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Cleavable Cationic Antibacterial Amphiphiles: Synthesis, Mechanism of Action, and Cytotoxicities

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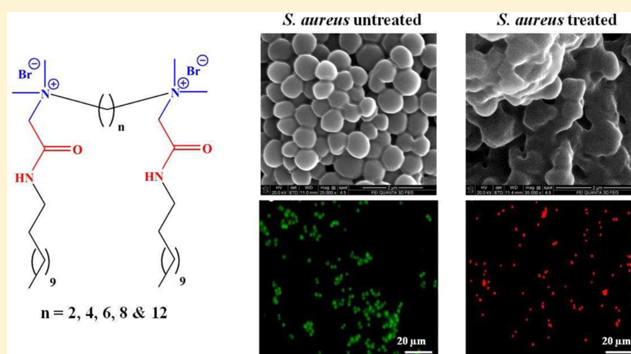
Supporting Information

ABSTRACT: The development of novel antimicrobial agents having high selectivity toward bacterial cells over mammalian cells is urgently required to curb the widespread emergence of infectious diseases caused by pathogenic bacteria. Toward this end, we have developed a set of cationic dimeric amphiphiles (bearing cleavable amide linkages between the headgroup and the hydrocarbon tail with different methylene spacers) that showed high antibacterial activity against human pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*) and low cytotoxicity. The Minimum Inhibitory Concentrations (MIC) were found to be very low for the dimeric amphiphiles and were lower or comparable to the monomeric counterpart. In the case of dimeric amphiphiles, MIC was found to decrease with the increase in the spacer chain length ($n = 2$ to 6) and again to increase at higher spacer length ($n > 6$). It was found that the compound with six methylene spacers was the most active among all of the amphiphiles (MICs = 10−13 μM). By fluorescence spectroscopy, fluorescence microscopy, and field-emission scanning electron microscopy (FESEM), it was revealed that these cationic amphiphiles interact with the negatively charged bacterial cell membrane and disrupt the membrane integrity, thus killing the bacteria. All of the cationic amphiphiles showed low hemolytic activity (HC_{50}) and high selectivity against both gram-positive and gram-negative bacteria. The most active amphiphile ($n = 6$) had a 10−13-fold higher HC_{50} than did the MIC. Also, this amphiphile did not show any cytotoxicity against mammalian cells (HeLa cells) even at a concentration above the MIC (20 μM). The critical micellar concentration (CMC) values of gemini surfactants were found to be very low (CMC = 0.30−0.11 mM) and were 10−27 times smaller than the corresponding monomeric analogue (CMC = 2.9 mM). Chemical hydrolysis and thermogravimetric analysis (TGA) proved that these amphiphiles are quite stable under both acidic and thermal conditions. Collectively, these properties make the newly synthesized amphiphiles potentially superior disinfectants and antiseptics for various biomedical and biotechnological applications.



INTRODUCTION

The shortage of antimicrobial agents and the emergence of drug-resistant bacteria have plagued human lives for centuries. Most of the existing medicines are failing because of bacterial resistance, causing an alarming depletion of the therapeutic armory.[1] Currently, the world witnesses around 300 million cases of severe bacterial infections and the death of 2 million children annually.[2] These shocking statistics pose an urgent need to develop novel antimicrobial agents with a lower propensity for bacterial resistance. These compounds need to act through a mechanism different from the existing classes of antibiotics. An antimicrobial agent that acts directly by disrupting the microbial cell membrane appears to be a better solution to curbing the development of bacterial resistance because bacteria are known to develop slow resistance against such agents.[3] Antimicrobial peptides (natural and synthetic mimics),[4] antimicrobial polymers,[5] cationic steroid antibiotics,[6] and quaternary ammonium compounds[7] were developed to combat microbial infection following this approach,.

Among them, small-molecular-weight quaternary ammonium compounds (QACs) are extensively used as antimicrobial agents in hospitals, in the food industry, and in treating human infections because they exhibit a wide spectrum of antimicrobial activity against bacteria (both gram-positive and gram-negative),[8] fungi,[9] and certain viruses.[10] These types of agents offer an additional advantage because they can be used to fabricate various surfaces and render them antimicrobial, which thus makes them suitable in biomedical applications.[11] Apart from possessing antimicrobial activity, these compounds also find application in skin and body care products,[12] vehicles for drug delivery,[13] and vectors for gene transfection.[14]

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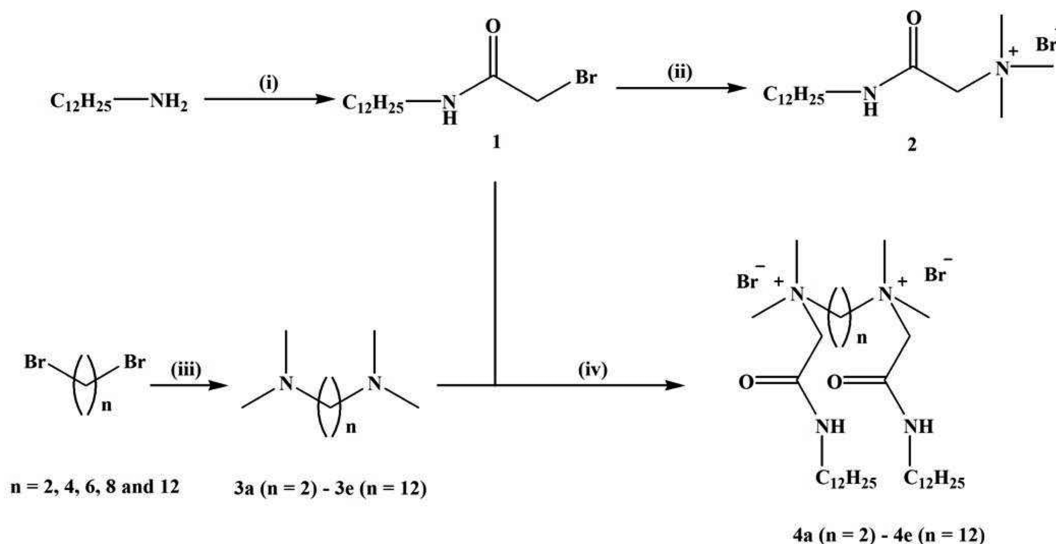
Scheme 1. Synthesis Scheme of Cationic Monomeric and Dimeric Amphiphiles Bearing Amide Linkages[a]



[a]Reagents, conditions, and yields: (i) $BrCH_2COBr$, K_2CO_3, DCM, H_2O; 5 °C, 30 min, room temperature (rt), 2 h; 100%. (ii) NMe_3, acetone; pressure tube, rt, 12 h; 99%. (iii) $NHMe_2$, tetrahydrofuran; rt, 24 h; 100%. (iv) Acetonitrile; pressure tube, 85 °C,12 h; 99%.

Conventional monomeric cationic amphiphiles derived from quaternary ammonium compounds show good antimicrobial activity, and their action is based on the ability to disrupt the bacterial membrane by a combined hydrophobic and electrostatic adsorption phenomenon at the membrane/water interface, followed by membrane disruption.[15] Because the bacterial cell membrane possess a higher percentage of negative charge as compared to mammalian cells,[16] the positive charge of the cationic amphiphiles facilitates greater interaction with the bacterial membrane. Haldar et al. has shown that with the increase in the number of cationic headgroups, the antibacterial activity of the cationic amphiphiles increases.[20b] However, much attention has been paid to the development of dimeric amphiphiles, which are composed of two hydrophilic cationic quaternary ammonium headgroups and two hydrophobic chains. These amphiphiles have various superior properties, including higher antibacterial activity, over monomeric amphiphiles, which possess only one headgroup and a hydrophobic tail.[17] However, this class of amphiphilic compounds exhibits high hemolytic activity[18] and causes skin irritation and hypersensitivity.[19] Furthermore, environmental concerns and aquatic toxicity, because of their nonbiodegradable nature, limit their practical use.

As a remedy to these side effects, the concept of "soft" antimicrobial agents was introduced.[20] These antimicrobial agents possess a cleavable moiety in the molecule, such as an ester of betaine or choline, that when subjected to base- or enzyme-catalyzed hydrolysis produces significantly fewer toxic components.[21] These new-generation ester-containing soft amphiphiles were found to exhibit improved biodegradation. However, these amphiphiles failed to possess sufficient chemical stability to have acceptable antibacterial activity. They are known to be hydrolyzed readily under acidic and basic conditions and also at high temperatures.[22] To be suitable for any biomedical application, the amphiphiles must be sufficiently stable under storage and sterilization conditions but should be nontoxic toward mammalian cells.[8a] An ideal solution to this issue lies in the incorporation of chemically more stable but biodegradable linkages, such as an amide moiety,[23] into the molecule.

Herein, we report the synthesis of a series of cationic dimeric amphiphiles (Scheme 1) bearing cleavable amide linkages in the side chain with different methylene spacers and their antibacterial activity against both *E. coli* and *S. aureus*. The hemolytic activity and cytotoxicity of these compounds were studied with human red blood cells (hRBC) and HeLa cells, respectively, and the selectivity of these compounds toward bacterial cells over mammalian cells was established. These compounds exhibit very high antibacterial activity and low toxicity. The mechanism of antibacterial action was investigated by both scanning electron microscopy (FESEM) and fluorescence microscopy, and the studies indicated that they interact with the cellular membrane of the microorganism. The mechanism was further corroborated spectroscopically by membrane (both outer- and inner-membrane) permeabilization assay. Both their chemical and thermal stability were investigated by acid-catalyzed hydrolysis and thermogravimetric analysis, respectively, and the results suggested that these amphiphiles are quite stable under both chemical and thermal conditions. This evidence suggests that these types of nontoxic and biodegradable systems can be used as novel antimicrobial agents for various biomedical applications.

■ EXPERIMENTAL SECTION

Materials. All of the solvents were reagent grade and dried prior to use. Dodecylamine, bromoacetyl-bromide, α,ω-dibromoalkanes, and *N,N,N′,N′*-tetramethyl-1,2-diaminoethane were purchased from Sigma-Aldrich and used as received. NMR spectra were recorded using a Bruker AMX-400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer. The chemical shifts (δ) are reported in parts per million downfield from the peak for internal standard TMS for ^1H NMR and ^{13}C NMR. Mass spectra were recorded on a Micromass Q-ToF micro mass spectrometer. Infrared (IR) spectra of the solid compounds were recorded on a Bruker IFS66 V/s spectrometer using KBr pellets. IR spectra of all of the liquid compounds were recorded on the same instrument using NaCl crystal. Elemental analyses were carried out with a Thermo Finnigan FLASH EA 1112 CHNS analyzer. Thermogravimetric analyses were performed on a TGA 850C thermogravimetric analyzer (Mettler Toledo). Fluorescence studies

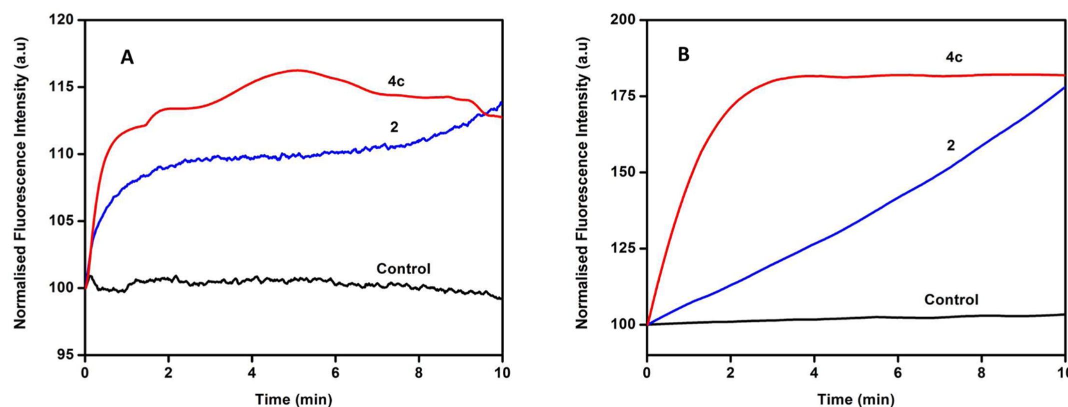


Figure 1. (A) Outer-membrane permeabilization of *E. coli* (10^8 cells/mL) by cationic amphiphiles **2** and **4c** measured by the increase in NPN fluorescence for 10 min. (B) Inner-membrane permeabilization of *E. coli* (10^8 cells/mL) by cationic amphiphiles **2** and **4c** measured by the increase in PI fluorescence for 10 min.

were carried out with a Perkin-Elmer Lambda 900 UV/vis/NIR spectrometer and a Perkin-Elmer LS 55 spectrometer. The optical density was measured with a TecanInfinitePro series M200 microplate reader. Fluorescence images were captured with a Leica DM 2500 fluorescence microscope. Scanning electron microscope images were obtained using a Quanta 3D FEG FEI field-emission scanning electron microscope.

Synthesis of *N*-Dodecyl-1-bromoethanamide (1). Dodecylamine (7 g, 37.83 mmol) was dissolved in dichloromethane (55 mL). Potassium carbonate, K_2CO_3 (7.8 g, 56.75 mmol), was dissolved in water, and the aqueous solution was added to the organic solution. The resulting two-phase solution was cooled to 5 °C. A solution of bromoacetyl bromide (11.45 g, 56.75 mmol) in dichloromethane (55 mL) was added dropwise to the cooled solution for about 30 min while maintaining the temperature at 5 °C. Then the reaction mixture was stirred at room temperature for about 2 h. The aqueous solution was separated and washed with dichloromethane (2 × 25 mL). All the organic solutions were mixed together and washed with water (2 × 50 mL) and passed over anhydrous Na_2SO_4 and concentrated to yield a white product quantitatively.

Synthesis of Dodecyl *N*-Ethanamide *N,N,N*-Trimethylammonium Bromide (2). *N*-Dodecyl-1-bromoethanamide (**1**) (1 g, 3.26 mmol) was reacted with excess dry NMe_3 gas in dry acetone (20 mL) in a screw-top pressure tube at room temperature for 12 h. After the reaction mixture was transferred to an RB under ice-cold conditions, excess NMe_3 gas was then removed by heating the reaction mixture and the precipitate was filtered and washed with cold acetone to get a white product quantitatively.

Synthesis of *N,N,N′,N′*-Tetramethyl-*α,ω*-diaminoalkanes (3a−3e). *N,N,N′,N′*-Tetramethyl-*α,ω*-diaminoalkanes (**3a**−**3e**) were synthesized via a slight modifications of the literature procedures.[24] Dry $NHMe_2$ gas was added to dry THF (45 mL) in a screw-top pressure tube at 0 °C until the volume of the resulting solution was roughly doubled (~90 mL). Dibromoalkane (6 g) was added through a syringe and stirred for 24 h at room temperature to complete the reaction. The pressure tube was cooled, and the reaction mixture was transferred to a RB and then it was washed with $CHCl_3$. A white precipitate appeared, and the solution was kept in a water bath to remove excess $NHMe_2$, followed by the removal of solvent. Then the reaction mixture was diluted with $CHCl_3$, followed by washing with NaOH (2 M, 100 mL) solution. A $CHCl_3$ layer was collected and passed through anhydrous Na_2SO_4 followed by drying to get a light-yellow gummy liquid in quantitative yield.

Synthesis of Dimeric Amphiphiles (4a−4e). *N*-Dodecyl-1-bromoethanamide (**1**) (1 g, 3.27 mmol) was reacted with a calculated amount of **3a**−**3e** (1.48 mmol) in dry acetonitrile (10 mL) in a screw-top pressure tube at 85 °C for about 12 h. After the reaction was over, the solvent was evaporated by rotary evaporation and the residue was dissolved in a minimum amount of $CHCl_3$. The product was precipitated by adding excess ethyl acetate and filtered. The white residue was washed repeatedly with ethyl acetate to yield **4a**−**4e** quantitatively (98−99%).

Microorganisms and Cell Culture Conditions. The antibacterial activity of the amphiphiles was determined against both gram-negative (*E. coli*; MTCC 443) and gram-positive (*S. aureus*; MTCC 737) bacteria. *E. coli* was cultured in Luria−Bertani broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1000 mL of sterile distilled water (pH 7), and *S. aureus* was grown in yeast−dextrose broth (1 g of beef extract, 2 g of yeast extract, 5 g of peptone, and 5 g of NaCl in 1000 mL of sterile distilled water). For solid media, 5% agar was used along with the above-mentioned composition. The bacterial samples were shipped from MTCC in freeze dried form, which were cultured on receiving and stored at −80 °C as glycerol stocks. Five microliters of these stocks was added to 3 mL of the nutrient broth, and the culture was grown for 6 h at 37 °C prior to the experiments. HeLa cells were grown at 37 °C in a humidified-air atmosphere (5% CO_2/95% air) in a DMEM complete medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, and 100 g/mL streptomycin.

Critical Micellar Concentration (CMC). The aggregation properties of the gemini surfactants in aqueous solution were investigated by static light scattering (SLS) measurements on a Perkin-Elmer LS-55 luminescence spectrometer.[24b] Surfactant solutions were prepared using Millipore water at room temperature at concentrations ~3 mM for gemini surfactants and ~8 mM for the monomeric counterpart. Two milliliters of this solution was used to measure the scattering intensity upon successive dilutions. Light scattering was evaluated by measuring the reflected light at an angle of 90°, fixing both the excitation and the emission at 400 nm at a scan speed of 10 nm/min. An excitation slit width of 10 nm and an emission slit width of 2.5 nm were used and kept constant throughout the experiment. The SLS signal is proportional to both the number of aggregated molecules and the size of the aggregates. To describe the dependence of the scattered signal on the surfactant concentration, the intensity of scattered light was plotted against the total surfactant concentration. The CMC was determined from the inflection point.[24b] The inflection point is the abscissa where the intensity rises steeply and decreases after reaching a local maximum (Figure S1).

Antibacterial Activity. Antibacterial activity was determined via slight modifications to literature procedures.[25] Water-soluble amphiphiles were assayed in a modified microdilution broth format. Stock solutions were made by serially diluting the compounds using autoclaved Millipore water. Bacteria to be tested were grown for 6 h in a suitable media and contained ~10^9 cfu/mL (determined by the spread plating method), which was then diluted to 10^5 cfu/mL using nutrient media. Fifty microliters of a serially diluted compound was added to a 96-well plate containing 150 μL of bacterial solutions. Two controls were used: one containing 150 μL of media and 50 μL of a compound and the other containing 200 μL of a bacterial solution. The plate was then incubated at 37 °C for 10 h, and MIC data was

recorded by measuring the OD value at 600 nm using a Tecan InfinitePro series M200 microplate reader. Each concentration had triplicate values, the whole experiment was done at least twice, and the MIC value was determined by taking the average of triplicate values for each concentration and subsequently plotting it against concentration (Figure S2). The data was then subjected to sigmoidal fitting. From the curve, the MIC value was determined as the point in the curve where the OD was similar to that of a control having no bacteria.

Outer Membrane Permeabilization Assay. The outer membrane permeabilization activity of amphiphiles **2** and **4c** was determined by an NPN (*N*-phenylnapthylamine) assay.[28] Midlog-phase *E. coli* cells (grown for 6 h, 10^8 cells/mL) were harvested (4000 rpm, 4 °C, 10 min), washed, and resuspended in 5 mM glucose/5 mM HEPES buffer at pH 7.2. Then, 10 μL of amphiphiles **4c** and **2** in water (which gives a working concentration 30 μM) was added to a cuvette containing 1.5 mL of cells and 10 μM NPN (30 μL from a 500 μM stock solution in acetone). The excitation and emission wavelengths used were 350 nm and 420 nm, respectively (slit width was 10 nm in both cases). The uptake of NPN as a measure of outer membrane permeabilization was monitored by the increase in fluorescence of NPN for 10 min (Figure 1A).

Inner-Membrane Permeabilization Assay. Midlog-phase *E. coli* cells (grown for 6 h, 10^8 cells/mL) were harvested (4000 rpm, 4 °C, 10 min), washed, and resuspended in PBS buffer at pH 7.2. Then, 30 μL of a 4.5 mM concentration of compounds in water was added to a cuvette containing 1.5 mL of cells and 15 μM propidium iodide (PI). The excitation wavelength was 535 nm (slit width: 10 nm), and the emission wavelength was 617 nm (slit width: 10 nm).The uptake of PI was measured by the increase in fluorescence of PI for 10 min as a measure of inner-membrane permeabilization (Figure 1B).[29]

Fluorescence Microscopy..[30,31] A 1.5 mL bacterial suspension containing 10^9 CFU/mL cells was centrifuged (12 000 rpm for 1 min) and the pellet was resuspended with PBS and 100 μM of **4c** to make a final volume of 1 mL of suspension (final concentration 1.5×10^9 cells/mL), and another 1 mL was left untreated as a control. The mixture was incubated for 1.5 h, centrifuged (12 000 rpm for 1 min), and resuspended in 50 μL of PBS. Five microliters of the bacterial suspension was combined with 20 μL of a fluorescent probe mixture containing 3.0 μM green fluorescent nucleic acid stain SYTO 9 (Invitrogen, USA) and 15.0 μM red fluorescent nucleic acid stain PI (Sigma-Aldrich, USA). The mixture was incubated in the dark for 15 min, and a 5 μL aliquot was placed on a glass slide, which was then covered with a coverslip, sealed, and examined under a fluorescence microscope. Excitation was carried out for SYTO 9 at 450−490 nm and for PI at 515−560 nm. Emission was collected using a band-pass filter for SYTO 9 at 500−550 nm and a long-pass filter for PI at 590−800 nm. In all cases, a 100× objective was used with immersion oil, giving a total magnification of 1000×. Images were captured with a Leica DM 2500 fluorescence microscope.

Scanning Electron Microscopy (SEM).[35] The bacterial cells were cultured for 6 h in suitable media (LB broth for *E. coli* and yeast dextrose broth for *S. aureus*) at 37 °C. The cells were centrifuged and resuspended in nutrient media at pH 7.4 (10^6 cells/mL). The suspension was divided into two portions. To one portion (1 mL) was added a suitable concentration (100 μM) of amphiphile **4c**. The other portion was left untreated as a control. The suspension was then incubated at 37 °C for 2 h (at 250 rpm shaking speed), and the cells from both tubes were harvested by centrifugation at 12 000 rpm. After treatment, the cells were dehydrated sequentially with 30, 50, 70, 80, 90, and 100% ethanol for 15 min. Later, 5 μL of dehydrated cells was dropped on a small piece of silicon wafer and dried at room temperature. Before being imaged, the silicon wafer containing *S. aureus* was sputter coated, and *E. coli* was used directly for imaging without sputtering. Images were recorded by using Quanta 3D FEG FEI field-emission scanning electron microscopy at 20 kV for *S. aureus* and 10 kV for *E. coli*.

Hemolytic Assay..[36,37] Human blood was freshly collected in heparinized vacoutainers and centrifuged at 3500 rpm for 5 min, which resulted in the separation of blood plasma and red blood cells. The supernatant was discarded, and the RBC pellet was washed twice with PBS (pH 7.4) and finally resupended to give 20 vol % RBC in PBS. In a 96-well microtiter plate, 150 μL of an erythrocyte suspension was added, followed by 50 μL of a serially diluted compound. Two controls were used: one without a compound and other with 1 vol % solution of Triton X-100. The plate was incubated for 1 h at 37 °C and then centrifuged at 3500 rpm for 5 min; 2 μL was then diluted to 200 μL in a fresh microtiter plate and the absorbance was measured at 414 nm. The percentage of hemolysis was determined from $\{(A - A_0)/(A_{total} - A_0)\} \times 100$, where A is the absorbance of the test well, A_0 is the absorbance of the negative controls, and A_{total} is the absorbance of 100% hemolysis wells, all at 414 nm.

Cytotoxicity Assay. HeLa cells were seeded onto polylysine-coated coverslips in a 24 well plate at a concentration of 100 000 cells per well. Cells were incubated at 37 °C under a 5% CO_2−95% air atmosphere until they reached 70−80% confluency. The cells were then treated with a 20 μM concentration of compound **4c** and incubated under the same conditions for 10 h. After 10 h, the medium was removed and the cells were thoroughly washed with 1× PBS buffer to remove unwanted media in the well. Two controls were used: one containing untreated cells and the other containing completely permeabilized cells. To achieve complete premeabilization, cells were first fixed with 4% paraformaldehyde at room temperature. This prevents the detachment of cells from the surface after treatment with Triton X. Cells were then treated with 0.1% Triton-X for 10 min at room temperature. After the incubation time was over, Triton was removed and the cells were washed with PBS. The cells were then stained with 2 μM calcein AM and 4.5 μM propidium iodide for 15 min at 37 °C under a 5% CO_2−95% air atmosphere. Dye was carefully removed, and the cells were washed and mounted onto glass slides. Images were captured using a band-pass filter for Calcein AM at 500−550 nm and a long-pass filter for PI at 590−800 nm. In all cases, a 40× objective was used and images were captured using a Leica DM2500 fluorescence microscope.

Thermogravimetric Analysis. Thermogravimetric analyses were performed on a TGA 850C thermogravimetric analyzer (Mettler Toledo). Samples (6 to 7 mg) of the dimeric amphiphiles were used for each experiment. The measurement was made from 30 to 550 °C at a constant heating rate H_R of 5 °C/min under a nitrogen atmosphere (40 mL/min, Figure S5).

Degradation Study (Chemical Hydrolysis). The degradation study (chemical hydrolysis) was performed by the acid-catalyzed hydrolysis of amide-containing surfactant **4c**. 500 μL of 3.6 mM solution of surfactant **4c** in DCl/KCl buffer (pD ∼ 2) was added to a 5 mm NMR tube. The tubes were held in a water bath at 40 °C, where proton NMR spectra were recorded every day for 15 days on Bruker AMX 400 MHz NMR spectrometer (Figure S6). The degree of hydrolysis at different times was calculated from the relative integrals originating from $-CH_2-$ protons (3.2 ppm) of the $-CONHCH_2-$ group of the intact or partially hydrolyzed surfactant and from α-CH_2- protons (δ 3.0 ppm) of the hydrolyzed product, protonated dodecylamine.

RESULTS AND DISCUSSION

Synthesis and Characterization. Both the monomeric (**2**) and dimeric amphiphiles (**4a−4e**) with varying methylene spacer length (n = 2, 4, 6, 8, and 12) were synthesized using an efficient synthesis methodology (Scheme 1). The synthesis of the monomeric amphiphile (dodecyl *N*-ethanamide *N,N,N*-trimethylammonium bromide (**2**)) began with the reaction of 1-aminododecane with bromoacetylbromide in the presence of aqueous K_2CO_3 in CH_2Cl_2 to afford *N*-dodecyl-1-bromoethanamide (**1**) in 100% yield. After that, **1** was quaternized using trimethylamine in dry acetone in a screw-top pressure tube with stirring at room temperature for 12 h. A white solid was formed, filtered, and repeatedly washed with dry acetone. Pure amphiphile **2** was obtained upon several recrystallizations from a mixture of $CHCl_3$/*n*-hexane in 99% yield.

Table 1. Antibacterial and Hemolytic Activities of Cationic Amphiphiles

amphiphiles	CMC (mM)	MIC (μM)		HC_{50} (μM)	selectivity (HC_{50}/MIC)	
		E. coli	*S. aureus*		*E. coli*	*S. aureus*
4a	0.30	100 ± 1	19 ± 6	154 ± 9	1.5	8.1
4b	0.23	30 ± 7	19 ± 1	135 ± 5	4.5	7.1
4c	0.19	13 ± 2	10 ± 4	128 ± 6	9.8	12.8
4d	0.14	14 ± 4	21 ± 1	122 ± 12	8.7	5.8
4e	0.11	34 ± 6	50 ± 1	100 ± 5	2.9	2
2	2.9	35 ± 12	43 ± 5	366 ± 10	10.5	8.5

For the synthesis of dimeric amphiphiles (**4a**−**4e**), α,ω-dibromoalkanes were reacted with dimethylamine in dry THF. This gave *N,N,N′,N′*-tetramethyl-α,ω-diaminoalkanes (**3a**−**3e**) in almost quantitative yield.[24] Compounds **3a**−**3e** were then reacted with the previously synthesized *N*-dodecyl-1-bromoethanamide (**1**) in dry acetonitrile in a screw-top pressure tube with stirring at 85 °C for 12 h (Scheme 1). The solvent was evaporated, and the residue was dissolved in a minimum amount of $CHCl_3$. The product was precipitated by adding excess ethyl acetate, filtered, and washed repeatedly with ethyl acetate to obtain **4a**−**4e** quantitatively. The products (**4a**−**4e**) were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, high-resolution mass spectrometry, and elemental analysis (Supporting Information).

Solubility. To perform various biological studies, we examined the solubility of these amphiphiles in water as well as in a nutrient medium. Monomeric and all of the dimeric amphiphiles except **4e** were found to be soluble in water at room temperature. Amphiphile **4e** was soluble at slightly higher temperatures (>30 °C). However, it was observed that amphiphiles **4a** and **4b** formed low-viscous solutions upon dissolution in water. The lower solubility of **4e** in water could be a consequence of the increased hydrophobicity of the amphiphile with a longer carbon chain length (n = 12). None of the compounds showed any precipitation in the nutrient medium in the concentration range of 0.09−100 μM.

Critical Micellar Concentration. To investigate the effect of the structure of the surfactants on the aggregation properties, the critical micellar concentration (CMC) was determined for each amide-bearing surfactant. The CMCs of all of the surfactants were determined by static light scattering, which is a tool that was recently developed by simultaneously scanning the excitation and emission monochromator of a common spectrofluorometer to measure the light-scattering signals.[24b] The SLS intensity was not linear with the surfactant concentration. With increasing surfactant concentration, the SLS intensity was found to increase to a certain point (local maximum), after which a steep decline was observed, and then it increased further with the addition of further surfactant, thereby resulting in an inflection point (Supporting Information). The sudden decline probably indicates the onset of aggregation, which continued until all of the free surfactant molecules were converted into aggregate form. After the CMC (inflection point), with the increase in concentration the number of micelles increased, which resulted in an increase in the SLS intensity (Figure S1). The CMC values for the gemini surfactants were found to be very low (0.30−0.11 mM) compared to that for the monomeric surfactant (2.9 mM). Because of the increased hydrophobic interactions, the gemini surfactant is known to have a very low CMC value compared to that of the monomeric surfactant, and the same behavior has been observed in the present study (Table 1).

Antibacterial Activity. The antibacterial activities of the cationic dimeric amphiphiles (**4a**−**4e**) were determined and compared to that of monomeric amphiphile **2**. The activity was established as the minimum inhibitory concentration (MIC) for both *S. aureus* and *E. coli* by the microbroth dilution method.[25] The MIC is defined as the lowest concentration of antimicrobial agent that inhibits bacterial growth, as determined by measuring the OD at 600 nm after 10 h of incubation at 37 °C (Figure S2). The bacterial inhibition effect of the amphiphiles was found to lie in the range of 13−100 μM in the case of *E. coli*, whereas for *S. aureus* it ranges from 10 to 50 μM (Table 1). Following the MIC values, a relationship between the systemic increase in the spacer unit and the antibacterial activity was also established, and it was found that the antibacterial activity of the dimeric amphiphiles followed a trend with the variation in the spacer chain length (Figure S3). Against *E. coli*, the MIC values initially decreased from 100 to 13 μM with the increase in the spacer chain length (n = 2 to 6) and later increased to 35 μM with the increase in spacer length (n = 12). Similarly, MIC values decreased from 19 to 10 μM with the increase in spacer chain length (n = 2 to 6) and then increased to 50 μM with the increase in spacer length (n = 12) against *S. aureus*. Among all of the dimeric amphiphiles, **4c** (n = 6) showed the maximum antibacterial activity at 13 and 10 μM against both *E. coli* and *S. aureus*, respectively. It is also found that amphiphile **4c** is three to four times more active than its monomeric counterpart **2** against either type of bacteria. However, it is interesting that cleavable cationic amphiphile **4c** (MIC values for *E. coli* and *S. aureus* are 13 and 10 μM respectively) showed higher or similar activity compared to that of well-known noncleavable cationic amphiphile cetyltrimethylamonium bromide, CTAB (MIC values for *E. coli* and *S. aureus* are 19 and 10 μM, respectively).

The antibacterial potency depends on the hydrophilic−hydrophobic balance of the cationic amphiphiles. Positively charged polar headgroups of the cationic amphiphiles interact with the negatively charged bacterial cell membrane, thus disrupting it with the aid of electrostatic and hydrophobic interactions leading to the release of K^+ and cytoplasmic constituents and finally cell death.[5d] The higher antibacterial activity of dimeric amphiphiles compared to that of their monomeric counterparts could be attributed to the greater number of positive charges (in a dimeric amphiphile, the number of positive charges is two whereas the same in a monomeric amphiphile is one) as well as higher hydrophobicity leading to better interaction with the bacterial cell surface.[8a] The lower antibacterial activity of dimeric amphiphiles **4a** and **4e** may be due to the formation of viscous solution and the poor solubility in water, respectively, thus leading to a greater binding affinity with a smaller number of cells.[20b]

However, it was observed that the antibacterial activity of the dimeric amphiphiles increases as the spacer chain length

increases from n = 2 to 6 and again decreases as the chain length increases. The role of the spacer in the dimeric amphiphiles in their antibacterial activity may be explained by the balance of hydrophilic/hydrophobic groups of these cationic amphiphiles. With the increase in the spacer chain length (from n = 2 to 6), the hydrophobicity of the cationic dimeric amphiphiles increases, leading to stronger interactions with the inner core of the bacterial cell membrane in addition to the electrostatic interactions.[26] Further increases in the spacer chain length (from n = 8 to 12) increase the hydrophobic character, which may be too high to facilitate transport through the bacterial cell membrane.[27]

Mechanism of Antibacterial Action. It is known that the cationic amphiphiles interact strongly with the negatively charged bacterial cell surface. After the successful determination of the antibacterial activity of the cationic amphiphiles, we studied the mechanism of their antibacterial action. Because the bacterial cell membrane is composed of mainly negatively charged phospholipids (as well as negatively charged lipopolysaccharides for gram-negative bacteria and negatively charged teichoic acid for gram-positive bacteria), it is therefore expected that these cationic amphiphiles will interact strongly with negatively charged cell surfaces of the bacteria. Furthermore, because these amphiphiles contain long hydrophobic alkyl chains, they can interact further with the hydrophobic lipid membrane and can disrupt the cellular membrane.

Membrane Permeabilization Assay. The disruption of membrane integrity is an important step in the mode of action of cationic amphiphiles because it can lead to the leakage of intracellular materials. To determine the physicochemical factors that influence membrane permeabilization, we performed both outer- and inner-membrane permeabilization assays for cationic amphiphiles **2** and **4c** as a model system (Figure 1).

We used 1-*N*-phenyl-naphthylamine (NPN) as a fluorescent probe (NPN fluoresces strongly only in a hydrophobic environment such as the hydrophobic segment of the lipid membrane).[28] Both amphiphiles examined at 100 μM showed a characteristic time-dependent rise in fluorescence intensity due to the increased uptake of NPN dye as a result of increased membrane permeabilization (Figure 1A). The rise in the fluorescence signal was gradual for compound **2**, and more rapid and highly intense for compound **4c**. Two different trends in the degree and rate of permeabilization are evident. First, between monomeric amphiphile **2** and dimeric amphiphile **4c**, **2** showed a smaller degree of outer-membrane permeabilization. Second, the rate of permeabilization was higher for dimeric amphiphile **4c**. These results clearly suggest that the degree and rate of permeabilization are influenced by charge and as well as the hydrophobicity of the amphiphiles.

The inner-membrane permeabilization was studied spectroscopically by measuring the uptake of fluorescent probe propidium iodide (PI). Unlike the outer-membrane permeabilization assay, bacteria were first treated with the amphiphiles for about 2 to 3 min and then the dye was added in order to record the fluorescence spectra of PI. Being positively charged, this dye can enter only cells with compromised cytoplasmic membranes and fluoresces upon binding to nucleic acids.[29] Figure 1B shows that the dimeric amphiphile **4c** has a strong ability to permeabilize the inner membrane. Furthermore, the rate of permeabilization of **4c** is higher than that of its monomeric analogue **2**. These results further prove the importance of the charge and hydrophobicity of the amphiphiles for having a greater, faster activity of dimeric amphiphiles.

Fluorescence Microscopy. To prove the mechanistic action of the cationic amphiphiles further, we carried out a LIVE/DEAD fluorescence study where a mixture of nucleic acid binding, green fluorescent dye SYTO 9, and red dye PI was used.[30] The microscopy technique used could distinguish intact cells and membrane-permeabilized cells. In general, SYTO 9 is membrane-permeable and thus labels all bacteria (those with intact and damaged membranes) in the population. In contrast, PI is membrane-impermeable and penetrates only when the bacterial membrane is permeabilized, causing a reduction in the SYTO 9 fluorescence when both dyes are present. Thus, bacteria with intact membranes, being exclusive to PI, will be stained with SYTO 9 and emit green fluorescence, whereas bacteria with permeabilized membranes will be stained with PI and emit red fluorescence.[31]

Fluorescence microscopy images of bacteria treated with amphiphile **4c** and without any treatment (as control) were captured. The effect of treating gram-negative and gram-positive bacteria with amphiphile **4c** is shown in Figure 2. The concentrations of **4c** used for this experiment were 20 and 100 μM for both *E. coli* and *S. aureus*, and the incubation time for the compound was 1.5 h. A similar experiment was performed without the compound, as control. The fluorescence microscopy images show that the cell viability in the case of the control samples is clearly seen by green fluorescence (Figure 2a,c for *E. coli* and *S. aureus*, respectively), and the cells treated with the compound show complete membrane permeabilization as indicated by red fluorescence (Figure 2b,d for *E. coli* and *S. aureus*, respectively). Complete bacterial killing was observed when bacteria were treated with 100 μM amphiphile **4c** for 1.5 h whereas more than 60% was seen at 20 μM for both bacteria.

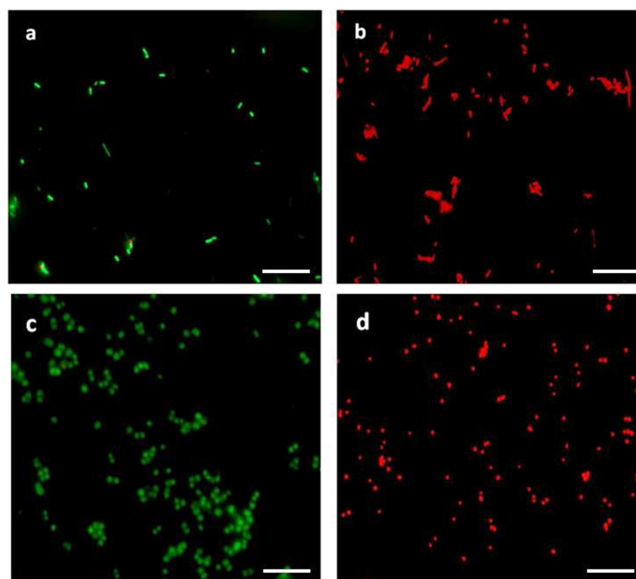


Figure 2. Fluorescence microscopy images of bacteria untreated {(a) *E. coli*; (c) *S. aureus*} and treated with compound **4c** {(b) *E. coli*; (d) *S. aureus*} for 1.5 h after staining with SYTO 9 and PI. Each image is a result of the superposition of an image taken for the green fluorescent dye (SYTO 9) and red fluorescent dye (PI) using the appropriate filters. The scale bar is 20 μm.

Scanning Electron Microscopy. To obtain visual insight into bacterial killing by amphiphiles, a scanning electron microscopy (FESEM) study was performed. FESEM images of both *E. coli* and *S. aureus* treated with cationic amphiphile **4c** (100 μM) for 2 h and without treatment with the amphiphile (as a control) were acquired (Figure 3). Both untreated bacteria showed the presence of normal cells (Figure 3a,c for *E. coli* and *S. aureus*, respectively) with preserved cell membranes. However, irregularly shaped and thus probably dead bacteria were observed upon treatment with **4c** (Figure 3b,d for *E. coli* and *S. aureus*, respectively). The cationic amphiphile therefore interacts with the bacterial cell membrane, makes holes, and disrupts the cell membrane, which may lead to the loss of cytoplasmic constituents and finally cell death.[32]

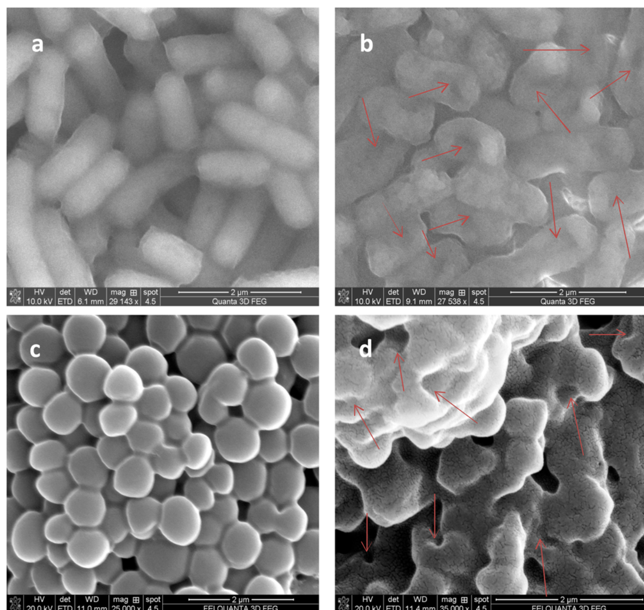


Figure 3. Scanning electron microscopy (SEM) images of *E. coli* {(a) untreated; (b) treated with amphiphile **4c** (100 μM)} and *S. aureus* for 2 h {(c) untreated; (d) treated with amphiphile **4c** (100 μM)}.

Toxicity. Biomedical applications of antimicrobial agents will be important only if they are lethal to pathogenic microorganisms but at the same time are nontoxic toward mammalian cells. In fact, it is very important to find agents that will widen this difference between antimicrobial activity and cytotoxicity to a greater extent. Here, we study the toxicity effect of the dimeric amphiphiles on mammalian cells (both hRBC and HeLa cells).

Hemolytic Activity. The hemolytic activity of the amphiphiles was evaluated and expressed as HC_{50}, the amphiphile concentration that induces the hemolysis of 50% of the total number of erythrocytes (Figure S4). Table 1 summarizes the HC_{50} values for all of the cationic amphiphiles. In general, these amphiphiles exhibited significantly lower hemolytic activity as evident from their higher HC_{50} values, which range from 100 to 154 μM, whereas the monomeric amphiphiles shows the least hemolytic activity with HC_{50} of 366 μM. Furthermore, the spacer chain length was observed to have a systematic effect on the hemolytic activities of the cationic dimeric amphiphiles (**4a**–**4e**). It was found that with the increase in spacer chain length the hemolytic activity increases gradually (Table 1). This trend was consistent with reports that an increase in hydrophobicity increases the hemolytic activity because of stronger hydrophobic component–lipid core interactions.[33]

However, it has been found that the HC_{50} values of all of the amphiphiles used in this study were higher than the

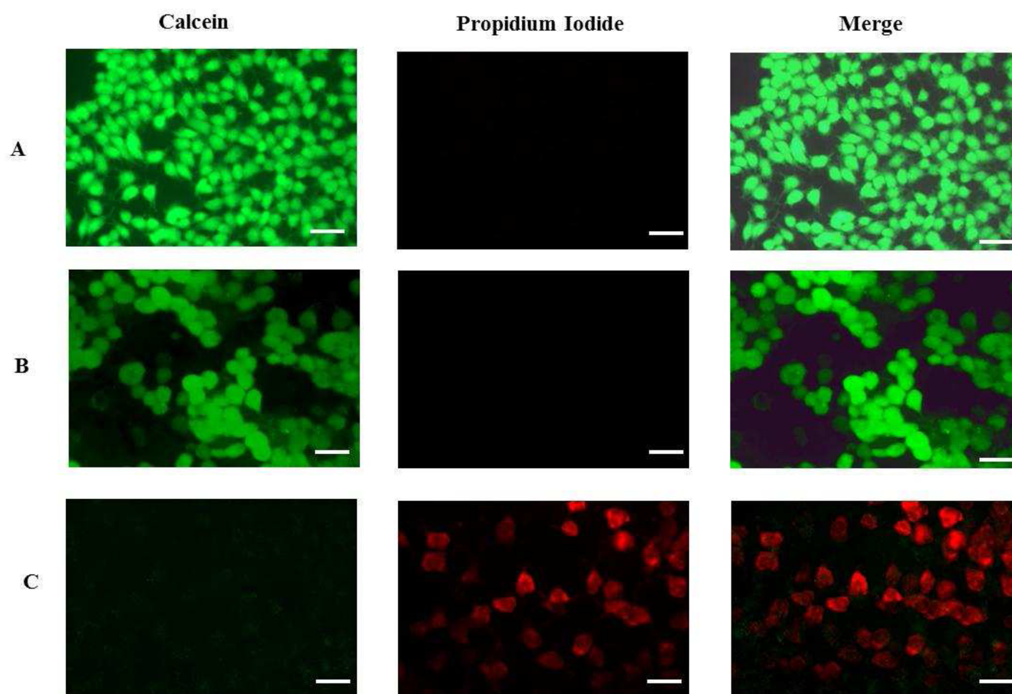


Figure 4. Fluorescence microscopy images of HeLa cells after treatment with amphiphile **4c** for 10 h and staining with calcein AM and propidium iodide (PI). (A) Nontreated cells (negative control); (B) cells treated with amphiphile **4c** (20 μM); and (C) cells treated with 0.1% Triton X (positive control). The scale bar is 20 μm.

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corresponding MIC values (Table 1), thus giving a selectivity, defined as the ratio of HC_{50}/MIC.[34] In general, all of the cationic amphiphiles possess antibacterial activity for both *E. coli* and *S. aureus* at lower concentrations than those in which they exhibit erythrocyte toxicity. The safe concentration range is remarkably wide in the case of monomeric amphiphile **2** and dimeric amphiphile **4c**. The observed HC_{50} of monomeric amphiphile **2** was 366 μM, a 9- to 10-fold higher concentration than the MIC values. However, the HC_{50} value observed for **4c**, the most potent amphiphile, was 128 μM, a 10-fold-higher concentration than the MIC for *E. coli* and a 13-fold-higher concentration than the MIC against *S. aureus*. It has been reported that the hydrophobic interaction controls the hemolytic activity, whereas charge interactions are suggested to be more important for antibacterial activity.[26a,34] These results show that the presence and balance of hydrophobic and hydrophilic groups decide the hemolytic activities of the amphiphilic cationic compounds.

Cytotoxicity. To strengthen our statement of the selectivity of dimeric amphiphiles toward bacteria over mammalian cells, we performed a cytotoxicity assay against HeLa cells using fluorescence microscopy with a dual-dye system. The dyes used were calcein AM and PI. Calcein AM is a nonfluorescent dye that permeates intact live cells and is hydrolyzed by the intracellular esterases, giving highly fluorescent calcein having excitation and emission wavelengths of 495 and 515 nm, respectively. Whereas PI is a membrane-impermeable dye and penetrates only when the cells have compromised cell membranes, live cells will fluoresce green and dead ones will emit red fluorescent light.

HeLa cells grown on polylysine-coated coverslips were treated with a 20 μM concentration (above the MIC) of amphiphile **4c**. Two controls were used: one where fixed cells were treated with 0.1% Triton X and the other where cells were left untreated. The images are shown in Figure 4. Untreated cells showed completely green fluorescence indicating live cells whereas Triton X-treated cells showed complete red fluorescence indicating cell death. HeLa cells show high green fluorescence and no trace of any red fluorescence even after being treated with compound **4c** for 10 h, which indicates that the dimeric amphiphile is not at all toxic toward human cells and possesses high selectivity only toward bacterial cells at the above-mentioned concentration.

Thermogravimetric Analysis. The study of the thermal stability of amphiphiles is required for their widespread application as antiseptics and disinfectants. The thermal stability of all of the amphiphiles was measured by thermogravimetric analysis. All of the amphiphiles used in this study were found to be thermally stable above 200 °C (Figure S5).

Chemical Degradation Study. In this article, we have studied the degradation properties of the amide-bearing dimeric amphiphile (**4c**, $n = 6$) under acidic conditions by ^{1}H NMR. An amphiphile (**4c**) solution (3.6 mM) was prepared in DCl−KCl buffer (pD ~2), and NMR spectra were recorded every day for about 15 days. All of the spectra were recorded at 40 °C. Degradation of the amide bond under acidic conditions will generate dodecylamine, which will eventually be protonated under hydrolysis conditions. The degree of hydrolysis at different times was calculated from the relative integrals originating from the $-CH_2-$ protons (3.2 ppm) of the $-CONHCH_2-$ moiety of the intact or partially hydrolyzed surfactant and from the α-CH_2- protons (3.0 ppm) of the hydrolyzed product, protonated dodecylamine (Figure S6).

The appearance of the peak at 3.0 ppm that corresponds to the α-CH_2- group of protonated dodecylamine confirmed the hydrolysis of the amide-containing dimeric amphiphile under acidic conditions. It was found that only 30−35% of the amide group was hydrolyzed after 15 days. These results indicate that the amide-containing cationic amphiphile degrades slowly under acidic conditions at higher temperature (40 °C). The susceptibility of the amide-bearing cationic dimeric amphiphile over that of normal amide-bearing surfactants[23] to acid-catalyzed hydrolysis is probably due to the presence of quaternary ammonium groups.

CONCLUSIONS

Dimeric amphiphiles with different spacer lengths bearing cleavable amide linkages between headgroups and hydrocarbon tails with varying methylene spacer lengths have been synthesized and characterized. The cationic amphiphiles showed high antibacterial activity against highly pathogenic bacteria such as *E. coli* and *S. aureus*. Dimeric amphiphile **4c** with six methylene spacers exhibited the highest activity against both types of bacteria. This could be due to its optimum hydrophilic/hydrophobic balance and higher solubility in water, which enable a better interaction with the bacterial cell surface, leading to the more efficient killing of bacteria. This premise was supported by scanning electron microscopy, membrane permeabilization assay, and fluorescence microscopy studies, which showed that these cationic amphiphiles interacted strongly with the bacterial cell surface, thereby causing membrane disintegrity. All of the cationic amphiphiles showed low hemolytic activity and high selectivity against both gram-positive and gram-negative bacteria. The most potent amphiphile, **4c**, had the highest selectivity and did not exhibit any cytotoxicity against mammalian cells (HeLa cells) at a concentration above the MIC. These amphiphiles were thermally stable above 200 °C and were slowly hydrolyzable under acidic conditions. For the widespread applications, these moderately stable, more eco-friendly and biodegradable amphiphiles can therefore replace the currently used antimicrobial amphiphilic agents.

ASSOCIATED CONTENT

Supporting Information

Characterization of all of the synthetic compounds. IR spectral data. ^{1}H NMR and ^{13}C NMR of *N*-dodecyl-1-bromoethanamide (**1**). ^{1}H NMR of *N,N,N′,N′*-tetramethyl-α,ω-diaminoalkanes (**3a**−**3e**). FTIR, ^{1}H NMR, and ^{13}C NMR spectra and spectra of cationic amide-containing dimeric amphiphiles *N,N′*-didodecylethanamide *N,N,N′,N′*-tetramethyl-*N,N′*-alkanediyl-lammoniumdibromide (**4a**−**4e**) including a monomeric amphiphile and dodecyl-*N*-ethanamide-*N,N,N*-trimethylammonium bromide (**2**). Plots of static light scattering as a function of surfactant concentration, the optical density of a bacterial suspension in media as a function of surfactant concentration, the % hemolysis of hRBC as a function of surfactant concentration, and the % weight loss of the surfactants as a function of temperature. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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REFERENCES

(1) (a) Walsh, C. Molecular mechanisms that confer antibacterial drug resistance. *Nature* **2000**, *406*, 775−781. (b) Levy, S. B.; Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* **2004**, *10*, S122−S129. (c) Alekshun, M. N.; Levy, S. B. Molecular mechanisms of antibacterial multidrug resistance. *Cell* **2007**, *128*, 1037−1050.

(2) (a) Inglesby, T. V. Updated recommendations for management. *J. Am. Med. Assoc.* **2002**, *287*, 2236−2252. Anthrax as a biological weapon, 2002: (b) Brecher, M. E.; Hay, S. N. Bacterial contamination of blood components. *Clin. Microbiol. Rev.* **2005**, *18*, 195−204. (c) Shorten, P. R.; Pleasants, A. B.; Soboleva, T. K. *Int. J. Food Microbiol.* **2006**, *108*, 369−375. (d) Ford, T. E.; Colwell, R. R. *A Global Decline in Microbiological Safety of Water: A Call for Action*; American Academy of Microbiology: Washington, DC, 1996; pp 1−40.

(3) Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389−395.

(4) (a) Hancock., R. E. W.; Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551−1557. (b) Goodman, C. M.; Choi, S.; Shandler, S.; DeGrado, W. F. Foldamers as versatile frameworks for the design and evolution of function. *Nat. Chem. Biol.* **2006**, *3*, 252−262. (c) Yanga, L.; Gordona, V. D.; Trinklea, D. R.; Schmidtb, N. W.; Davisa, M. A.; DeVriesa, C.; Somc, A.; Cronan, J. E., Jr; Tew, G. N.; Wong, G. C. L. Mechanism of a prototypical synthetic membrane active antimicrobial: efficient hole-punching via interaction with negative intrinsic curvature lipids. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 20595−20600.

(5) (a) Sambhy, V.; Peterson, B. R.; Sen, A. Antibacterial and hemolytic activities of pyridinium polymers as a function of the spatial relationship between the positive charge and the pendant alkyl tail. *Angew. Chem., Int. Ed.* **2008**, *47*, 1250−1254. (b) Tiller, J. C.; Liao, C. J.; Lewis, K.; Klibanov, A. M. Designing surfaces that kill bacteria on contact. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5981−5985. (c) Wang, Y.; Corbitt, T. S.; Jett, S. D.; Tang, Y.; Schanze, K. S.; Chi, E. Y.; Whitten, D. G. Direct visualization of bactericidal action of cationic conjugated polyelectrolytes and oligomers. *Langmuir* **2012**, *28*, 65−70. (d) Song, A.; Walker, S. G.; Kathlyn, A.; Parker, K. A.; Sampson, N. S. Antibacterial studies of cationic polymers with alternating, random, and uniform backbones. *ACS Chem. Biol.* **2011**, *6*, 590−599. (e) Ji, E.; Parthasarathy, A.; Corbitt, T. S.; Schanze, K. S.; Whitten, D. G. Antibacterial activity of conjugated polyelectrolytes with variable chain lengths. *Langmuir* **2011**, *27*, 10763−10769.

(6) (a) Li, C.; Peters, A. S.; Meredith, E. L.; Allman, G. W.; Savage, P. B. Design and synthesis of potent sensitizers of gram-negative bacteria based on a cholic acid scaffolding. *J. Am. Chem. Soc.* **1998**, *120*, 2961−2962. (b) Li, C.; Budge, L. P.; Driscoll, C. D.; Willardson, B. M.; Allman, G. W.; Savage, P. B. Incremental conversion of outer-membrane permeabilizers into potent antibiotics for gram-negative bacteria. *J. Am. Chem. Soc.* **1999**, *121*, 931−940. (c) Ding, B.; Guan, Q.; Walsh, J. P.; Boswell, J. S.; Winter, T. W.; Winter, E. S.; Boyd, S. S.; Li, C.; Savage, P. B. Correlation of the antibacterial activities of cationic peptide antibiotics and cationic steroid antibiotics. *J. Med. Chem.* **2002**, *45*, 663−669.

(7) (a) Hugo, W. B.; Russel, A. D. In *Types of Antimicrobial Agents: Principles and Practice of Disinfection, Preservation*, 2nd ed.; Russel, A. D., Hugo, W. B., Ayliffe, G. A. J., Eds.; Blackwell Scientific Publications: Oxford, U.K., 1992; pp 7−68. (b) Merianos, J. J. Quaternary Ammonium Antimicrobial Compounds. In *Disinfection, Sterilization, and Preservation*; Block, S. S., Ed.; Philadelphia: Lea & Febiger, 1991; pp 225−255. (c) McDonnell, G.; Pretzer, D. In *Disinfection, Sterilization, and Preservation*; Block, S. S., Ed.; Lippincott Williams & Wilkins: Philadelphia, PA, 2001. (d) Wang, Y.; Jones, E. M.; Tang, Y.; Ji, E.; Lopez, G. P.; Chi, E. Y.; Schanze, K. S.; Whitten, D. G. Effect of polymer chain length on membrane perturbation activity of cationic phenylene ethynylene oligomers and polymers. *Langmuir* **2011**, *27*, 10770−10775.

(8) (a) Colomer, A.; Pinazo, A.; Manresa, M. A.; Vinardell, M. P.; Mitjans, M.; Infante, M. R.; Pérez, L. Cationic surfactants derived from lysine: effects of their structure and charge type on antimicrobial and hemolytic activities. *J. Med. Chem.* **2011**, *54*, 989−1002. (b) Martins, L. M. S.; Mamizuka, E. M.; Carmona-Ribeiro, A. M. Cationic vesicles as bactericides. *Langmuir* **1997**, *13*, 5583−5587. (c) Viscardi, G.; Quagliotto, P.; Barolo, C.; Savarino, P.; Barni, E.; Fisicaro, E. Synthesis and surface and antimicrobial properties of novel cationic surfactants. *J. Org. Chem.* **2000**, *65*, 8197−8203.

(9) (a) Vieira, D. B.; Carmona-Ribeiro, A. M. Cationic lipids and surfactants as antifungal agents: mode of action. *J. Antimicrob. Chemother.* **2006**, *58*, 760−767. (b) Ng, C. K. L.; Obando, D.; Widmer, F.; Wright, L. C.; Sorrell, T. C.; Jolliffe, K. A. Correlation of antifungal activity with fungal phospholipase inhibition using a series of bisquaternary ammonium salts. *J. Med. Chem.* **2006**, *49*, 811−816.

(10) (a) Haldar, J.; An, D.; de Cienfuegos, L. A..; Chen, J.; Klibanov, A. M. Polymeric coatings that inactivate both influenza virus and pathogenic bacteria. *Proc. Natl. Acad. Sci.U.S.A.* **2006**, *103*, 17667−17671. (b) Wong, Y. L.; Hubieki, M. P.; Curfaman, C. L.; Doncel, G. F.; Dudding, T. C.; Savle, P. S.; Gandour, R. D. A structure-activity study of spermicidal and anti-HIV properties of hydroxylated cationic surfactants. *Bioorg. Med. Chem.* **2002**, *10*, 3599−3608. (c) Hsua, B. B.; Wongb, S. Y.; Hammond, P. T.; Chenc, J.; Klibanov, A. M. Mechanism of inactivation of influenza viruses by immobilized hydrophobic polycations. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 61−66.

(11) (a) Melo, L. D.; Palombo, R. R.; Petri, D. F. S.; Bruns, M.; Pereira, E. M. A.; Carmona-Ribeiro, A. M. Structure−activity relationship for quaternary ammonium compounds hybridized with poly(methyl methacrylate). *ACS Appl. Mater. Interfaces* **2011**, *3*, 1933−1939. (b) Pereira, E. M. A.; Kosaka, P. M.; Rosa, H.; Vieira, D. B.; Kawano, Y.; Petri, D. F. S.; Carmona-Ribeiro, A. M. Hybrid materials from intermolecular associations between cationic lipid and polymers. *J. Phys. Chem. B* **2008**, *112*, 9301−9310.

(12) Macián, M.; Seguer, J.; Infante, M. R.; Selve, C.; Vinardell, M. P. Preliminary studies of the toxic effects of derived from lysine. *Toxicology* **1996**, *106*, 1−9.

(13) (a) Stephenson, B. C.; Rangel-Yagui, C. O.; Pessoa, A.; Tavares, L. C.; Beers, K.; Blankschtein, D. Experimental and theoretical investigation of the micellar-assisted solubilization of ibuprofen in aqueous media. *Langmuir* **2006**, *22*, 1514−1525. (b) Bombelli, C.; Caracciolo, G.; Profio, P. D.; Diociaiuti, M.; Luciani, P.; Mancini, G.; Mazzuca, C.; Marra, M.; Molinari, A.; Monti, D.; Toccacieli, L.; Venanzi, M. Inclusion of a photosensitizer in liposomes formed by DMPC/gemini surfactant: correlation between physicochemical and biological features of the complexes. *J. Med. Chem.* **2005**, *48*, 4882−4891.

(14) (a) Llies, M. A.; Seitz, W. A.; Johnson, B. H.; Ezell, E. L.; Miller, A. L.; Thompson, E. B.; Balaban, A. T. Lipophilic pyrylium salts in the synthesis of efficient pyridinium-based cationic lipids, gemini surfactants, and lipophilic oligomers for gene delivery. *J. Med. Chem.*

2006, *49*, 3872−3887. (b) Kirby, A. J.; Camilleri, P.; Engberts, J. B. F. N.; Feiters, M. C.; Nolte, R. J. M.; Söderman, O.; Bergsma, M.; Bell, P. C.; Fielden, M. L.; Rodríguez, C. L. G.; Guédat, P.; Kremer, A.; McGregor, C.; Perrin, C.; Ronsin, G.; van Eijk, M. C. P. Gemini surfactants: new synthetic vectors for gene transfection. *Angew. Chem., Int. Ed.* **2003**, *42*, 1448−1457. (c) Heyes, J. A.; Nicolescu-Duvaz, D.; Cooper, R. G.; Springer, C. J. Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer. *J. Med. Chem.* **2002**, *45*, 99−114.

(15) Castillo, J. A.; Pinazo, A.; Carilla, J.; Infante, M. R.; Alsina, M. A.; Haro, I.; Clapeés, P. Interaction of antimicrobial arginine-based cationic surfactants with liposomes and lipid monolayers. *Langmuir* **2004**, *20*, 3379−3387.

(16) Oren, Z.; Hong, J.; Shai, Y. A repertoire of novel antibacterial diastereomeric peptides with selective cytolytic activity. *J. Biol. Chem.* **1997**, *272*, 14643−14649.

(17) (a) Zana, R.; Talmon, Y. Dependence of aggregate morphology on structure of dimeric surfactants. *Nature* **1993**, *362*, 228−230. (b) Menger, F. M.; Keiper, J. S. Gemini surfactants. *Angew. Chem., Int. Ed.* **2000**, *39*, 1906−1920. (c) Bajaj, A.; Kondiah, P.; Bhattacharya, S. Design, synthesis, and in vitro gene delivery efficacies of novel cholesterol-based gemini cationic lipids and their serum compatibility: a structure-activity investigation. *J. Med. Chem.* **2007**, *50*, 2432−2442.

(18) (a) Shalel, S.; Streichman, S.; Marmur, A. Monitoring surfactant induced hemolysis by surface tension measurement. *J. Colloid Interface Sci.* **2002**, *252*, 66−76. (b) Funasaki, N.; Ohigashi, M.; Hada, S.; Neya, S. Surface tensiometric study of multiple complexation and hemolysis by mixed surfactants and cyclodextrins. *Langmuir* **2000**, *16*, 383−388. (c) Vieira, D. B.; Carmona-Ribeiro, A. M. Cationic lipids and surfactants as antifungal agents: mode of action. *J. Antimicrob. Chemother.* **2006**, *58*, 760−767.

(19) Effendy, I.; Maibach, H. Surfactants and experimental irritant contact dermatitis. *Contact Dermititis* **1995**, *33*, 217−225.

(20) (a) Bodor, N.; Kaminski, J. K.; Selk, S. Soft drugs. 1. Labile quaternary ammonium salts as soft antimicrobials. *J. Med. Chem.* **1980**, *23*, 469−474. (b) Haldar, J.; Kondaiah, P.; Bhattacharya, S. Synthesis and antibacterial properties of novel hydrolyzable cationic amphiphiles. Incorporation of multiple head groups leads to impressive antibacterial activity. *J. Med. Chem.* **2005**, *48*, 3823−3831.

(21) (a) Linstedt, M.; Allenmark, S.; Thompson, R. A.; Edebo, L. Antimicrobial activity of betaine esters, quaternary ammonium amphiphiles which spontaneously hydrolyze into nontoxic components. *Antimicrob. Agents Chemother.* **1990**, *34*, 1949−1954. (b) Ahlstrom, B.; Chelminska-Bertilsson, M.; Thompson, R. A.; Edebo, L. Long-chain alkanoylcholines, a new category of soft antimicrobial agents that are enzymatically degradable. *Antimicrob. Agents Chemother.* **1995**, *39*, 50−55.

(22) (a) Thorsteinsson, T.; Masson, M.; Kristinsson, K. G.; Hjalmarsdottir, M. A.; Hilmarsson, H.; Loftsson, T. Soft antimicrobial agents: synthesis and activity of labile environmentally friendly long chain quaternary ammonium compounds. *J. Med. Chem.* **2003**, *46*, 4173−4181. (b) Tehrani-Bagha, A. T.; Holmberg, K. Cleavable surfactants. *Curr. Opin. Colloid Interface Sci.* **2007**, *12*, 81−91.

(23) Stjerndahl, M.; Holmberg, K. Synthesis, stability, and biodegradability studies of a surface-active amide. *J. Surf. Deterg.* **2005**, *8*, 331−336.

(24) (a) Spencer, T. A.; Onofrey, T. J.; Cann, R. O.; Russel, J. S.; Lee, L. E.; Blanchard, D. E.; Castro, A.; Gu, P.; Jiang, G.; Shechter, I. Zwitterionic sulfobetaine inhibitors of squalene synthase. *J. Org. Chem.* **1999**, *64*, 807−818. (b) Zhao, H. W.; Huang, C. Z.; Li, Y. F. Validation of micelle formation of proteins and determination of their critical micelle concentrations by measuring synchronous light scattering signals using a common spectrofluorometer. *Chem. Lett.* **2006**, *35*, 418−419.

(25) (a) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **2008**, *3*, 163−175. (b) Liu, D.; DeGrado, W. F. De-novo design, synthesis, and characterization of antimicrobial *β*-peptides. *J. Am. Chem. Soc.* **2001**, *123*, 7553−7559.

(26) (a) Dathe, M.; Schümann, M.; Wieprecht, T.; Winkler, A.; Beyermann, M.; Krause, E.; Matsuzaki, K.; Murase, O.; Bienert, M. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* **1996**, *35*, 12612−12622. (b) Andreu, D.; Rivas, L. Animal antimicrobial peptides: an overview. *Biopolymers* **1998**, *47*, 415−433.

(27) Willemen, H. M.; de Smet, L. C. P. M.; Koudijs, A; Stuart, M. C. A.; Jong, I. G. A. M. H.; Marcelis, A. T. M.; Sudhölter, E. J. R. Micelle formation and antimicrobial activity of cholic acid derivatives with three permanent ionic head groups. *Angew. Chem., Int. Ed.* **2002**, *41*, 4275−4277.

(28) Je, J. Y.; Kim, S. K. Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. *J. Agric. Food Chem.* **2006**, *54*, 6629−6633.

(29) Anantharaman, A.; Sahal, D. Reverse engineering truncations of an antimicrobial peptide dimer to identify the origins of potency and broad spectrum of action. *J. Med. Chem.* **2010**, *53*, 6079−6088.

(30) Milović, N. M.; Wang, J.; Lewis, K.; Klibanov, A. M. Immobilized *N*-alkylated polyethylenimine avidly kills bacteria by rupturing cell membranes with no resistance developed. *Biotechnol. Bioeng.* **2005**, *90*, 715−722.

(31) Dutta, S.; Shome, A.; Kar, T.; Das, P. K. Counter ion-induced modulation in the antimicrobial activity and biocompatibility of amphiphilic hydrogelators: influence of in-situ-synthesized Ag nanoparticle on the bactericidal property. *Langmuir* **2011**, *27*, 5000−5008.

(32) Mitra, R. N.; Shome, A.; Paul, P.; Das, P. K. Antimicrobial activity, biocompatibility and hydrogelation ability of dipeptide-based amphiphiles. *Org. Biomol. Chem.* **2009**, *7*, 94−102.

(33) FiratIlker, M.; Nüsslein, K.; Tew, G. N; Coughlin, E. B. Tuning the hemolytic and antibacterial activities of amphiphilic polynorbornene derivatives. *J. Am. Chem. Soc.* **2004**, *126*, 15870−15875.

(34) Liu, D. H.; Choi, S.; Chen, B.; Doerksen, R. J.; Clements, D. J.; Winkler, J. D.; Klein, M. L.; DeGrado, W. F. Nontoxic membrane-active antimicrobial arylamide oligomers. *Angew. Chem., Int. Ed.* **2004**, *43*, 1158−1162.

(35) Liu, S.; Wei, L.; Hao, L.; Fang, N.; Chang, M. W.; Xu, R.; Yang, Y.; Chen, Y. Sharper and faster "nano darts" kill more bacteria: a study of antibacterial activity of individually dispersed pristine single-walled carbon nanotube. *ACS Nano* **2009**, *3*, 3891−3902.

(36) Chongsiriwatana, N. P.; Patch, J. A.; Czyzewski, A. M.; Dohm, M. T.; Ivankin, A.; Gidalevitz, D.; Zuckermann, R. N.; Barron, A. E. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2794−2799.

(37) Patch, J. A.; Barron, A. E. Helical peptoid mimics of magainin-2 amide. *J. Am. Chem. Soc.* **2003**, *125*, 12092−12093.