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Synthesis and characterization of dimethacrylates containing quaternary ammonium functionalities for dental applications

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Abstract

Objectives—The widespread incidence of recurrent caries highlights the need for improved dental restorative materials. The objective of this study was to synthesize low viscosity ionic dimethacrylate monomers (IDMAs) that contain quaternary ammonium groups (antimicrobial functionalities) and are compatible with existing dental dimethacrylate-based monomers. Such monomers have the potential to copolymerize with other methacrylate monomers and produce antibacterial polymers.

Methods—Two monomers (IDMA-1 and IDMA-2) were synthesized using the Menshutkin reaction and incorporated at 0% to 30% (by mass) into a 1:1 (by mass) bisphenol A glycerolate dimethacrylate (BisGMA):triethylene glycol dimethacrylate (TEGDMA) resin. Resin viscosity was quantified using rheology, and polymer degree of conversion (DC) and surface charge density were measured using Fourier transform infrared spectroscopy (FTIR) and fluorescein binding, respectively. Effects of IDMA-1 on initial attachment of *Streptococcus mutans* and on viability and enzymatic activity (formazan reduction) of RAW 264.7 macrophage-like cells were quantified.

Results—IDMA-1 and IDMA-2 were prepared and characterized. IDMA-1 was miscible with BisGMA:TEGDMA and slightly increased the resin viscosity and DC. As expected, polymeric surface charge density increased with increasing IDMA-1. Incorporation of 10% IDMA-1 into BisGMA:TEGDMA reduced bacterial colonization without affecting viability or enzymatic activity of mammalian cells. Increasing IDMA-1 up to 30% had no additional effect on bacterial coverage, but $\geq 20\%$ IDMA-1 significantly reduced macrophage density, viability, and enzymatic activity. Leachables from polymers containing IDMA-1 were not cytotoxic.

Significance—The Menshutkin reaction provides a facile, convenient means to synthesize new monomers with quaternary ammonium groups for dental and medical applications.

Keywords

bacterial adhesion; cytotoxicity; dental restorative material; degree of conversion; dimethacrylate monomer; Menshutkin reaction; MTT assay; quaternary ammonium salts; *Streptococcus mutans*; surface charge density

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1. Introduction

Since their introduction in the 1960s, crosslinking (meth)acrylic resins, such as bisphenol A glycerolate dimethacrylate (BisGMA), have been increasingly used in many dental applications, including restorative composites, protective sealants, and adhesive bonding agents [1]. Over the past several decades, considerable efforts, including the utilization of photopolymerization methods to yield crosslinked polymers, have been expended to improve the quality and durability of polymeric restorative materials [2]. These efforts, coupled with improvements in dental adhesive systems for bonding composites to tooth structure, have increased the clinical utility of polymeric restorative materials to the point that they are currently the material of choice for direct, esthetic restorations. For all applications, one major concern remains susceptibility to bacteria colonization. Biofilm growth is especially problematic when restorative composites interfacially debond from tooth structure to create a gap prone to the development of secondary (recurrent) caries. When a conventional bonding agent is used, as in the case of orthodontic brackets, biofilm growth at the tooth-adhesive junction can lead to rapid demineralization of the tooth [3].

One approach to prevent recurrent caries is to use dental materials, such as polymeric adhesives or composites, with antibacterial properties. Charged moieties, such as quaternary ammonium compounds, have been shown to be effective in reducing bacterial growth in a wide range of applications including medical devices, water purification systems, and textiles [4]. This tactic of utilizing quaternary ammonium compounds has carried over to dental materials, where cationic, monomethacrylate monomers have been developed to impart antibacterial activity to polymeric dental materials [5,6]. For instance, 12-methacryloyloxydodecylpyridinium bromide (MDPB) [7] and methacryloxyethyl cetyl dimethyl ammonium chloride (DMAE-CB) [8] are two monomers that have demonstrated bactericidal activity in dental adhesives. MDPB was also bacteriostatic in dental resins [9]. Unlike the dimethacrylates typically used in dental resins and adhesives, these cationic monomers generally have only one methacrylate group. Incorporating high concentrations of monomethacrylate could significantly affect the overall polymer network structures and properties. In addition, some monomethacrylates with pendant quaternary ammonium moieties may present miscibility problems with hydrophobic dimethacrylates commonly used in dental composites.

Another approach to incorporate high concentrations of quaternary ammonium groups into dental composites is through the use of filler particles. Polymers that contain cationic monomers have been pulverized into a fine powder to be used as filler particles with bacteriostatic properties [10]. In addition, quaternary ammonium poly(ethylenimine) nanoparticles have also been incorporated into resin composites to impart antibacterial properties [11]. While this approach using filler particles has shown some promise in reducing biofilm growth, the effectiveness may be difficult to reproduce, as the activity depends on particle size and uniformity of dispersion. There is also the potential for particle loss due to leaching or wear processes experienced by the composites.

The classical Menshutkin [12] reaction (the addition reaction of tertiary amines with organo-halides) provides a facile approach to produce a wide variety of potentially antibacterial monomers, oligomers, and polymers that have potential applications in a range of dental and biomedical materials. For instance, step-growth polymerization based on the Menshutkin reaction resulted in multiple quaternary ammonium functional groups in the backbone of these ionic polymers (termed ionenes) and imparted antimicrobial properties to the linear polymers [13,14].

The objective of this study was to adapt the Menshutkin reaction for the synthesis of free radical, photo-crosslinking, dimethacrylate monomers containing quaternary ammonium functionalities. These novel, reactive monomers can be designed to have solubility parameters similar to common dental resins and therefore are expected to be miscible with such resins. The resultant copolymers are expected to have lower monomer leachability and degradability due to multiple vinyl groups that lead to tighter polymer networks with increased crosslink density. Further, the class of monomers can be designed to contain one or more quaternary ammonium functionalities for reduced bacterial growth. In this study, we demonstrate the synthesis of two dimethacrylates with quaternary ammonium salts. The monomers were characterized by proton nuclear magnetic resonance (^1H NMR) and Fourier transform infrared (FTIR) spectroscopy. The viscosity of these monomers when incorporated into common dental monomers was characterized to assess how they affect the processability. The degree of conversion (DC) of the photo-crosslinked materials was determined by near infrared (NIR) spectroscopy, and the surface charge density was determined using a fluorescein binding assay. A model dimethacrylate system containing various amounts of one quaternary ammonium monomer was used to conduct a preliminary evaluation of the polymers' biological response in terms of both bacterial colonization and mammalian cell viability.

2. Materials and methods¹

2.1. Materials

2-(*N,N*-dimethylamino)ethyl methacrylate (DMAEMA), 2-bromoethyl methacrylate (BEMA), 2,2'-bis(bromomethyl)-1,1'-biphenyl (BbmBP), camphorquinone, ethyl 4-*N,N*-dimethylaminobenzoate, and anhydrous ethanol (EtOH) were purchased from Sigma-Aldrich. BisGMA and triethylene glycol dimethacrylate (TEGDMA) were obtained from Esstech Inc. Culture reagents were purchased from Invitrogen Corp. All reagents were used as received.

2.2. Synthesis and characterization of ionic dimethacrylates (IDMAs)

The synthesis of bis(2-methacryloyloxyethyl)dimethylammonium bromide [IDMA-1] is shown in Figure 1A. DMAEMA (1.57 g, 10 mmol), BEMA (1.93 g, 10 mmol), and 3 g ethanol were added to a tared vial equipped with a magnetic stir bar. The vial was capped and stirred for 24 h at 60 °C. After removal of the solvent and residual reagents by evaporation with the aid of a moderate (7 KPa) vacuum, a clear, colorless, viscous product (IDMA-1) was isolated with a yield > 95 %. 2,2'-bis(methacryloyloxyethyl)dimethylammonium bromide-1,1'-benzyl (IDMA-2) was synthesized in virtually quantitative yield in a similar manner from DMAEMA and BbmBP (Fig. 1B).

FTIR spectra of the starting materials and the low viscosity products were collected between ThBr₁ plates in the 4000 cm^{-1} to 400 cm^{-1} region with a wavenumber expanded uncertainty of 0.5 cm^{-1} . The spectrum of solid BbmBP was obtained from the ground powder, about 10 μm in particle size, in a KBr pellet. Water bands from the KBr pellet and from the atmosphere were removed from all spectra by subtraction. All spectra for IDMA-1 (Figure 2A) were normalized to the same carbonyl absorbance at 1720 cm^{-1} . For IDMA-2, the total area of the three bands at 776 cm^{-1} , 771 cm^{-1} , and 756 cm^{-1} of BbmBP was set approximately equal ($\pm 5\%$) to the area of the 770 cm^{-1} band of reacted BbmBP in the product IDMA-2.

¹Certain commercial materials and equipment are identified in this article to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by NIST or that the material or equipment identified is necessarily the best available for the purpose.

High-resolution, 270 MHz ^1H NMR spectra were taken on a 6.35 T JEOL GX270 spectrometer manufactured by JEOL, Ltd. (Akishima, Japan). Deuterated chloroform was used as a solvent and the monomer concentrations varied between 2.5 % and 3.0 % by mass fraction. All spectra were run at room temperature, 15 Hz sample spinning, 45° tip angle for the observation pulse, and a 10 s recycle delay, for 64 scans. The standard relative uncertainty for molecular mass calculated via ^1H NMR arises from the choice of baseline and is estimated to be 8 %.

2.3. Resin formulation and viscosity

IDMA-1 was mixed with BisGMA:TEGDMA (50:50 mass fraction) for final IDMA-1 concentrations of 10 %, 20 %, and 30 % (by mass). Control resins contained no IDMA-1. Viscosity measurements were performed at room temperature on an ARES rheometer (TA Instruments) with a cone-and-plate geometry (25 mm diameter). Steady shear viscosity was determined from 0.1 s^{-1} to 10 s^{-1} . No changes in viscosity were detected as a function of shear rate. The relative standard uncertainty for the viscosity measurements is 5 %.

2.4. Photopolymerization and DC

Resins containing IDMA-1 were activated for blue light (470 nm) photopolymerization with camphorquinone (0.2 % by mass) and ethyl 4-N,N-dimethylaminobenzoate (0.8 % by mass). Polymer disks (diameter 4 mm, height 1 mm) were prepared by irradiating (Dentsply Triad 2000, 250 W, 120 V) the activated resin blends between two clean glass slides (1 min per side) [15]. DC was quantified using transmission NIR spectroscopy on a Nicolet Magna 550 FTIR spectrometer (Madison, WI) configured with a white light source, CaF_2 beam splitter, and InSb detector. Spectra of the activated resin and the photopolymerized polymer disks were acquired over 7000 cm^{-1} to 4000 cm^{-1} from 64 co-added scans at 6 cm^{-1} resolution. The methacrylate peak height (4743 cm^{-1}) was normalized against the aromatic peak height (4623 cm^{-1}), and the reduction in the normalized value due to polymerization was calculated as the DC [16].

2.5. Surface charge density of polymers

The density of quaternary ammonium groups present on the polymer surfaces was quantified using fluorescein dye [17]. Sample diameters ($\sim 7 \text{ mm}$ to 8 mm) were measured using calipers, and samples were placed in a 48-well plate. Sample heights were 1 mm. Fluorescein sodium salt (200 μL of 10 mg/mL in deionized (DI) water) was added, and samples were left for 10 min at room temperature in the dark. After removing the fluorescein solution and rinsing extensively with DI water, each sample was placed in a new well, and 200 μL of 0.1 % (by mass) cetyltrimethylammonium chloride (CTMAC) in DI water was added. Samples were shaken for 20 min at room temperature in the dark to desorb the bound dye. The CTMAC solution was supplemented with 10 % (by volume) 100 mM phosphate, pH 8.0 (0.94 mg/mL monosodium phosphate – monohydrate and 13.2 mg/mL disodium phosphate – anhydrous in DI water). Sample absorbance was read at 501 nm using a standard plate reader. The fluorescein concentration was calculated using Beers Law and an extinction coefficient of $77 \text{ mM}^{-1} \text{ cm}^{-1}$ [17]. Using a ratio of 1:1 for fluorescein molecules to accessible quaternary ammonium groups, the surface charge density was calculated as the total molecules of charge per exposed surface area (sum of top, bottom and side edge area, measured independently for each polymer disk due to slight variations in disk diameters).

2.6. Bacteria inoculation and imaging

Streptococcus mutans (*S. mutans*) Clarke UA159 from the American Type Culture Collection (ATCC) were cultured in brain heart infusion (BHI) broth with $0.5 \mu\text{g}/$

mL bacitracin. Polymers were sterilized with 70 % (volume fraction) ethanol for 20 min, soaked in phosphate buffered saline (PBS) overnight, and inoculated with *S. mutans* prepared at an optical density (OD₆₀₀) of 0.06 in PBS containing 100 mg/L MgCl₂ 6H₂O and 100 mg/L CaCl₂. After incubating at 37 °C, 5 % CO₂ (by volume) for 4 h, samples were washed 3X to remove nonadherent bacteria, fixed with 37 mg/mL formaldehyde, and stained for 1 h with 1 µmol/L SYTOX green [15]. Samples were not passed through the air-liquid interface during the rinsing and fixing steps. Samples were imaged using a Zeiss LSM 510 laser scanning confocal microscope with a 40× water immersion objective. Three samples were evaluated for each IDMA-1 concentration. Five image stacks were collected at random locations on each sample, and projection images were prepared using the manufacturer's software. Custom macros in Image-Pro Plus software (Media Cybernetics, Inc.) were used to quantify each image in terms of the total surface area covered by bacteria, the area of each object, and the object density. Object density and surface coverage were plotted as a percentage of the control (no IDMA-1).

2.7. Macrophage viability and enzymatic activity

Cytotoxicity experiments were performed using the murine RAW 264.7 macrophage-like cell line (passages 8–12, ATCC TIB-71). Polymers containing 0 % to 30 % IDMA-1 were prepared and sterilized with 70 % ethanol (by volume) and seeded with RAW 264.7 macrophages (18 000 cells/cm²). Negative controls consisted of cells seeded on tissue culture polystyrene (TCPS). After 24 h, samples were evaluated for viability by staining for 10 min with 2 µmol/L calcein acetoxymethyl ester (calcein AM, live cells), 2 µmol/L ethidium homodimer-1 (EthD-1, dead cells), and 10 µmol/L Hoechst 33342 (H33342, all nuclei) [16]. Cells were visualized on a Leica DMA upright epifluorescent microscope (Leica Microsystems AG). Images were captured using a digital camera (Hamamatsu Photonics K.K.) and analyzed with Image-Pro Plus. Four separate fields of view were imaged on each of three samples per composition, and data were combined from two separate experiments (total images per composition = 24). Cell density was calculated as the number of cells per image. Cell viability was determined as the number of live cells divided by the total number of cells per image.

Cellular activity was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, a colorimetric assay measuring the enzymatic reduction of MTT to formazan [18]. Prior to sample sterilization, the average diameter of each polymer disk was determined from three separate diameter measurements using digital calipers. Disks were then sterilized, placed in 24-well TCPS plates, and seeded with 90 000 cells/cm². Controls included wells with polymers only (no cells), wells with cells only (no polymers) and wells without cells or polymers (blanks). After 24 h, the growth medium was removed. The polymer disks were transferred to a new 24-well plate, and 300 µL MTT solution (0.5 mg/mL MTT in PBS) was added to each polymer disk and each TCPS well from which the disks were removed. The plates were incubated for 1 h at 37 °C, 5 % CO₂. The MTT solution was then removed and replaced with 300 µL DMSO. The plates were incubated at room temperature with gentle mixing for 20 min. After brief mixing via pipetting, 200 µL from each well was transferred to a 96-well plate and the optical density at 540 nm was measured. Results were normalized to the surface area exposed to cells (for disks: polymer disk area; for TCPS: well area minus the polymer disk area). Controls without cells and polymers were used as blanks and subtracted from all sample readings. Two separate experiments, each with three samples, were completed (total n = 6).

2.8. Statistical analysis

Rheology, DC, charge density, and bacterial object area data were analyzed using One-Way Analysis of Variance (ANOVA) and a post-hoc Bonferroni multiple comparison test

(significance level of 0.05), with charge density and bacterial object area data log-transformed prior to analysis to homogenize the variance and to approximate normality, respectively. The surface area covered by bacteria and the bacterial object density were analyzed using the non-parametric Kruskal-Wallis test at a 95 % confidence interval to compare populations, as the data were not found to conform to an established statistical distribution. For RAW 264.7 cells, viability, density, and MTT assay data were analyzed using One-Way ANOVA and the Tukey/Kramer multiple comparison test at 95 % confidence, with cell density data log-transformed to approximate normality prior to analysis.

3. Results

3.1. Synthesis and characterization of IDMA-s

The Menshutkin reaction was indicated to be successful, based on the characterization of IDMA-1 and IDMA-2 using FTIR and ^1H -NMR. For IDMA-1, FTIR showed the disappearance of the C-Br absorption bands from BEMA (575 cm^{-1} , 512 cm^{-1}) as well as the $\text{N}(\text{CH}_3)_2$ bands (2822 cm^{-1} , 2771 cm^{-1}) from DMAEMA (Fig. 2A). For IDMA-2, the disappearance of these bands was also observed (Fig. 2B). The presence of two C-Br bands each for BEMA and BbmBP arose from isomeric conformations of each compound [19]. Two C-H stretching bands found in DMAEMA (2822 cm^{-1} and 2771 cm^{-1}) are caused by decreases in the C-H force constants effected by the CH trans-bonding to the nonbonding electron pair on the nitrogen atom [19,20]. IDMA-1 and IDMA-2 both display new bands in the 1100 cm^{-1} to 700 cm^{-1} region (1089 cm^{-1} , 1048 cm^{-1} , 886 cm^{-1} , and 858 cm^{-1} , and for IDMA-2 also at 981 cm^{-1} and 712 cm^{-1}). Most of these bands plausibly arise from vibrations of the NR_4^+ complexes, as spectra of compounds containing NR_4^+ groups are known to have bands in the 1100 cm^{-1} to 450 cm^{-1} region [21]. Additional broad bands at 3412 cm^{-1} and 550 cm^{-1} are from hydrogen-bonded water. The ^1H -NMR confirmed the assigned structures of IDMA-1 and IDMA-2. The peak assignments δ (ppm) for the ^1H NMR of IDMA-1 are as follows: 3.46 (6H, s, CH_3N), 5.52, 6.03 (4H, s, $\text{CH}_2\text{CCH}_3\text{C}$), 4.6 (2H, s, $\text{CH}_2\text{CH}_2\text{N}$), 4.13 (2H, s, $\text{CH}_3\text{CH}_2\text{O}$).

3.2. Resin viscosity

The viscosities of the monomer solutions containing various amounts of IDMA-1 were determined using a rheometer (Fig. 3A). The addition of IDMA-1 into BisGMA:TEGDMA (50:50) slightly increased the viscosity of the mixture from $(0.25 \pm 0.01)\text{ Pa}\cdot\text{s}$ for 0 % IDMA-1 to $(0.63 \pm 0.01)\text{ Pa}\cdot\text{s}$ for 30 % IDMA-1. The increase in viscosity was linear ($R^2 = 0.9988$) with respect to the amount of IDMA-1 incorporated over the range examined.

3.3. Polymer characterization

DC, quantified using NIR spectroscopy, increased slightly upon incorporation of IDMA-1 into the polymers, with statistically significant increases with 20 % and 30 % IDMA-1 (Fig. 3B). The increase in DC was nearly linear with respect to the IDMA-1 concentration ($R^2 = 0.9857$). Fluorescein binding to the cationic quaternary groups revealed statistically significant increases in the levels of quaternary ammonium sites present on the surfaces of polymers containing 20 % and 30 % IDMA-1 (Table 1). Samples with 30 % IDMA-1 had approximately 2 orders of magnitude more quaternary ammonium sites present on the surfaces as compared to all other concentrations. Samples with 0 % IDMA-1 had slight nonspecific interaction with the fluorescein salt.

3.4. Bacterial attachment

Incorporation of IDMA-1 reduced bacteria colonization for all tested concentrations. As Fig. 4A indicates, the surface coverage of the adherent bacteria depended upon the IDMA-1 concentration, showing reduced coverage for all samples containing IDMA-1. Also, bacterial morphology on the surfaces was altered on polymers containing 30 % IDMA-1. Image analysis of the objects (single fluorescent entities) revealed no significant differences in object area due to IDMA-1 (Fig. 4B) but confirmed that both object density (Fig. 4C) and surface coverage (Fig. 4D) were significantly reduced in the presence of IDMA-1 as compared to 0 % IDMA-1 controls. There were no significant differences among the IDMA-1 concentrations for any of the parameters quantifying bacterial adhesion.

3.5. Macrophage viability and enzymatic activity

Viability staining was used to assess macrophage density and viability at 24 h (Fig. 5A). The incorporation of 10 % IDMA-1 significantly reduced cell density at 24 h, and 20 % and 30 % IDMA-1 further reduced the density as compared to both 0 % and 10 % IDMA-1 (Fig. 5B). Interestingly, cell viability was unchanged at 10 % IDMA-1 but significantly reduced by 20 % and 30 % IDMA-1 (Fig. 5C). Thus, although the cell density was much lower with 10 % IDMA-1 as compared to 0 % IDMA-1, the cell viability was not affected.

Macrophages on polymer disks (disk cells) as well as cells in the same wells as the polymer disks but adherent to the TCPS substrate (TCPS cells) were evaluated separately. The results for the disk cell MTT activity agreed well with the viability results, in that 10 % IDMA-1 did not have a significant effect on MTT activity, whereas both 20 % and 30 % IDMA-1 resulted in statistically significant reductions in activity (Fig. 6A). The results for the TCPS cells showed no significant changes in MTT activity as a result of the inclusion of IDMA-1 (Fig. 6B).

4. Discussion

The classical Menshutkin reaction was successfully adapted for the synthesis of resin-compatible, crosslinking methacrylic monomers with quaternary ammonium functionality as a way to impart antimicrobial properties (reduced bacterial colonization and growth) to dental resins. It is expected that these monomers would exert similar effects in other resin-based dental materials, including sealants, adhesives, and composites. The successful synthesis of two low viscosity ionic monomers using the Menshutkin reaction demonstrates the versatility of this reaction scheme for generating charged dimethacrylate compounds. The monomer IDMA-1 contains a single quaternary ammonium functionality and has a lower viscosity than monomer IDMA-2, which has two quaternary ammonium sites and a biphenylene structure that potentially provides improved mechanical properties. These new, low viscosity monomers are highly compatible with BisGMA and TEGDMA and are also expected to be miscible with other dimethacrylate resins. Moreover, the combination of the quaternary ammonium functionality with two methacrylate end groups provides surface antimicrobial activity while maintaining desirable material properties, including high DC, good processability, and low leachable levels.

Additional critical aspects, including mechanical properties of composites containing IDMA-1 or IDMA-2, are not expected to change substantially upon IDMA incorporation, primarily due to the similar chemical nature of these monomers to common dental monomers (all dimethacrylates). IDMA-1 was selected as a representative from this class of monomers and further evaluated upon incorporation into a common dental resin system of BisGMA:TEGDMA (1:1 by mass). As most dental adhesives and composites contain two or more dimethacrylate monomers, IDMA-1 was incorporated into the BisGMA:TEGDMA

mixture at a maximum of 30 % (by mass) to simulate the properties of a ternary resin system. As expected, IDMA-1 mixed into and copolymerized with BisGMA:TEGDMA without difficulty. BisGMA:TEGDMA without IDMA-1 was used for control samples.

An important consideration for monomer design and selection is processability. A lower monomer viscosity tends to lead to improved processability, increased range of filler loadings, and better handleability of the corresponding composite paste. The viscosity of IDMA-1 is slightly higher, but in the same range, as that of the 50:50 BisGMA:TEGDMA mixture. Therefore, incorporating IDMA-1 into the BisGMA:TEGDMA resin led to an increased viscosity, but the increase was less than three-fold, even with 30 % IDMA-1. These results indicate that the addition of IDMA-1 should not drastically alter the processability of the corresponding composite pastes. Furthermore, the linear correlation between IDMA-1 content and viscosity validates the miscibility of the resins.

DC also increased slightly as a function of IDMA-1 concentration up to 30 % IDMA-1. IDMA-1, a tetrafunctional monomer, is expected to be incorporated into the polymer network in a similar way to BisGMA and TEGDMA. It is well-known that DC is governed largely by the vitrification point and that resins containing a large amount of the more rigid BisGMA lead to lower DC [22]. Addition of lower viscosity diluent monomers can delay the vitrification point and thereby increase the DC. Specifically, for BisGMA:TEGDMA based resins of varying mass ratios, DC increases with increasing amounts of the diluent monomer TEGDMA [16]. The slight increase in DC with IDMA-1 incorporation is likely due to an overall reduction in the amount of BisGMA as the IDMA-1 concentration increases. These results indicate that the addition of IDMA-1 does not adversely affect the DC of BisGMA:TEGDMA resins and that the majority of the IDMA-1 is incorporated into the polymer network.

The fluorescein assay confirmed the presentation of quaternary ammonium functionalities on the polymer surface as it measured an increase in surface charge density with increasing IDMA-1 concentration (Table 1), particularly for materials containing higher IDMA-1 concentrations. Samples were sterilized prior to the fluorescein assay to ensure that the surface evaluated for charge density was the same as that exposed to the bacteria and macrophages. The increase in surface charge density at the 10 % IDMA-1 incorporation level may be too small to be detected with the fluorescein assay.

Bacterial surface coverage on polymers containing IDMA-1 was not dose-dependent under the conditions tested. Samples with only 10 % IDMA-1, which did not have a detectable level of cationic charges on the surface, were sufficient to reduce bacterial coverage as compared to 0 % IDMA-1. These data suggest that IDMA-1 is active even at concentrations below the detectable limit of the fluorescein assay. Increases in the IDMA-1 concentration did not further reduce the bacterial coverage, bacteria size (object area), or bacteria density. However, slight morphological changes in the bacteria were evident with 20 % IDMA-1 and even more so with 30 % IDMA-1 (Fig. 4A). The significant jump (almost 100-fold) in surface charge density from 20 % IDMA-1 to 30 % IDMA-1 may contribute to the bacterial clustering seen with 30 % IDMA-1 and is likely to reduce the viability of these bacteria. The bacteriostatic activity of these dimethacrylate systems containing IDMA-1 is similar to that seen with MDPB incorporated into similar BisGMA:TEGDMA based dimethacrylate systems [9]. While MDPB has been shown to be bacteriocidal as a homopolymer and as a copolymer with acrylamide, it is primarily bacteriostatic when copolymerized with hydrophobic dental resins [23]. Thus, IDMA-1 may also have increased bacterial effects when incorporated into other polymer systems.

Determining the response of eukaryotic cells is an important component in the thorough evaluation of a potentially antimicrobial material. Having confirmed the ability of IDMA-1 to reduce early bacterial colonization, cytotoxicity experiments were performed on cells directly contacting the polymers and cells exposed to the leachables without contacting the polymers. RAW 264.7 macrophage-like cells were chosen for this study as a model cell type involved in the first line of defense against foreign materials. In the direct contact cytotoxicity studies, polymers with 10 % IDMA-1 reduced cell density without affecting viability or total metabolic activity, indicating that while there are significantly fewer cells, their viability is high and their metabolic activity is very high on a per cell basis. IDMA-1 concentrations of 20 % or greater significantly reduced viability, metabolic activity, and density. Combining this concentration-dependent response seen in macrophages with the bacterial response to IDMA-1 indicates that 10 % IDMA-1 is sufficient to reduce bacterial adhesion while also minimizing direct toxicity to mammalian cells at the 24 h time point. IDMA-1 concentrations ≥ 20 % (by mass) reduced bacterial coverage, but they also showed significant direct cytotoxicity and therefore are not recommended for use in applications having direct contact with eukaryotic cells. In applications such as dental restorations which often have minimal contact with cells, an IDMA-1 concentration greater than 10 % may be acceptable.

It should be noted that for 10 % IDMA-1, the lower number of cells that are highly active may indicate a detrimental effect on the attached cells that would manifest at later timepoints. Thus, studies to evaluate other aspects of the cell response, such as cytokine production in macrophages, cell apoptosis, and gene expression analyses, could help to better determine additional, subtle effects of IDMA-1 on the eukaryotic cell response after longer direct exposure to the polymers.

The leachables did not affect the cells, demonstrating that the toxicity of high concentrations of IDMA-1 is due to direct contact between the macrophages and the polymer disks and not to the presence of cytotoxic material leaching from the disks. Macrophages cultured in the presence of the polymer disks (and therefore any leachables they released) but adherent to the tissue culture polystyrene showed no changes in MTT activity as a function of IDMA-1 concentration (Fig. 6B), indicating a lack of toxic leachables even for polymers containing 30 % IDMA-1. Leachables are a concern in dimethacrylate-based polymers and composites, especially when one of the monomers purposefully contains a quaternary ammonium group. However, statistically speaking, IDMA-1, being a dimethacrylate monomer, should result in less leachable material as compared to other monomethacrylate monomers that contain quaternary ammonium groups, due to the multiple reactive groups increasing the likelihood of monomer incorporation into the crosslinked polymer network. In addition, since the addition of IDMA-1 did not affect the DC values, the leachables are not expected to increase due to increasing concentrations of IDMA-1. The current study only considered the effects of leachables from the first 24 h, since the majority of leachables are known to release during that time frame [24]. Toxic levels of leachables at later time points are not expected, particularly in dynamic *in vivo* settings such as the oral environment.

The lack of cytotoxic leachables after 24 h incubation also suggests that the leachables are not likely to be responsible for the bacterial response during this time frame and that the bacteriostatic effect is instead due to direct surface contact. The bacterial and mammalian cell results tracked similarly with respect to the IDMA-1 concentration, and other studies with cationic monomers have found that the minimum inhibitory concentration (MIC) for *S. mutans* is similar to the cytotoxic concentration for mammalian cells. For instance, the MIC for DMAE-CB in *S. mutans* was reported as 2.4 $\mu\text{g/mL}$ [6] and is in the same range as the cytotoxic concentration via the MTT assay (2 $\mu\text{g/mL}$) [25]. Likewise, MDPB was also shown to have a similar MIC for *S. mutans* (62.5 $\mu\text{g/mL}$) and cytotoxic concentration (40

µg/mL) [26]. Since DMAE-CB, MDPB, and IDMA-1 likely work via similar mechanisms, one would expect that if the concentration of IDMA-1 present in the leachables does not alter the cells (Fig. 6B), then it should not alter the bacteria either. Additional studies are needed to verify that this is the case for IDMA-1.

While many studies, including the present one, have shown that surfaces containing quaternary ammonium functionalities reduce bacterial growth, the mechanism responsible for their antibacterial activity has yet to be fully understood. One proposed mechanism is the penetration of surface-available quaternary ammonium functionalities into the bacterial cell membrane, damaging the membrane and eventually leading to cell death [27]. Other studies have proposed a mechanism of electrostatic disruption of the cell membrane due to ion exchange, where divalent counterions that stabilize the membrane are displaced by the cationic charges present on the substrate [28]. For the latter mechanism, surface charge density is a critical component and has been shown to be independent of chain length [29]. These mechanisms may apply to different scenarios, depending on the surface presentation of the quaternary ammonium functional group. For IDMA-1, the quaternary ammonium functionality is incorporated into the polymer and remains active and accessible on the surface. Further, due to chemical cross-linking, neither the polymer chains nor the quaternary ammonium functionality is expected to be able to extend sufficiently beyond the material surface to penetrate fully through the bacterial cell membranes. Thus, an electrostatic-based mechanism may explain the biological response to IDMA-1-containing polymers. The same mechanism of electrostatic interactions proposed to cause toxicity in prokaryotic cells may also be responsible for toxicity seen in eukaryotic cells.

5. Conclusions

In summary, the Menshutkin reaction can provide a facile synthetic route to flowable dimethacrylates with varying chemistries including one or more quaternary ammonium functionalities. The two dimethacrylates produced in this study were miscible with common dental resins, and the incorporation of IDMA-1 only slightly increased the viscosity of the BisGMA-TEGDMA 50:50 resin system. DC and surface charge density both increased with the addition of IDMA-1, indicating that the charged monomer was well incorporated into the polymer network. Polymers containing as little as 10 % IDMA-1 reduced bacterial growth on their surfaces, and ≥ 20 % IDMA-1 resulted in significant cytotoxicity to macrophages cultured directly on the polymers. Thus, the IDMA-1 concentration selected for some applications may need to balance the antibacterial effects with the cytotoxicity to mammalian cells. This example using IDMA-1, oral bacteria, and macrophages demonstrates the potential of these new classes of bioactive monomers synthesized via the Menshutkin reaction to aid in the development of improved biomaterials with reduced bacterial adhesion and perhaps in the identification of mechanisms responsible for antimicrobial activity.

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Figure 1.
Reaction scheme for the synthesis of low viscosity ionic dimethacrylates containing quaternary ammonium functionalities: (A) IDMA-1, (B) IDMA-2.

Figure 2.

FTIR spectra of reactants and products for low viscosity ionic dimethacrylate monomers (A) IDMA-1 and (B) IDMA-2.

Figure 3. Effect of IDMA-1 incorporation on (A) resin viscosity and (B) DC. Each error bar represents one standard deviation. The data points represent the average values, and the lines are the best linear fit of the data.

Figure 4.

S. mutans evaluation at 4 h. (A) Images of *S. mutans* cultured on polymer disks containing increasing amounts of IDMA-1. Scale bars = 10 μm. (B) Individual object area as a function of IDMA-1 concentration. (C) Object density normalized to density on 0 % IDMA-1 disks (control). (D) Fraction of surface covered normalized to 0 % IDMA-1 disks. * *P*-value < 0.05 as compared to 0 % IDMA-1. Each error bar represents one standard deviation and serves as the estimate of standard uncertainty.

Figure 5.

RAW 264.7 viability at 24 h. (A) Images of viability staining. (B) Cell density as a function of IDMA-1 concentration. (C) Viable cell fraction as a function of IDMA-1. * P -value < 0.05 compared to 0 % IDMA-1. ** P -value < 0.05 compared to both 0 % and 10 % IDMA-1. Each error bar represents one standard deviation and serves as the estimate of standard uncertainty.

Figure 6.

MTT assay results for RAW 264.7 macrophages at 24 h. (A) MTT activity for cells cultured directly on the polymer disks (disk cells). ** P -value < 0.05 compared to 0 % IDMA-1 and 10 % IDMA-1. (B) MTT activity for cells cultured in the same well as the polymer disks but growing on the TCPS well plate (TCPS cells). No significant differences were present for TCPS cells (p -value > 0.05). Each error bar represents one standard deviation and serves as the estimate of standard uncertainty.

Table 1

Surface charge density of polymer disks containing IDMA-1.

IDMA-1 (mass %)	Charge Density (N ⁺ /cm ²)
0	$(1.40 \pm 0.87) \times 10^{13}$
10	$(2.04 \pm 0.92) \times 10^{13}$
20	$(6.73 \pm 4.87) \times 10^{13}$ *
30	$(5.54 \pm 1.85) \times 10^{15}$ **

* P-value < 0.005 as compared to 0 % IDMA-1

** P-value < 0.005 as compared to 0 %, 10 %, and 20 % IDMA-1