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ORIGINAL ARTICLE

Antibacterial Activities of Five Cationic Gemini Surfactants with Ethylene Glycol Bisacetyl Spacers

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Abstract A series of cationic gemini surfactants containing two dimethylalkylammonium chains linked by ethylene glycol bisacetyl spacers were synthesized [$G_{m\text{-}AnA\text{-}m}$, G = gemini surfactant, m = 12 ($-C_{12}H_{25}$), 14 ($-C_{14}H_{29}$), or 16 ($-C_{16}H_{33}$), A = acetyl, and n = 2, 3, or 4 is the number of ethylene glycol units in the spacers]. Because of the inductive effect of the oxygen atom in the spacer, acylation can take place using chloroacetyl chloride instead of bromoacetyl bromide which helps to limit the use of environmentally harmful reagents. Critical micelle concentrations were determined using conductivity measurements. The antibacterial activities of the surfactants against Gram-positive bacterium *Staphylococcus aureus* and Gram-negative bacterium *Escherichia coli* were evaluated from the minimum inhibitory concentration (MIC), minimum bacterial concentration, a time–kill study, and the inhibitory zone. Increasing the length of the spacer did not result in an obvious change of antibacterial activity. However, increasing the length of the alkyl chain apparently increased the antibacterial activity against *S. aureus* but decreased the antibacterial activity against *E. coli*. The $G_{12\text{-}A2A\text{-}12}$ surfactant had the lowest CMC of 1.26 mmol L^{-1} and exhibited the best antibacterial activity with a MIC of 32 μg mL^{-1} toward *S. aureus* and 64 μg mL^{-1} toward *E. coli* in the presence of 10^5 CFU of bacteria. This work indicated that these cationic gemini surfactants have potential applications as antibacterial agents and emulsifiers.

Keywords Quaternary ammonium salt · Cationic gemini surfactant · Ethylene glycol bisacetyl spacer · Spacer chain length · Alkyl chain length · Antibacterial activity

Abbreviations

QACs	Quaternary ammonium compounds
$G_{12\text{-}A2A\text{-}12}$	Diethylene glycol bisacetateyl-α,ω-*bis* (dimethydodecylammonium choride)
$G_{12\text{-}A3A\text{-}12}$	Triethylene glycol bisacetateyl-α,ω-*bis* (dimethydodecylammonium choride)
$G_{12\text{-}A4A\text{-}12}$	Tetraethylene glycol bisacetateyl-α,ω-*bis* (dimethydodecylammonium choride)
$G_{14\text{-}A2A\text{-}14}$	Diethylene glycol bisacetateyl-α,ω-*bis* (dimethytetradecylammonium choride)
$G_{16\text{-}A2A\text{-}16}$	Diethylene glycol bisacetateyl-α,ω-*bis* (dimethyhexadecylammonium choride)
^{1}H NMR	^{1}H Nuclear magnetic resonance
FTIR	Fourier transform infrared spectroscopy
CMC	Critical micelle concentration
α_{cb}	Counterion dissociation constant
β_{cb}	Counterion binding coefficient
E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
MIC	Minimum inhibitory concentration
MBC	Minimum bacterial concentration
TSA	Tryptic soy agar
TSB	Tryptic soy broth
LPS	Lipopolysaccharide

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Introduction

We are inevitably exposed to a variety of bacteria, fungi, and other microorganisms. The resistance of pathogens to antibacterial agents is a growing concern in public health [1]. Antimicrobial materials are a group of functional materials that possess properties that can kill or inhibit microbial growth and have applications in medicine, household articles, and food packaging among others [2, 3]. However, like other chemical syntheses, methods for the preparation of antibacterial materials are sought which minimize the impact on the environment.

During the past two decades, numerous antimicrobial agents, such as quaternary ammonium compounds (QACs) [4, 5], fluoroquinolones [6], oxazolidinones [7], antibacterial polymers [8, 9], and transition metal complexes [10, 11], have been developed. Among these, QACs and polymers with QA substituents have been extensively used because of their many merits. In general, QACs possess both a positively charged hydrophilic ammonium group and a long hydrophobic alkyl chain [12] which provides a hydrophobic segment compatible with the bilayer of the outer cell wall [13, 14]. QACs have several advantages over other antibacterial agents, including excellent cell membrane penetration properties, low toxicity, environmental stability, low skin irritability, low corrosiveness, extended residence time, and favorable biological activity. The antibacterial activity of QACs strongly depends on the nature of the organic groups attached to the nitrogen, the number of nitrogen atoms present, and the alkyl chain length. Many experiments have shown that QACs containing one long alkyl chain substituent of at least eight carbon atoms are effective biocides [15]. However, the use of conventional QACs is still limited by the development of microbial resistance [16]. Recently, particular attention has been directed toward cationic gemini surfactants as new types of QACs which exhibit better antibacterial activities than conventional surfactants [17, 18].

Gemini surfactants are bis-QACs composed of two hydrophilic polar head groups and two hydrophobic hydrocarbon chains covalently connected by a spacer [19, 20]. Their generally accepted mechanism of action is that the cationic sites of the QACs are adsorbed onto anionic sites of the cell wall surface by electrostatic interactions. Then a significant lipophilic component promotes diffusion through the cell wall and the QAC binds to the cell membrane, disrupting the cytoplasmic membrane and causing the release of electrolytes and nucleic materials which results in bacteria death [21, 22]. Adsorption to negatively charged bacterial cell surfaces is expected to increase with an increase in the charge density of the cationic biocides. Because of the spacer chain in gemini surfactants, the two hydrophilic polar head groups are so close that the charge densities of these surfactants are higher than those of conventional surfactants. Therefore, it is reasonable to assume that gemini surfactants would be able to more rapidly adsorb to the bacteria surface than conventional surfactants and result in bacteria death. There are many reports discussing the antimicrobial activity of QACs, but a complete understanding of their structure–activity relationship is lacking [23]. In this work, the effects of different lengths of the alkyl chain and spacer chain in five gemini surfactants on the bactericidal activities were evaluated by determining the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the surfactants, performing a time–kill study, and evaluating the inhibitory zone against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*.

Experimental

Materials

Chloroacetyl chloride was purchased from Shanghai Jinshan Ting New Chemical Plant Reagent Factory, Shanghai, China. Monochloroacetic acid was purchased from Chengdu Kelong Chemical Reagent Factory, Chengdu, China. Diethylene glycol, triethylene glycol, tetraethylene glycol, dodecyldimethyl tertiary amine, tetradecyldimethyl tertiary amine, and hexadecyldimethyl tertiary amine were purchased from Aladdin, Shanghai, China. Acetone and petroleum ether were obtained from Tianjin Bodi Chemical Co. Ltd., Tianjin, China. All chemicals were reagent grade and used without further purification except for acetone which was purified by reflux distillation. The bacterial strains used for antibacterial activity tests, *S. aureus* RN4220 and *E. coli* TOP10, were by the Faculty of Life Science, Hubei University, Wuhan, China. Tryptic soy agar (TSA) and tryptic soy broth (TSB) were purchased from Aladdin, Shanghai, China. Sodium chloride was purchased from Sinopharm Chemical Reagent Co., Ltd., Tianjin, China. Peptone and yeast extract powder were purchased from Beijing Twin Twist Microbial Culture Medium Products Factory, Beijing, China. Agar was purchased from Biosharp, Japan. The 96-well microtiter plates were from WHB, Shanghai, China.

Synthesis

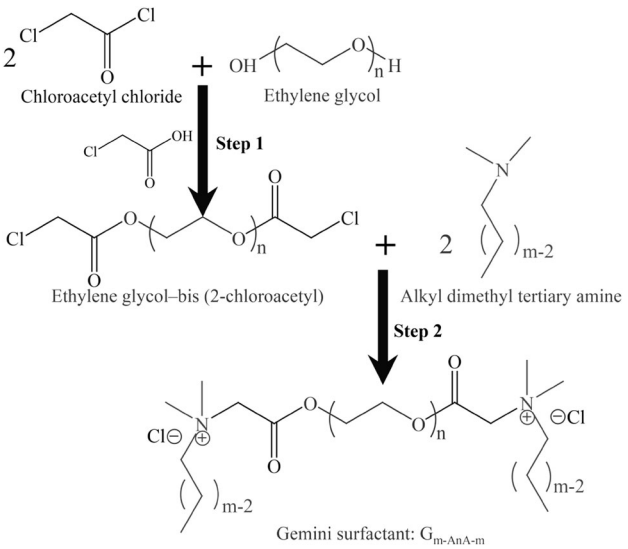
Synthesis of Diethylene Glycol-bis(2-Chloroethanoate), Triethylene Glycol-bis(2-Chloroethanoate), and Tetraethylene Glycol-bis(2-Chloroethanoate) (Step 1: Acylation)

Monochloroacetic acid (0.6 mol) and chloroacetyl chloride (0.88 mol) were added to a 250-mL flask equipped with

reflux condenser, dropping funnel, and thermometer fixed on a magnetic stirrer. The solution mixture was stirred and the temperature was adjusted to 55 °C to dissolve the monochloroacetic acid completely, and then diethylene, triethylene, or tetraethylene glycol (0.4 mol) was added from a dropping funnel over 2 h. After 1 h, the reaction was stopped and the mixture was poured into ice water with stirring until the excess chloroacetyl chloride was completely hydrolyzed. Finally, the ethylene glycol-bis(2-chloroethanoate) was obtained by extraction, separation, and drying. The light yellow liquids, diethylene glycol-bis(2-chloroethanoate) (65.7 %), triethylene glycol-bis(2-chloroethanoate) (63.2 %), and tetraethylene glycol-bis(2-chloroethanoate) (57.8 %), were obtained in the indicated yields [24].

Synthesis of Ethylene Glycol Bisacetyl-α,ω-bis(dimethylalkylammonium Choride) (Step 2: Quaternization)

Dodecyldimethyl tertiary amine, tetradecyldimethyl tertiary amine, or hexadecyldimethyl tertiary amine (0.36 mol) and acetone (50 g) were added into a 250-mL flask equipped with a reflux condenser fixed on a magnetic stirrer. The solution mixture was stirred and then the glycol-bis(2-chloroethanoate) (0.15 mol) was added to the flask. After refluxing for 24 h, the mixture was placed at 4 °C until the product precipitated. The precipitate was washed several times with petroleum ether. It was dried in a vacuum oven at 45 °C for 12 h to remove any trapped solvents to give the final product. The yellow viscous liquids [$G_{12\text{-}A2A\text{-}12}$, 96.2 % yield based on diethylene glycol-bis(2-chloroethanoate); $G_{12\text{-}A3A\text{-}12}$, 95.1 % yield based on triethylene glycol-*bis*-(2-chloroethanoate); $G_{12\text{-}A4A\text{-}12}$, 93.5 % yield based on tetraethylene glycol-bis(2-chloroethanoate); $G_{14\text{-}A2A\text{-}14}$, 92.9 % yield based on diethylene glycol-bis(2-chloroethanoate); and $G_{16\text{-}A2A\text{-}16}$, 91.3 % yield based on diethylene glycol-bis(2-chloroethanoate)] were obtained [24, 25] (Scheme 1).



Scheme 1 Synthesis of bis-quaternary ammonium cationic gemini surfactants. Step 1: synthesis of ethylene glycol-bis(2-chloroethanoate) (acylation). Step 2: synthesis of ethylene glycol bisacetyl-α,ω-dimethylalkylammonium chloride (quaternization). $G_{12\text{-}A2A\text{-}12}$ ($n = 2$, A = $-C_{12}H_{25}$, $m = 12$), $G_{12\text{-}A3A\text{-}12}$ ($n = 3$, A = $-C_{12}H_{25}$, $m = 12$), $G_{12\text{-}A4A\text{-}12}$($n = 4$, A = $-C_{12}H_{25}$, $m = 12$), $G_{14\text{-}A2A\text{-}14}$ ($n = 2$, A = $-C_{14}H_{29}$, $m = 14$), $G_{16\text{-}A2A\text{-}16}$ ($n = 2$, A = $-C_{16}H_{33}$, $m = 16$)

Characterization

FTIR spectra were obtained on a Perkin-Elmer Spectrum One. ^{1}H NMR spectra were recorded on a Varian INOVA 600 MHz NMR spectrometer with $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal reference. The critical micelle concentration (CMC) was determined using a DDSJ-308A conductivity meter.

FTIR of Diethylene Glycol-bis(2-Chloroethanoate) and $G_{12\text{-}A2A\text{-}12}$

FTIR spectroscopy was used to follow the synthetic reactions of diethylene glycol-bis(2-chloroethanoate) and $G_{12\text{-}A2A\text{-}12}$. The peak assignments for the glycol-bis(2-chloroethanoate) were as follows:

(a) Diethylene glycol: O–H stretching (3,391 cm^{-1}); C–H symmetric and asymmetric stretching (2,874 cm^{-1}); C–H bending (1,355–1,460 cm^{-1}); H_2C–C–O–C (1,064, 1,120, 1,248 cm^{-1}).

(b) Diethylene glycol-bis(2-chloroethanoate): O–H stretching (3,491 cm^{-1}); C–H symmetric and asymmetric stretching (2,860, 2,960 cm^{-1}); C=O stretching (1,755 cm^{-1}); C–H bending (1,354–1,456 cm^{-1}); O=C–O–C symmetric and antisymmetric stretching (1,170–1,317 cm^{-1}), H_2C–C–O–C (1,034, 1,132 cm^{-1}); C–Cl stretching (700 cm^{-1}).

(c) $G_{12\text{-}A2A\text{-}12}$: C–H symmetric and asymmetric stretching (2,850–2,920 cm^{-1}); C=O stretching (1,750 cm^{-1}); C–H bending (1,350–1,450 cm^{-1}); O=C–O–C symmetric and antisymmetric stretching (1,180–1,300 cm^{-1}), H_2C–C–O–C (1,035, 1,137 cm^{-1}); O–H stretching (3,400 cm^{-1}) probably arising from water because these surfactants were very difficult to dehydrate.

Appearance of the C–Cl stretching band in the FTIR spectra (b) compared with (a) confirmed the successful synthesis of the intermediate product. Disappearance of this band in the spectra for (c) confirmed that the syntheses of these products was likely successful. Other FTIR spectra of gemini surfactants were similar to those of $G_{12\text{-}A2A\text{-}12}$.

Further confirmations of successful syntheses were obtained by ^{1}H NMR spectroscopy.

^{1}H NMR of Diethylene Glycol-bis(2-Chloroethanoate), Triethylene Glycol-bis(2-Chlorotetraethanoate), and Ethylene Glycol-bis(2-Chloroethanoate)

(a) Diethylene glycol-bis(2-chloroethanoate): ^{1}H NMR ($CDCl_3$, ppm), 4.12 (s, 4H, a), 4.35 (t, 4H, b), 3.74 ppm (t, 4H, c) were assigned to $ClCH_2^a COOCH_2^b CH_2^c OCH_2^c CH_2^b OOCCH_2^a Cl$.

(b) Triethylene glycol-bis(2-chloroethanoate): ^{1}H NMR ($CDCl_3$, ppm), 4.11 (s, 4H, a), 4.35 (t, 4H, b), 3.74 (t, 4H, c), 3.66 (t, 4H, d) were assigned to $ClCH_2^a COOCH_2^b CH_2^c OCH_2^d$ $CH_2^d OCH_2^c CH_2^b OOCCH_2^a Cl$.

(c) Tetraethylene glycol-bis(2-chloroethanoate): ^{1}H NMR ($CDCl_3$, ppm), 4.06 (s, 4H, a), 4.30 (t, 4H, b), 3.69 (t, 4H, c), 3.61 (m, 8H, d) were assigned to $ClCH_2^a COOCH_2^b CH_2^c OCH_2^d$ $CH_2^d OOCH_2^d$ $CH_2^d OCH_2^c$ $CH_2^b OOCCH_2^a Cl$.

^{1}H NMR of Gemini Surfactants

(a) Diethylene glycol bisacetyl-α,ω-bis (dimethyldodecylammonium chloride) ($G_{12\text{-}A2A\text{-}12}$): ^{1}H NMR ($CDCl_3$, ppm), 0.89 (t, 6H, a), 1.25–1.35 (m, 36H, b), 1.76 (m, 4H, c), 3.80 (t, 4H, d), 3.56 (m, 12H, e), 5.32 (s, 4H, f), 4.34 (t, 4H, g), 3.75 (t, 4H, h) were assigned to $[CH_3^a (CH_2^b)_9 CH_2^c CH_2^d N^+ (CH_3^e)_2 CH_2^f COOCH_2^g CH_2^h OCH_2^h$ $CH_2^g OOCCH_2^f N^+ (CH_3^e)_2 CH_2^d CH_2^c (CH_2^b)_9 CH_3^a] \cdot 2Cl^-$.

(b) Triethylene glycol bisacetyl-α,ω-bis (dimethyldodecylammonium chloride) ($G_{12\text{-}A3A\text{-}12}$): ^{1}H NMR ($CDCl_3$, ppm), 0.89 (t, 6H, a), 1.25–1.35 (m, 36H, b), 1.76 (m, 4H, c), 3.65 (t, 4H, d), 3.62 (m, 12H, e), 5.28 (s, 4H, f), 4.37 (t, 4H, g), 3.80 (t, 4H, h), 3.72 (t, 4H, i) were assigned to $[CH_3^a (CH_2^b)_9 CH_2^c$ $CH_2^d N^+ (CH_3^e)_2 CH_2^f COOCH_2^g CH_2^h OCH_2^i CH_2^{\,i} OCH_2^h$ $CH_2^g OOCCH_2^f N^+ (CH_3^e)_2 CH_2^d CH_2^c (CH_2^b)_9 CH_3^a] \cdot 2Cl^-$.

(c) Tetraethylene glycol bisacetyl-α,ω-bis (dimethyldodecylammonium chloride) ($G_{12\text{-}A4A\text{-}12}$): ^{1}H NMR ($CDCl_3$, ppm), 0.88 (t, 6H, a), 1.25–1.35 (m, 36H, b), 1.75 (m, 4H, c), 3.76 (t, 4H, d), 3.58 (m, 12H, e), 5.20 (s, 4H, f), 4.40 (t, 4H, g), 3.80 (t, 4H, h), 3.67 (m, 8H, i) were assigned to $[CH_3^a (CH_2^b)_9 CH_2^c CH_2^d$ $N^+ (CH_3^e)_2 CH_2^f COOCH_2^g CH_2^h OCH_2^i CH_2^i OCH_2^i CH_2^{\,i}$ $OCH_2^h CH_2^g OOCCH_2^f N^+ (CH_3^e)_2 CH_2^d CH_2^c (CH_2^b)_9 CH_3^a] \cdot$ $2Cl^-$.

(d) Diethylene glycol bisacetyl-α,ω-bis (dimethyltetradecylammonium chloride) ($G_{14\text{-}A2A\text{-}14}$): ^{1}H NMR ($CDCl_3$, ppm), 0.89 (t, 6H, a), 1.25–1.77 (m, 44H, b), 2.85 (m, 4H, c), 3.13 (t, 4H, d), 3.56(m, 12H, e), 5.39 (s, 4H, f), 4.33 (t, 4H, g), 3.75 (t, 4H, h) were assigned to $[CH_3^a (CH_2^b)_{11} CH_2^c CH_2^d N^+ (CH_3^e)_2 CH_2^f$ $COOCH_2^g CH_2^h OCH_2^{\,h} CH_2^g OCOCH_2^f N^+ (CH_3^e)_2 CH_2^d CH_2^c$ $(CH_2^b)_{11} CH_3^a] \cdot 2Cl^-$.

(e) Diethylene glycol bisacetyl-α,ω-bis (dimethylhexadecylammonium chloride) ($G_{16\text{-}A2A\text{-}16}$): ^{1}H NMR ($CDCl_3$, 400 ppm), 0.88 (t, 6H, a), 1.25–1.76 (m, 52H, b), 2.84 (m, 4H, c), 2.95 (t, 4H, d), 3.54 (m, 12H, e), 5.31 (s, 4H, f), 4.34 (t, 4H, g), 3.75 (t, 4H, h) were assigned to $[CH_3^a (CH_2^b)_{13} CH_2^c CH_2^d N^+ (CH_3^e)_2$ $CH_2^f COOCH_2^g CH_2^h OCH_2^h CH_2^g OOCCH_2^f N^+ (CH_3^e)_2$ $CH_2^d CH_2^c (CH_2^b)_{13} CH_3^a] .2Cl^-$.

Antimicrobial Test

S. aureus and *E. coli* were lifted off with a wire loop and placed in 5 mL of TSB and incubated with shaking at 37 °C until the cultures became turbid. The turbid cultures were then 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 of 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 approximately 10^9 CFU/mL and were used for antibacterial tests.

MIC and MBC Testing

The MIC and MBC tests against *S. aureus* and *E. coli* were performed using different concentrations of gemini surfactants. A range of concentrations (2–1,024 μg mL^{-1}) for each surfactant were prepared with 0.9 % sterilized, saline water. The tested organisms (5×10^5 CFU, 50 μL of TSB) were added to a 96-well microtiter plate. Each well contained 350 μL of surfactant, 50 μL of TSB, and the test organism. The 96-well plate was incubated at 37 °C for 24 h. The 96-well plates were evaluated for growth of bacterial cells and the lowest concentration of the antibacterial agent where no visible growth of bacteria was observed was determined to be the MIC. A small amount of the mixture from each well was removed and spread on solid agar plates. The plates were incubated at 37 °C for 24 h to obtain MBC values. The MIC and MBC tests were repeated at least four times for each antibacterial surfactant. A aliquots of 400 μL of saline water and saline water/TSB containing each test bacterium mixture (350/50 μL) were used as negative and positive controls, respectively [26].

Time–Kill Study

The time–kill study was performed to investigate the killing kinetics of the antibacterial surfactants against *S.*

aureus and *E. coli*. A total of 4.5 mL of six continuous mass concentrations of each antibacterial agent solution were mixed with 0.5 mL of 0.9 % sterilized saline water containing the bacterial suspension (5×10^6 CFU) in culture tubes and used as the blank control. The tubes were incubated at 37 °C for 24 h with shaking. After 24 h, a small amount of solution was removed and the surviving bacteria were then counted by dilution and spread-plate methods. An aliquot of 45 mL of an accurate concentration of the antibacterial agent solution was combined with 5 mL of the 0.9 % sterilized saline water containing the bacterial suspension (5×10^6 CFU) in a conical flask. The mixed solution was incubated with shaking at 37 °C. After 10, 30, 60, 90, 120, 150, and 240 min of incubation, the surviving bacteria were counted. These tests were repeated at least three times [27].

Inhibitory Zone Method

A total of 15 mL of melted TSA was poured into a plate. Then, 300 μL of cell suspension containing 2×10^6 CFU was evenly spread on the surface of the agar plate. Round filter paper ($D = 6$ mm) was placed on the agar plate and then surfactants were added to the filter paper. Every plate included a blank control. All plates were incubated at 37 °C for 3 days and the inhibition zone diameters were then measured with vernier calipers. The inhibitory zone test was repeated at least five times for each antibacterial surfactant [28].

Results

CMC Testing

The plot for conductance of these five gemini surfactants in water is shown in Fig. 1. The conductivities of a range of concentrations for each surfactant solution were found to vary linearly with the surfactant concentration in both premicellar and postmicellar regions. The ratio of the slopes of the postmicellar to the premicellar region provided the counterion dissociation constant (α_{cb}) and $1 - \alpha_{cb}$ gives the counterion binding coefficient β_{cb}. The concentration at the intersection of these two lines was the CMC of the corresponding surfactant [29]. The degree of binding was related to the surface area per head group in the ionic micelles. A lower value of the counterion binding coefficient means a higher head group area [30]. β_{cb} values of $G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, $G_{12\text{-}A4A\text{-}12}$, $G_{14\text{-}A2A\text{-}14}$, and $G_{16\text{-}A2A\text{-}16}$ were 0.723, 0.698, 0.656, 0.706, and 0.651, respectively. These results indicated that increasing the length of the ethylene glycol bisacetyl spacer and hydrophobic alkyl chain caused a decrease of β_{cb}. CMCs of

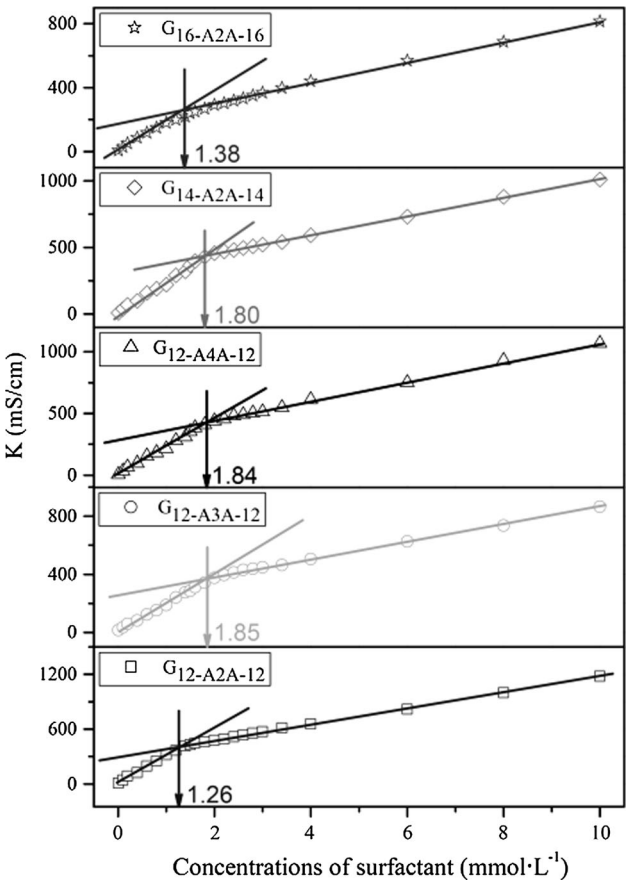


Fig. 1 CMC values of the cationic gemini surfactants. CMCs were determined from electrical conductivity data (25 °C)

$G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, $G_{12\text{-}A4A\text{-}12}$, $G_{14\text{-}A2A\text{-}14}$, and $G_{16\text{-}A2A\text{-}16}$ were 1.26, 1.85, 1.84, 1.80, and 1.38 mmol L^{-1}, respectively. All five surfactants have low critical micelle concentrations [29]. It was observed that the CMC values did not vary much with an increase in the length of the ethylene glycol bisacetyl spacer and hydrophobic alkyl chain.

Antibacterial Tests

MIC and MBC Testing

As shown in Table 1 (left panel), the MIC was the lowest concentration with no visible growth. An aliquot from each well was removed, spread on solid agar plates, and incubated at 37 °C for 24 h to determine the MBC values (Table 1, middle panel). Interestingly, the MIC values obtained for *E. coli* or *S. aureus* were identical to the obtained MBC values. Overall, the MIC and MBC results showed that the antibacterial activities of the surfactants against *S. aureus* were higher than those against *E. coli*. The MIC and MBC values for $G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, and $G_{12\text{-}A4A\text{-}12}$ were not dependent on an increase in the glycol

Table 1 MIC values, MBC values, and inhibitory zone diameters of five surfactants against *S. aureus* and *E. coli*

Sample	MIC (μg mL^{-1})		MBC (μg mL^{-1})		Inhibitory zone diameter (mm)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
Blank[a]	–	–	–	–	7.00 ± 0.02	7.00 ± 0.02
$G_{12\text{-}A2A\text{-}12}$	64	128	64	128	24.01 ± 0.68	12.33 ± 0.70
$G_{12\text{-}A3A\text{-}12}$	64	128	64	128	20.05 ± 0.44	11.35 ± 0.52
$G_{12\text{-}A4A\text{-}12}$	64	128	64	128	21.2 8 ± 0.38	10.83 ± 0.26
$G_{14\text{-}A2A\text{-}14}$	64	128	64	128	23.18 ± 0.75	12.18 ± 0.56
$G_{16\text{-}A2A\text{-}16}$	4	512	4	512	14.82 ± 0.20	7.08 ± 0.14

[a] 0.9 % Saline water

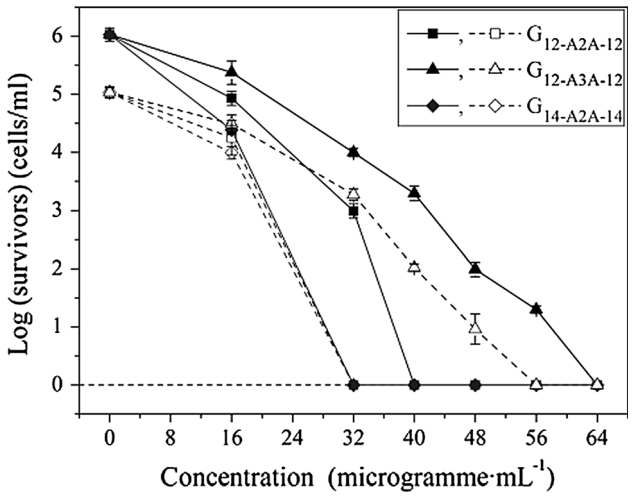


Fig. 2 Plots of log (survivors) vs. concentration of $G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, and $G_{14\text{-}A2A\text{-}14}$ against *S. aureus* after 24 h. *Solid symbols* and *solid lines* cell suspension contained 10^6 cells/mL; *hollow symbols* and *dotted lines* cell suspension contained 10^5 cells/mL

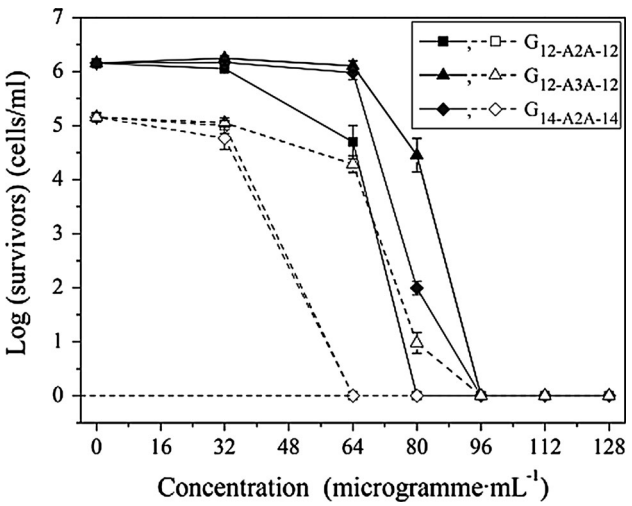


Fig. 3 Plots of log (survivors) vs. concentration of $G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, and $G_{14\text{-}A2A\text{-}14}$ against *E. coli* after 24 h. *Solid symbols* and *solid lines* cell suspension contained 10^6 cells/mL; *hollow symbols* and *dotted lines* cell suspension contained 10^5 cells/mL

bisacetyl spacer chain length. Conversely, the MIC and MBC values for $G_{16\text{-}A2A\text{-}16}$ against *S. aureus* were significantly lower than those for $G_{12\text{-}A2A\text{-}12}$. However, the values for $G_{16\text{-}A2A\text{-}16}$ against *E. coli* were higher than for $G_{12\text{-}A2A\text{-}12}$ (see Supplementary Figs. 1 and 2).

Time–Kill Study

To further compare the antibacterial activity of the surfactants, the effects of various surfactant concentrations of $G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, and $G_{14\text{-}A2A\text{-}14}$ on the survival of *S. aureus* were determined. As shown in Fig. 2, 100 % lethality toward 10^5 CFU of *S. aureus* was achieved after treatment with 32 μg mL^{-1} of $G_{12\text{-}A2A\text{-}12}$, 56 μg mL^{-1} of $G_{12\text{-}A3A\text{-}12}$, and 32 μg mL^{-1} of $G_{14\text{-}A2A\text{-}14}$, whereas 40 μg mL^{-1} of $G_{12\text{-}A2A\text{-}12}$, 64 μg mL^{-1} of $G_{12\text{-}A3A\text{-}12}$, and 32 μg mL^{-1} of $G_{14\text{-}A2A\text{-}14}$ were required for similar lethality in the presence of 10^6 CFU. Therefore, the different net increases of surfactant concentrations required to obtain lethality reflected the antibacterial activities of the surfactants: $G_{14\text{-}A2A\text{-}14} > G_{12\text{-}A2A\text{-}12} > G_{12\text{-}A3A\text{-}12}$. In parallel, the differences in the net increase in surfactant concentrations toward both 10^5 CFU and 10^6 CFU of *E. coli* (Fig. 3) suggested an antibacterial activity order of $G_{12\text{-}A2A\text{-}12} > G_{14\text{-}A2A\text{-}14} > G_{12\text{-}A3A\text{-}12}$. A total of 64 μg mL^{-1} $G_{12\text{-}A2A\text{-}12}$, 96 μg mL^{-1} $G_{12\text{-}A3A\text{-}12}$, and 64 μg mL^{-1} $G_{14\text{-}A2A\text{-}14}$ gave 100 % lethality toward 10^5 CFU of *E. coli*, whereas 80 μg mL^{-1} $G_{12\text{-}A2A\text{-}12}$, 96 μg mL^{-1} $G_{12\text{-}A3A\text{-}12}$, and 96 μg mL^{-1} $G_{14\text{-}A2A\text{-}14}$ were required to achieve the same lethality against 10^6 CFU.

The plots of surviving *S. aureus* cells vs contact time for 10 μg mL^{-1} of $G_{12\text{-}A2A\text{-}12}$, $G_{14\text{-}A2A\text{-}14}$, and $G_{16\text{-}A2A\text{-}16}$ showed that it required only 10 min for $G_{16\text{-}A2A\text{-}16}$ to kill all bacteria, whereas the percentage survival of *S. aureus* in the presence of $G_{12\text{-}A2A\text{-}12}$ or $G_{14\text{-}A2A\text{-}14}$ was still considerable, even when exposed for 4 h (Fig. 4). However, the sterilizing rate of $G_{14\text{-}A2A\text{-}14}$ (about 80 %) was higher than that of $G_{12\text{-}A2A\text{-}12}$ (about 60 %). This result indicated that the antibacterial activity of $G_{16\text{-}A2A\text{-}16}$ against *S. aureus* was significantly higher than that of either $G_{12\text{-}A2A\text{-}12}$ or $G_{14\text{-}A2A\text{-}14}$. In addition, the bacteriolytic effect of $G_{14\text{-}A2A\text{-}14}$ was slightly better than that of $G_{12\text{-}A2A\text{-}12}$. The

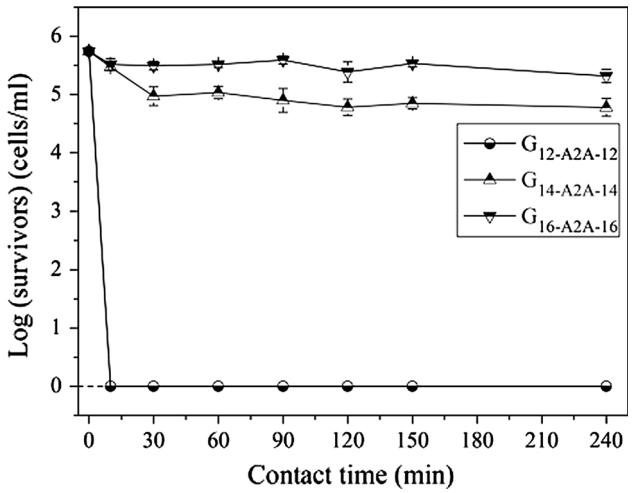


Fig. 4 Plots of log (*S. aureus* survivors) vs. contact time in a suspension containing 5×10^5 CFU cells/mL in contact with 10 μg mL^{-1} of $G_{12\text{-}A2A\text{-}12}$, $G_{14\text{-}A2A\text{-}12}$, and $G_{16\text{-}A2A\text{-}16}$

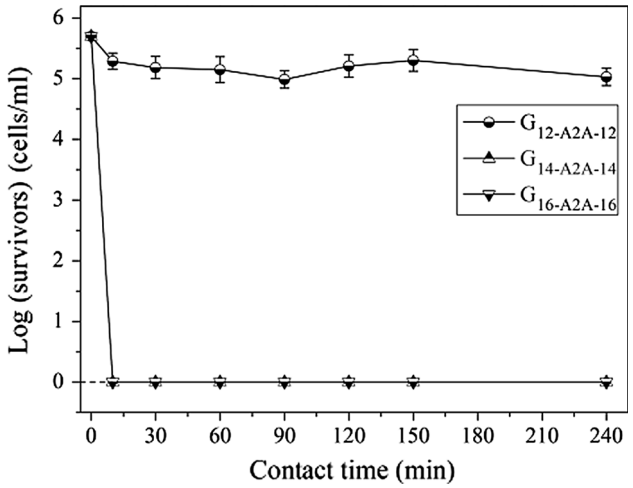


Fig. 5 Plots of log (*E. coli* survivors) vs contact time in a suspension containing 5×10^5 CFU cells/mL in contact with 150 μg mL^{-1} of $G_{12\text{-}A2A\text{-}12}$, $G_{14\text{-}A2A\text{-}12}$, and $G_{16\text{-}A2A\text{-}16}$

plots of surviving *E. coli* vs contact time for 150 μg mL^{-1} of $G_{12\text{-}A2A\text{-}12}$, $G_{14\text{-}A2A\text{-}14}$, and $G_{16\text{-}A2A\text{-}16}$ showed that after 10 min, virtually all bacteria were killed by $G_{12\text{-}A2A\text{-}12}$ and $G_{14\text{-}A2A\text{-}14}$ (Fig. 5). However, $G_{16\text{-}A2A\text{-}16}$ showed limited antibacterial activity (about 60 %) against *E. coli* with 4 h of contact time at the same concentration, suggesting that the antibacterial activity of $G_{12\text{-}A2A\text{-}12}$ and $G_{14\text{-}A2A\text{-}14}$ against *E. coli* was much higher than that of $G_{16\text{-}A2A\text{-}16}$ under similar conditions. Therefore, an increase in the alkyl chain length conferred a corresponding increase in the antibacterial activity against *S. aureus* but a decrease in the activity against *E. coli*.

Inhibitory Zone Method

The suppressing effects of the surfactants toward both *S. aureus* and *E. coli* were further illustrated by measuring the inhibitory zone diameters of the bacterial cultures after exposure to the surfactants. The results confirmed that *S. aureus* was more sensitive to these antibacterial agents compared with *E. coli* (Table 1, right panel), which was in good agreement with the results from the MIC/MBC testing. On the basis of the size of inhibitory zone diameters, all surfactants exhibited antibacterial activities against *S. aureus* in the order $G_{12\text{-}A2A\text{-}12} > G_{14\text{-}A2A\text{-}14} > G_{12\text{-}A4A\text{-}12} > G_{12\text{-}A3A\text{-}12} > G_{16\text{-}A2A\text{-}16}$, whereas against *E. coli* they were in the order $G_{12\text{-}A2A\text{-}12} > G_{14\text{-}A2A\text{-}14} > G_{12\text{-}A3A\text{-}12} > G_{12\text{-}A4A\text{-}12} > G_{16\text{-}A2A\text{-}16}$. The surfactants could also be grouped as $G_{12\text{-}A2A\text{-}12}$/$G_{14\text{-}A2A\text{-}14}$, $G_{12\text{-}A3A\text{-}12}$/$G_{12\text{-}A4A\text{-}12}$, and $G_{16\text{-}A2A\text{-}16}$, representing those with high sensitivity, medium sensitivity, and low sensitivity to the tested bacteria, respectively (see Supplementary Fig. 3).

Discussion

In this work, a series of cationic gemini surfactants containing two dimethylalkylammonium chains (two $–C_{12}H_{25}$, $–C_{14}H_{29}$, or $–C_{16}H_{33}$) linked by diethylene glycol bisacetyl, triethylene glycol bisacetyl, or tetraethylene glycol bisacetyl spacers were systematically synthesized by two reactions, acylation and quaternization. Unlike previous approaches, the synthetic methods discussed herein allowed the study of different lengths of hydrophobic chains and spacers [24, 25]. Acylation occurred via chloroacetyl chloride instead of bromoacetyl bromide [16], which produces environmentally harmful HBr. The yield of the intermediate product ethylene glycol-bis(2-chloroethanoate) was decreased by using chloroacetyl chloride; however, the inductive effect of the oxygen atom of the ether bond in the glycol does help to increase the yield. Conversely, quaternization was a very safe and high yielding reaction. Therefore, the advantages of this synthetic strategy are safety, simplicity, and environmentally friendly performance. The five products were obtained as yellow viscous liquids at room temperature and improved thermal stability (up to temperatures of 170 °C), similar to ionic liquids [25].

The cell wall teichoic acid in *S. aureus* cells, the lipopolysaccharide (LPS) in *E. coli* cells, as well as the phospholipids of both cells are rich in surface-exposed negative charges. These negative charges allow the bis-quaternary ammonium salt cationic gemini surfactants linked by a ethylene glycol bisacetyl spacer to associate with the outer surface of the target bacterial cells via electrostatic interactions. Furthermore, the hydrophobic group of the

cationic gemini surfactants provided compatibility with the hydrophobic end of the phospholipid of the cytoplasmic membrane and allowed the cationic gemini surfactants to be inserted into the cell membrane, resulting in disruption of cell membrane integrity and eventually leading to leakage of the intracellular lysate and dissolution of the cytoplasmic membrane. Consequently, these surfactants behaved as efficient sterilizers [30, 31]. Interestingly, the MBC tests yielded values similar to the MIC values for both bacteria. The bacteria were killed completely in the time–kill study after only a 10-min exposure, indicating that neither *E. coli* nor *S. aureus* was resistant to the gemini surfactants [32]. On the basis of the results, the antibacterial activities of the five surfactants against *S. aureus* were higher than those against *E. coli*. Our current hypothesis to explain this result is that because *E. coli* has a more complicated overall cell wall structure than that of *S. aureus* owing to its additional outer membrane, it is more protected from antibacterial agents. Additionally, the phospholipids in the *E. coli* outer membrane may make it more difficult for the antibacterial agents to diffuse through the membrane. As a result, the antibacterial activities of these cationic gemini surfactants against *E. coli* were lower than against *S. aureus* [33]. The order of antibacterial activities against *S. aureus* was $G_{16\text{-}A2A\text{-}16} \gg G_{14\text{-}A2A\text{-}14} > G_{12\text{-}A2A\text{-}12}$, whereas the order of activities against *E. coli* was $G_{12\text{-}A2A\text{-}12} > G_{14\text{-}A2A\text{-}14} \gg G_{16\text{-}A2A\text{-}16}$. The antibacterial activity of $G_{16\text{-}A2A\text{-}16}$ against *S. aureus* was significantly higher than that of $G_{12\text{-}A2A\text{-}12}$, and appeared to be related to the longer alkyl chains of $G_{16\text{-}A2A\text{-}16}$ that may make it more compatible with the phospholipid of the cell membrane. However, the antibacterial activity of $G_{16\text{-}A2A\text{-}16}$ against *E. coli* was lower than that of $G_{12\text{-}A2A\text{-}12}$. This is likely because the hydrophobic substances on the cationic gemini surfactants will make it more compatible with the outer membrane of *E. coli*. The phospholipid of the outer membrane in the cell wall of *E. coli* provides better compatibility with $G_{16\text{-}A2A\text{-}16}$, resulting in a higher degree of association of $G_{16\text{-}A2A\text{-}16}$ with the outer membrane leading to only a small amount of $G_{16\text{-}A2A\text{-}16}$ diffusion through the cell membrane. The length of the alkyl chain of $G_{12\text{-}A2A\text{-}12}$ and $G_{14\text{-}A2A\text{-}14}$ did not play a remarkable role in the antibacterial activities against *E. coli* and *S. aureus*, which were not significantly different. This was likely because the structure of $G_{14\text{-}A2A\text{-}14}$ results in it having limited hydrophobicity which is the primary contributing factor to the antibacterial activity. The antibacterial activities of $G_{12\text{-}A2A\text{-}12}$, $G_{12\text{-}A3A\text{-}12}$, and $G_{12\text{-}A4A\text{-}12}$ did not vary with increasing the ethylene glycol bisacetyl spacer length. The probable reason was that the interaction between the N atoms of the hydrophilic group and the ester group of the spacer caused the distance of the two nitrogen atoms of the hydrophilic portion to decrease and distort the linking group. Therefore, changing the length of the spacer failed to induce a change in the charge density of the hydrophilic portion.

Cationic gemini surfactants act as emulsifier and antibacterial agents when added to emulsions [34]; their low CMC and MIC/MBC values show that low additive amounts can produce the expected antibacterial emulsions. $G_{12\text{-}A2A\text{-}12}$ had the lowest CMC, 1.26 mmol L^{-1}, and exhibited the best MICs of 32 μg mL^{-1} toward *S. aureus* and 64 μg mL^{-1} toward *E. coli* in the presence of 10^5 CFU of bacteria. Therefore, the optimized cationic gemini surfactant $G_{12\text{-}A2A\text{-}12}$ containing two dodecyl chains linked by a diethylene glycol bisacetyl spacer may have potential applications as a quick-acting and efficient antibacterial agent and emulsifier.

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