Synthesis and Antimicrobial Activities of New Water-Soluble Bis-Quaternary Ammonium Methacrylate Polymers

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ABSTRACT: New methacrylate monomers containing pendant quaternary ammonium moieties based on 1,4-diazabicyclo-[2.2.2]-octane (DABCO) were synthesized. The DABCO group contains either a butyl or a hexyl pendant group comprising the hydrophobic segment of the monomers and one tether group to the methacrylate moiety. The monomers were homopolymerized in water by using 2,2′-azobis(2-methylpropionamide) dihydrochloride (V-50) as an initiator. The monomers and polymers were characterized by elemental analysis, thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), FTIR, and ^{13}C-NMR. The antimicrobial activities of the corresponding small molecules (bis-quaternary ammonium monocarboxylates) and polymers were investigated against *Staphylococcus aureus* and *Escherichia coli*. Although the small molecules did not show any antimicrobial activity, the polymers were moderately effective against both Gram-positive and Gram-negative bacteria. The minimum inhibitory concentration (MIC) values of the polymers with butyl and hexyl hydrocarbon chains against *S. aureus* and *E. coli* were found to be 250 and 62.5 μg/mL, respectively. The minimum bactericidal concentration (MBC) value for the polymer with the butyl group was higher than 1 mg/mL, whereas the MBC value for the polymer with hexyl group was found to be 62.5 μg/mL. Thus, an increase of the alkyl chain length from 4 to 6 significantly increased the antimicrobial activity of the polymer. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 94: 635–642, 2004

Key words: antimicrobial; quaternary ammonium; water-soluble polymers; biopolymers; NMR

INTRODUCTION

Quaternary ammonium compounds (QACs) are some of the most commonly used antimicrobials. Common characteristics among QACs are that they possess both a positive charge and a hydrophobic segment.[1–3] Classification and biological activity of QACs depend upon the nature of the organic groups attached to nitrogen, the number of nitrogen atoms present, and the counterion.[1] QACs usually contain four organic groups linked to nitrogen, which may be similar or different in chemistry and structure. The organic substituents are either alkyl, aryl, or heterocyclic.[4] At least one of the organic substituents should be a long alkyl chain to provide a hydrophobic segment compatible with the bilayer of the outer cell wall.[5–7] It has been shown that an increase of the alkyl chain length of an amphiphilic compound (i.e., to 14 carbon alkyl chains) is followed by an increase in the hydrophobic interaction with the lipid bilayer of the cell wall, which in turn increases the antimicrobial activity of the compound.[7] QACs containing one long alkyl chain substituent of at least eight carbon atoms were shown to be very active biocides in water.[8] The number of nitrogen atoms can vary in the molecule depending on the starting materials used in the synthesis. Both mono- and bis-quaternary ammonium compounds are currently in use. Any anion may be attached to the cation to form a salt, although the chloride and bromide salts are most commonly used.[2]

QACs are usually white, crystalline powders that are very soluble or dispersible in water. As the chain lengths of the substituents increase, the solubility of QACs in polar solvents decreases, whereas their solubility in nonpolar solvents increases.[9] QACs have a broad spectrum of antimicrobial activity and often display extended biological activity because they may leave long-lived residues on treated surfaces.[2] They are effective against both Gram-positive and Gram-negative bacteria at medium concentrations and also have moderate effectiveness against viruses, fungi, and algae.[7,10] Some of the advantages of QACs over other antimicrobial agents are that they are more sta-

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ble, less corrosive, nonirritating to the skin, and have low mammalian toxicity.[2]

Continuous effort has been made during the last two decades to synthesize polymers with QAC substituents.[1,3,4,5,11–13] The literature suggests that the polymers containing QACs either in the backbone or as pendant groups show enhanced efficacy over corresponding small molecule QACs plus reduced residual toxicity, increased efficiency and selectivity, and prolonged lifetime.[1,5,14–16] Polymeric antimicrobial agents also have the advantage that they are nonvolatile, chemically stable, and do not permeate through the skin. As a result, they significantly reduce losses associated with volatilization, photolytic decomposition, and migration.[17]

Antimicrobial polymers have been used as coatings in many areas such as food processing,[18,19] biomedical devices,[20] filters,[21] and additives for antifouling paints.[22] The use of cationic antimicrobial polymers can eliminate bacterial infection of implanted devices such as catheters.[23] These polymers can be used in paints on hospital room walls and everyday objects such as doorknobs, children's toys, computer keyboards, and telephones. This renders them antiseptic and thus less able to transmit bacterial infections.[24] They are used in the textile industry to form antimicrobial fibers[25,26] and as disinfectants and preservatives in pharmaceuticals.[27] Other likely uses of these polymers are as cleaning solutions for contact lenses[28] as well as coatings and chemically bound components of biomaterials.[29]

In addition to the wide array of mono-quaternary ammonium compounds, 1,4-diazabicyclo-[2.2.2]-octane (DABCO)-based QACs with two ammonium groups were shown to have excellent antimicrobial activity. For example, DABCO derivatives containing one long alkyl chain were active against Gram-positive and Gram-negative bacteria as well as fungi.[30,31] When attached to insoluble carbohydrate and protein-based materials, they also showed very high antimicrobial activities.[31–33] However, the activity of the DABCO group depends on the length of the alkyl chain attached to it, and an increase in alkyl chain length was shown to increase the antimicrobial activity.[30,33] Moreover, the DABCO moiety, with its ability to have two positive charges (with both nitrogens reacted), has higher fixed charge density than other commonly used cationic antimicrobial agents. This increases its ability to interact with negatively charged bacterial cell surfaces. Several cationic antimicrobial polymers were shown to have higher charge densities (local cation concentration) compared to their monomers or corresponding small molecules, which apparently contribute to the higher antimicrobial activities of the polymers.[1,23,34,35] Thus, polymers containing DABCO pendant groups can be expected to have higher antimicrobial activities compared to the corresponding small molecules and/or monomers.

In this article, we describe the syntheses and characterization of new acrylate-based polymers having pendant DABCO moieties. Both butyl and hexyl chains were attached to one of the DABCO nitrogens. The other was reacted with 11-bromoundecanoic acid and then with ethyl α-chloromethyl acrylate (ECMA) to form new methacrylate monomers. These monomers were homopolymerized by conventional free-radical polymerization techniques. The obtained polymers were tested for antimicrobial activities against *Staphylococcus aureus* and *Escherichia coli*. The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) tests were performed by using broth dilution[36] and spread plate methods,[37] respectively.

EXPERIMENTAL

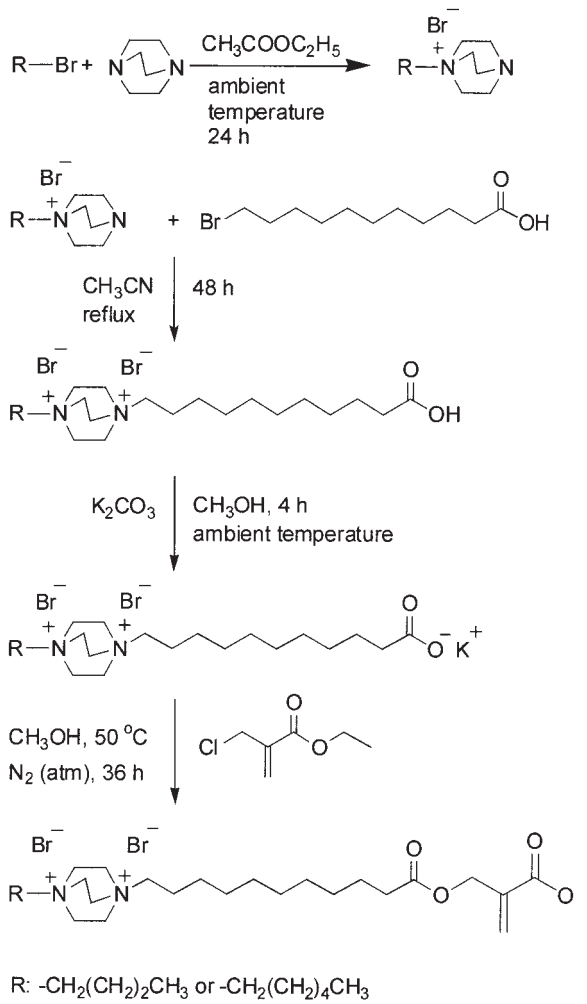
Materials and bacterial strains

DABCO is a white crystalline powder purchased from Air Products and Chemicals, Inc. All alkyl halides and solvents used in the synthesis were purchased from Acros Chemical Co., Fisher, or Aldrich Chemical Co. 11-Bromoundecanoic acid was purchased from Acros Organics. 2,2′-Azobis(2-methylpropionamide) dihydrochloride (V-50) is a water-soluble azo initiator, which was purchased from Wako Chemical. Ethyl α-chloromethyl acrylate was synthesized according to a procedure described in the literature.[38] All other chemicals were used as received.

Tryptic soy agar (TSA) was purchased from Difco Laboratories. It contained 15.0 g pancreatic digest of casein, 5.0 g enzymatic digest of soybean meal, 5.0 g sodium chloride, and 15.0 g agar. Tryptic soy broth (TSB) was also purchased from Difco Laboratories. It contained 17.0 g pancreatic digest of casein, 3.0 g enzymatic digest of soybean meal, 2.5 g dextrose, 5.0 g sodium chloride, and 2.5 g dipotassium phosphate. Bacterial strains used for antimicrobial activity tests included *S. aureus RN4220* and *E. coli* TOP10 strain. The strains were kept at −80°C in a freezer.

Measurements

^{13}C-NMR spectra were collected on a Varian 200 MHz NMR in $CDCl_3$, DMSO-d_6, and D_2O. Fourier transform infrared spectroscopy (FTIR) spectra were recorded on a Nicolet 5DX by using pressed KBr pellets. Thermal analyses were performed on a TA Instruments 9900 analyzer equipped with 910 differential scanning calorimeter (DSC) and 952 thermal gravimetric analyzer (TGA) cells by using heating rates of 10°C/min under nitrogen purge. Elemental analysis



Scheme 1 Synthesis of the monomers.

results were obtained from Quantitative Technologies Inc.

Procedures for the synthesis of new DABCO-based QACs

The general route for the syntheses of the monomers is shown in Scheme 1.

Reaction of DABCO with bromoalkanes

DABCO (11.39 g, 0.102 mol) was dissolved in 80 mL ethyl acetate in a 100-mL round-bottomed flask. The solution mixture was stirred for 10 min to dissolve DABCO completely, and then 1-bromobutane (17.82 g, 0.140 mol) was added to the flask containing the DABCO solution. The flask was closed with a rubber septum to prevent the absorption of water by the salt being formed. 1-Bromobutane was used in excess to ensure that all DABCO reacted, as it is more easily removed from the final product than unreacted DABCO. After 5 min, the formation of a white solid product was observed and the reaction was continued for 24 h. The salt formed was filtered from the solution by suction filtration and washed once with ethyl acetate and then three times with diethyl ether. It was dried over P_2O_5 at room temperature in a vacuum oven to remove trapped solvents to give product (C4-D) (22.35 g) in 93% yield.

This same procedure was applied to the reaction of DABCO with 1-bromohexane. DABCO (5.61 g, 0.05 mol) and 1-bromohexane (9.905 g, 0.06 mol) reacted in ethyl acetate (50 mL) to give product C6-D (12.49 g) in 90% yield.

Reaction of monocationic-DABCO-QAC with 11-bromoundecanoic acid

1-Bromobutane-DABCO-QAC (C4-D) (6.03 g, 0.024 mol) and 11-bromoundecanoic acid (9.62 g, 0.036 mol) were dissolved in 30 mL CH_3CN in a 50-mL round-bottomed flask (excess 11-BUA was used). The flask was closed with a rubber septum and N_2 was passed through the flask by using syringe needles. The mixture was refluxed for 48 h. The final product precipitated slowly during this time. More CH_3CN (10 mL) was added to the flask at the end of the reaction and the mixture was stirred for an additional hour. The mixture was gravity filtered and the solid product was washed twice with hot CH_3CN. It was then put into a 250-mL Erlenmeyer flask containing diethyl ether (200 mL) and stirred for 2 h to remove any residual reactants and solvents. The product was filtered and washed with diethyl ether. Finally, it was dried at 60°C for 6 h in a vacuum oven to remove trapped solvents to give product C4-DA (11.15 g) in 90% yield.

This same procedure was applied to the reaction of C6-D with 11-bromoundecanoic acid. Thus, C6-D (8.07 g, 0.029 mol) and 11-bromoundecanoic acid (11.58 g, 0.0437 mol) were reacted in 50 mL acetonitrile to give product C6-DA (11.77 g) in 75% yield.

The reaction of bis-quaternary ammonium monocarboxylates with ECMA

Bis-quaternary ammonium monocarboxylate C4-DA (2.57 g, 0.005 mol) was dissolved in 20 mL methanol in a 100-mL round-bottomed flask. Excess K_2CO_3 (2.16 g, 0.016 mol) was added to the flask and the mixture was stirred for 4 h. The clear starting solution turned to white on addition of K_2CO_3. After completion of the reaction, the methanol was removed with a rotary evaporator and 40 mL ethanol was added to the flask. A white precipitate (excess K_2CO_3 and $KHCO_3$) formed and was filtered. The ethanol was removed with a rotary evaporator to give a sticky white product in the flask.

Methanol (10 mL) and ECMA (1.13 g, 0.0076 mol) were added to the flask containing the sticky product.

TABLE I
Elemental Analysis Results of the Monomers

Monomers	Anal. Calc.			Found		
	C %	H %	N %	C %	H %	N %
C4-DAM.3H_2O	47.65	8.3	4.12	47.71	8.59	4.01
C6-DAM.3/2H_2O	51.1	8.44	4.11	51.42	9.00	4.09

The flask was closed with a rubber septum and N_2 was passed through the flask by using syringe needles. The mixture was stirred at 50°C for 36 h. A white precipitate (KCl) formed, which was removed by filtration. The solution left was precipitated into diethyl ether. The mixture was gravity filtered and the solid product was washed with diethyl ether twice. Finally, the monomer was dried in a vacuum oven at ambient temperature for 48 h to give monomer C4-DAM (3.13 g, 0.0046 mol) in 93% yield.

This same procedure was applied to the reaction of C6-DA with potassium carbonate and then ECMA. The reaction gave a white solid monomer C6-DAM (5.21 g, 0.0076 mol) in 75% yield.

Elemental analysis results for the monomers are shown in Table I. C, H, and N% found in the elemental analysis tests of the monomers are in good agreement with the calculated values. The water contents of C4-DAM and C6-DAM were calculated to be 3 and 3/2 mol for each molecule, respectively.

Synthesis of polymers

The general route for the polymerization of the monomers is shown in Scheme 2.

Monomer C4-DAM (0.501 g, 0.80 mmol) was dissolved in a small test tube containing 3 mL double-distilled water and V-50 (3.4 mg). The tube was closed with a rubber septum and three standard freeze–evacuate–thaw procedures were applied to remove air inside the tube. Then, the tube was placed into an oil bath at 45–50°C. After 24 h the reaction was stopped and the mixture was precipitated into acetone. The white solid product was filtered, dissolved in methanol (5 mL), and precipitated into diethyl ether (100 mL). Finally, polymer C4-DAP was dried in a vacuum oven at 50°C for 24 h to remove trapped solvents and give polymer (0.210 g) in 42% yield.

This same procedure was applied to the polymerization of C6-DAM. Thus, C6-DAM (0.504 g, 0.73 mmol) was heated at 50°C with V-50 (3.3 mg) in 6 mL water for 24 h to give polymer C6-DAP (0.201 g) in 40% yield.

Antimicrobial assessment

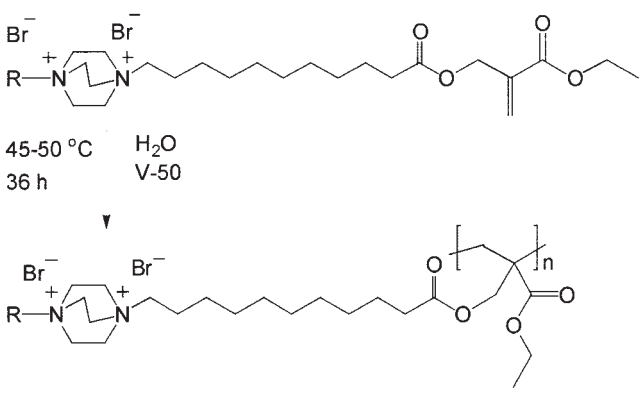
S. aureus and *E. coli* were streaked out on TSA plates and incubated at 37°C for 24 h. A representative colony was lifted off with a wire loop and placed in 5 mL TSB, which was then incubated with shaking at 37°C for 24 h. At this stage, the cultures of *S. aureus* and *E. coli* contained $\sim 10^9$ colony forming units (CFU) per milliliter. Cultures of *S. aureus* and *E. coli* containing 10^7 CFU/mL were prepared by dilution with TSB, which were used for antimicrobial tests.

The MIC values of the new QACs were determined by the broth dilution method by using geometric two-fold dilutions in TSB.[37] The polymer concentrations ranged from 1 mg/mL to 3.9 μg/mL. Each solution in the series was mixed with 10^5 CFU of the test organism in a 96-well microtiter plate. The 96-well plate was incubated at 37°C for 24 h. The MIC test was repeated at least four times for each antimicrobial agent. The dilutions showing no growth were cultured on TSA plates and incubated at 37°C overnight to determine the MBC.[4]

RESULTS AND DISCUSSION

Characterization of monomers and polymers

Figure 1 shows ^{13}C-NMR results of DABCO, monocationic DABCO-based QAC (C4-D), and bis-quaternary ammonium monocarboxylate (C4-DA). DABCO has an intense peak at 47.5 ppm. When it reacts with 1-bromobutane, this peak splits into two peaks at 45.6 and 52.2 ppm because of the differences in electron densities of carbons near the quaternary ammonium and neutral amine groups. Butyl carbon peaks are seen at 14.2, 19.9, 23.7, and 63.5 ppm. The carbon in the α-position to bromine in 1-bromobutane normally shows up at 33.4 ppm, but shifts to 63.5 ppm after reaction. This shift, and the splitting of DABCO peaks, confirms formation of the monocationic QAC. Only one nitrogen of DABCO reacts, partly because the monocationic DABCO salt precipitates from the solution before the other nitrogen can react, and partly



Scheme 2 Synthesis of the polymers.

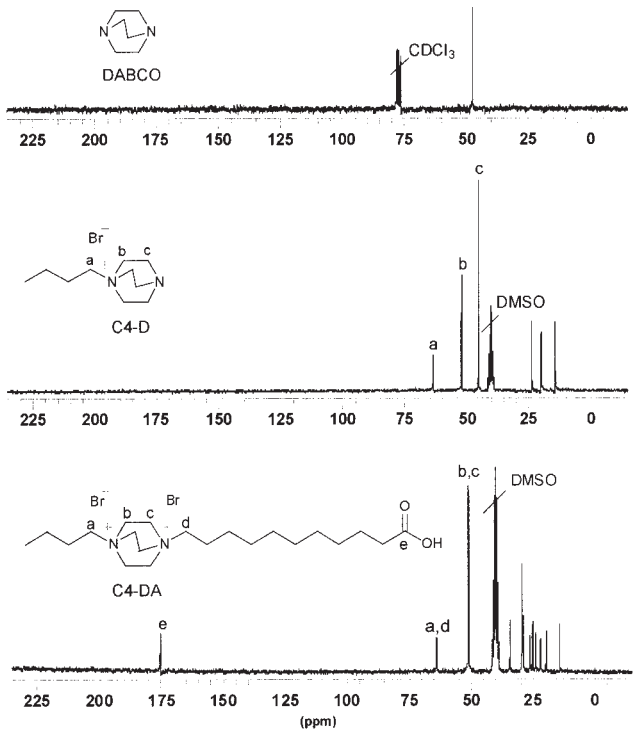


Figure 1 ^{13}C-NMR spectra of DABCO, monocationic-QAC (C4-D), and bis-quaternary ammonium monocarboxylate (C4-DA).

because of reduced reactivity of the second nitrogen due to through-space and through-bond interactions with the quaternary nitrogen.

The ^{13}C-NMR spectrum (Fig. 1) of bis-quaternary ammonium carboxylate (C4-DA) demonstrates that the splitting pattern seen in the first reaction disappears in the reaction of C4-D with 11-bromoundecanoic acid. The DABCO peaks now have the same electron densities around them, and only one intense peak for them is seen at 51.0 ppm. The carbonyl peak at 175.6 ppm verifies the formation of bis-quaternary ammonium carboxylate.

In Figure 2, ^{13}C-NMR spectra of the monomer (C4-DAM) and polymer (C4-DAP) in D_2O are shown.

Two new peaks for the double bond carbons of reacted ECMA in the monomer are seen at 128.2 and 136.1 ppm. There is a new peak in the monomer spectrum for the conjugated carbonyl carbon at 165.6 ppm. The peak for the carbon in the α-position to chloride in the ECMA, which normally shows up at 42.8 ppm, shifts to 62.5 ppm in the monomer. Other peaks verifying the formation of the monomer are *k* and *l* peaks at 61.3 and 14.2 ppm, which are the peaks for ester carbons. Upon the polymerization, the peaks for the double bond carbons of the monomer disappear and backbone peaks of the polymer are seen at 46

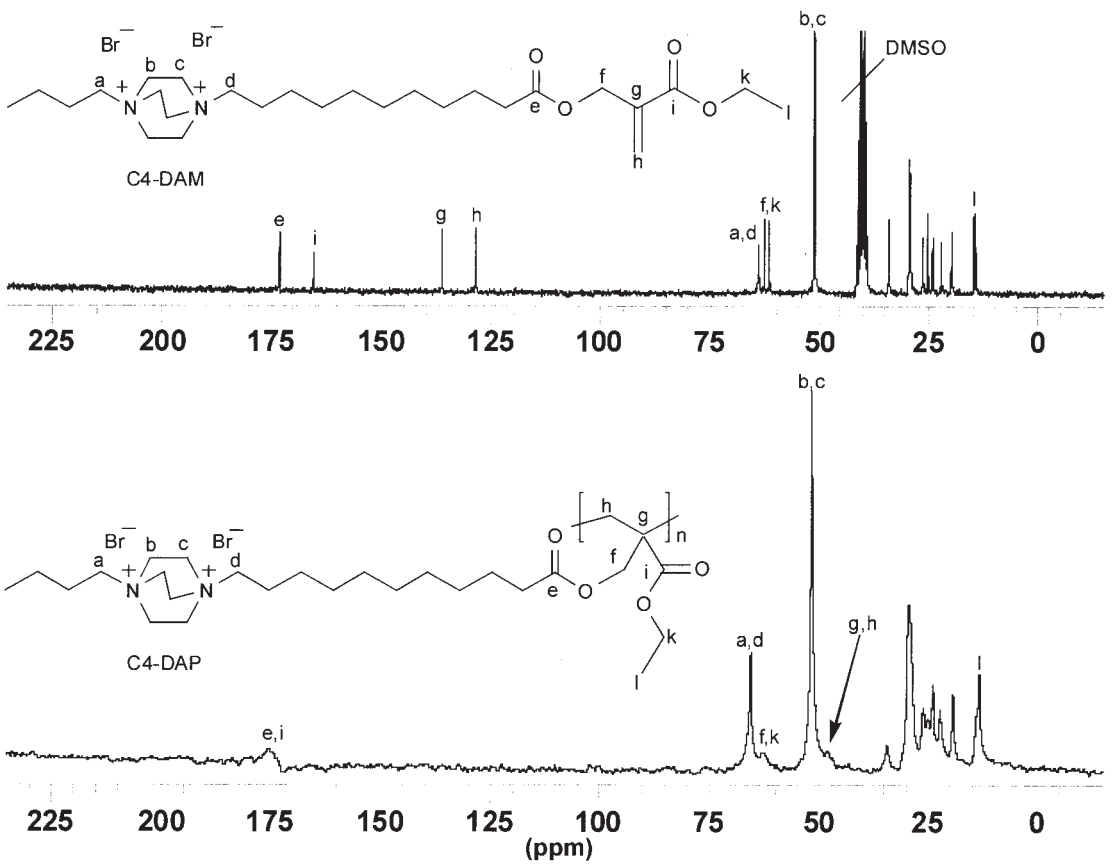


Figure 2 ^{13}C-NMR spectra of monomer C4-DAM and its polymer C4-DAP.

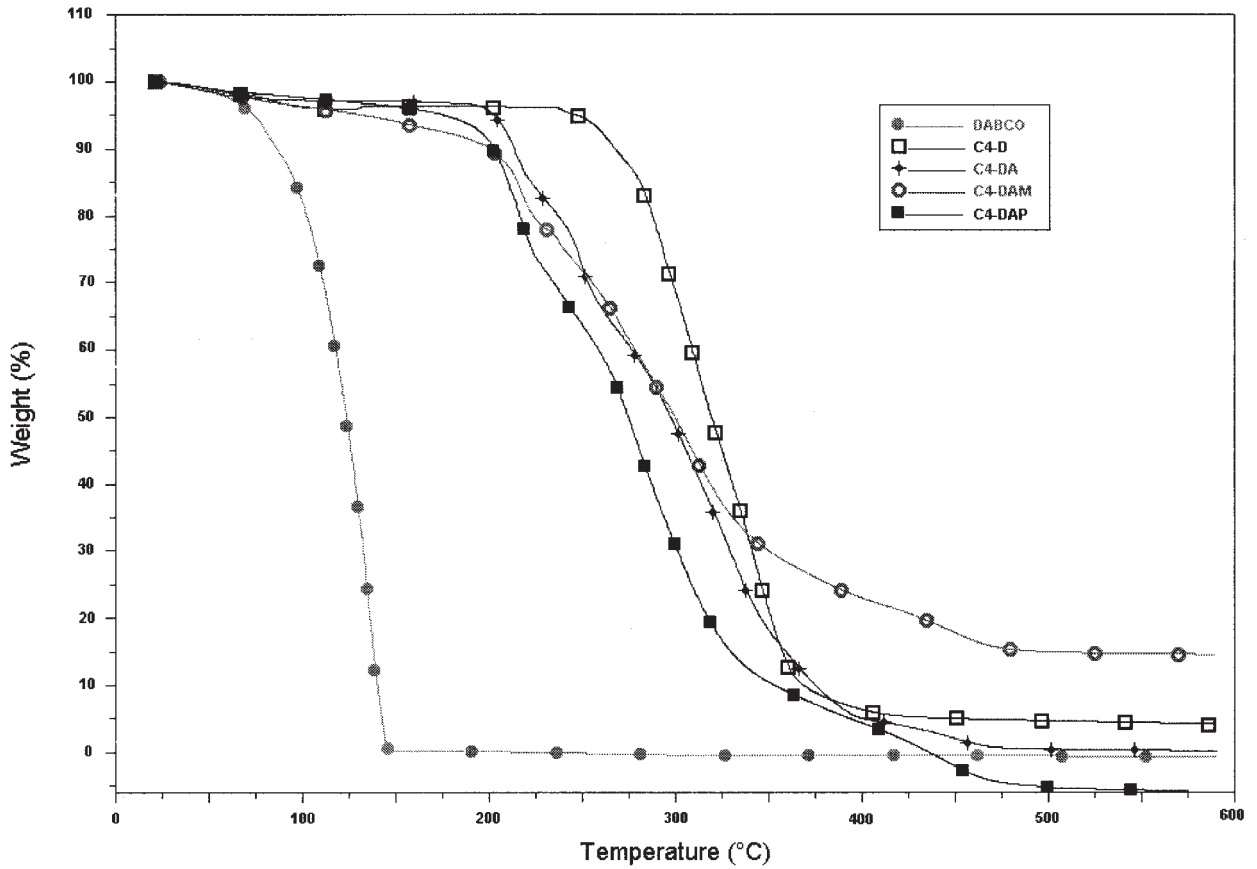


Figure 3 TGA results of DABCO, C4-D, C4-DA, C4-DAM, and C4-DAP.

and 48 ppm. The carbonyl peaks of the polymer are seen together around 175 ppm. The other peaks of the monomer are seen in the same chemical shifts in the polymer.

FTIR was utilized to follow the reaction of C4-D and C6-D with 11-BUA as well as the formation of the monomers and polymers. Appearance of an intense carbonyl stretching peak around 1720 cm^{-1} in the bis-quaternary ammonium carboxylate spectrum shows that the reaction between C4-D and 11-BUA proceeded as shown in Figure 1. It was expected that disappearance of the double bond peaks of the monomers would confirm polymerization; however, the intense H_2O band at around 1600–1650 cm^{-1}, which is the same frequency region as the —C═C— alkene peak (~ 1640 cm^{-1}), makes it difficult to see the latter peak (spectra not shown). On the other hand, the new carbonyl peak of the ester in the FTIR spectra of the monomers appeared at 1740 cm^{-1}. FTIR spectra of the species synthesized all have large water bands around 3500 cm^{-1}, which confirms their hygroscopic nature.

TGA and DSC were used for thermal analysis of both small molecules and polymers. Figure 3 shows the TGA thermograms of DABCO, C4-D, C4-DA, C4-DAM, and C4-DAP. It is seen that the mono-QACs are more stable than the bis-QACs, with the former decomposing at ~ 245°C, and the latter decomposing at ~ 200°C. The monomers and polymers with both butyl and hexyl chains decomposed at temperatures ranging from 190 to 200°C. No glass transition (T_g) or melting temperatures (T_m) were observed by DSC for any monomer or polymer, probably because of plasticization by residual moisture.

Antimicrobial assessment

The screening of the compounds for antimicrobial activity was done by using *S. aureus* and *E. coli* as test organisms because they represent Gram-positive and Gram-negative bacteria, respectively. *S. aureus* and *E. coli* are also two of the most common nosocomial (originating in a hospital) pathogens.[39,40] All QACs containing butyl and hexyl groups were water-soluble and the antimicrobial tests were carried out in water. The MIC values for these compounds were determined by using the broth dilution method. We used test bacterium as negative control and TSB inoculated with test bacterium as positive control. The polymer concentrations ranged from 1 mg/mL to 3.9 μg/mL, which were obtained by twofold serial dilutions. Each solution in the series was mixed with 10^5 CFU of the test organism in a 96-well microtiter plate. The 96-well

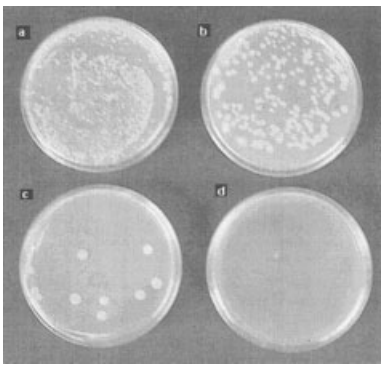


Figure 4 MBC test results: growth of *S. aureus* at (a) 250 μg/mL C4-DAP, (b) 500 μg/mL C4-DAP, (c) 1000 μg/mL C4-DAP, and (d) 62.5 μg/mL C6-DAP.

plate was incubated at 37°C for 24 h. The MIC was the lowest concentration with no visible growth. The growth of bacteria was observed in the cells with lower concentrations than MIC. The solutions with no visible growth were then spread on agar plates and incubated at 37°C for 24 h to obtain MBC values. The MIC and MBC concentrations were also determined for bis-quaternary ammonium monocarboxylates. However, they did not have any antimicrobial activity against *S. aureus* and *E. coli* at concentrations studied.

The C4-DAP inhibited the growth of *S. aureus* and *E. coli* at 250 μg/mL concentration. The MBC value of C4-DAP against *S. aureus* was the same as its MIC value. The MBC value of the C4-DAP against *E. coli* was found to be higher than 1 mg/mL. However, increasing the concentration of C4-DAP from 250 to 500 and 1000 μg/mL decreased the number of CFU on the TSA plates, as shown in Figure 4a–c. C4-DAP has a moderate activity against these two types of bacteria.

The MIC values of C6-DAP were much lower than for C4-DAP and were found to be 62.5 μg/mL against both *S. aureus* and *E. coli*. The MBC values for C6-DAP against both bacteria types were the same as its MIC values. There was no growth on TSA plates at 62.5 μg/mL polymer concentration against both bacteria types as shown in Figure 4d. The MIC and MBC values of C6-DAP are good compared to those of other commonly used quaternary ammonium compounds, which have MIC values of 59–156 μg/mL.[41] The polymer with a hexyl pendant group gives surprisingly good results with a linker group based on 11-bromoundecanoic acid, which has a similar structure to the hydrophobic part of the phospholipids located in the cell membrane of bacteria. This structural similarity may affect the incorporation of the polymers into the bacterial cell membrane, which in turn can change the diffusion characteristics of the polymers. As a result, the antimicrobial activities of the polymers may show a different trend on changing the alkyl chain length attached to one of the nitrogens of DABCO.

The lethal action of cationic biocides is mechanistically complex. Biocide target sites are the cytoplasmic membranes of bacterial cells and the following elementary processes were identified as modes of action: (1) adsorption onto the bacterial cell surface; (2) diffusion through the cell wall; (3) binding to the cytoplasmic membrane; (4) disruption of the cytoplasmic membrane; (5) release of the cytoplasmic constituents such as K^+ ions, DNA, RNA; and (6) death of the cell.[1,8] An increase in charge density of the cationic biocides increases their adsorption to negatively charged bacterial cell surfaces. Therefore, it is reasonable to assume that the adsorption to bacterial cell surfaces is enhanced for DABCO-based QACs compared to monocationic antimicrobial agents. Going from monomers to polymers also increases local charge density enormously. The formation of polymers results in a concentrated assembly of pendant groups, which should increase the ionic interaction of the polymers with bacterial cell surfaces. Better incorporation of the DABCO-based polymers due to structural similarities of the polymer pendant group and the lipid bilayer will also enhance their diffusion into the bacterial cell wall due to combined ionic and van der Walls interactions. Enhancement in ionic interactions and the incorporation should also boost up the binding of the polymers to the cytoplasmic membrane of the bacteria because there are many negatively charged species present in the cytoplasmic membrane, such as acidic phospholipids and membrane proteins. The disruption of the cytoplasmic membrane and the release of the cytoplasmic constituents should also be favored for polymeric DABCO-based QACs because the concentration of cationic groups bound to the cytoplasmic membrane will be higher with these compounds.

CONCLUSION

New methacrylate polymers were synthesized and tested for antimicrobial activities. They showed antimicrobial activities against *S. aureus* and *E. coli* and the activity increased as the alkyl chain length attached to one of the nitrogens increased from four to six carbons. The results are very encouraging because QACs with short chains are generally not active against bacteria. C6-DAP especially has good MIC and MBC values against both *S. aureus* and *E. coli*. MIC and MBC tests for corresponding small molecules (bis-quaternary ammonium monocarboxylates) showed no antimicrobial activity.

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