

TEMPERATURE RESPONSIVE METHACRYLAMIDE POLYMERS WITH ANTIBACTERIAL ACTIVITY*

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Abstract A new methacrylamide monomer, hexylamine methacrylamide (MAHA), was synthesized and used in polymerizations. The homopolymer of MAHA and its copolymers were synthesized by free radical polymerization techniques with *N*-isopropyl acrylamide (NIPAAm) in two different compositions. The quaternization of the homopolymer and copolymers were carried out using 1-bromopropane. The copolymers with NIPAAm and a low MAHA content showed temperature-responsive behavior in an aqueous environment. The lower critical solution temperatures (LCSTs) of these polymers varied between 32°C and 44°C. The LCSTs of quaternized copolymers were higher than those of neutral copolymers because they were more hydrophilic. The obtained homopolymers and copolymers were tested for antibacterial activities against *S. aureus* and *E. coli*. The quaternized water-soluble copolymers showed antibacterial activities against *S. aureus*. The quaternization resulted in the synthesis of both antibacterial and temperature-responsive copolymers.

Keywords: Methacrylamide; NIPAAm; Antibacterial; Temperature-responsive.

INTRODUCTION

Antibacterial polymers have the advantages of enhanced antibacterial activity, reduced residual toxicity, increased efficiency and improved selectivity^[1, 2]. Antibacterial polymers have been used as coatings in many areas such as food processing, filters and biomedical devices^[3]. They have also been used as preservatives and disinfectants in the pharmaceutical field and in the textile industry in the form of antibacterial fibers. Among the commonly used low-molecular-weight antibacterial agents, quaternary ammonium compounds have been the most widely used agents^[4]. They have advantages over other antibacterial agents in terms of better cell membrane penetration, lower toxicity and corrosivity, good environmental stability, lack of skin irritation and extended biological activity^[5]. In order to be utilized in biomedical applications, *e.g.*, controlled-release drugs, it would be better to have the lower critical solution temperature (LCST) of the polymer near body temperature. The homopolymer of *N*-isopropyl acrylamide (NIPAAm), PNIPAAm, is one of the most extensively studied polymers with an LCST of 32°C^[6, 7]. Several copolymers of NIPAAm have been synthesized to change the LCST. It has been found that the use of hydrophilic comonomers with NIPAAm increases the LCST of the copolymers, whereas the use of hydrophobic monomers decreases the LCST^[7, 8].

The purpose of this study was to design antibacterial and temperature-responsive polymers. In this study, we first synthesized a new acrylamide monomer, hexylamine methacrylamide (MAHA), then copolymerized it with NIPAAm in two different compositions by conventional free radical polymerization techniques^[9, 10]. The copolymers with a high NIPAAm content and a low MAHA content showed temperature-responsive behavior in an aqueous environment. We then prepared antibacterial quaternary ammonium salts of methacrylamide

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polymers with temperature-responsive behavior. The quaternization of these polymers was carried out using bromopropane and resulted in the synthesis of both antibacterial and temperature-responsive polymers^[3, 11]. The obtained polymers were tested for antibacterial activities against *S. aureus* and *E. coli*. The minimum bactericidal concentration values were determined for water-soluble copolymers using broth dilution^[12] and spread plate methods^[13].

The synthesized polymers were characterized by FT-IR and NMR to identify the chemical structures. TGA and DSC were used to measure the thermal properties. Their temperature-responsive and antibacterial properties are presented and discussed.

EXPERIMENTAL

Materials

Chemicals of high purity were purchased from various commercial sources, which include methacrylic anhydride (Aldrich), 1,6-diaminohexane (Aldrich), 2,2'-azobis-isobutyronitrile (AIBN) (Showa, Japan), *N*-isopropylacrylamide (NIPAAm) (Aldrich), triethylamine (Tedia) and 1-bromopropane (Fluka). Solvents: methanol, ether, dichloromethane, 1,4-dioxane, petroleum ether, *N,N*-dimethylformamide (DMF) and *n*-hexane (all from Acros) were used as received.

Synthesis of Hexylamine Methacrylamide (MAHA)

1,6-Diaminohexane (29.1 g, 0.25 mol) and triethylamine (25.3 g, 0.25 mol) were mixed in methylene chloride (80 mL) in a 500 mL round-bottom flask. The flask was kept in an ice bath. Methacrylic anhydride (38.6 g, 0.25 mol) was added to the mixture dropwise over 30 min. The mixture was allowed to react for 30 min at 0°C and at ambient temperature overnight. After completion of the reaction, the reaction mixture was extracted with water, and the organic layer was separated and extracted two more times with water. Finally, the methylene chloride was evaporated using a rotary evaporator to give a solid product. Yield: 89%.

Synthesis of Homopolymer

MAHA (18.6 g, 0.1 mol) and AIBN (0.16 g, 1.0 mmol) were dissolved in 80 mL of 1,4-dioxane in a 250 mL round-bottom flask. The flask was purged with nitrogen. The temperature was raised to 70°C, and the mixture was stirred for 24 h. 1,4-Dioxane was evaporated using a rotary evaporator. The solid product was dissolved in DMF and precipitated in ether. The product was then reprecipitated from methanol with petroleum ether twice. The final white solid product was dried in a vacuum oven at room temperature to give the homopolymer. Yield: 51%.

Synthesis of NIPAAm/MAHA Copolymer (90/10 Composition)

In a 250 mL round-bottom flask, MAHA (1.85 g, 0.01 mol), NIPAAm (10.2 g, 0.09 mol), and AIBN (0.32 g) were dissolved in 120 mL of DMF. The flask was purged with nitrogen, the temperature was maintained at 70°C, and the mixture was stirred for 24 h. The final mixture was precipitated into 50 mL of diethyl ether. It was then dissolved in methanol (50 mL), and reprecipitated in petroleum ether. A rotary evaporator was used to remove the solvents. The final product was dried in a vacuum oven at ambient temperature to give the 90/10 copolymer. Yield: 56%.

Quaternization of NIPAAm/MAHA 90/10 Copolymer

In a 250 mL round-bottom flask purged with nitrogen, NIPAAm/MAHA 90/10 copolymer (6 g, 0.05 mol) and 1-bromopropane (6.15 g) were mixed in a solvent mixture of methanol (30 mL) and acetonitrile (18 mL). The mixture was stirred at 60°C for 48 h. At the end of the reaction, the mixture was precipitated into diethyl ether. The solid product obtained was dissolved in 50 mL of methanol and reprecipitated in ether. A rotary evaporator was used to remove solvents. Finally, the product was dried in a vacuum oven at 40°C for 8 h and at room temperature overnight.

Synthesis of NIPAAm/MAHA Copolymer (70/30 Composition)

In a 250 mL round-bottom flask, MAHA (5.58 g, 0.03 mol), NIPAAm (7.93 g, 0.07 mol) and AIBN (0.16 g) were dissolved in 120 mL DMF. The flask was purged with nitrogen, the temperature was maintained at 70°C,

and the mixture was stirred for 24 h. The final mixture was precipitated into 50 mL diethyl ether. The mixture was then dissolved in methanol, and reprecipitated into petroleum ether. A rotary evaporator was utilized to remove solvents, and *n*-hexane was added to solidify the product. The solid product was dried in a vacuum oven at ambient temperature. Yield: 55%.

A similar quaternization procedure was used for the 70/30 copolymer.

Characterization

Fourier transfer infrared (FTIR) spectra were recorded on a Bio-Rad Digilab FTS-40 spectrometer. ^1H -NMR spectra were performed on a Bruker AMX-400 spectrometer with DMSO-d_6 as the solvent. Thermogravimetric analysis (TGA) was performed with a Perkin-Elmer Pyris 1 TGA at a heating rate of 20 K/min in N_2 . Differential scanning calorimetry (DSC) data were obtained from a Perkin-Elmer Pyris Diamond DSC. Samples were scanned at a heating rate of 10 K/min under N_2 . The T_g values were measured as the change of the specific heat in the heat flow curves. Molecular weight was determined by gel permeation chromatography (GPC) with polystyrene calibration using a Perkin-Elmer series 200 HPLC system equipped with a Jordi Gel DVB column at 40°C in THF.

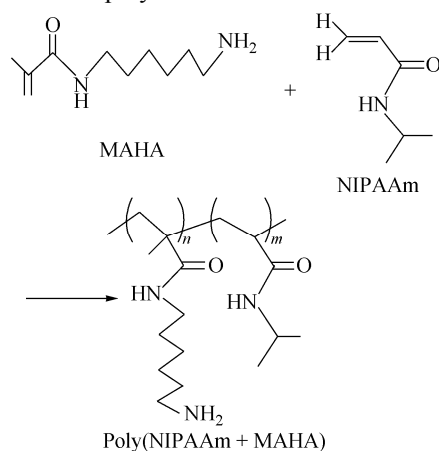
Antibacterial Evaluation

The antibacterial activities of the water-soluble copolymers against *S. aureus* and *E. coli* were determined using broth dilution and spread plate methods. The species used for *E. coli* and *S. aureus* were ATCC 25922 and ATCC 25923, respectively. *E. coli* and *S. aureus* were streaked out on tryptic soy agar (TSA) plates and incubated at 30–35°C for 24 h. Cultures of *S. aureus* and *E. coli* containing 10^7 colony-forming units (CFU) per mL were lifted off with a wire loop and placed in 5 mL of tryptic soy broth (TSB) that were used for antibacterial tests. A range of concentrations from 20000 $\mu\text{g/mL}$ to 39 $\mu\text{g/mL}$ of quaternary ammonium salt of 90/10 copolymers were prepared using sterile deionized water in a 96-well microtiter plate (ELISA plate). The test organisms (2×10^5 CFU, 20 μL TSB) were added into each well. In the end, each well contained 180 μL of water, 20 μL of TSB, and the test organism. The ELISA plates were incubated at 30–35°C for 24 h. At the end of this time period, a small amount of the mixture from each well was pulled out and spread on agar plates using a swab. The plates were incubated at 35°C for 48 h with CO_2 breathing (since both organisms are facultatively anaerobic bacteria) and the growth of bacterial cells was observed on agar plates. The lowest concentration of antibacterial copolymer at which no growth was observed was determined as the minimum bactericidal concentration (MBC). The same procedure was used for the quaternary ammonium salt of 70/30 copolymer.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Monomer and Polymers

The synthesis of monomer MAHA and its copolymers is illustrated in Scheme 1.



Scheme 1 Synthesis of the monomer MAHA and its copolymers

FT-IR was utilized to follow the polymerizations and quaternization of copolymers. The FT-IR spectrum of MAHA is shown in Fig. 1. The peak assignments were made as follows: (1) C=C stretching in $\text{CH}_2=\text{R}_1\text{R}_2$ (928 cm^{-1}), (2) symmetric C—N stretching vibration (1372 cm^{-1}), (3) CH_2 scissors vibration and CH_3 antisymmetric deformation (1447 cm^{-1}), (4) N—H deformation bend (1540 cm^{-1}), (5) C=C stretching in methacrylamide (1615 cm^{-1}), (6) C=O stretching (1656 cm^{-1}), (7) aliphatic CH, CH_2 , CH_3 symmetric and antisymmetric stretching ($2850\text{--}3000\text{ cm}^{-1}$), and (8) N—H stretching (3331 cm^{-1}). Upon polymerization, the C=C stretching peaks 1 and 5 of MAHA disappeared. The polymers had broader amide C=O stretching (peak 6) compared to the monomer MAHA. For the polymers, the absorption bands of these FT-IR spectra were quantified by both absorption heights and areas. Band 7 from $2850\text{--}3000\text{ cm}^{-1}$ represented C—H absorption from methyl and methylene groups. Band 6 at 1656 cm^{-1} represented C=O bands of amide groups and was used as an internal reference to facilitate semi-quantitative analysis. Absorption band height and absorption area were highly correlated, with a correlation coefficient of 0.98. For absorption area, the band 7/6 ratio was positively correlated with the amount of MAHA in the polymers. The NH stretching peak of the monomer appeared at around 3330 cm^{-1} (peak 8). This peak was broader with higher intensity in the polymers compared to the monomers.

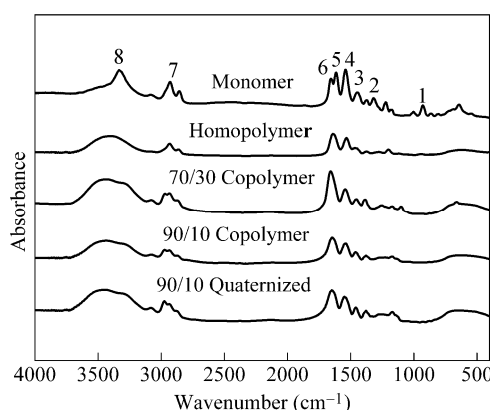


Fig. 1 FT-IR spectra of MAHA and its copolymers

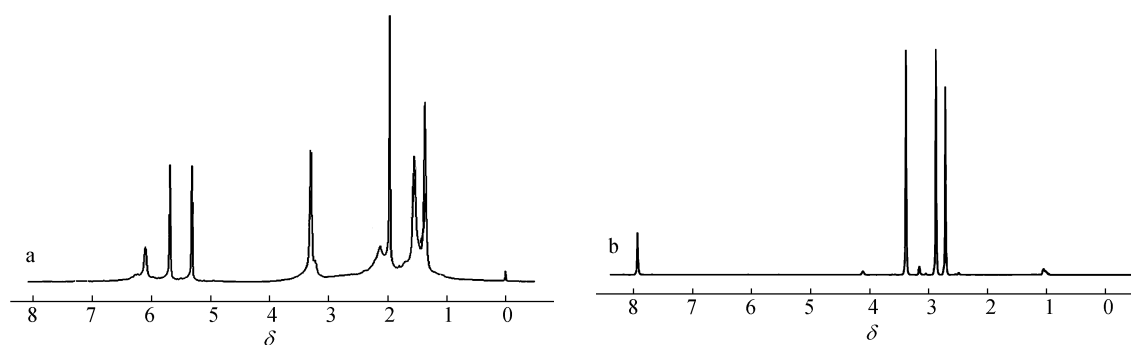


Fig. 2 ^1H -NMR spectra of the (a) monomer MAHA and (b) homopolymer

In Fig. 2, the ^1H -NMR spectra of the monomer and homopolymer are shown. Upon polymerization, the monomer MAHA double-bond peaks observed at $\delta = 5.3$ and 5.7 disappeared. The amide peak shifted from $\delta = 6.1$ in the monomer to $\delta = 7.9$ in the polymers. In the polymers, the methylene peak next to the amide group shifted upfield and broadened. The methyl peak of the monomer shifted from $\delta = 1.9$ to 1.1 in the polymers. For the ^1H -NMR spectra of the 90/10 and 70/30 copolymers, as the content of the MAHA increased from 10% to 30%, the intensities increased for the amide peak, methyl peak and methylene peak next to the amide in the

MAHA. The other peaks of the NIPAAm unit were observed in the copolymer spectrum at $\delta = 1.0$ (as showed in Fig. 3). As the neat copolymer was quaternized with bromopropane, the methylene peak was observed in the $^1\text{H-NMR}$ spectra of quaternized copolymers at $\delta = 1.2$.

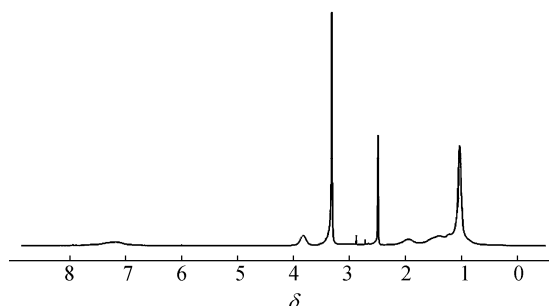


Fig. 3 $^1\text{H-NMR}$ spectra of the 90/10 copolymer

TGA and DSC were used for thermal analysis of the polymers. Figure 4 shows both the TGA thermograms and 10% decomposition temperatures ($T_{d,10}$) of neat and quaternized polymers. The $T_{d,10}$ values for the 90/10 and 70/30 copolymers, and quaternized 90/10 and 70/30 copolymers were 383°C, 386°C, 355°C, 348°C, respectively. The $T_{d,10}$ values for the quaternized polymers were lower than those of the neat polymers. The hydrogen bonding between the methacrylamide groups of the polymers was more dominant than the van der Waals interactions between the aliphatic side chains in determining the T_g values of the copolymers. The T_g value for MAHA homopolymer was 133°C, while no T_g s were observed for the 90/10 and 70/30 copolymers and quaternized copolymers. This might be due to the plasticization effect of the aliphatic side chains and/or water that was absorbed by the samples.

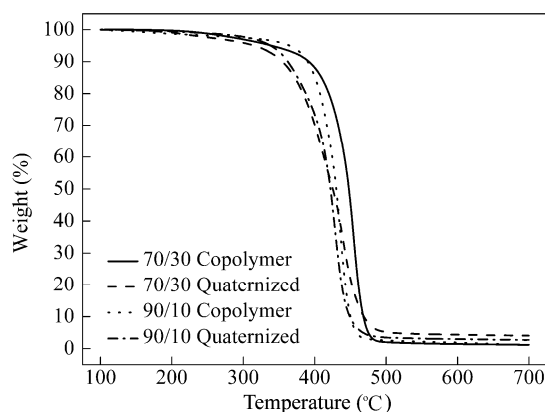


Fig. 4 TGA thermograms of the 90/10 and 70/30 copolymers, and quaternized 90/10 and 70/30 copolymers

Figure 5 demonstrates that the 90/10 copolymer showed a transition at temperatures between 32°C and 36°C. The copolymer was water-soluble below 32°C and water-insoluble above 36°C. As the temperature was increased above 36°C, a white dispersion was formed. The phase transition temperatures were also visibly observed for the quaternized 90/10 and 70/30 copolymers (Fig. 5). As we know^[14], at temperatures below LCST, PNIPAAm hydrogels show water-swollen property due to the hydrogen bond interactions between water and hydrophilic amide groups of the polymer. At temperatures near or higher than LCST, hydrophobic interactions between the isopropyl pendant groups increase and the hydrogels appear to be hydrophobic and the PNIPAAm start to aggregate and phase separation takes place. Upon quaternization of the 90/10 copolymer, the

phase transition temperature increased. This could be explained by the fact that quaternized polymers are more hydrophilic than the neat copolymers. The phase transition temperature decreased as the content of MAHA increased. This is due to the fact that the increased hydrophobicity of the copolymer resulted in a decrease of the phase transition temperature.

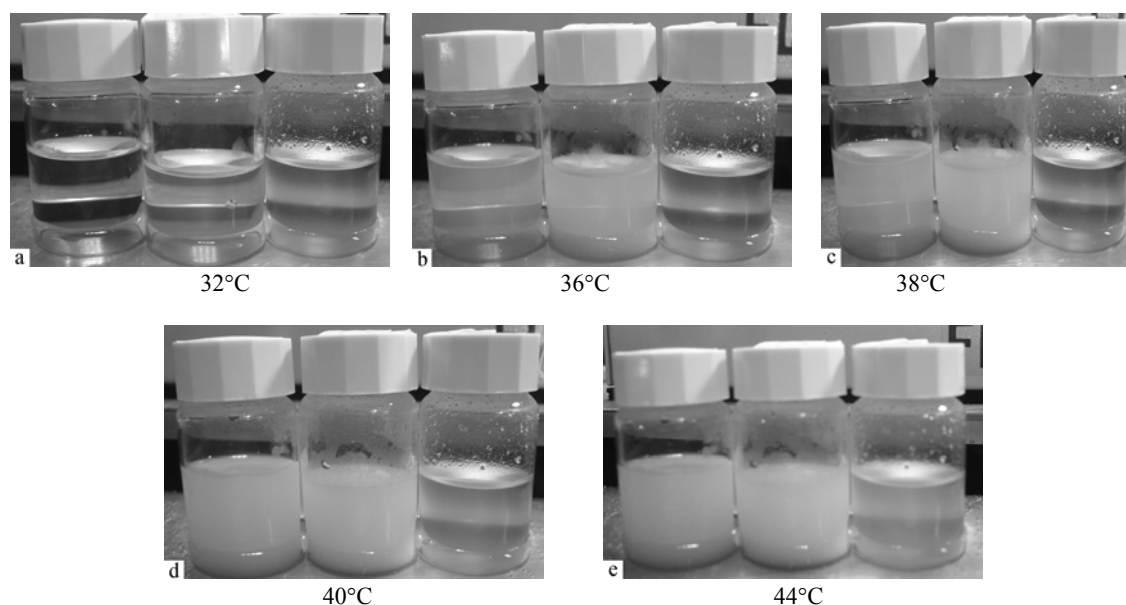


Fig. 5 Phase transition temperatures for copolymers (left to right in each photo: 90/10 neutral copolymer, 70/30 quaternized, 90/10 quaternized)

Antibacterial Evaluation

The antibacterial activities of the water-soluble copolymers against *S. aureus* and *E. coli* were determined by testing different concentrations of the copolymers using broth dilution and spread plate methods. A range of concentrations of each copolymer was prepared according to the experimental procedure described earlier. The addition of TSB into the wells of an ELISA plate containing different concentrations of the copolymers in water resulted in precipitation of the copolymers. The agar plates showing the MBC results of quaternized water-soluble 90/10 copolymers against *S. aureus* and *E. coli* are shown in Fig. 6. The minimum bactericidal concentration (MBC) values of the quaternized water soluble copolymers obtained from broth dilution and spread plate tests are summarized in Table 1. For the antibacterial activity against *S. aureus*, the 90/10 and 70/30 quaternized water-soluble copolymers had MBC values of 625 and 1250 $\mu\text{g/mL}$, respectively. There was no antibacterial activity against *E. coli* for both quaternized copolymers. This could be attributed to the fact^[15] that *S. aureus* has better absorption of the quaternary ammonium compounds, while *E. coli* has decreased absorption of which from its cell wall. So, even though *E. coli* has a much thinner cell wall, it does not absorb quaternary ammonium compounds, making these compounds less effective. The 70/30 quaternized copolymer had higher MBC value against bacteria. This could be explained by the fact that the copolymer precipitated at the test temperature (35°C). Solid copolymers interact less with bacteria when compared to the solution state, and antibacterial activities might be reduced. However, considering that only around 10% of the copolymer composition contains MAHA, the copolymer shows relatively good antibacterial activities against *S. aureus*.

Table 1. Minimum bactericidal concentrations of the water-soluble quaternized copolymers against *S. aureus* and *E. coli*

Quaternized polymer	MBC ($\mu\text{g/mL}$)	
	<i>S. aureus</i>	<i>E. coli</i>
90/10	625	> 5000
70/30	1250	> 5000

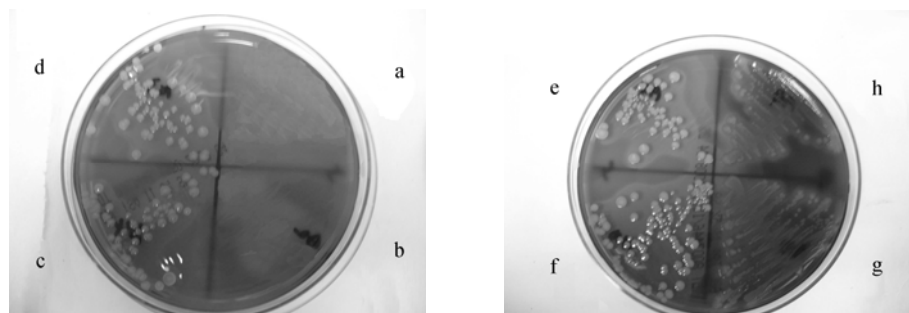


Fig. 6 The MBC results of the quaternized water-soluble 90/10 copolymer against *S. aureus* (a–f) and *E. coli* (g–h). The concentrations for *S. aureus* are (a) 1250, (b) 625, (c) 312.5, (d) 156.25, (e) 78.125, and (f) 39 µg/mL; The concentrations for *E. coli* are (g) 2500 and (h) 5000 µg/mL.

The antibacterial mechanism of polymers containing quaternary ammonium compounds is complex. The target sites of these polymers are the cytoplasmic membranes of bacterial cells. The polymers are adsorbed and diffuse through the surface of the bacterial cell wall, bind to the cytoplasmic membrane, and disrupt the membrane. This causes the release of the bacterial cytoplasmic constituents and results in death of the bacteria^[16, 17]. The same type of mechanism is expected for the synthesized temperature responsive polymers. As the results demonstrate, the water-soluble polymers produce better antibacterial activity compared to water-insoluble polymers.

CONCLUSIONS

A new methacrylamide monomer (MAHA) was synthesized, then homopolymerized and copolymerized with NIPAAm at two different compositions by free radical polymerization. The quaternization of the copolymers was performed using bromopropane. The quaternized copolymers with high NIPAAm and low MAHA content were water-soluble and showed temperature responsive behavior. The quaternized water-soluble copolymers showed good antibacterial activities against *S. aureus*. The neutral and water-insoluble polymers did not show any antibacterial activities, as expected. These polymers may be candidates for biomedical applications.

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