methacrylate) support and 5 μm-thick sections were cut from the face of the slab using a tungsten carbide knife mounted on a Polycut S “sledge” microtome (Leica, Deerfield, IL, USA). The sections were mounted on glass microscope slides previously treated with Haupt's adhesive (1% gelatin in water with 2% phenol crystals and 15% glycerine), which is

used to keep the sections attached to the glass slide during the subsequent staining procedures. Differential staining of the microtomed sections was accomplished with Goldner's trichrome. Stained sections were dehydrated, cover-slipped with mounting media and observed under a Nikon E 800 light microscope. The width of the layer stained by the Goldner's trichrome was determined by measuring directly from photomicrographs whose exact magnification was established with a stage micrometer.

Scanning electron microscopy (SEM)

The *in vitro* penetration of adhesive resin into the dentin and the micromorphology of the resin-dentin interface were observed by scanning electron microscopy. The specimens were prepared according to the following protocol.9 The sectioned specimens were treated with 5N HCl for 15s and 5% NaOCl for 30 min. After rinsing with distilled water, the specimens were dehydrated using a graded series of ethyl alcohol solutions and air-dried in a fume hood overnight. Following drying, the specimens were mounted on 12 mm aluminum stubs and sputter-coated with gold-palladium. Specimens were then examined at a variety of magnifications using a Field Emission Philips XL30 ESEM-FEG 515 electron microscope (Philips Electron Optics Inc., Hillsboro, OR) at 15 kV.

Enzymatic degradation studies

Five adhesive discs with a surface area of ~2.0 cm^2/ml were placed in sterile vials and pre-washed in sterile 0.05M phosphate buffer saline (PBS) with pH 7.4 for three days to remove unreacted monomer. Following the pre-wash, adhesive discs were incubated in 1 mL of 0.2 M phosphate buffer solution containing 30 U/mL porcine liver esterase (PLE, EC 3.1.1.1., Sigma E3019) at 37°C for 8 days with shaking; concurrent analysis without enzyme consisted of incubations of test specimens in 0.2M phosphate buffer (PB). Daily changes with PLE enzyme were necessary to maintain its optimum activity. PLE was selected for its non-specific effect on ester bonds and its activity was routinely checked at zero and 24 hours using ethyl butyrate. One unit of fresh PLE hydrolyzed 1.0 μmole of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0/25°C; after 24 hours, the activity was 96-98% of this value. Solution samples obtained each day were immediately centrifuged (15 min × 13,400 rpm) to remove the enzyme. The supernatants were then stored at -20 °C until analysis by HPLC. The methacrylic acid content (MAA) was determined by reverse phase HPLC using a 600E system controller, a 717 plus autosampler and a 484 tunable wavelength UV (208 nm) detector from Waters (Milford, MA)30. Samples were thawed and centrifuged again prior to injection into the HPLC system for analysis. An enzyme-free solution at pH 7 and 37 °C served as a negative control and as a measure of the non-enzymatic hydrolysis of each material. A Phenomenex Luna 5 μm C_{18} 4.6 × 250 mm (Phenomenex, Torrance, CA) column and security guard cartridge were used to isolate the products. The mobile phase was CH_3CN: 10 mM potassium phosphate buffer (60:40, v/v) at a flow rate of 1.0 mL/min. MAA concentrations were determined by comparing peak areas with a calibration curve prepared using MAA standards of 50, 100, 250, 500, and 1000 μM concentration. Relative retention times of HPLC peak of the standard solution were found to be 1.9 min for MAA.

Results

Characterization of the synthesized MPE

The structure of the newly synthesized trimethacrylate monomer (MPE) was identified using FTIR and ^1H NMR/^{13}C NMR (FT-400 MHz Bruker Spectrometer, $CDCl_3$ as solvent) spectroscopy. The characteristic FTIR peaks for MPE are: 3342.9 cm^{-1} (NH stretching on CONH), 1710.5 cm^{-1} (C=O stretching on OCONH and OCO, where both carbonyl peaks overlap), 1637.0 cm^{-1} (C=C bending on methacrylate group), 1536.4 cm^{-1} (amide II, CONH), 1208.4 cm^{-1} (C-O stretching), 815 cm^{-1} (C=C twisting). Disappearance of the –

NCO band at 2250 cm^{-1} and appearance of the C=C stretching band at 1637.0 cm^{-1} confirmed the formation of the new methacrylate monomer. The ^{1}H and ^{13}C NMR spectra of MPE (Figure 2) show chemical shifts consistent with the desired structure.

In the ^{1}H-NMR spectrum (Figure 2A), the chemical shifts of MPE were (ppm): a, 7.9 (^{1}H, -OCONH-); b, 7.1 (4H, C_6H_4-); c, 6.20 and 5.60 (2H, -CH_2=$C(CH_3)$COO-); d, 4.28 (2H, -$NHCH_2CH_2O$-); e, 3.4 (2H, -$NHCH_2CH_2O$-); f, 2.10 (3H, $CH_3C(C_6H_4)_3$-); g, 1.9 (3H, -CH_2=$C(CH_3)$COO-). In the ^{13}C -NMR spectrum (Figure 2B), the chemical shifts of MPE were (ppm): m, 166.9 (-CH_2=$C(CH_3)$COO-); l, 155.0 (-OCONH-); k, 149.0 (-C_6H_4-); j, 146.3 (-C_6H_4-); i, 136.0 (-CH_2=$C(CH_3)$COO-); h, 129.9 (-C_6H_4-); g, 126.3,(-CH_2=$C(CH_3)$COO-); f, 121.2 (-C_6H_4-); e, 63.6 (-N-C-C-O-); d, 51.3 (-$CH_3C(C_6H_4)_3$-); c, 40.0 (-N-C-C-O-); b, 18.0 (CH_2=$C(CH_3)$COO-); a, ($CH_3C(C_6H_4)_3$-). The methacrylate and aromatic groups are supported by the presence of two singlets at 6.1 and 5.6 ppm for the double bond, by multiplets at 7.1 ppm for the benzene ring on the ^{1}H-NMR spectrum, by the peaks at 136.2 and 125.5 ppm for the double bond, and by peaks at 121.0, 129.9, 146.0, and 149.6 ppm for the benzene ring in the ^{13}C –NMR spectrum.

Degree of conversion of adhesives

The kinetics of photopolymerization of the adhesives are shown in Figure 3. Conversion of all the adhesives tested approached a plateau at 80 s after light initiation. The adhesives were light-cured for 40 sec at room temperature using a commercial visible-light-curing unit (Spectrum® 800, Dentsply, Milford, DE, USA) at an intensity of 550 mW cm^{-2}.

There were no marked differences in the DC values for *in situ* photopolymerization of control and experimental adhesives cured in the absence of water. The polymerization conversion was ~60 % when cured in the absence of water. When cured in the presence of 8 or 16% water, the DC values were in the range of 70 – 77 %, which is significantly ($P < 0.05$) greater than those cured without water.

Mechanical properties of adhesive resins

The mechanical properties of dentin adhesives cured in the absence or presence of water are summarized in Table I and the comparisons of ultimate tensile strength (UTS) are shown in Figure 4.

The ultimate tensile strength (UTS) value of all the samples tested was in the range of 32.4 – 49.0 MPa. UTS values for control adhesives (C0, C8 and C16) stored for 24 hours in water were significantly lower ($P<0.05$) than for samples stored in air. However, experimental adhesives (MPE0, MPE8 and MPE16) showed similar UTS values irrespective of the presence of water and storage conditions (Figure 4). For example, the UTS of C0 stored in air was 44.6 MPa, which is similar to that of MPE0 (45.7 MPa), while the value for MPE0 (45.1 MPa) stored in water was significantly ($p < 0.05$) greater than that of the corresponding control (C0 stored in water, 37.6 MPa). The experimental adhesives cured in the presence of 8 wt% and 16 wt% water showed significantly greater UTS values than those of controls (Table I) irrespective of storage conditions. UTS values of control air-stored samples decreased with an increase in water content, while experimental adhesives showed no significant difference (Table I). Moduli of all specimens were in the range of 0.62 ~ 1.18 MPa. For both adhesives, specimens stored in air exhibited significantly ($p < 0.05$) higher moduli than samples stored in water (0.62 ~ 0.80), following the trend observed in the UTS tests. Moduli of the experimental adhesives were significantly ($p < 0.05$) higher when stored in water than those of control. Toughness values for the air-stored adhesives were relatively unaffected by water content, but increased with water storage for 24 h. Control adhesives showed somewhat less toughness than the experimental adhesives (Table

I). Elongation of the resins was in the range of 0.06 – 0.14 %. There was no significant difference ($P > 0.05$) in elongation between control and experimental adhesives.

Observations of dentin/adhesive interfaces

Representative SEM micrographs of the dentin/adhesive interfaces are shown in Fig. 5A. The exposure technique, in which the sectioned specimen was treated with 5N HCl for 15s and 5% NaOCl for 30 min, has been commonly used to determine the adhesive penetration into the dentin. Numerous resin tags were observed in both control and experimental adhesives and these were formed by the photopolymerization of adhesive resins that penetrated into the dentinal tubules, indicating good resin infiltration into the prepared dentin surface. Both experimental and control adhesives exhibited a distinct hybrid layer (HL) zone. The thickness of the HL formed by the control adhesives in dentin was approximately 2.5 μm. For the experimental adhesive, the HL thickness was ~3.5 μm. In the micrographs of the experimental adhesive (Figure 5A; MPE0), some resin tags cut by a water-cooled low speed diamond saw are seen on the front side, due to the orientation of the dentinal tubules. The resin tags also showed small lateral branches. Thus, there were no marked differences in the control and experimental adhesives on SEM evaluation.

Representative optical micrographs of Goldner's trichrome stained sections of the adhesive/dentin interface are shown in Figure 5B (control: C0; experimental: MPE0). Using this staining technique, mineralized dentin collagen is stained green, unprotected exposed collagen/protein is stained red and pure adhesive is either stained pale yellow or not stained[31,32]. Both micrographs clearly showed an interface which connected the dentin structure with the adhesive resin (Figure 5B). The color of the interface zone is dark red when treated with control adhesive and the width is about 2.4 μm, while the experimental adhesive is orange/red and 3.5 μm in width. The difference in color represents the extent of exposed collagen at the interface. The red color represents exposed collagen at the interface that is available for reaction with stains. The orange color indicates the resin-infiltrated layer where exposed collagen was slightly more encapsulated with adhesive.

Enzymatic biodegradation of adhesive resins

Net cumulative release of MAA in the presence of PLE is shown in Figure 6 and was obtained by subtracting the MAA release measured in buffer [$MAA_{in\ PLE} - MAA_{in\ PB}$].

The net cumulative release of MAA for control adhesives cured in the presence of water (C8, 1352 μg/mL; C16, 1586 μg/mL) was significantly greater ($p < 0.05$) than for control adhesives cured in the absence of water (C0, 574 μg/mL). The experimental adhesives showed similar MAA release regardless of the presence of water in the resin mixture (approximately 300 μg/mL for MPE0, MPE8 and MPE16). In addition, the net cumulative MAA release from the experimental adhesives containing the new monomer was significantly less ($p < 0.05$) than the controls, indicating that the new adhesives have greater esterase resistance than conventional adhesives.

Discussion

A new trimethacrylate monomer, MPE, containing a urethane-linked group for use as a co-monomer in dentin adhesives was synthesized and characterized. The trifunctional MPE was readily synthesized in good yields, 90%. Experimental adhesives contained HEMA and BisGMA in addition to the novel MPE monomer. Adhesives were photopolymerized in the presence of 0, 8 and 16 wt% water to simulate the wet conditions of the mouth and were compared to control adhesives (HEMA/BisGMA, 45/55 w/w, at 0, 8 and 16 wt% water).

The extent of polymerization influences the physical and mechanical properties of the polymer, and may also contribute to their susceptibility to enzymatic degradation.4,7 In this study, the degree of conversion (DC) of samples polymerized in the presence of 8 wt% and 16 wt% water was greater than those cured without water, which may be due to enhanced mobility of reactive species in lower viscosity solutions containing water. There was no significant difference in the DC of control and experimental adhesives (Figure 3), suggesting that the experimental adhesives reach DC comparable to that of the control, regardless of the presence or absence of water.

Clinically, dentin adhesives are placed on the moist dentin surface and subsequently light-cured. Residual water on the dentin surface may dilute the adhesive monomers prior to polymerization, possibly influencing the formation of the polymer network and the resulting mechanical properties. Because of its small size, water is expected to penetrate into nano/micrometer-size free volume spaces between polymer chains33, or cluster around functional groups that are capable of hydrogen bonding.34 The water penetration may alter mechanical properties observed at the macro scale Thus, it is important to determine the mechanical properties of samples polymerized under moist conditions. Here, the adhesive formulated using the newly synthesized trimethacrylate monomer (MPE) showed mechanical properties that were comparable or superior to the control (Table I). Interestingly, the UTS values for the experimental adhesive were not influenced by the water that was present during photopolymerization. In contrast, the controls showed a reduction in UTS when cured in the presence of water (Figure 4). The reduction in UTS for controls cured in water may be related in part to the higher cohesive energy density of the hydroxyl groups (2980 J cm^{-3}) compared with the urethane groups (1425 J cm^{-3}).35 In control samples containing more hydroxyl groups, hydrogen bonding between water molecules and the hydroxyl groups of the polymer network36 may disrupt the interchain hydrogen bonds, with adverse effects on mechanical properties.37

The formation of the hybrid layer is due to the application of acids or self-etching acidic primers to the dentin, followed by adhesive resin penetration into the decalcified zone.38,39 The complete penetration of adhesive monomers into the demineralized dentin is essential to create strong adhesion and to envelop the collagen fibers.40 In this study, SEM and staining/light microscopy were used to evaluate the morphology and quality of the interface between adhesives and dentin. SEM observations of both control and experimental adhesives showed numerous resin tags and small lateral branches, suggesting good resin penetration into the dentinal tubules. There were no significant differences in the morphology of the adhesive/dentin interfaces for the control and experimental adhesives. SEM observation involves time-consuming specimen preparation and is very sensitive to sample preparation techniques that may alter or even destroy the interface. Many of the disadvantages associated with the SEM specimen preparation technique can be overcome using the staining/light microscopic method. The light micrographs of adhesive/dentin interfaces stained with Goldner's trichrome clearly show an interface in which the dentin structure is connected with the adhesive resin (Figure 5B). These thin sections were differentially stained using Goldner's trichrome, a conventional bone stain. The width of the interface was 2.4 and 3.5 μm for the control and experimental adhesives, respectively, consistent with the SEM observations. However, Finger *et al.*41 found no correlation between interface thickness and the bond strength of adhesive resins, suggesting that bond strength is determined by the quality of the interface rather than its thickness. The SEM images for experimental and control adhesives showed no separation between the adhesive and dentin along the length of the interface (Figure 5A), indicating overall structural integrity of the interface.

Although many factors may contribute to the premature breakdown of methacrylate-based adhesives, their chemical “Achilles heel” may prove to be the ester linkages in the

methacrylate matrix since these linkages are susceptible to attack by water and esterases. Each monometharylate contributes one ester bond, and each dimethacrylate contributes two, so that ester linkages are numerous and widely distributed in the network. Esters anchor the cross-linking dimethacrylates and the pendant monomethacrylate side chains to the vinyl chains, forming critical structural bonds. When exposed to oral fluids, the ester bonds are vulnerable to two forms of hydrolytic attack: (i) chemical hydrolysis catalyzed by acids or bases, and (ii) enzymatic hydrolysis catalyzed by salivary enzymes, particularly esterases. Both require the presence of water in close association with the bond that will be hydrolyzed.

The mechanism described above suggests several strategies for reducing hydrolytic degradation of methacrylate adhesives. First, the rate and extent of water ingress into the matrix should be minimized. This can be accomplished by the use of relatively hydrophobic monomers, by increasing cross-link density and/or by a high degree of conversion during polymerization. A drawback to this approach is the reduced water compatibility of hydrophobic monomers. In particular, our research has shown very poor interfacial integrity with wet dentin and limited durability of the adhesive/dentin (a/d) bond with hydrophobic monomers.11 A second strategy involves selectively modifying methacrylate side chains to create branched15,30 and/or urethane23 functional groups that are poor esterase substrates while retaining some hydrophilic character (e.g., by balancing with incorporating polar functional groups such as hydroxyl, urethane, ether, etc.). Clearly, any change in the chemical structure intended to increase esterase resistance is likely to alter other chemical and physical properties of the matrix. The optimal adhesive will be produced by balancing the desired physical, chemical and mechanical properties of the matrix with the need for esterase resistance.

This study tested the effect of enzyme-exposure on the release of MAA from adhesives formulated in water to simulate wet bonding conditions. Esterases known to activate ester hydrolysis include salivary esterases, cholesterol esterase, pseudocholinesterase, porcine liver esterase and acethylcholinesterase.30 Based on our previous investigation14,15, PLE was selected for its non-specific effect on ester bonds and high concentration of 30 U/mL porcine liver esterase was used as a rapid screening method. The control adhesives cured in the presence of water showed greater net cumulative MAA release than the control cured in the absence of water. This result may be linked to the BisGMA structure and adhesive phase separation that occurred when the materials were photopolymerized in the presence of water. BisGMA has a relatively unhindered ester bond as compared to the new monomer and has two pendant hydroxyl groups, which are responsible for the high water sorption, and may increase its susceptibility to hydrolytic degradation. Due to adhesive phase separation, poorly polymerized hydrophilic polymer domains may degrade rapidly in the aqueous oral environment.11,12

In the present work, both the high functionality and urethane functional group of MPE were expected to contribute to increased esterase resistance of resins containing this material, especially in the presence of water. The factors affecting the enzymatic degradation of methacrylate resins include the DC, crosslink density, monomer structure and morphology of polymer network. The improved esterase resistance of the experimental adhesive in this study could be explained in terms of a greater degree of crosslinking due to the higher functionality of the new monomer and/or minimizing the enzyme's access23 to the ester bond due to both the intra- and intermolecular hydrogen bonds (NH of urethane and C=O of ester) of the new monomer in the polymer matrix.

Conclusions

A new trimethacrylate monomer with a urethane linkage (MPE) was synthesized and used as a co-monomer in dentin adhesives. Adhesives containing the new monomer were formulated in the presence of water to simulate the behavior of these materials within the wet demineralized dentin matrix. The new experimental adhesives showed a degree of double bond conversion and properties comparable with the control. On exposure to porcine liver esterase, the net cumulative MAA release from the experimental adhesives containing new monomer was significantly less than the controls, indicating that the new adhesive has greater esterase resistance when formulated under wet conditions simulating the oral cavity than adhesives that model current commercial dentin adhesives. MPE thus shows promise as a component of durable, esterase-resistant water-compatible dentin adhesives.

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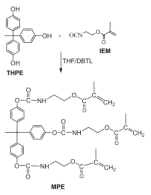

Figure 1.
Reaction scheme for synthesis of 1,1,1-tri-[4-(methacryloxyethylamino-carbonyloxy)-phenyl]ethane (MPE).

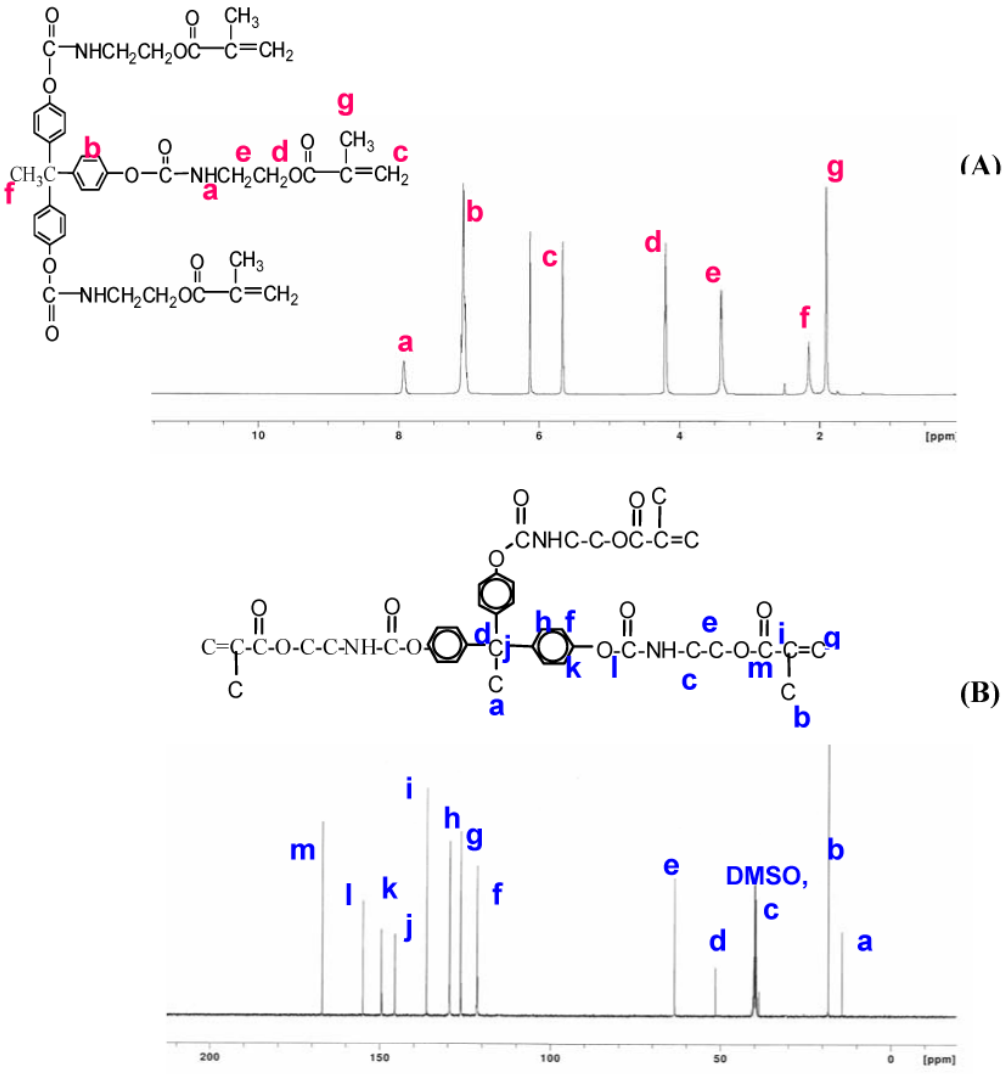

Figure 2.
^{1}H (A) and ^{13}C (B) NMR Spectra in DMSO of new monomer, MPE.

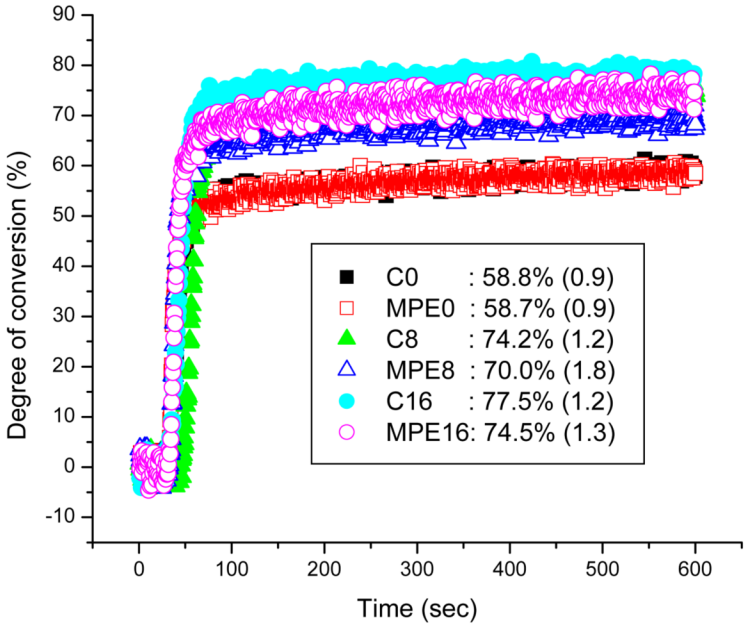

Figure 3.
Real-time conversion of control (C0, C8, C16) and experimental adhesives (MPE0, MPE8, MPE16). The adhesives were light-cured for 40 sec at room temperature using a commercial visible-light-curing unit (Spectrum® 800, Dentsply, Milford, DE, USA) at an intensity of 550 mW cm^{-2}.

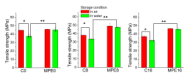

Figure 4.
Ultimate tensile strength of control and experimental adhesives cured in clinically relevant moist conditions. C0, C8, C16 = control formulations polymerized in the presence of 0, 8 or 16 wt% water; MPE0, MPE8, MPE16 = experimental formulations containing MPE polymerized in the presence of 0, 8 or 16 wt% water. n = 5 +/- S.D. (* = significant difference ($P < 0.05$) in UTS of control adhesives stored in water and air; ** = significant difference ($P < 0.05$) in UTS of control and experimental adhesives stored in water.

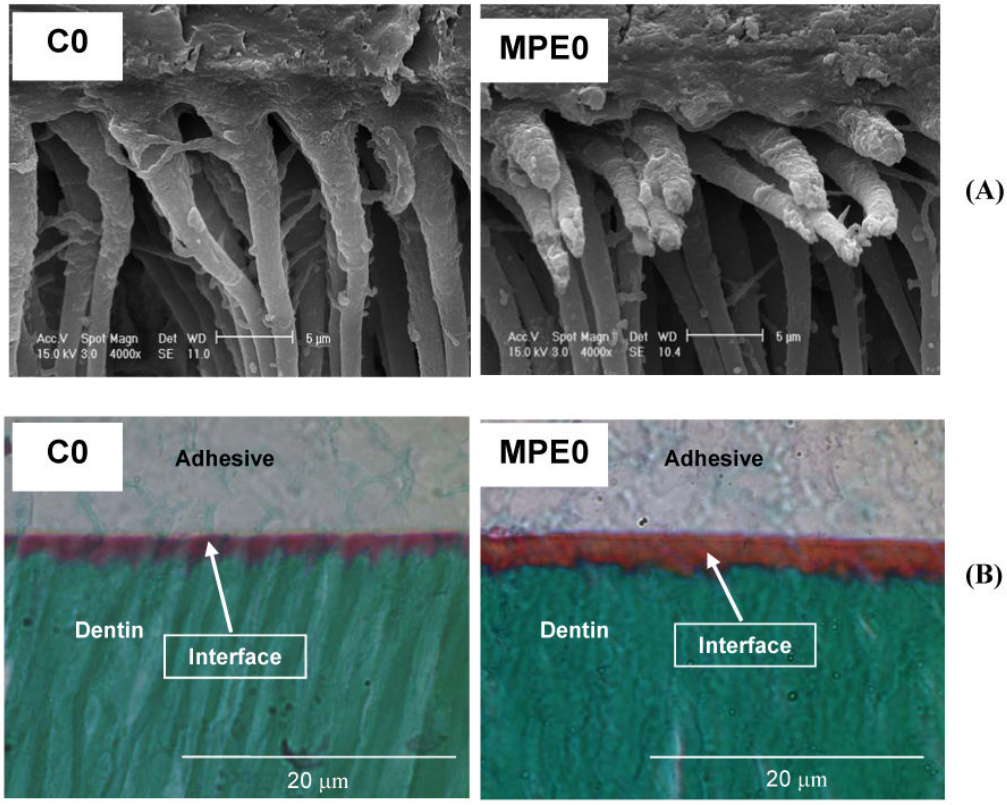

Figure 5.

Representative SEM micrographs (A) and staining/light microscopy (B) of dentin/adhesive interface for control (C0) and experimental (MPE0) resins. The SEM images indicated good resin penetration into the prepared dentin surface for both adhesive formulations. The staining light micrographs of adhesive/dentin interfaces stained with Goldner's trichrome clearly show an interface in which the dentin structure is connected with the adhesive resin. Adhesive resin composition: (C0 : HEMA/BisGMA=45/55 w/w ratio + 40 wt% EtOH; MPE0: HEMA/BisGMA/MPE =45/30/25 w/w ratio + 40 wt% EtOH).

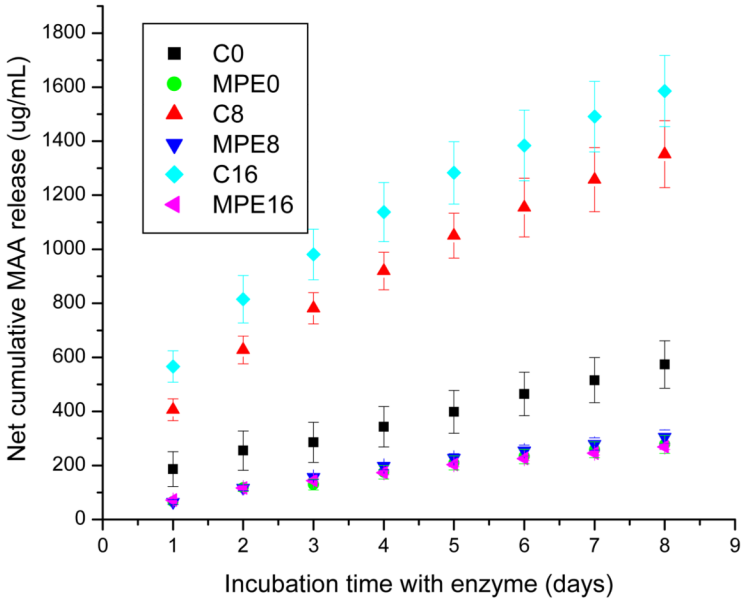

Figure 6.

Net cumulative MAA release from dentin adhesives as a function of incubation time on exposure to esterase for control formulations and experimental formulations. Net cumulative MAA release was obtained by subtracting the MAA release measured without PLE in phosphate buffer [$MAA_{in\ PLE} - MAA_{in\ PB}$]. C0, C8, C16 = control formulations polymerized in the presence of 0, 8 or 16 wt% water; MPE0, MPE8, MPE16 = experimental formulations containing MPE polymerized in the presence of 0, 8 or 16 wt% water. N = 3 +/- S.D.

Table I

Mechanical Properties of Adhesive Resins.

Sample	Water Content (wt%)	Storage and Test Condition	Toughness (MN/m^3)	Elongation (%)	Ultimate Tensile Strength (MPa)	Modulus of Elasticity (GPa)
C0	0	In air[b]	2.5(0.1) [a]	0.08(0.01)	44.6(0.3)	1.00(0.16)
		In water[c]	5.2(2.5)	0.12(0.02)	37.6(2.5)	0.62(0.11)
MPE0	0	In air	2.7(0.6)	0.06(0.01)	45.7(0.3)	1.18(0.02)
		In water	6.1(2.6)	0.11(0.02)	45.1(2.5)	0.80(0.11)
C8	8	In air	1.7(0.7)	0.07(0.02)	38.1(0.3)	0.78(0.20)
		In water	2.7(0.2)	0.08(0.02)	33.6(0.9)	0.70(0.02)
MPE8	8	In air	2.6(0.2)	0.09(0.01)	49.0(3.0)	0.91(0.07)
		In water	4.1(1.6)	0.11(0.02)	47.6(1.3)	0.80(0.05)
C16	16	In air	1.3(0.3)	0.06(0.01)	37.0(0.8)	0.77(0.02)
		In water	1.9(0.1)	0.11(0.01)	32.4(0.8)	0.69(0.05)
MPE16	16	In air	2.9(0.5)	0.10(0.02)	46.4(0.8)	0.89(0.07)
		In water	2.9(0.3)	0.14(0.03)	45.6(1.5)	0.78(0.06)

[a] Entries are mean values of five specimens with standard deviations in parentheses.

[b] 24 hr storage in air at room temperature after polymerization, the specimens were subjected to mechanical testing under dry conditions.

[c] 24 hr storage in air at room temperature and 24 hr storage in water and then tested while wet after polymerization.