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Effects of Quaternary Ammonium Chain Length on Antibacterial Bonding Agents

ABSTRACT

The objectives of this study were to synthesize new quaternary ammonium methacrylates (QAMs) with systematically varied alkyl chain lengths (CL) and to investigate, for the first time, the CL effects on antibacterial efficacy, cytotoxicity, and dentin bond strength of bonding agents. QAMs were synthesized with CL of 3 to 18 and incorporated into Scotchbond Multi-Purpose (SBMP) bonding agent. The cured resins were inoculated with *Streptococcus mutans*. Bacterial early attachment was investigated at 4 hrs. Biofilm colony-forming units (CFU) were measured after 2 days. With CL increasing from 3 to 16, the minimum inhibitory concentration and minimum bactericidal concentration were decreased by 5 orders of magnitude. Incorporating QAMs into SBMP reduced bacterial early attachment, with the least colonization at CL = 16. Biofilm CFU for CL = 16 was 4 log lower than SBMP control ($p < .05$). All groups had similar dentin bond strengths ($p > .1$). The new antibacterial materials had fibroblast/odontoblast viability similar to that of commercial controls. In conclusion, increasing the chain length of new QAMs in bonding agents greatly increased the antibacterial efficacy. A reduction in *Streptococcus mutans* biofilm CFU by 4 log could be achieved, without compromising bond strength and cytotoxicity. New QAM-containing bonding agents are promising for a wide range of restorations to inhibit biofilms.

KEY WORDS: antibacterial activity, *Streptococcus mutans*, quaternary ammonium compounds, dentin bonding, fibroblasts, odontoblasts.

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INTRODUCTION

Secondary caries is a primary reason for dental restoration failure (Mjör and Toffeneti, 2000; Sakaguchi, 2005). Replacing failed restorations accounts for 50% to 70% of all restorations performed (Frost, 2002; Bagramian *et al.*, 2009). Biofilm acids could lead to caries (ten Cate, 2006); hence antibacterial resins were developed to inhibit biofilms (Imazato *et al.*, 1999; Imazato, 2003; Beyth *et al.*, 2006; Namba *et al.*, 2009; Cheng *et al.*, 2012a). Indeed, previous studies investigated the copolymerization of quaternary ammonium methacrylates (QAMs) to develop antibacterial resins (Imazato, 2003; Li *et al.*, 2009; Xie *et al.*, 2011; Antonucci *et al.*, 2012; Xu *et al.*, 2012; Zhang *et al.*, 2012).

Composites are the principal materials for tooth-cavity restorations (Ferracane, 2011) which are bonded in cavities *via* adhesives (Ferracane *et al.*, 2010; Spencer *et al.*, 2010; Pashley *et al.*, 2011). Residual bacteria could exist in the prepared cavity, and microleakage could allow bacteria to invade the tooth-restoration interfaces. Therefore, it is beneficial for primer/adhesive to be antibacterial (Imazato *et al.*, 1998; Imazato, 2003; Li *et al.*, 2009; Hiraishi *et al.*, 2010; Cheng *et al.*, 2012b). Quaternary ammonium can cause bacteria lysis by binding to membranes (Beyth *et al.*, 2006). A previous study revealed that long polymeric chains with positive charges and hydrophobicity effectively killed bacteria (Lin *et al.*, 2002). When the alkyl chain length (CL) was increased, the hydrophobicity increased, thereby enhancing its propensity to penetrate the hydrophobic bacterial membrane (Tiller *et al.*, 2001). Long cationic polymers can penetrate bacterial cells to disrupt membranes, like a needle bursting a balloon (Tiller *et al.*, 2001; Murata *et al.*, 2007). Therefore, the CL of QAMs is important. However, to date, there has been no report on the effects of CL of QAMs in dental adhesives on the antibacterial efficacy.

The objectives of this study were to synthesize a series of new QAMs with CL from 3 to 18, and to investigate, for the first time, the CL effects on antibacterial efficacy, cytotoxicity, and dentin bond strength of bonding agents. It was hypothesized that: (1) antibacterial potency of new QAMs will increase with increasing CL; (2) bacteria early attachment and biofilm CFU will decrease with increasing CL; and (3) dentin bond strength and fibroblast/odontoblast viability will be similar to those of commercial control.

MATERIALS & METHODS

QAMs were synthesized by a Menschutkin reaction *via* the addition reaction of tertiary amines with organo-halides (Antonucci *et al.*, 2012; Cheng *et al.*,

2012a). 2-(dimethylamino) ethyl methacrylate (DMAEMA) was the methacrylate-containing tertiary amine. To synthesize dimethylaminododecyl methacrylate (DMADDM) with CL = 12, 10 mmol of DMAEMA, 10 mmol of 1-bromododecane (BDD) (TCI America, Portland, OR, USA), and 3 g of ethanol were added to a vial, which was capped and stirred at 70°C for 24 hrs (Cheng *et al.*, 2013). After the reaction was completed, ethanol was removed *via* evaporation. This yielded DMADDM as a clear liquid, which was verified *via* Fourier transform infrared spectroscopy (Cheng *et al.*, 2013). Similarly, 6 QAMs were synthesized (Fig. 1).

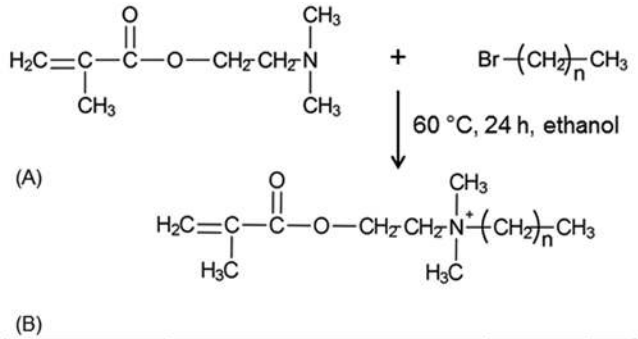
Each monomer was dissolved in Brain Heart Infusion broth (BHI, Becton, Sparks, MD, USA) (concentration = 50 mg/mL), which was then two-fold serially diluted. A microtiter plate assay determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) with *S. mutans* (ATCC700610, American Type Culture Collection, Manassas, VA, USA). MIC was the lowest concentration of an antimicrobial agent where bacterial growth was completely inhibited. MBC was the lowest concentration of the antimicrobial agent that killed 99.9% of bacteria. An aliquot of 10 μL of *S. mutans* inoculum was added to wells of 96-well plates (Corning, Corning, NY, USA), which contained 200 μL of a series of monomer dilution broths. MIC was determined by measurement of the absorbance at 600 nm before and after incubation at 37°C for 24 hrs (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). For MBC, 10-μL aliquots from the wells where bacterial growth was inhibited were spread onto BHI agar and cultured for 48 hrs. Measurements were repeated 3 times on different days.

Scotchbond Multi-Purpose (SBMP, 3M, St. Paul, MN, USA) primer contained 35% to 45% 2-hydroxyethylmethacrylate (HEMA), 10% to 20% acrylic/itaconic acids, and 40% to 50% water. SBMP adhesive contained 60% to 70% bisphenol-glycerolate dimethacrylate (BisGMA) and 30% to 40% HEMA. Each QAM was mixed at a QAM/(SBMP primer+QAM) mass fraction of 10% (Cheng *et al.*, 2012b). SBMP adhesive also contained 10% QAM (Zhang *et al.*, 2012). Seven bonding agents were tested:

[1] SBMP control;
[2] 10% DMAPM in SBMP (SBMP+CL3);
[3] 10% DMAHM in SBMP (SBMP+CL6);
[4] 10% DMANM in SBMP (SBMP+CL9);
[5] 10% DMADDM in SBMP (SBMP+CL12);
[6] 10% DMAHDM in SBMP (SBMP+CL16); and
[7] 10% DMAODM in SBMP (SBMP+CL18).

The cover of a 96-well plate (Corning) was used as the mold for specimen preparation (Li *et al.*, 2009). A 10-μL quantity of primer was placed in the mold. After the sample was dried with a stream of air, a 20-μL quantity of adhesive was applied and photo-polymerized to obtain a disk (approximate diameter = 8 mm, thickness = 0.5 mm). The disks were immersed in water and agitated for 1 hr to remove uncured monomers (Imazato *et al.*, 1998), then sterilized with ethylene oxide (AN-74i, Andersen, Haw River, NC, USA).

The density of quaternary ammonium groups on resin surfaces was quantified by a fluorescein-dye method (Tiller *et al.*, 2001). Disks were placed in 48-well plates. Fluorescein-sodium salt [200 μL of 10 mg/mL in de-ionized (DI) water] was added, and specimens were left for 10 min in darkness. Then, each sample was placed in a new well, and a 200-μL quantity of 0.1% cetyltrimethylammonium chloride (CTMAC) in DI water was added. Sample absorbance was measured at 501 nm (Tiller *et al.*, 2001). With 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/surface (sum of top, bottom, and side areas of disks) (Antonucci *et al.*, 2012).



(B)

Tertiary Amine	Alkyl Organo-Halide	Product	CL
2-dimethylamino ethyl methacrylate (DMAEMA)	1-bromopropane (BP) (n=2)	DMAPM	3
	1-bromohexane (BH) (n=5)	DMAHM	6
	1-bromononane (BN) (n=8)	DMANM	9
	1-bromododecane (BDD) (n=11)	DMADDM	12
	1-bromohexadecane (BHD) (n=15)	DMAHDM	16
	1-bromooctadecane (BOD) (n=17)	DMAODM	18

Figure 1. Synthesis of quaternary ammonium methacrylates (QAMs) with various alkyl chain lengths (CL). **(A)** Reaction scheme. The alkyl chain has the form of -(CH_2-) which repeats n times and ends with a single CH_3 group. The CL is equal to the number of CH_2 groups (given by n) plus 1 (the CH_3 group). **(B)** A series of QAMs with various CL synthesized in this study.

Resin disks were placed in 24-well plates with 1.5 mL *S. mutans* suspension in BHI. To examine early bacterial attachment, we incubated samples at 37°C and 5% CO_2. An incubation time of 4 hrs was chosen to test the early attachment and contact-killing effects, because the initial biofilm formation in oral cavity usually occurred in 2 to 4 hrs (Montanaro *et al.*, 2004). Samples were fixed with 37 mg/mL formaldehyde, stained with 1 μmol/L SYTOX-green (Invitrogen, Carlsbad, CA, USA), and examined with epifluorescence microscopy (TE2000-S, Nikon, Melville, NY, USA). Image-J software (NIH, Bethesda, MD, USA) was used to quantify bacterial area coverage.

Disks were placed in 24-well plates with 1.5 mL of BHI supplemented with 0.2% sucrose. A 10-μL quantity of *S. mutans* suspension was inoculated into each well. After incubation in 5% CO_2 at 37°C for 8 hrs, disks were transferred to new 24-well plates with fresh medium. After 16 hrs, disks were transferred to new 24-well plates and incubated for 24 hrs. The 2-day biofilms on disks were harvested by sonicating/vortexing, serially diluted, and spread onto agar plates for CFU analysis (Cheng *et al.*, 2012b).

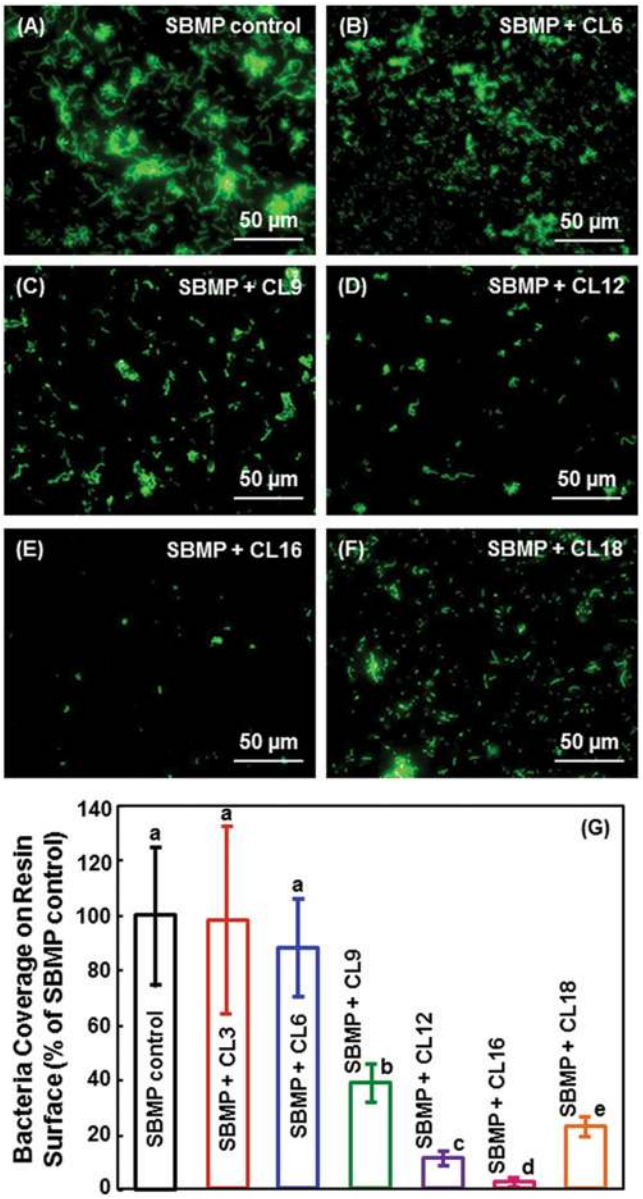


Figure 2. *S. mutans* early attachment and contact-killing effects of antibacterial bonding agents. Images of *S. mutans* attachment at 4 hrs on cured resin disks: **(A)** SBMP control, **(B)** SBMP plus 10% of QAM with alkyl chain length CL = 6, **(C)** CL = 9, **(D)** CL = 12, **(E)** CL = 16, and **(F)** CL = 18. Three disks, with 5 images/disk at random locations, yielded 15 images *per* group. SBMP with QAM at CL = 16 had the least bacteria colonization. **(G)** Quantification of resin surface area coverage by bacteria (mean ± SD; n = 15), normalized by bacteria coverage on SBMP control (as 100%). Increasing the CL of QAM greatly decreased bacterial attachment, reaching a minimum at CL = 16 ($p < .05$). Values with dissimilar letters are significantly different from each other ($p < .05$).

Human molars were collected with University of Maryland approval. Mid-coronal dentin was exposed, polished with 600-grit SiC, and etched with 37% phosphoric acid gel for 15 sec. A primer was applied, and then an adhesive was applied and light-cured for 10 sec. A composite (TPH, Caulk/Dentsply, Milford, DE, USA) was applied and light-cured for 60 sec. After storage in water at 37°C for 24 hrs, each tooth was vertically sectioned into 0.9 x 0.9 mm composite-dentin beams (Li *et al.*, 2012). Each beam was loaded in uniaxial tension (MTS, Eden Prairie, MN, USA). Load-at-failure divided by cross-sectional area yielded the microtensile bond strength (Li *et al.*, 2012).

To test cytotoxicity, we cultured human gingival fibroblasts (HGF, ScienCell, San Diego, CA, USA) in fibroblast medium (FM) supplemented with 2% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Odontoblast-like MDPC-23 mouse cells (University of Michigan, Ann Arbor, MI, USA) were cultured in growth medium comprised of α-minimum essential medium (α-MEM, Invitrogen) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). HEMA, triethylene glycol dimethacrylate (TEGDMA), and BisGMA (Esstech, Essington, PA, USA) were tested as controls. Each monomer, except BisGMA, was dissolved in medium at concentrations of: 0, 0.5, 1, 2, 5, 10, 20, 40, 60, and 100 µg/mL (Imazato *et al.*, 1999). BisGMA was dissolved in dimethylsulfoxide (DMSO) and diluted with medium because this monomer was highly hydrophobic. The final concentration of DMSO in medium was < 1 mg/mL and had no influence on cell proliferation in the preliminary study. Seeding density in 96-well plates was 5,000 cells/well (Huang *et al.*, 2011). Cells were cultured with 100 µL of monomer-containing medium for 48 hrs. Then, a 20-µL quantity of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution (5 mg/mL) was added. After 4 hrs, a 150-µL quantity/well of dimethylsulfoxide (DMSO) was added. Absorbance was measured (SpectraMax M5) at 492 nm (Huang *et al.*, 2011).

Each resin disk was immersed in 50 mL medium and agitated for 24 hrs at 37°C to obtain eluents (Huang *et al.*, 2011). Original extract was diluted with fresh medium at 2- to 64-fold. HGF or odontoblasts were seeded into 96-well plates at 5,000 cells/well with 100 µL of eluent-containing medium. Cells were cultured for 48 hrs, and the viability was measured *via* the MTT assay (Huang *et al.*, 2011).

One-way analysis of variance (ANOVA) was performed to detect the significant effects of variables. Tukey's multiple comparison was used to compare individual groups at $\alpha = 0.05$.

RESULTS

MIC (mg/mL) was 40 for DMAPM, 5 for DMAHM, 0.0195 for DMANM, 0.0049 for DMADDM, 0.0006 for DMAHDM, and 0.0049 for DMAODM. Hence, the antibacterial potency increased with increasing CL till CL = 16, then decreased at CL = 18. MBC (mg/mL) was 80 for DMAPM, 10 for DMAHM, 0.0391 for DMANM, 0.0097 for DMADDM, 0.0012 for DMAHDM, and 0.0097 for DMAODM (n = 3).

Incorporating QAMs into SBMP reduced early attachment bacteria (Figs. 2A-2F). Resin area coverage by bacteria decreased with increasing CL ($p < .05$), reaching a minimum at CL16, and then increased at CL18 (Fig. 2G).

Resin surface charge density (Fig. 3A) first increased with CL ($p < .05$), then there were no significant differences between CL of 12 to 18 ($p > .1$). Biofilm CFU/disk decreased with increasing

CL (Fig. 3B). CFU for CL = 16 was 4 log lower than SBMP control ($p < .05$). Antibacterial effects were obtained without sacrificing dentin bond strength (Fig. 3C, $p > .1$).

Increasing monomer concentration in medium decreased viability for HGF (Fig. 4A) and odontoblast-like cells (Fig. 4B). The main results are: (1) Increasing monomer concentration increased cytotoxicity; (2) increasing CL increased cytotoxicity; (3) all antibacterial monomers had less cytotoxicity than BisGMA; and (4) at monomer concentrations ≤ 2 μg/mL, all antibacterial monomers with CL ≤ 16 had cytotoxicity matching HEMA and TEGDMA ($p > .1$).

For cured resin eluents on fibroblast viability (Fig. 4C), at each concentration, all antibacterial groups had viability similar to that of SBMP control ($p > .1$). For odontoblasts (Fig. 4D), all groups had viability similar to that of control medium without resin eluents ($p > .1$).

DISCUSSION

This study represents the first report on the development of antibacterial bonding agents containing new QAMs with systematically varied CL, and investigation of the CL effects on bacterial attachment, biofilm growth, dentin bond strength, and cytotoxicity. Caries is a dietary carbohydrate-modified bacterial infectious disease caused by biofilm acids (ten Cate, 2006). Secondary caries is the main reason for restoration failures, costing tens of billions of dollars annually (Beazoglou *et al.*, 2007). Antibacterial adhesives could kill residual bacteria in the oral cavity and inhibit bacterial invasion at tooth-restoration interfaces. Antibacterial primers/adhesives would be especially useful for minimal-intervention dentistry with less removal of tooth structures (Lynch *et al.*, 2011), which could leave behind more bacteria and carious tissues in the tooth cavity. In the present study, bacterial early attachment was reduced, and antibacterial potency increased with increasing CL. Biofilm CFU was reduced by 4 log *via* CL16, compared with commercial adhesive, without compromising dentin bond strength. Therefore, these new antibacterial bonding agents are promising inhibitors of biofilms/caries.

The positively charged quaternary amine N^+ of QAMs can attract the negatively charged cell membrane of bacteria, which can disrupt membranes and cause cytoplasmic leakage (Beyth *et al.*, 2006; Namba *et al.*, 2009). For short-chained quaternary ammonium, the antimicrobial activity relies solely on a positively charged ammonium group coupling with a negatively charged bacterial membrane to disrupt membrane functions, alter the balance of essential ions (*i.e.*, K^+, Na^+, Ca^{2+}, and Mg^{2+}), interrupt protein activity, and damage bacterial DNA (Simoncic and Tomcis, 2010). Long-chained quaternary ammonium compounds have double-killing effects: (1) the positive charges; and (2) the additional antimicrobial activity by insertion into the bacterial membrane, resulting in physical disruption (Simoncic and Tomcis, 2010). Indeed, a recent study on glass ionomers showed stronger antibacterial activity with longer chain length (Xie *et al.*, 2011). These results are consistent with those of the present study on a new series of QAMs in bonding agents, showing increasing antibacterial potency with increasing CL from 3 to 16. However, when

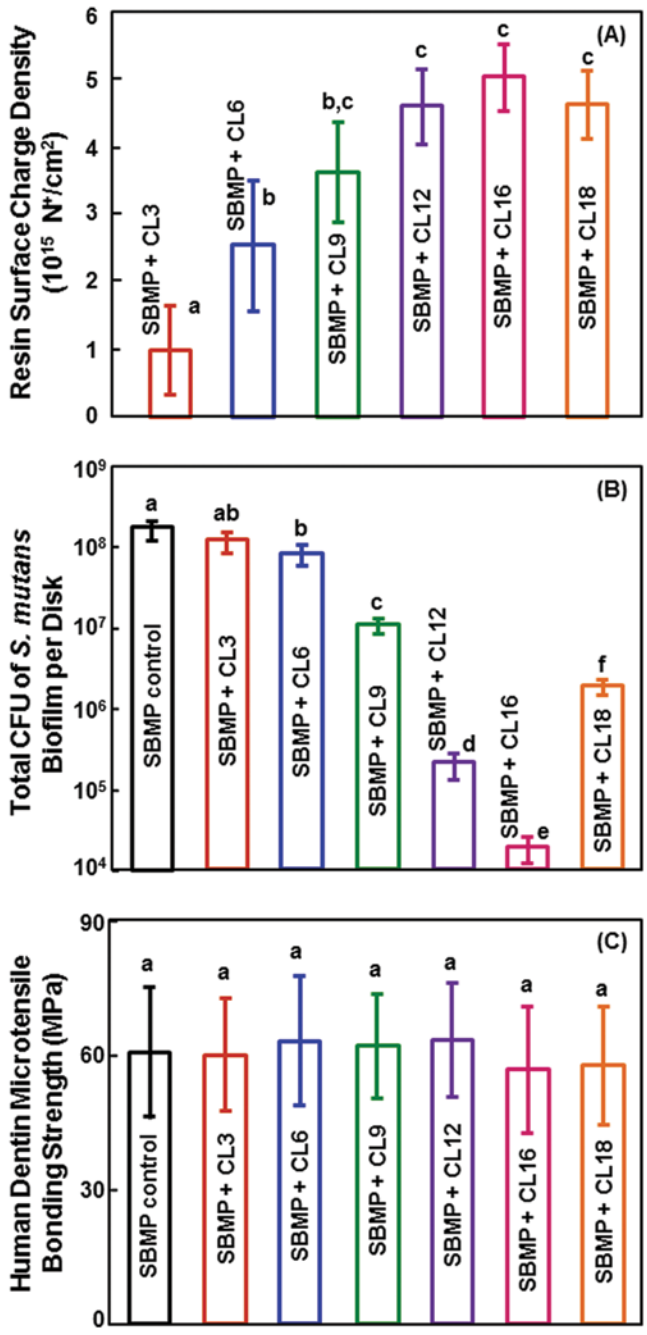


Figure 3. Anti-biofilm and dentin bond strength properties. **(A)** Surface charge density of cured resin disks (mean ± SD; n = 6). Fluorescein binding to cationic quaternary groups revealed increases in quaternary ammonium sites with chain length. Control without QAM had slight non-specific interaction with the fluorescein salt, and its value was subtracted from all groups to obtain net contribution from charges. **(B)** Colony-forming units (CFU) of biofilms at 2 days on resin surfaces (mean ± SD; n = 6). **(C)** Human dentin microtensile bond strength (mean ± SD; n = 40). Antibacterial efficacy increased with CL from 3 to 16 and then decreased at CL = 18. Biofilm CFU for SBMP with QAM at CL = 16 was reduced by 4 orders of magnitude, compared with SBMP control ($p < .05$). For dentin bond test, 8 teeth were used for each group. Five microtensile beams were obtained from each tooth, yielding 40 beams/group. In each plot, values with dissimilar letters are significantly different from each other ($p < .05$).

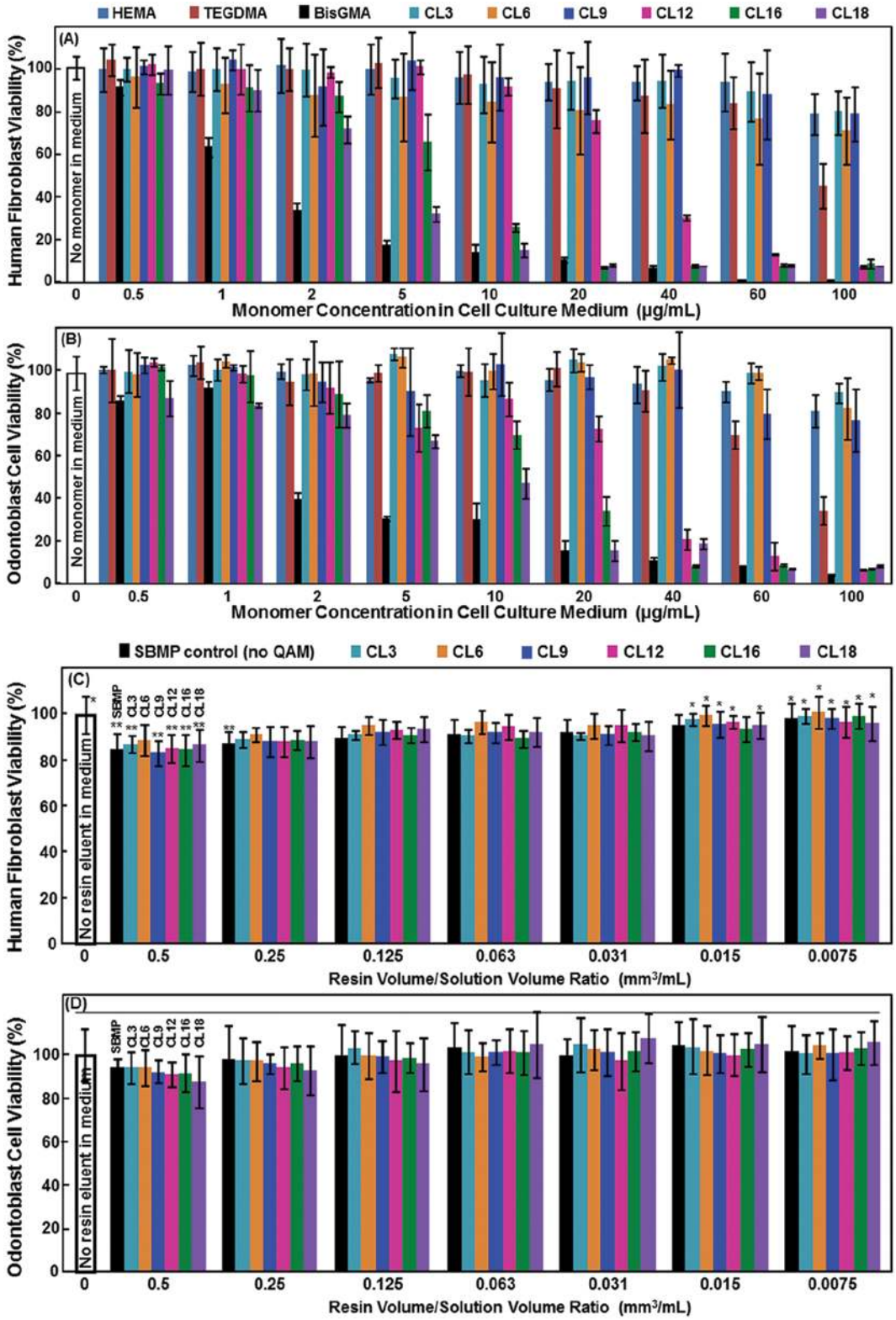


Figure 4. Cytotoxicity against fibroblasts and odontoblast-like cells. **(A,B)** Uncured monomers; **(C,D)** cured resin eluents. In (A) and (B), increasing monomer concentrations in medium decreased cell viability (mean ± SD; n = 4). High concentrations of up to 100 μg/mL were used to determine cell viability trends (Imazato *et al.*, 1999). Realistically, a typical tooth cavity would use about 20 μg of bonding agent. Even in the worst-case scenario, where all 20 μg were leached out in 1 day, human saliva flow of 1,000 to 1,500 mL/day would yield a monomer concentration of only 0.02 μg/mL. At a much higher concentration of 0.5 μg/mL, cell viability was excellent for all new QAMs. In (C) and (D), the original extract was obtained with a resin disk of 8 mm in diameter and 0.5 mm in thickness in 50 mL of culture medium, yielding a resin volume/solution ratio of 0.5 mm^3/mL. In (C), values with a single * are higher than values with double ** ($p < .05$). All the other values are not significantly different from each other and from those with * or ** ($p > .1$). At each concentration, all groups with new QAMs had viability similar to that of SBMP control ($p > .1$). In (D) the horizontal line indicates statistically similar values ($p > .1$).

CL was further increased to 18, the antibacterial efficacy decreased. It is possible that when the alkyl chain is too long, it may bend/curl and hence cover the positively charged quaternary ammonium groups, thereby blocking the electrostatic interactions with bacteria and diminishing the antibacterial potency. CL16 may be the critical point for the alkyl chain to extend without burying the quaternary ammonium sites, while CL18 may be too long with bending to form barriers between positively charged nitrogen and bacteria. Further study is needed to understand the CL effect.

Since all samples had the same 10% mass fraction of QAM, the samples with longer CL monomers (higher molecular weight) would have a lower molar fraction *per* sample. However, samples with longer CL exhibited a higher charge density (Fig. 3A). Hence, samples with longer CL (fewer positively charged molecules) actually had higher surface charge densities. This may be because, with increasing CL, the monomer became more hydrophobic. With a short CL, the adhesive would be more hydrophilic, slightly compromising its conversion degree (Cadenaro *et al.*, 2008). This leaves more monomers unpolymerized, to leach out during the rinsing/immersion process and during incubation for charge density determination. A hydrophilic resin would have more water sorption/solubility, which contributes to leach-out. Hence, although samples with shorter CL had more positively charged molecules, some of these molecules were lost and did not contribute to charge density. Therefore, increasing CL appeared to have double benefits: (1) longer chains inserting into bacterial membranes to disrupt bacteria; and (2) higher surface charge density for bonding agent, even with the same QAM mass fraction. Hence, while CL was directly altered in this study, variations in CL could lead to changes in other physical properties, such as hydrophilicity and surface charge density, thereby altering the antibacterial effects of adhesives, which requires further investigation.

When QAM is incorporated into resin, upon photo-polymerization the QAM is copolymerized by forming covalent bonds with the polymer network (Imazato, 2003; Li *et al.*, 2009; Antonucci *et al.*, 2012). Therefore, the antibacterial agent is immobilized in resin and not lost over time, thus providing a durable antibacterial capability (Imazato, 2003). The lack of significant QAM leach-out minimizes cytotoxicity. The present study tested uncured monomers and cured resin eluents, showing acceptable cytotoxicity similar to that of clinically used monomers. In Figs. 4A and 4B, it should be noted that the higher concentrations are unlikely to occur *in vivo*. A typical tooth cavity would use approximately 20 μg of bonding agent. In the unrealistic worst-case scenario, assume that all 20 μg were leached out in 1 day. Human saliva flow is 1,000 to 1,500 mL/day (Humphrey and Williamson, 2001). This would yield a monomer concentration of 0.02 μg/mL. Figs. 4A and 4B show that, at 0.5 μg/mL, which is more concentrated than in the worst-case scenario, cell viability was still excellent for all QAMs. They matched the cell viability of HEMA, TEGDMA, and control medium without monomer. For cured resin eluents (Figs. 4C, 4D), cell viability of QAMs matched that of SBMP control at all concentrations tested. While a recent study showed that there was no decrease in antibacterial potency after 6 mos of water-aging, which indicated copolymerization (Zhang *et al.*, 2013), further study is needed to investigate how well QAMs were copolymerized in bonding agents. Further study should also investigate the incorporation of QAMs, especially CL16, into pit/fissure sealants, orthodontic bracket cements, and composites to inhibit biofilms/caries.

In summary, the effects of alkyl chain length on antibacterial bonding agents were systematically investigated for the first time. A series of new QAMs was developed, and the antibacterial efficacy of bonding agents increased with CL from 3 to 16, then decreased at CL = 18. Bacterial early attachment was substantially reduced, and biofilm CFU was decreased by 4 log, compared with control. The new antibacterial bonding agents had dentin bond strength and fibroblast/odontoblast cytotoxicity similar to those of commercial controls. The new QAMs (especially CL16) are promising for a wide range of restorations to inhibit biofilms and caries.

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