Synthesis, antibacterial activity, and biocompatibility of new antibacterial dental monomers

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ABSTRACT: Purpose: To synthesize a small library of antibacterial dental monomers based on quaternary ammonium salts and to test their antibacterial activity against cariogenic bacteria. **Methods:** Five new antibacterial monomers were synthesized and characterized by NMR, IR and HRMS. **Results:** Cytotoxicity assays using human gingival fibroblast cells showed that these new antibacterial monomers were biocompatible at concentrations of 10^{-5} M and displayed less cytotoxicity than BisGMA, a common dental monomer. When analyzed in vitro, all new monomers demonstrated strong inhibitory activity against biofilm formation by cariogenic *Streptococcus mutans* and *Lactobacillus casei*. Results indicated that antibacterial monomers containing a long alkyl (i.e. hexadecyl) chain are superior to their shorter-chain counterparts. The cross-linking monomers based on glycerol dimethacrylate also consistently outperformed their monomethacrylate analogs. Finally, the ammonium salts containing the dimethylbenzyl moiety were superior to the similar structures containing 1,4-diazabicyclo[2.2.2]octane (DABCO) in some cases. (*Am J Dent* 2018;31(Sp Is B):17B-23B).

CLINICAL SIGNIFICANCE: All five new monomers were deemed biocompatible at concentrations of 10^{-5} M or less, and most had better biocompatibility than BisGMA. Dimethacrylate monomers 5 and 6 generally demonstrated high antibacterial activities, with the highest activity shown for the most lipophilic monomer 6, and these new antibacterial monomers have potential future application in dental composites and bonding agents.

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Introduction

Resin-based dental composites consisting of BisGMA and other methacrylate dental monomers have been widely used in dentistry to restore decayed teeth. Composite restorations have limited service life (typically 5-7 years). The occurrence of secondary (recurrent) caries caused by bacterial biofilms accumulated at the restoration margin is the leading cause of failure and replacement of dental restorations. To inhibit bacterial biofilms and reduce recurrent caries, new composites and bonding agents that exhibit antibacterial activity have been developed.[1-5] Antibacterial restorative dental materials generally fall into two categories: those with releasable agents and those with non-releasable antibacterial monomers. Common releasable antibacterial agents in dental materials include silver[6] and chlorhexidine.[2] Materials with releasable agents often show very high antibacterial activity over a short time span (< 1 week) followed by little to no activity as the material leaches out. The release of compounds such as chlorhexidine can also result in a significant reduction of mechanical properties over time, likely due to the formation of a porous structure and increased water sorption.[7] As a result, the probability of restoration failure due to fracture is increased.

Dental materials containing non-releasable antibacterial monomers have been under investigation.[3,4,9] Many of these monomers contain a methacrylate group and a long-chain alkyl ammonium or pyridinium salt. These monomers show bactericidal activity in the uncured state and a bacteriostatic and/or bactericidal (contact-kill) effect in the cured state against oral pathogens including *Streptococcus mutans*.[4,10] Since the antibacterial functional group is immobilized (polymerized) in the material, such materials usually have long-term antibacterial effect without significant adverse effect on the physical and mechanical properties. For example, the monomer methacryloyloxydodecylpyridinium bromide (MDPB) shows bactericidal activity against *S. mutans* in the uncured state, and the composites containing MDPB at concentrations of up to 2.83 wt% show antibacterial activity with no adverse effects on mechanical properties. Increasing the concentration of MDPB in the composite beyond this wt% results in a deterioration of mechanical properties.[3,11] Thus, striking a balance between (maximizing) antibacterial capability of the monomers and (minimizing) detrimental effects on the mechanical properties of the material is of great importance.

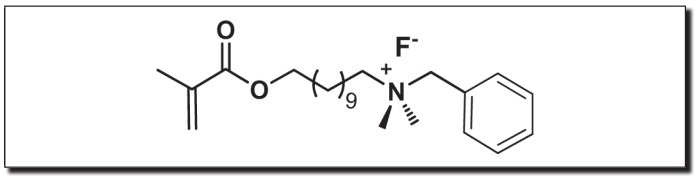

Fig. 1. Structure of antibacterial monomer methacryloyloxyundecyldimethylbenzylammonium fluoride 1.[12]

The synthesis of a fluoride-releasing antibacterial monomer, methacryloyloxyundecyldimethylbenzylammonium fluoride 1 (Fig. 1), which exhibits antibacterial activity against *S. mutans* was previously reported.[12] This new monomer, which incorporates the dimethylbenzylammonium moiety, exhibited overall better bactericidal activity against *S. mutans* biofilm than did the corresponding pyridinium salt and a dodecyltrimethylammonium methacrylamide monomer. This new monomer also can serve as a fluoride source and counter ion for antibacterial fluoride-releasing dental monomers.[13] Additionally, composites containing this monomer maintained good mechanical properties with antibacterial monomer concentrations of up to 3 wt%. Unfortunately, at high concentration (6 wt%), mechanical properties of the composite were significantly decreased over time.

To improve the overall performance of antibacterial composites, we sought to improve both the efficacy of this antibacterial monomer and the mechanical properties of composites containing a higher amount of this monomer. Observed differences in the activity of antibacterial dental monomers based on the structure of the ammonium group led us to examine a broader structure-activity relationship for this class of compounds, covering varied alkyl chain lengths, ammonium salts based on 1,4-diazabicyclo[2.2.2]octane (DABCO) and cross-linking antibacterial monomers. The alkyl chain length of ammonium salts has a significant impact on bactericidal activity, with longer chains (up to 18 C atoms) conferring the best effects.[14] Moreover, alkyl ammonium salts derived from DABCO have been synthesized previously and have demonstrated antibacterial effects.[15,16] However, to the best of our knowledge, DABCO based ammonium salts have not been incorporated into dental monomers. Furthermore, cross-linking antibacterial dental monomers are rare in comparison with their monounsaturated counterparts (monomethacrylates).[17,18] This is of particular importance because monounsaturated antibacterial monomers can increase water sorption and decrease mechanical properties of composite. As a result, the useful concentration of such antibacterial monomers in dental composites is very limited (ca. 3%).[11,12] Therefore, new cross-linking antibacterial monomers would be desirable for dental composites because they would allow a higher content of the antibacterial component while maintaining physical and mechanical properties of the material.

The cytotoxicity and the bactericidal activity of the antibacterial monomers changes after polymerization. However, determination of antibacterial activity in monomer form is important because removal of carious material from the tooth structure can be incomplete, leaving behind cariogenic bacteria such as *S. mutans*.[19] During the restoration process before polymerization, uncured monomer can potentially kill bacteria that are still present in the existing tooth structure, thus decreasing the likelihood of restoration failure due to secondary caries formation.[20]

Building upon our previous results, we report here the synthesis of five new (three cross-linking dimethacrylate) antibacterial monomers and the comparison of the structure-activity relationships of these and the previously reported monomer 1 in terms of cytotoxicity and antibacterial activity against four bacteria species: *S. mutans*, *L. casei*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Among these four species, *S. mutans* and *L. casei* are known for their role in caries formation, and *S. aureus* and *P. aeruginosa* are opportunistic pathogens involved in various systemic infections, especially in aging and immunocompromised patients.[3,21] The antibacterial activities of the synthesized monomers against the latter two bacteria will explore their potential applications in other biomedical materials such as implants, feeding tubes and catheters.

Materials and Methods

Monomer synthesis - All solvents were dried over 3Å molecular sieves and reactions were run under N_2 atmosphere. All synthesized intermediates and products were purified by column chromatography. ^{1}H- and ^{13}C-NMR spectra were recorded at room temperature with a Varian Unity Plus 400 MHz[a] instrument. High resolution mass spectra were obtained with a Waters Synapt HD[b] mass spectrometer with a nano-electrospray source. FI-IR spectra were recorded with a Thermo-Nicolet 670 FT-IR[c] spectrometer (resolution: 4 cm^{-1}, number of scans: 128).

2-(1,3-dimethacryloyloxy)propyl 10-bromodecanoate (2). To a 50 mL round bottom flask containing 1,3-glycerol-dimethacryate (1.9302 g, 8.4569 mmol), 10-bromodecanoic acid (0.5339 g, 2.216 mmol) and 4-dimethylaminopyridine (DMAP) (0.0250 g, 0.205 mmol) under N_2 atmosphere, 5 mL dichloromethane was added followed by dicyclohexyl-carbodiimide (DCC) (0.4839 g, 2.345 mmol). A white precipitate formed immediately. After 3-hour stirring, the slurry was filtered over a coarse (60 M) frit and the filtrate collected. The solvent was removed under reduced pressure. Purification by chromatography (2 × 16 cm silica) and elution with acetone/hexanes 1:19-1:9 v/v, R_f ~ 0.45 (1:9) yielded the product as a yellow oil (0.7490 g, 1.623 mmol, yield 76%).

^{1}H NMR ($CDCl_3$, δ)[24] 6.11 (br, 2H, 2C*H*H'), 5.61-5.59 (m, 2H, 2CH*H*'), 5.44-5.34 (m, 1H, $(CH_2)_2$C*H*OR), 4.44-4.22 (m, 4H, $(CH_2)_2$CHOR), 3.40 (t, $^3J_{HH}$ = 6.8 Hz, 2H, CH_2Br), 2.32 (pseudo td, $^3J_{HH}$ = 7.5 Hz, $^3J_{HH}$ = 2.7 Hz, 2H, CH_2CO$_2$R), 1.94 (s, 6H, 2CH_3), 1.85 (pent, $^3J_{HH}$ = 7.5 Hz, 2H, CH_2CH$_2$Br), 1.64-1.57 (m, 2H, CH_2CH$_2$CH$_2$Br), 1.46-1.37 (m, 2H, CH_2CH$_2$CH$_2$CH$_2$Br), 1.29 (br, 8H, 4CH_2); 13C{^{1}H} 173.4, 173.0, 166.9, 166.5, 136.0, 135.91, 135.90, 126.6, 126.53, 126.51, 69.5, 69.0, 62.8, 62.6, 62.2, 34.3, 34.2, 34.1, 32.9, 29.4, 29.3, 29.2, 29.1, 28.8, 25.03, 24.99, 18.42, 18.40.

IR (cm^{-1}) 2928(m), 2855(w), 1720(s, C=O), 1638(w, C=C), 1453(m), 1292(m), 1144(s), 941(m).
HRMS calculated for $C_{21}H_{32}O_6BrNa^+$: 483.1353; found: 483.1369.

2-(1,3-dimethacryloyloxy)propyl 16-bromohexadecanoate (3). To a 50 mL round bottom flask containing 1,3-glycerol-dimethacryate (4.0808 g, 17.879 mmol), 16-bromohexadecanoic acid (3.0068 g, 8.9670 mmol) and DMAP (0.0560 g, 0.458 mmol) under N_2 atmosphere, 20 mL dichloro-methane was added and the solution cooled to 0°C. DCC (2.0251 g, 9.8149 mmol) was added dropwise as a solution in dichloromethane (4 mL) and a white precipitate formed. After 5-hour stirring, the slurry was filtered over a coarse (60 M) frit and the filtrate collected. The solvent was removed under reduced pressure. Purification by chromatography (4 × 15 cm silica) and elution with acetone/hexanes 1:19 v/v, R_f ~ 0.5 (1:9) yielded the product as an oily white solid (4.1082 g, 7.5304 mmol, yield 84%).

^{1}H NMR ($CDCl_3$, δ)[23] 6.12 (br, 2H, 2C*H*H'), 5.61-5.59 (m, 2H, 2CH*H*'), 5.42-5.35 (m, 1H, (CH_2)2C*H*OR), 4.42-4.22 (m, 4H, $(CH_2)_2$CHOR), 3.41 (t, $^3J_{HH}$ = 6.9 Hz, 2H, CH_2Br), 2.32 (pseudo td, $^3J^{HH}$ = 7.6 Hz, $^3J_{HH}$ = 2.8 Hz, 2H, CH_2CO$_2$R), 1.94 (br, 6H, 2CH$_3$), 1.85 (pent, $^3J_{HH}$ = 7.6 Hz, 2H, CH_2CH$_2$Br), 1.64-1.57 (m, 2H, CH_2CH$_2$CH$_2$Br), 1.45-1.38 (m, 2H, CH_2CH2CH$_2$CH$_2$Br), 1.33-1.23 (m, 20H, 10CH2); 13C{1H} 173.5, 173.0, 166.9, 166.5, 136.0, 135.91, 135.89, 126.6, 126.5, 126.4, 69.6, 69.0, 62.8, 62.6, 62.2, 34.4, 34.2, 34.1, 33.0, 29.79, 29.77, 29.76, 29.7, 29.61, 29.60, 29.4, 29.25, 29.21, 28.9, 28.3, 25.1, 25.0, 18.40, 18.38.

IR (cm^{-1}) 2922(s), 2852(m), 1722(s, C=O), 1655(m, C=C), 1453(m), 1293(m), 1148(s), 941(m).
HRMS calculated for $C_{27}H_{45}O_6Br$: 567.2292; found: 567.2291.

2-(1,3-dimethacryloyloxy)propyl 10-(1-(1-azonia-4-azabicylco-[2.2.2]octyl))decanoate bromide (4). To a 50 mL round bottom flask containing 2 (1.2948 g, 2.8063 mmol) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (0.3169 g, 2.8249 mmol) under N_2 atmosphere, 3 mL dichloromethane was added and the solids dissolved. After 18.5 hours, the solvent was removed under vacuum. Purification by chromatography (2 × 15 cm silica) and elution with dichloromethane/methanol 1:9 v/v, R_f ~ 0.1 yielded the product as a clear oil (0.5961 g, 1.039 mmol, yield 37%).

^{1}H NMR ($CDCl_3$, δ)23 6.10 (br, 2H, 2C*H*H'), 5.61-5.59 (m, 2H, 2CH*H'*), 5.39-5.32 (m, 1H, $(CH_2)_2CHOR$), 4.40-4.20 (m, 4H, $(CH_2)_2CHOR$), 3.65 (t, $^3J_{HH}$ = 7.3 Hz, 6H, $3N^+CH_2CH_2N$), 3.54-3.49 (m, 2H, N^+CH_2), 3.26 (t, $^3J_{HH}$ = 7.3 Hz, 6H, $3N^+CH_2CH_2N$), 2.31 (pseudo td, $^3J_{HH}$ = 7.5 Hz, $_3J_{HH}$ = 2.8 Hz, 2H, CH_2CO_2R), 1.92 (s, 6H, $2CH_3$), 1.75 (br, 2H, CH_2), 1.62-1.54 (m, 2H, CH_2), 1.36-1.32 (m, 4H, CH_2), 1.27 (br, 6H, $3CH_2$); $^{13}C\{^1H\}$ 173.5, 173.1, 167.0, 166.6, 135.85, 135.83, 135.81, 126.8, 126.7, 126.6, 69.5, 68.9, 64.8, 62.7, 62.7, 62.2, 52.7, 45.5, 34.3, 34.1, 29.3, 29.2, 29.1, 29.0, 26.5, 25.0, 24.9, 22.3, 18.4, 18.42.

IR (cm^{-1}) 3411(m, br, H_2O), 2927(m), 2856(w), 1719(s, C=O), 1637(w, C=C), 1455(m), 1293(m), 1149(s), 943(m).
HRMS calculated for $C_{27}H_{45}O_6N_2^+$: 493.3272; found: 493.3283

2-(1,3-dimethacryloyloxy)propyl 16-(1-(1-azonia-4-azabicylco-[2.2.2]octyl))hexadecanoate bromide (5). To a 50 mL round bottom flask containing **3** (1.0061 g, 1.8442 mmol) and DABCO (0.3169 g, 2.8249 mmol) under N_2 atmosphere, 3 mL ethyl acetate was added and the solids dissolved. After 6 days, the solvent was removed under vacuum. Purification by chromatography (2 × 15 cm silica) and elution with dichloro-methane/methanol 1:9 v/v, Rf ~ 0.1 yielded the product as a clear oil (0.8000 g, 1.216 mmol, yield 66%).

^{1}H NMR ($CDCl_3$, δ)[23] 6.06 (br, 2H, 2C*H*H'), 5.55 (br, 2H, 2C*H*H'), 5.36-5.28 (m, 1H, $(CH_2)_2CHOR$), 4.36-4.15 (m, 4H, $(CH_2)_2CHOR$), 3.62 (t, $^3J_{HH}$ = 7.1 Hz, 6H, $3N^+CH_2CH_2N$), 3.46-3.38 (m, 2H, N^+CH_2), 3.24 (t, $^3J_{HH}$ = 7.1 Hz, 6H, $3N^+CH_2CH_2N$), 2.30-2.23 (m, 2H, CH_2CO_2R), 1.88 (s, 6H, $2CH_3$), 1.71 (br, 2H, CH_2), 1.59-1.50 (m, 2H, CH_2), 1.33-1.26 (m, 4H, $2CH_2$), 1.19 (br, 16H, $8CH_2$); $^{13}C\{^1H\}$ 173.5, 173.1, 167.0, 166.5, 135.89, 135.86, 135.8, 126.7, 126.6, 126.5, 69.5, 68.9, 64.8, 62.8, 62.6, 62.2, 53.7, 52.7, 45.6, 34.4, 34.2, 29.82, 29.79, 29.7, 29.6, 29.4, 29.3, 29.2, 26.6, 25.1, 25.0, 22.4, 18.43, 18.42.

IR (cm-1) 3402(m, br, H_2O), 2922(m), 2852(m), 1721(s, C=O), 1637(w, C=C), 1456(w), 1293(m), 1152(s), 941(m).
HRMS calculated for $C_{33}H_{57}O_6N_2^+$: 577.4211; found: 577.4190.

2-(1,3-dimethacryloyloxy)propyl 16-N,N-dimethylbenzyl-ammoniumhexadecanoate bromide (6). To a 50 mL round bottom flask containing 3 (1.0288 g, 1.8858 mmol) and dimethylbenzylamine (0.285 mL, 0.256 g, 1.90 mmol) under N_2 atmosphere, 2 mL acetonitrile was added and the mixture heated to 50°C. After 48 hours, the reaction was allowed to cool to room temperature and the solvent was removed under vacuum. Purification by chromatography (2 × 15 cm silica) and elution with dichloro-methane/methanol gradient, 3%-10% v/v, Rf ~ 0.5 yielded the product as a clear oil (1.0920 g, 1.6041 mmol, 85%).

^{1}H NMR ($CDCl_3$, δ)[23] 7.64 (t, $^3J_{HH}$ = 7.9 Hz, 2H, Ph), 7.52-7.40 (m, 3H, Ph), 6.10 (br, 2H, 2C*H*H'), 5.59 (br, 2H, 2CH*H'*), 5.41-5.33 (m, 1H, $(CH_2)_2CHOR$), 5.03 (s, 2H, CH_2Ph), 4.41-4.20 (m, 4H, $(CH_2)_2CHOR$), 3.54-3.49 (m, 2H, CH_2N^+), 3.28 (s, 6H, $N^+(CH_3)_2$), 2.31 (pseudo td, $^3J_{HH}$ = 7.5 Hz, $^3J_{HH}$ = 2.8 Hz, 2H, CH_2CO_2R), 1.79 (br, 2H, CH_2), 1.67 (s, 6H, $2CH_3$), 1.63-1.54 (m, 2H, CH_2), 1.36-1.29 (m, 4H, $2CH_2$), 1.23 (br, 16H, $8CH_2$); $^{13}C\{^1H\}$ 173.5, 173.1, 166.9, 166.5, 135.83, 135.80, 135.77, 133.4, 130.8, 129.3, 127.6, 126.7, 126.6, 126.5, 69.5, 68.9, 67.5, 63.9, 62.7, 62.6, 62.1, 49.8, 34.3, 34.2, 29.72, 29.70, 26.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.4, 25.0, 24.9, 23.0, 18.4, 18.3.

IR (cm^{-1}) 3404(w, br, H_2O), 2923(m), 2852(m), 1720(s, C=O), 1637(w, C=C), 1455(m), 1293(m), 1151(s), 940(m).
HRMS calculated for $C_{36}H_{58}O_6N$+: 600.4259; found: 600.4247.

16-bromohexadecanol (7b). A 100 mL round bottom flask equipped with magnetic stirring bar was charged with 16-bromohexadecanoic acid (1.68 g, 5 mmol) in THF (20 mL) and BH3/THF was added dropwise at 0°C. The reaction mixture was allowed to slowly warm to room temperature and was stirred overnight. 30 mL water was added then the product was extracted using ether (3 × 25 mL). The organic layer was washed by water and brine, dried over anhydrous Na_2SO_4, filtered and the solvent was removed under vacuum to give 7b as a white solid (1.472 g, 4.6 mmol, 92%). A similar reaction starting with 11-bromoundecanoic acid yielded 11-bromoundecanol (7a, 97%)

^{1}H NMR ($CDCl_3$, δ) 3.62 (t, 2H, CH_2OH), 3.39 (t, 2H, CH_2Br), 1.86-1.82 (m, 2H, CH_2CH_2OH), 1.55-1.41 (m, 2H, CH_2CH_2Br), 1.30-1.25 (m, 24H, $12CH_2$); $^{13}C\{^1H\}$ 63.3, 34.3, 33.1, 30.1, 29.9, 29.8, 29.7, 29.0, 28.4, 26.0.

IR (cm^{-1}) 3277(m, OH), 2916(s), 2848(s), 1473(m), 1462(m), 1122(w), 731(m).

16-(1-(1-azonia-4-azabicylco[2.2.2]octyl))-1-hexadecanol bromide (9). A 100 mL round bottom flask equipped with magnetic stirring bar was charged with 1,4-diazabicyclo[2.2.2]-octane (DABCO, 4 mmol), 16-bromohexadecanol (7 b, 1.28 g, 4 mmol) and EtOAc (30 mL). A white solid precipitated and was collected by filtration, washed with cold EtOAc and dried under vacuum to give 9 as a white solid (1.32 g, 3.06 mmol, 77%). A similar reaction of DABCO with 7a yielded 8 (83%).

^{1}H NMR ($CDCl_3$, δ) 3.55-3.52 (t, 2H, CH_2OH), 3.40-3.36 (t, 6H, $3CH_2N^+$), 3.27-3.17 (m, 8H, $3CH_2N$, CH_2N^+), 1.72-1.48 (m, 4H, $2CH_2$), 1.39-1.24 (m, 24H, $12CH_2$); $^{13}C\{^1H\}$ 61.8, 52.33, 52.27, 52.2, 44.9, 32.5, 29.6, 29.5, 29.44, 29.39, 29.2, 29.0, 25.8, 21.6.

IR (cm^{-1}) 3282(m, OH), 2916(s), 2847(s), 1470(m, C=C), 1462(m), 1056(s), 720(m).
HRMS calculated for $C_{22}H_{45}ON_2$: 353.3526; found: 353.3562.

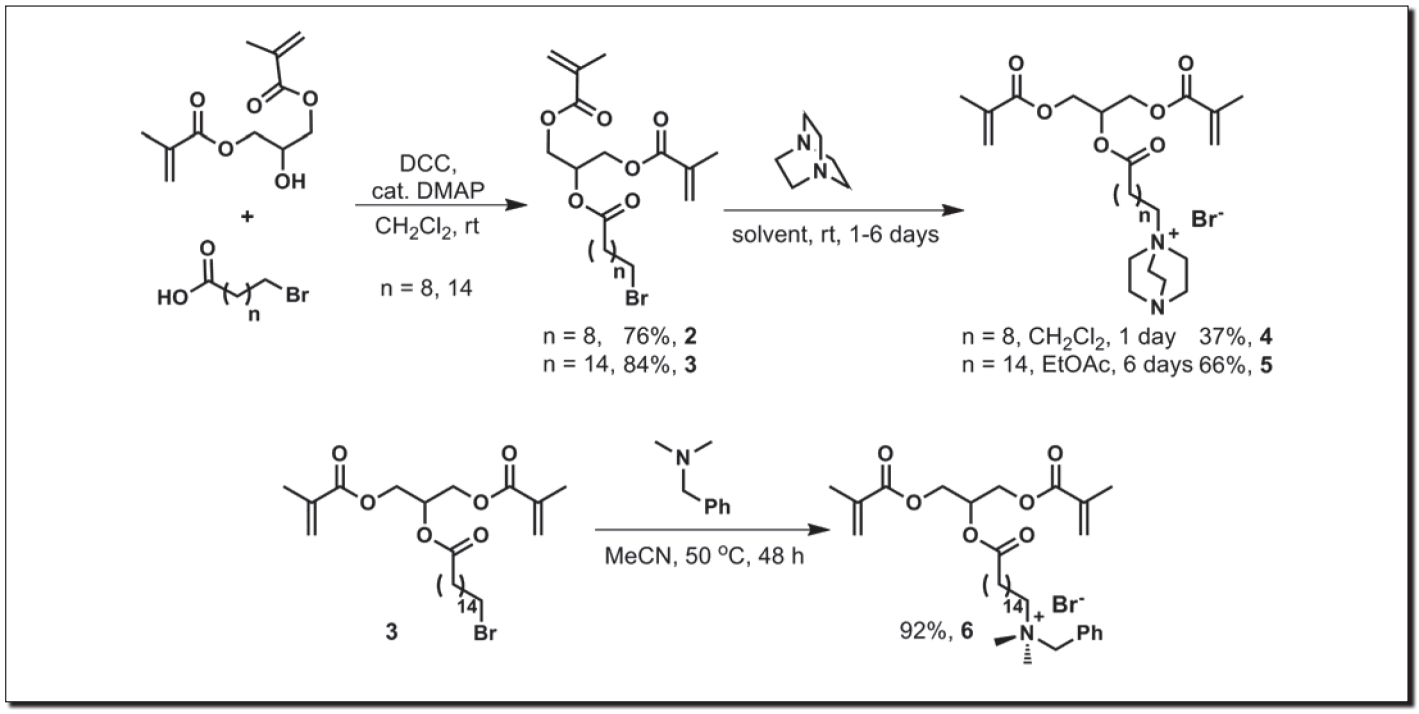

Fig. 2. Synthesis of the new dimethacrylate monomers.

16-(1-azonia-4-azabicylco[2.2.2] octyl)hexadecylmethacrylate bromide (11). A 100 mL round flask equipped with magnetic stirring bar was charged with 1-(16-(hydroxyhexadecyl-4-azaoniabicyclo[2.2.2]octane)) bromide (9) 1.3 g, 3 mmol and dichloromethane (30 mL) and was placed in an ice bath. After the reaction flask was cooled for 15 minnutes, methacryloyl chloride (3.2 mmol) was added via syringe over 10 minutes. The reaction mixture was stirred at 0°C for 2 hours and then room temperature overnight. The reaction mixture was quenched by adding saturated aqueous K_2CO_3 (150 mL). The aqueous layer was extracted with chloroform (3 × 30 mL). The combined organic extract was washed sequentially with saturated aqueous $NaHCO_3$ (2 × 20 mL) and brine (2 × 20 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified on silica gel column with EtOAc:MeOH (3:1) as mobile phase. After removal of the solvent under vacuum, 11 was isolated as a waxy, white solid (1.14 g, 2.28 mmol, 76%). A similar reaction of 8 with methacryloyl chloride yielded monomer 10 (70%).

^{1}H NMR ($CDCl_3$, δ) 6.08 (s, 1H, C=C*H*H'), 4.14 (t, 2H, CH_2O), 5.60 (s, 1H, C=CH*H*'), 3.40-3.36 (m, 6H, 3CH_2), 3.27-3.18 (m, 8H, 4CH_2), 1.93 (s, 3H, CH_3), 1.71-1.64 (m, 4H, 2CH_2), 1.39-1.30 (m, 24H, 12CH_2); ^{13}C{^{1}H} 167.6, 137.0, 124.8, 64.8, 62.4, 52.3, 52.22, 52.18, 29.54, 29.51, 29.48, 29.46, 29.4, 29.3, 29.13, 29.09, 28.5, 25.9, 21.6, 17.2.

IR (cm^{-1}) 3365(m, br, H2O), 2923(m), 2850(m), 1723(s, C=O), 1635(w, C=C), 1467(m) 1152(s), 905(w).
HRMS calculated for $C_{26}H_{49}O_2N_2$: 421.3789; found: 421.3792.

Cytotoxicity test - Human gingival fibroblasts were obtained from extracted molars from patients with healthy gingiva following informed consent as prescribed in an approved IRB protocol. Gingival fibroblasts were maintained in MEMα containing 10% fetal calf serum (FCS) and 200 units/mL penicillin and 200 µg/mL streptomycin. Cells were grown in 48-well plates for 24 hours prior to exposure to the synthesized antibacterial monomers. Growth media containing 0.1% dimethylsulfoxide (DMSO) were supplemented with 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M concentrations of the five newly synthesized monomers (4-6, 10, 11) and added to the cells for 24 hours. MEMα served as a control for cytotoxicity. Cell survival was visualized using a fluorescent esterase substrate (Calcein-AM[d]) and a Nikon TE2000[e] inverted fluorescent microscope. Cell survival was quantified using a BioTek Synergy 2[f] fluorescent multi-well plate reader.

Evaluation of antimicrobial activity - *S. mutans* UA159 and *L. casei* ATCC 4646, two major cariogenic bacteria, were used for antibacterial activity assessment. *S. mutans* was grown in brain heart infusion broth (BHI[g]), and *L. casei* was grown in MRS medium.[g] In an effort to find out the breadth of the antibacterial activity of the monomers and their potential in other medical applications, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853, two bacteria commonly associated with a range of medical conditions such as wounds and abscesses, were also tested. *S. aureus* and *P. aeroginosa* were grown in Tryptic Soy Broth (TSB[g]). All bacteria were maintained under static conditions in a 37°C aerobic chamber with (for *S. mutans* only) or without 5% CO_2. For antibacterial activity assay, these bacteria were cultivated using a semi-defined medium (BM) with glucose (18 mM) and sucrose (2 mM) (BMGS) as supplemental carbohydrate sources.

Antimicrobial efficacy was measured using a Bioscreen C,[h] which is an automated system that provides constant temperature and automatic optical density (OD) measurement.[24] Overnight cultures were transferred to fresh BMGS medium and allowed to grow to mid-exponential phase, at which point they were properly diluted in BMGS and allowed to grow in Bioscreen C with and without inclusion of different concentrations of antimicrobial monomers. All antimicrobial

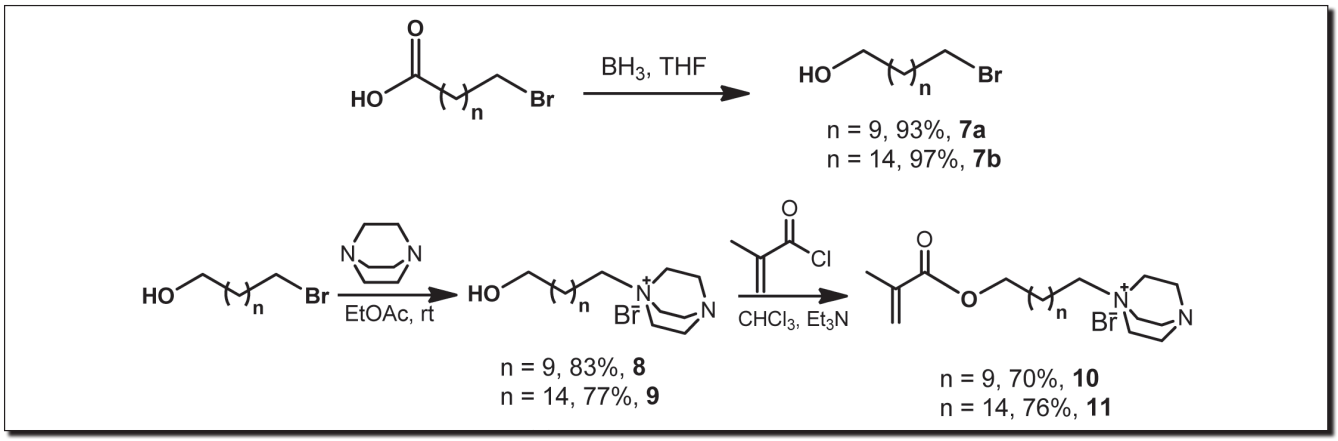

Fig. 3. Synthesis of methacrylate monomers containing DABCO.

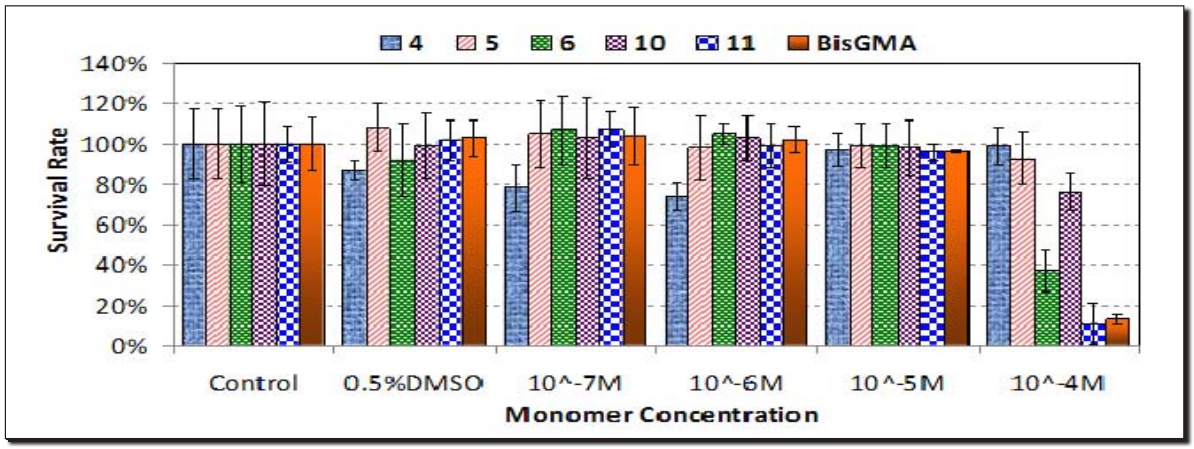

Fig. 4. Cytotoxicity of synthesized antibacterial monomers to human gingival fibroblast cells. The survival rate near 100% indicate no or low cytotoxicity. Lower survival rate indicates higher cytotoxicity.

monomers were dissolved in DMSO at 10^{-2} M concentration and serial dilutions were made to achieve the desired concentrations (10^{-4} M - 10^{-7} M). Chlorhexidine, an antibacterial agent commonly used in oral infection and disease control, were used as a positive control. Negative controls received equal volume of DMSO. The optical density of the cultures with and without antibacterial agents included were measured every 30 minutes for 48 hours, and all experiments were run in triplicate.

Data analysis - The data were analyzed using one-way ANOVA and Tukey's Studentized Range (HSD) Test for multiple pairwise comparison (α= 0.05).

Results

Monomer synthesis – Fig. 2 and Fig. 3 outline the synthesis of the new monomers. For the monomers based on glycerol dimethacrylate (GDMA), the appropriate ω-bromocarboxylic acid was reacted with GDMA in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 at room temperature (Fig 2).[23] The corresponding esters were then isolated in high yield by column chromatography (76-84%). Bromoesters 2 and 3 were then reacted with 1,4-diazabicyclo[2.2.2]octane (DABCO) at room temperature in CH_2Cl_2 or ethyl acetate (EtOAc) for 1-6 days to give monomers 4 and 5, respectively.[15] For compound 6 bearing the dimethylbenzylammonium group, more forcing conditions were necessary. Reaction of the alkyl bromide with the amine took place in acetonitrile (MeCN) at 50°C over 2 days. The ammonium bromide monomers were all isolated by column chromatography.

In the case of the monomethacrylates, the bromoalcohol was reacted with the appropriate amine under conditions similar to those described for the dimethacrylates (Fig 3). Following isolation by chromatography, the alcohol was esterified by reaction with methacryloyl chloride in $CHCl_3$ in the presence of triethyl amine. The hexadecyl compound 7 was produced by reduction of the acid with borane in THF prior to reaction with the amine.

All of the new monomers and intermediates were characterized by NMR (^{1}H, ^{13}C), IR and HRMS (ESI). Formation of the product cations was most clearly seen by the strong molecular ion peak visible in the ESMS spectra. Additionally, a downfield shift of the protons α- to the ammonium N atom clearly shows the formation of the cations. In the IR spectra, the carbonyl stretches fall in the range 1,720-1,722 cm^{-1}, in accord with the assigned structures. For the dimethacrylates, a mixture of isomers was formed, consistent with the starting GDMA isomer ratio.

Cytotoxicity test - Cytotoxicity of the new monomers was tested by adding solutions of the monomers to human gingival fibroblast cells at various concentrations (10^{-4} M - 10^{-7} M) and measuring cell survival. As shown in Fig. 4, toxicity was generally low for all monomers tested, only becoming apparent at high

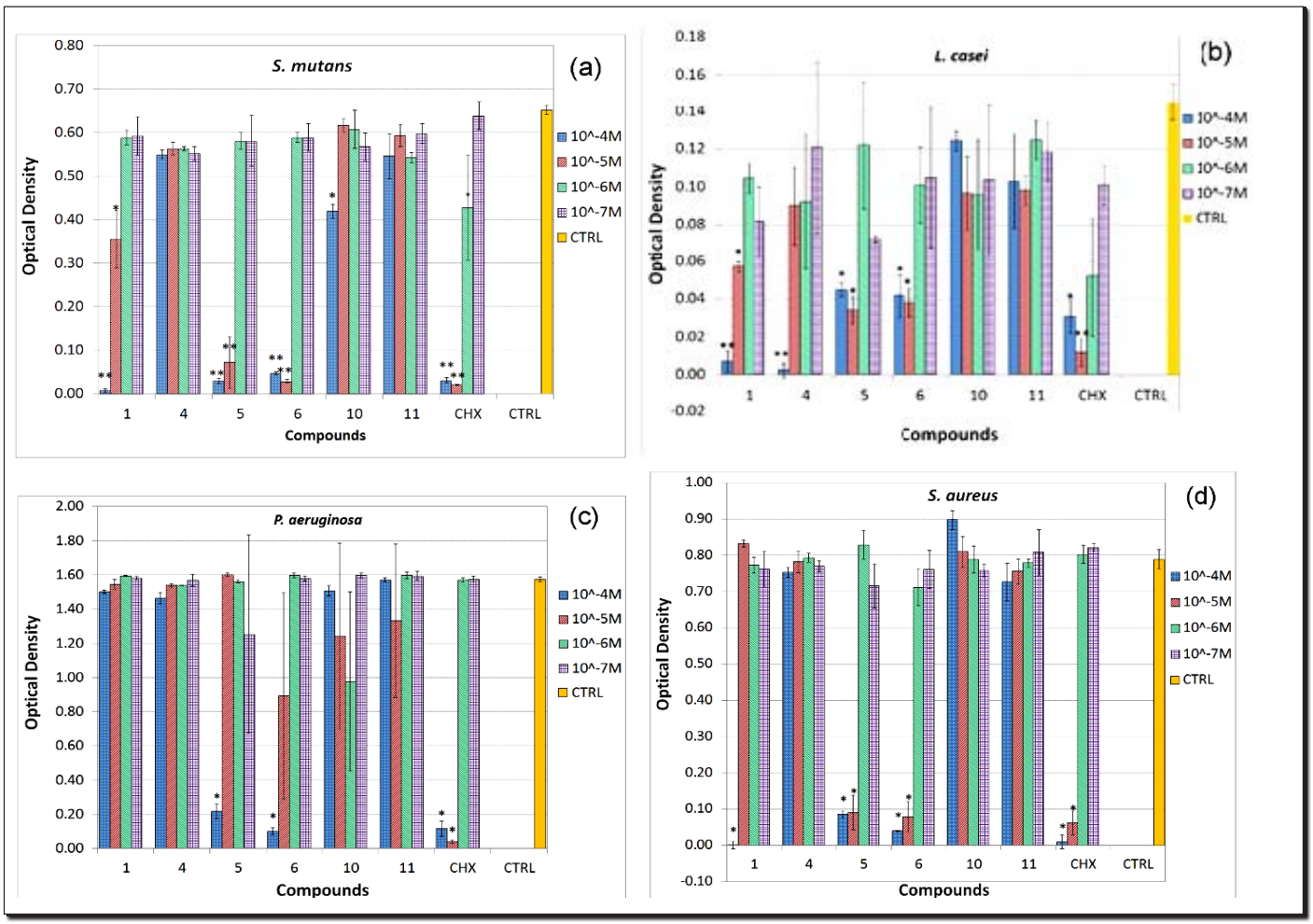

Fig. 5. Effects of antibacterial monomers on the growth of four bacteria: (a) *S. mutans*, (b) *L. casei*, (3) *P. aeruginosa*, and (d) *S. aureus*. Bacteria were grown in Bioscreen C with and without inclusion of monomers (1, 4, 5, 6, 10, 11,) or chlorhexidine (CHX) as positive control. Bar graphs represent the average maximum optical densities of the cultures. Those with *and ** indicate significant difference at the level of P< 0.05 when compared to the control (CTRL). Those with ** also indicate significant difference from those with *.

(10^{-4} M) concentration. The hexadecyl dimethacrylate (6) and hexadecyl monomethacrylate (11), both having C16 aliphatic chain, showed the highest toxicity (similar to BisGMA). Their counterparts with shorter (C11) aliphatic chain monomers 4 and 10, respectively, have better biocompatibility than BisGMA.

Test of antibacterial activity – Figure 5 shows, of the six antibacterial monomers, including previously synthesized monomer 1 and five newly synthesized, all except 10 and 11, displayed effective antibacterial activity, although the effective concentrations varied with the different monomers against different bacteria. As compared to the negative control that received solvent DMSO, Chlorhexidine (positive control) was effective against all four bacteria at the concentrations of 10^{-5} M and above (P< 0.001), which is expected. Previously synthesized monomer 1 showed strong inhibitory activity against *S. mutans* and *L. casei* at the level of 10^{-4} and 10^{-5} M (P< 0.05), and was effective against *S. aureus* at the concentration of 10^{-4}M (P< 0.001). However, it showed no effect against *P. aeruginosa* at any concentration tested (P> 0.05) (Fig. 5c). Similarly, the newly synthesized monomer 5 and monomer 6 also showed strong inhibitory activity against *S. aureus*, *S. mutans* and *L. casei* at the concentration 10^{-5} M and above (P< 0.001). However, unlike monomer 1, both were effective against *P. aeruginosa* at 10^{-4} M (P< 0.001). Monomer 4 was shown to be strongly effective against *L. casei* at the concentration of 10^{-4} M, but not to the other bacteria tested. However, neither monomer 10 nor monomer 11 displayed any major effects against the bacteria tested (P> 0.05).

Discussion

Importantly, any antibacterial component of new dental materials must show sufficiently low cytotoxicity to healthy cells in order to make it a clinically viable product. In an earlier study,[12] monomer 1 showed good biocompatibility at 10^{-4} M concentration (the highest concentration tested in the Bioscreen analysis). The five new monomers described here were also tested against human gingival fibroblast cells at concentrations varying from 10^{-4} M to 10^{-7} M. As shown in Fig. 4, at 10^{-4} M concentration, monomers 4 and 5 showed little cytotoxicity; monomer 10 showed moderate cytotoxicity; and monomers 6 and 11 showed severe cytotoxicity, Nevertheless, all of the synthesized monomers have similar or lower cytotoxicity than BisGMA. BisGMA is a currently widely used monomer in dental composites, bonding agents, sealants and other resin-based dental materials. These dental materials have been used in dental clinics on millions of patients without significant side effects. After proper cure (polymerization) of the material and

removal of oxygen inhibition layer on the surface, the concentration of the monomers released from the dental materials into saliva is rather low ($<10^{-5}$ M) and further decreases with time. Therefore, in general, as long as the vitro cytotoxicity of a monomer is not higher than that of BisGMA, it is considered safe and acceptable.

The structure-activity relationship of the various monomers in the Bioscreen analysis against pathogens, including *S. mutans* and *L. casei*, two major cariogenic bacteria, revealed several things of interest. Firstly, as previously observed, the activity of the compounds is dependent upon chain length, with longer chain alkyls (i.e. hexadecyl) showing higher activity than their shorter chain counterparts.[14] Additionally, the nature of the ammonium group is clearly an important factor in determining antibacterial activity. The dimethylbenzylammonium salts outperform the corresponding trimethyl, pyridyl and DABCO based salts in many cases.[12] The most surprising result, however, is the difference in activity between the mono- and dimethacrylates (5 and 11). The hexadecyl DABCO monomethacrylate 11 exhibited little to no activity against the four bacteria tested. By contrast, the structurally related dimethacrylate DABCO monomer 5 showed relatively higher activity, bested only slightly by the corresponding dimethylbenzyl compound 6. Chlorhexidine showed greater activity than the synthesized monomers against S. mutans and *P. aeruginosa*, but only slightly in comparison to monomers 5 and 6 (Fig. 2). Further studies to determine the new monomers' ability to inhibit biofilm formation will provide further information concerning the clinical viability of these newly synthesized antibacterial monomers.

In summary, five monomers based on quaternary ammonium salts bearing a long alkyl chain were synthesized. Biocompatibility of the monomers was tested against human gingival fibroblast cells and all monomers were deemed biocompatible at concentrations of 10^{-5} M or less. Most of them have better biocompatibility than BisGMA. In Bioscreen analysis against four opportunistic human pathogens, dimethacrylate monomers 5 and 6 generally demonstrated high antibacterial activities. These results further suggest that lipophilicity of the monomers plays a significant role in their antibacterial activity, with the highest activity shown for the most lipophilic monomer 6. Monomers 5 and 6 are also cross-linking monomers, and therefore, they should have less negative effect on the physical and mechanical properties of the dental composite and can be used at higher concentrations than the monomethacrylate antibacterial monomer (1). The applications of these two new antibacterial monomers in dental composites and bonding agents are under investigation.

a. Varian, Santa Clara, CA, USA.
b. Waters, Milford, MA, USA.
c. Thermo Scientific, West Palm Beach, FL, USA.
d. Calcein-AM, Thermo Fisher Scientific, Waltham, MA, USA.
e. Nikon, Tokyo, Japan.
f. BioTek, Winooski, VT, USA.
g. Difco Laboratories, Detroit, MI, USA.
h. OY Growth Curves AB Ltd, Helsinki, Finland.

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