Hydrogelation Through Self-Assembly of Fmoc-Peptide Functionalized Cationic Amphiphiles: Potent Antibacterial Agent

Sisir Debnath, Anshupriya Shome, Dibyendu Das, and Prasanta Kumar Das*

Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata - 700 032, India

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The present work reports a new class of antibacterial hydrogelators based on anti-inflammatory *N*-fluorenyl-9-methoxycarbonyl (Fmoc) amino acid/peptides functionalized cationic amphiphiles. These positively charged hydrogelators were rationally designed and developed by the incorporation of a pyridinium moiety at the *C*-terminal of Fmoc amino acid/peptides, because the pyridinium-based amphiphiles are a known antibacterial agent due to their cell membrane penetration properties. The Fmoc amino acid/peptide-based cationic amphiphiles efficiently gelate (minimum gelation concentration ~0.6−2.2%, w/v) water at room temperature. Judicious variation of amino acid and their sequences revealed the architectural dependence of the molecules on their gelation ability. Several microscopic techniques like field-emission scanning electron microscopy (FESEM) and atomic force microscopy (AFM) were used to obtain the visual insight of the morphology of the gel network. A number of spectroscopic techniques like circular dichroism, FTIR, photoluminescence, and XRD were utilized to know the involvement of several noncovalent interactions and participation of the different segments of the molecules during gelation. Spectroscopic results showed that the $\pi-\pi$ interaction and intermolecular hydrogen bonding are the major responsible factors for the self-assembled gelation process that are oriented through an antiparallel β-sheet arrangement of the peptide backbone. These Fmoc-based cationic molecules exhibited efficient antibacterial activity against both Gram-positive and Gram-negative bacteria.

Introduction

Low molecular weight hydrogels (LMWHs), a novel class of soft materials, have attracted large interest for the last two decades due to their wide-ranging applications from biomedicines to advanced materials.[1−5] Noncovalent interactions like electrostatic, dipole−dipole, van der Waals, $\pi-\pi$ stacking, and hydrogen bonding between the small molecules leads to the formation of a self-assembled three-dimensional (3D) network that can immobilize water.[6] Among several LMWHs, peptide-based gelators are finding great importance because of their potential biocompatibility and applications in drug delivery, tissue engineering, cell culture, and so on.[7] These self-assembled peptide hydrogels are known to form through varied supramolecular structural motifs like α-helix, β-sheet, β-hairpin, coiled-coil, and so on.[8] Often it is seen that a small peptide or even an amino acid with modified *C*- and *N*-termini either by aromatic ring or a long alkyl chain can self-assemble to form supramolecular gel.[9]

To this end, peptides containing aromatic moieties like *N*-fluorenyl-9-methoxycarbonyl (Fmoc) are known to induce excellent self-assembling properties resulting in hydrogelation.[7a−c,10] Interestingly, the Fmoc amino acid/dipeptides possess intrinsic anti-inflammatory properties.[11] In this context, lipopolysaccharide (LPS), the key constituents of outer layer membrane of Gram-negative bacteria, is also known to be one of the major causes for stimulating proinflammatory responses that may lead to sepsis.[12] Therefore, the design of anti-inflammatory agent having strong antibacterial activity will surely eliminate the possibility of LPS-induced inflammation as well as bacterial infections. Also for the biomedicinal use of hydrogels, it is preferable the gelator to be antibacterial to avoid the material associated nosocomial infection. Thus, to incorporate the antibacterial property, judicious functionalization (for example, inclusion of cationic charge) in the molecular architecture of the Fmoc-based peptides is necessary. These cationic peptides would exterminate the microbial cell membranes resulting in the failure of the innate defense mechanism of microorganisms.[13] In this regard, pyridinium group is known for its proven antimicrobial efficacies.[13c] Additionally, the planar aromatic moiety can also induce $\pi-\pi$ stacking interactions, a key factor for the formation of supramolecular gels. Hence, the self-assembly of a rationally designed molecule comprising of a pyridium group, along with the intrinsic properties of Fmoc amino acids/peptides will lead to the formation of a multifaceted antimicrobial gel.

In the present work, we have designed and developed a class of Fmoc amino acid/peptide functionalized cationic amphiphiles (Figure 1) having pyridinium moiety at the *C*-terminal. These amphiphiles exhibited excellent gelation ability (minimum gelation concentration (MGC) ~0.6−2.2%, w/v) in plain water over a wide range of pH at room temperature. The aliphatic/aromatic residues of the amino acids and their sequences were altered to find out the respective roles on the gelation efficiency of these Fmoc-based amphiphiles. These cationic amphiphilic peptides were arranged as antiparallel β-sheets as confirmed from XRD, FTIR, and fluorescence studies. The changes in the supramolecular arrangement of the hydrogels with structural variation at the molecular level were investigated using several spectroscopic and microscopic techniques. Intriguingly, these molecules showed excellent bactericidal activities having similar

* To whom correspondence should be addressed. Fax: +(91)-33-24732805. E-mail: bcpkd@iacs.res.in.

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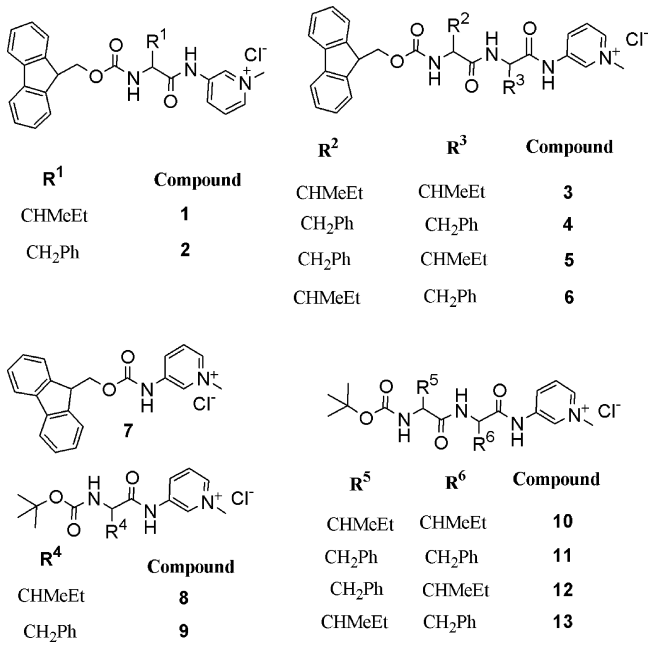

Figure 1. Structure of amphiphiles **1**−**13**.

efficacies against both Gram-negative and Gram-positive bacteria. The antibacterial property strongly depends on the structures of the gelators reaching up to a minimum inhibitory concentration (MIC) of 10 μg/mL.

Experimental Section

Materials. All amino acids, di-*tert*-butyl dicarbonate (Boc anhydride), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBT), Fmoc chloride, and solvents were procured from SRL (India). Trifluoro acetic acid (TFA) was purchased from Spectrochem, India. $CDCl_3$ was obtained from Aldrich Chemical Co. Thin layer chromatography were performed on Merck precoated silica gel 60-F_{254} plates. ^{1}H NMR spectra were recorded in AVANCE 300 MHz (BRUKER) spectrometer. Mass spectrometric data were acquired by the electron spray ionization (ESI) technique on a Q-tof-micro quadruple mass spectrometer (Micromass). The specific rotations of the synthesized compounds were measured in Perkin-Elmer (model 341LC) polarimeter. Fluorescence and FT-IR experiments were done on Varian Cary Eclipse luminescence spectrometer and Perkin-Elmer Spectrum 100 FT-IR spectrometer, respectively.

Synthetic Procedure. Synthetic scheme for all the amphiphiles (**1**−**13**) are given in Figure S1 (Supporting Information).

Synthesis of Amphiphile 1 and 2. Required amino acid was protected with *tert*-butyloxycarbonyl (Boc) group. Free COOH group of this Boc protected L-amino acid was coupled with *m*-amino pyridine using DCC and HOBt in DCM at room temperature for 12 h. The reaction mixture was washed with alkali followed by brine solution. Then DCM was evaporated and the pure product was isolated by column chromatography using 100−200 mesh silica gel with 1% MeOH/$CHCl_3$ as eluent. The product was subjected to deprotection by TFA (2 equiv) in dry DCM. After 2 h of stirring, solvents were removed on a rotary evaporator and the mixture was taken in ethyl acetate. The EtOAc part was thoroughly washed with aqueous 10% sodium carbonate solution followed by brine to neutrality. The organic part was dried over anhydrous sodium sulfate and concentrated to get the corresponding amine. Then Fmoc protection was done by the reaction of this amine with Fmoc chloride in dry DCM in presence of 1.1 equiv dry triethylamine for 30 min. After the reaction the DCM solution was washed with brine for several times and the pure Fmoc-protected product was obtained by column chromatography using 100−200 mesh silica gel and MeOH/$CHCl_3$ as eluent. Next, the quaternization at the pyridine "N" was done by the stirring with excess iodomethane in dry DCM for 6 h. The reaction mixture was taken in $CHCl_3$ and washed with aqueous thiosulphate solution and water, respectively. The pure quaternized iodide product was obtained by column chromatography using 60−120 mesh silica gel with MeOH/$CHCl_3$ as solvent. The iodide salt thus obtained was subjected to ion exchange on Amberlite Ira-400 chloride resin column to get the pure chlorides (**1** and **2**). Overall yield was ∼50−60%.

Synthesis of Amphiphile 3−**6.** The amino acid coupled with *m*-amino pyridine at "C" terminal end with free *N*-terminal after deprotection (Boc) was obtained as described earlier. This amine was further coupled with another Boc-protected amino acid using DCC and HOBt in dry DCM at room temperature for 12 h. The product was purified in a similar way as mentioned earlier and also Boc group was removed using TFA. Then the Fmoc protection was done at the free *N*-terminal of the dipeptide in a similar ways as described above. After completion of the reaction, the product was precipitated out from the solvent DCM and it was obtained by simple filtration and several time washing with DCM. The product was further purified by column chromatography using 100−200 mesh silica gel with MeOH/$CHCl_3$ as eluent. Then the quaternization at the pyridine "N" was done by stirring with excess iodomethane in dry $CHCl_3$/MeOH mixture for 6 h and purified in a similar way as mentioned above. The iodide salt thus obtained was subjected to ion exchange on Amberlite Ira-400 chloride resin column to get the pure chlorides (**3**−**6**). Overall yield was ∼40−50%.

Synthesis of Amphiphile 7. *m*-Amino pyridine is coupled with Fmoc chloride in dry DCM in presence of 1.1 equiv dry triethylamine for 1 h. The reaction mixture was worked up and purified in a similar way as described above. Also, the quaternization at the pyridine "N" was done exactly following the same protocol as mentioned above. The pure quaternized iodide product was obtained by column chromatography using 60−120 mesh silica gel and MeOH/$CHCl_3$ as solvent. The iodide salt thus obtained was subjected to ion exchange on Amberlite Ira-400 chloride resin column to get the pure chloride (**7**). Overall yield was ∼70−80%.

Synthesis of Amphiphile 8−**13.** The pure Boc-protected amino acid/peptide coupled with *m*-amino pyridine was prepared as described above. Then the quaternization at the pyridine "N" was done exactly following the same protocol as mentioned above. The pure quaternized iodide product was obtained by column chromatography using 60−120 mesh silica gel and MeOH/$CHCl_3$ as solvent. The iodide salt thus obtained was subjected to ion exchange on Amberlite Ira-400 chloride resin column to get the pure chloride (**8**−**13**). Overall yield was ∼70−80. Characterization data for all the amphiphiles are given in Supporting Information.

Preparation of Hydrogel. Required amount of the compounds were added in 1 mL water in a screw-capped vial with internal diameter (i.d.) of 10 mm and slowly heated until the solid was completely dissolved. Then the solutions were cooled to room temperature with out any disturbance. After 1 h, formation of gel was confirmed by stable to inversion of the glass vial. Only compound **2** gave gelation after keeping the solution for 12 h at room temperature.

Microscopic Studies. FESEM was performed on JEOL-6700F microscope. A piece of hydrogel was mounted on glass slide and dried for few hours under vacuum before imaging. The morphology of the dried gels of **2** and **4** was studied using atomic force microscopy (Veeco, modelAP0100) in noncontact mode. A piece of gel was mounted on a silicon wafer and dried for a few hours under vacuum before imaging.

Circular Dichroism (CD). CD spectra of the aqueous solutions of **2**, **4**, and **11** at varying concentration were recorded by using a quartz cuvette of 1 mm path length in a Jasco J-815 spectropolarimeter. Varying temperature CD spectra were also recorded for compound **2** and **4** at concentration 0.025%, w/v from 20 to 70 °C.

FTIR Measurements. FTIR measurements of the gelators **2** and **4** in $CHCl_3$ solution and in gel state in D_2O were carried out in a Perkin-Elmer Spectrum 100 FT-IR spectrometer using KBr and CaF_2 windows, respectively, with 1 mm Teflon spacers at their MGC.

Fluorescence Spectroscopy. The emission spectra of the compounds **2** and **4** were recorded on Varian Cary Eclipse luminescence spectrometer in a concentration range from 0.0025%, w/v, to above MGC. A super stock solution of **2** and **4** was prepared, which was diluted as required. Solutions were excited at $\lambda_{ex} = 280$ nm. The excitation and emission slit width were 5 and 5 nm, respectively.

X-ray Diffraction (XRD). XRD measurements were taken in Seifert XRD 3000P diffractometer and the source was Cu Kα radiation ($\alpha = 0.15406$ nm) with a voltage and current of 40 kV and 30 mA, respectively. Gel of **4** was mounted on the glass slide and dried under vacuum. The xerogel was scanned from 1−40°.

Microorganisms and Culture Conditions. The in vitro antimicrobial activity of all the cationic amphiphiles was investigated against representative Gram-positive and Gram-negative bacteria. Gram-positive bacteria used in the present study were *Bacillus subtilis* and *Staphylococcus aureus*. Gram-negative bacteria investigated include *Escherichia coli* and *Pseudomonas aeruginosa*. Investigations of antibacterial activities were performed by both broth dilution and spread plate method. The LB medium (tryptone (10 g), yeast extract (5 g), and NaCl (10 g) in 1 L sterile distilled water at pH 7.0) was used as a liquid medium and LB agar (tryptone (10 g), yeast extract (5 g), NaCl (10 g), and agar (15 g) in 1 L of sterile distilled water at pH = 7.0) was used as a solid medium in all antibacterial experiments. All the microbial strains were purchased from Institute of Microbial Technology, Chandigarh, India. The stock solutions of all the amphiphiles as well as the required dilutions were made in autoclaved sterile water.

The freeze-dried ampules of all bacterial strains were opened and a loopful of culture was spread to give single colonies on the respective solid LB agar media and incubated for 24 h at 37 °C. A representative single colony was picked up with a wire loop and was spread on an agar slant to give single colonies. The slants were incubated at 37 °C for the respective time. These incubated cultures of all the bacteria were diluted as required to give a working concentration in the range of 10^6-10^9 colony forming units (cfu)/mL before every experiment.

Antimicrobial Studies. Minimum inhibitory concentrations (MICs) of **1**−**6** were estimated by both broth dilution and spread plate method. MIC was measured using a series of test tubes containing the amphiphiles (0.05−200 μg/mL) in 5 mL of liquid medium. Diluted microbial culture was added to each test tube in identical concentration to obtain the working concentration of bacteria as for *B. subtilis*, $7.5 \times 10^7-1 \times 10^8$ cfu/mL; for *S. aureus*, $5 \times 10^6-7.5 \times 10^6$ cfu/mL; for *E. coli*, $3.75 \times 10^7-7.5 \times 10^7$ cfu/mL; and for *P. aeruginosa*, $9 \times 10^7-1.2 \times 10^8$ cfu/mL. All the test tubes were then incubated at 37 °C for 24 h. The optical density of all the solutions was measured at 650 nm before and after incubation. Liquid medium containing microorganisms was used as a positive control. All the experiments were performed in triplicate and repeated twice.

FESEM of Amphiphile 4 Treated *E. coli*. *E. coli* ($3.75 \times 10^7-7.5 \times 10^7$ cfu/mL) cells (1 mL) were treated with compound **4** above MIC at 200 μg/mL. The bacterial cells with and without (untreated cells as control) amphiphile were incubated for 30−40 min. After incubation, the mixtures were centrifuged at 5000 rpm for 5 min at 4 °C. The media was removed completely and the cells were redispersed in 0.9 wt % saline. Finally, 5 μL of the redispersed samples were mounted on a glass slide and dried under vacuum for 4 h, and the SEM images were taken on JEOL-6700F scanning electron microscope.

Fluorescence Microscopic Study. The Live/Dead BacLight TM Bacterial Viability Kit was used to examine bacterial cell viability under a fluorescence microscope. The kit contains a mixture of two nucleic acid binding stains, specifically referred to as SYTO 9 and propidium iodide. The kit was stored at −20 °C in dark, which is taken out and thawed at room temperature just prior to assay. *E. coli* ($3.75 \times 10^7-7.5 \times 10^7$ cfu/mL) and *S. aureus* ($5 \times 10^6-7.5 \times 10^6$ cfu/mL) cells (1 mL) were treated with four above MIC and also untreated cells were taken in centrifuge tube. The mixtures were centrifuged at 5000 rpm for 5 min at 4 °C. Then, media was removed completely and the cells were redispersed in 0.9 wt % saline. Finally, 3 μL of the BacLight dye mixture was added and incubated in dark at room temperature for 15−20 min. After incubation, 5 μL of the solution mixture was mounted over microscope slides, which then air-dried and viewed under the light microscope (BX61, Olympus) using an excitation filter of BP460−495 nm and a band absorbance filter covering wavelength below 505 nm.

Results and Discussion

Rational design and synthesis of low molecular weight gelators (LMWGs) with a correlation between the structure and gelation property are intensified in recent years due to their potential applications in diversified scientific arena. To develop functional hydrogels, herein we have synthesized a series of *N*-terminal protected Fmoc amino acids/peptides that have pyridinium moiety at the *C*-terminal (**1**−**6**, Figure 1). Cationic charge within these compounds were introduced through the coupling of *m*-amino pyridine at the *C*-terminal of Fmoc-protected amino acids or peptides followed by methylation at the heterocyclic nitrogen of the pyridine ring (Figure S1, Supporting Information). The gelation ability of the amphiphiles was tested by stable to inversion of glass vial at room temperature (Table 1).

Gelation Studies. To check the gelation efficiency we started with Fmoc-protected aliphatic (L-isoleucine, **1**) and aromatic (L-phenylalanine, **2**) amino acid compounds functionalized at *C*-terminal. Amphiphile **1** with aliphatic side chain substituted amino acid was found to be a nongelator. Amphiphile **2** comprising of an aromatic moiety at the side chain exhibited gelation ability with a MGC value of 1%, w/v (Table 1), while 12 h was required for this gelation. This result indicates that, in addition to the two aromatic segments, Fmoc and the pyridinium (Py^+) moiety, the presence of another $\pi-\pi$ stacking unit (phenyl ring of **2**) is important to induce gelation for single amino acid based amphiphiles. At this point, we were interested to know whether the presence of a dipeptide unit instead of the

TABLE 1: Gelation Results of 1−13 in Plain Water

compound	state[a]	MGC (%, w/v)
1	S	
2	TG	1
3	TG	1.7
4	TG	0.6
5	TG	1.5
6	OG	2.2
7	S	
8	S	
9	S	
10	S	
11	TG	3.0
12	S	
13	S	

[a] S: solution (soluble up to 10%, w/v); TG: transparent gel; OG: opaque gel.

single amino acid between the Fmoc and the Py^+ moieties can improve the gelation. To this end, we have synthesized different dipeptide compounds (**3**−**6**, Figure 1) comprising both aromatic (L-phenylalanine) and aliphatic (L-isoleucine) amino acids in varying combinations. Fascinatingly, all these compounds showed excellent hydrogelation ability (Table 1), and the gelation occurs within a few minutes of dissolving the compounds in water. Interestingly, the dipeptide gelator **3** with two aliphatic isoleucine amino acids showed MGC at 1.7%, w/v (Table 1). This result indicates the importance of the additional hydrogen bonding peptide linkage within the dipeptide moiety in hydrogelation. Even the absence of an aromatic moiety in the amino acid unit can be compensated with the presence of an extra hydrogen-bonding moiety between two aliphatic amino acids that converted the nongelator (**1**) to an efficient gelator (**3**). So the presence of an aromatic side chain for single amino acid (**2**) or at least a peptide bond (**3**) is crucial toward hydrogelation of the Fmoc amino acid/peptide functionalized amphiphiles. On this basis, we have synthesized Fmoc dipeptide functionalized cationic amphiphile, **4**, having a diphenylalanine unit with the expectation of better gelation efficiency. Indeed, the amphiphilic dipeptide **4** showed superior gelation efficiency with a MGC value of 0.6%, w/v.

To investigate the effect of dipeptide moiety comprising both aliphatic and aromatic side chains substituted amino acids in the gelation efficiency, compounds **5** and **6** were prepared. The positions of L-isoleucine and L-phenylalanine at *N*- and *C*-termini were swapped in these amphiphiles (Figure 1). It would be intriguing to know which position is preferred by the aromatic moiety in the molecular architecture of the gelator for displaying better gelation efficiency. In the case of **5**, where the aromatic amino acids was closer to the *N*-terminal Fmoc segment and the aliphatic amino acid was attached to the Py^+ unit, MGC value was 1.5%, w/v (Table 1), which is slightly lower than that found in case of the **3** (MGC = 1.7%, w/v). However, as the dipeptide sequence in the gelator **5** was reversed to form compound **6** having aliphatic amino acid at the *N*-terminal attached to Fmoc moiety, gelation efficiency decreased (MGC = 2.2%, w/v) with the formation of an opaque gel. This result indicates that the presence of a nonplanar aliphatic substitution closer to the Fmoc unit possibly hinders the $\pi-\pi$ stacking of the extended aromatic rings resulting decline in the gelation efficiency of **6**. The importance of the amino acid/peptide moiety in the hydrogelation by Fmoc functionalized compounds was further established when compound **7** (Figure 1), having no amino acid unit between Fmoc and Py^+ unit, was found to be a nongelator.

Furthermore, the importance of the planar aromatic ring of Fmoc on the gelation efficiency of these cationic amphiphiles (**1**−**6**) was also investigated. For this purpose, *tert*-butyloxycarbonyl (Boc) unit, which neither had the aromaticity nor the planarity, was utilized as the protecting agent at the *N*-terminal of amino acid/peptide. Keeping all other structural motif identical as in amphiphiles **1**−**6**, only the Fmoc group was replaced by the Boc moiety in **8**−**13** (Figure 1), respectively. All the Boc containing cationic amino acids or dipeptides were found to be nongelators except **11** that showed the ability to form a transparent gel having MGC = 3.0%, w/v. The loss of ordered supramolecular arrangement in presence of nonplanar Boc moiety is the most obvious reason for the nongelation of all the Boc-protected cationic amphiphiles. However, the two aromatic rings of the diphenylalanine peptide in compound **11** might have helped in the restoration of the ordered supramolecular arrangement through its $\pi-\pi$ interaction during gelation. This observation indicates the important participation of $\pi-\pi$ stacking of diphenylalanine units in the self-assembling process that resulted in the hydrogelation of **11**. However, the importance of the extended aromatic ring of Fmoc was stressed once again as the gelation efficiency of **11** (MGC = 3.0%, w/v) is five times lower than the corresponding Fmoc analogue, **4** (MGC

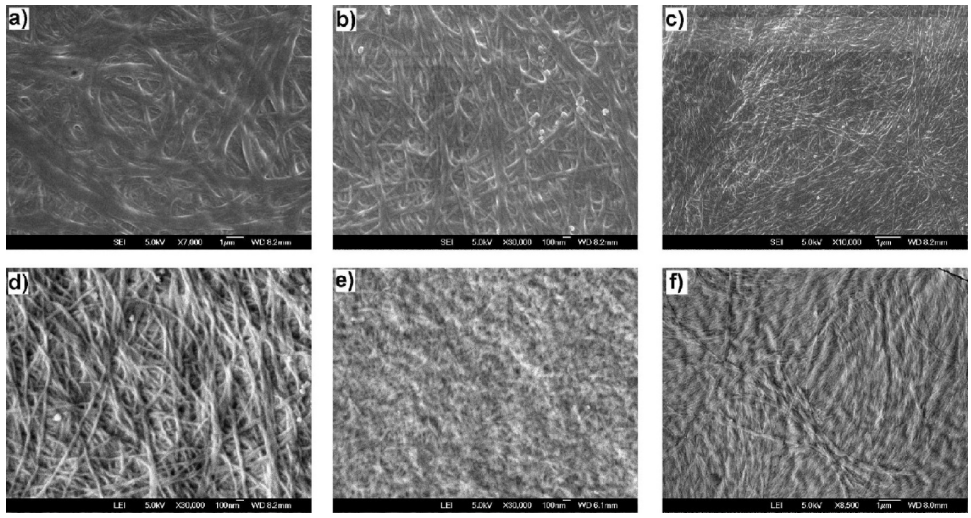

Figure 2. (a−f) FESEM images of the dried gels of **2**−**6** and **11**, respectively at MGC.

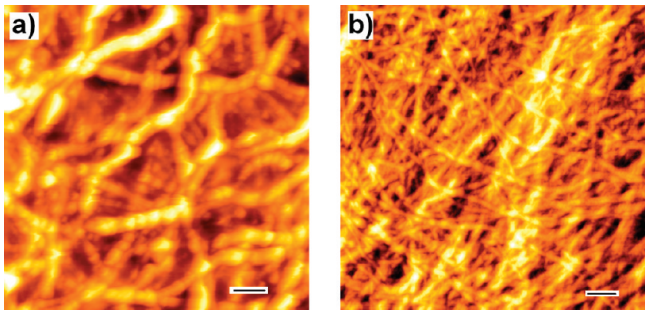

Figure 3. (a,b) AFM images of **2** and **4** at their respective MGC (scale bars = 500 nm).

= 0.6%, w/v). All the hydrogels were thermoreversible and the gel-to-sol transition temperature was ~50−60 °C at their MGC.

Microscopy. Supramolecular morphologies of the gelators **2**−**6** and **11** at their MGC was investigated by field emission scanning electron microscopy (FESEM). All the dried gel showed fibrous networks (Figure 2a−f), although their nature and size were different due to the variation in the supramolecular arrangement, depending on the structure of LMWGs. Gelator **2**, having one amino acid (L-phenylalanine) unit, showed entangled fibrous morphology with an individual fiber diameter of ~60 nm. Some of the fibers were coiled together to form thicker fibers of ~300 nm diameter (Figure 2a). Xerogels of dipeptides **3** (aliphatic−aliphatic) and **4** (aromatic−aromatic) also gave an intertwined fibrous network having a fiber thickness of ~80 and 150 nm, respectively (Figure 2b,c). The length of the fibers of gelator **4** (Figure 2c) was found to be several micrometers with uniform diameter. This intertwined fibrous network of **4** with a large length and relatively thicker diameter possibly helps to entrap more water molecules within its supramolecular structure leading to the formation of most efficient hydrogel among all the amphiphiles. Compound **5** also showed similar intertwined fibrous morphology having fiber thickness ~100 nm (Figure 2d). However, amphiphile **6** exhibited very thin fibrous network having fiber thickness ~40 nm, while the only Boc-protected gelator **11** showed a similar thin scattered fibril structure (Figure 2e,f). The thin fibrous morphology of compounds **6** and **11** made them relatively weak gelators with a MGC value of 2.2 and 3%, w/v, respectively. The fibrous morphology of xerogels of **2** and the best gelator **4** has been further confirmed from their atomic force microscopy (AFM) images (Figure 3a,b). In concurrence with the observed FESEM images, xerogel of **2** showed intertwined fibrous network having fiber diameter ~300 nm (Figure 3a) and the dipeptide **4** gave several micrometer length fiber of 140 nm diameter (Figure 3b).

Circular Dichroism (CD) Spectroscopy. The supramolecular arrangement in the organized aggregates was further confirmed from circular dichroism (CD) spectroscopy of the gelators **2**, **4**, and **11** (Figure 4a−c) in water. Compound **2** showed negative cotton effects in the CD spectra having double minima at 190 and 210 nm (Figure 4a). The nature of the spectra is analogous to that was generally observed for the helical structure of protein by its pattern[14] but not by the positions of the double minima. However, the supramolecular arrangements of dipeptide gelators (**4**, **11**) are different (Figure 4b,c) from that of the single amino acid containing **2**. The CD spectrum of gelator **4** showed a negative peak at 218 nm and a positive peak at 197 nm (Figure 4b). This particular pattern of CD spectra confirmed that the self-assembled structure of compound **4** is mostly dominated with β-sheet arrangement of the peptide backbone.[15] Gelator **11** having same dipeptide moiety with Boc protection also

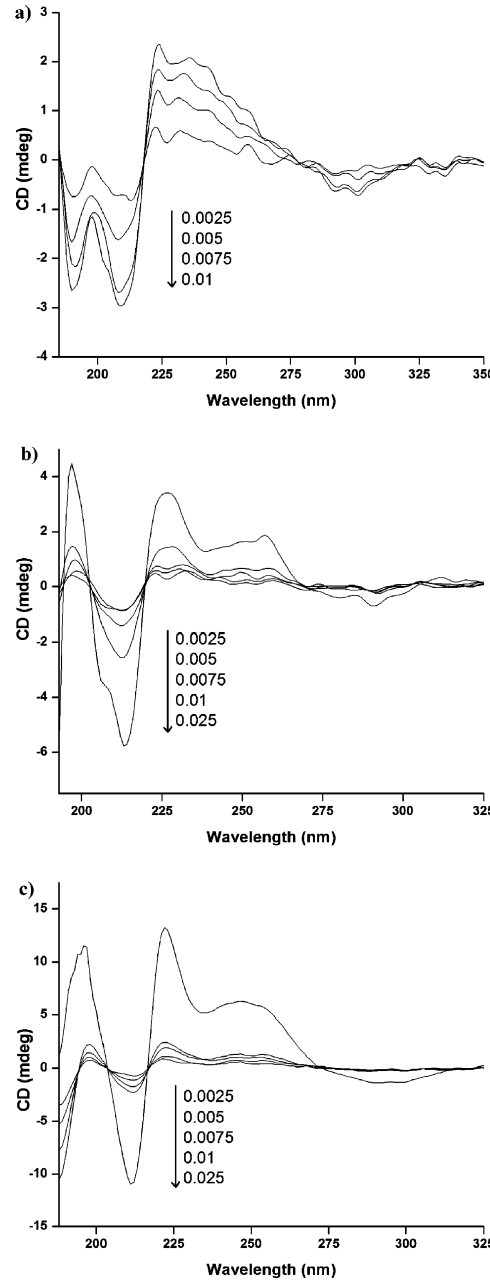

Figure 4. CD spectra of **2**, **4**, and **11** with varying concentration (%, w/v) in plain water at 25 °C.

showed similar CD spectra to that of **4**, which proves that the dipeptide unit is the key factor for this β-sheet dominated supramolecular structure. In all the cases, the formation of a higher ordered structure was originated from the supramolecular association of individual molecules that showed a sharp increase in the peak intensity with the increasing gelator concentration. The noncovalent packing of the molecules was further confirmed from the temperature dependent CD experiment of **2** and **4** at 0.025%, w/v (Figure S2, Supporting Information) in water. In both cases, intensity of the CD peak gradually decreased with an increase in temperature from 20 to 70 °C due to the destruction of the 3D-aggregated structure.

FT-IR Spectroscopy. To investigate the involvement of hydrogen bonding of the peptide (amide) moiety during the self-assembly of gelators, FTIR spectra of **2** and **4** were taken at the self-assembled state in D_2O and at non-self-assembled state in chloroform solution. Compound **2** in chloroform gives carbonyl-stretching frequency (amide-I) at 1708 cm^{-1}, which

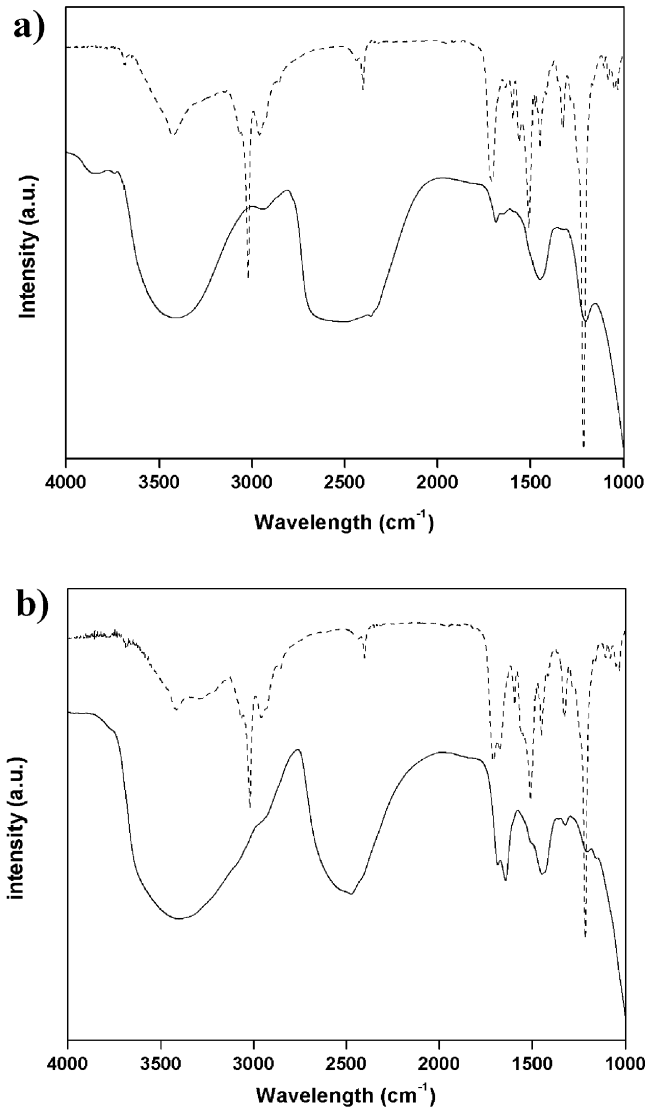

Figure 5. (a,b) FT-IR spectra of **2** and **4** in $CHCl_3$ solution (---) and in D_2O at the gel state (—).

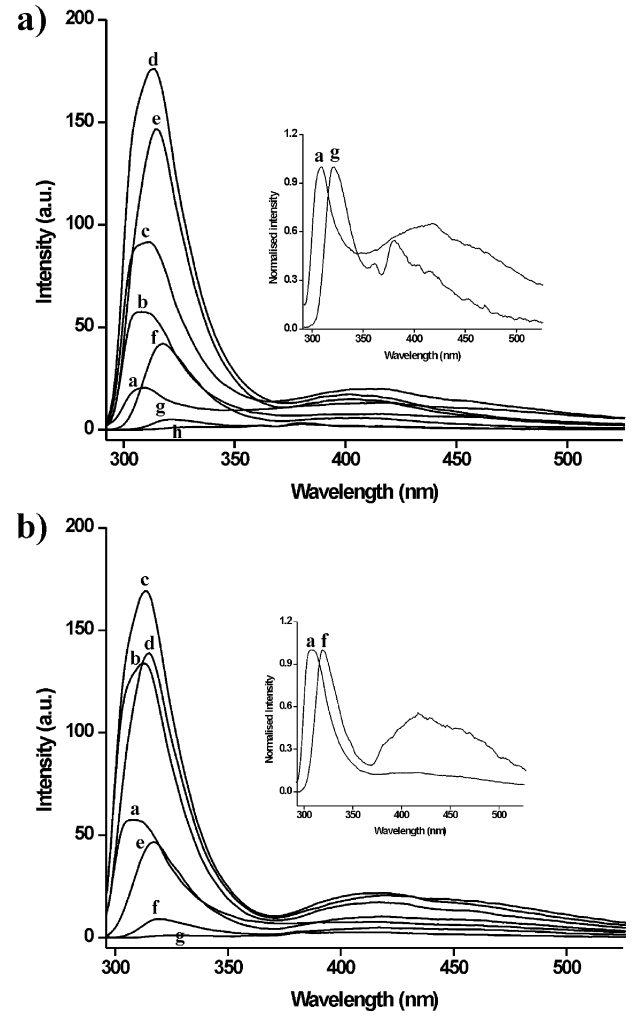

Figure 6. (a) Luminescence spectra of compound **2** at varying concentrations in water at room temperature at λ_{ex} 280 nm: [**2**] (%, w/v) $a = 0.0025$; $b = 0.005$; $c = 0.01$; $d = 0.05$; $e = 0.1$; $f = 0.5$; $g = 1$; $h = 1.5$; Inset is the normalized spectra of 0.0025 and 1%, w/v, of **2**. (b) Luminescence spectra of compound **4** at varying concentrations in water at room temperature at λ_{ex} 280 nm: [**4**] (%, w/v) $a = 0.0025$; $b = 0.005$; $c = 0.01$; $d = 0.05$; $e = 0.1$; $f = 0.5$; $g = 1$; Inset is the normalized spectra of 0.0025 and 0.5%, w/v, of **4**.

at the gel state in D_2O decreased to 1655 cm^{-1} (Figure 5a). This shifting indicates the formation of intermolecular H-bonding of carbonyl group of one molecule with the amide-NH of another molecule at the gel state. Existence of the intermolecular interactions was also seen for **4** as the two amide-I peaks at 1708 and 1678 cm^{-1} in $CHCl_3$ solution shifted to 1684 and 1636 cm^{-1}, respectively, at the gel state in D_2O (Figure 5b). The presence of this two characteristic peaks designate the β-sheet structure of the supramolecular aggregates with antiparallel arrangement of the peptide backbone.[16]

Luminescence Studies. Participation of the fluorenyl moiety of the Fmoc group in the gelation was investigated by taking the luminescence spectra of gelators **2** and **4** with varying concentrations in water (Figure 6). The molecules were excited at 280 nm. At very dilute concentration (0.0025%, w/v), compounds **2** and **4** were found to remain at the molecular state, as it showed emission at $\lambda_{em} = 307$ nm. With the increase of concentration, the intensity of the peaks initially increases followed by decrease with continuous red shift of the λ_{em} of fluorenyl group. At a concentration of 1%, w/v of both the gelators, the emission was observed at $\lambda_{em} = 323$ nm, which confirms the self-assembly of the fluorenyl moiety where this extended aromatic rings were overlapped in an antiparallel orientation.[17] The red shift of the short wavelength emission (normalized intensity) with increase in concentration of gelator is shown in the inset of the corresponding figure. Also, at higher concentration of the amphiphiles, a broad phosphorescence peak appeared at 425 nm. This indicates the more efficient overlapping of fluorenyl as well as the phenyl ring at the self-assembled state.[17c] At 1%, w/v, of compound **2**, a small peak at 361 nm is generated, which is the characteristic peak for parallel orientation of fluorenyl group.[17b] Thus, in the gel state, these Fmoc functionalized cationic amphiphiles self-assemble mostly by the antiparallel overlapping through $\pi-\pi$ stacking of the aromatic rings.

Wide Angle X-ray Scattering (WAXS). To establish further the molecular packing and the possible orientation of the gelator in the supramolecular fibrous networks, we have taken the wide-angle X-ray diffraction (WXRD) spectra of the dried gels of **4**. Xerogel obtained from gel **4** gave a peak at $2\theta = 20.05°$ corresponding to *d*-spacing 4.6 Å, which is accompanied by another peak at $2\theta = 9.4°$ with a *d*-spacing of 9.5 Å (Figure 7). These two peaks indicate the presence of the cross β-structure between the dipeptide moieties with an antiparallel alignment in the peptide strand.[18] This observation is also supported from the results obtained from the CD and FTIR spectroscopy where

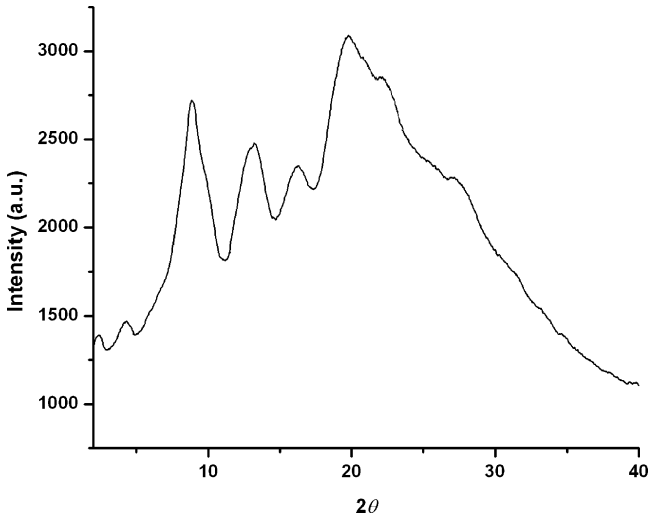

Figure 7. XRD diagrams of the dried gels of **4**.

the presence of the β-structure between the dipeptide moieties was indicated. The periodicity of 4.6 Å arises from spacing between the peptide chains with in a β-sheet where as the other peak of periodicity 9.4 Å was due to the spacing between two stacked β-sheet layers. Another peak at *d*-spacing 3.5 Å (2θ = 23.01°) further established the presence of $\pi-\pi$ stacking between two antiparallel Fmoc group in the self-assembled β-sheet structure.[19] The observed spectroscopic and microscopic studies suggest an ordered arrangement of the Fmoc-peptide functionalized cationic amphiphile involving the H-bonding, $\pi-\pi$ stacking interaction, and the higher order packing in the 3D network of the hydrogel (representative example is given for **4** in Figure 8).

Antibacterial Activity. As mentioned earlier that biomedicinal applications of these anti-inflammatory Fmoc-peptide-based cationic amphiphiles can be dramatically enhanced if they also become simultaneously antibacterial. To this end, "cationic peptide antibiotics", as well as quaternary ammonium and pyridinium amphiphiles, are known to exhibit antibacterial activity.[13] As the Fmoc-based compounds possess positively charged pyridinium moiety, they also could be potential small molecule antibacterial agents. Antibacterial activities of all the Fmoc-based peptides (**1**−**6**) were checked against both types of Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria. Minimum inhibitory concentrations (MIC), the lowest amphiphile concentration at which no viable bacterial cell is present, are presented in Table 2. Interestingly, almost all the Fmoc-based cationic amphiphiles showed killing efficacy against both Gram-positive and Gram-negative bacteria. Compound **1** having nonaromatic L-isoleucine showed relatively low antibacterial activity against Gram-positive bacteria (MIC 150 and 200 μg/mL for *B. subtilis* and *S. aureus*, respectively). MICs of **1** against Gram-negative bacteria *E. coli* and *P. aerugenosa* were 150 and 75 μg/mL, respectively. However, dipeptide of L-isoleucine (**3**) showed better activity than **1** (Table 1), but it cannot kill *E. coli* up to 200 μg/mL. Among all the Fmoc-based amphiphiles, **2**, **4**, and **5** are most effective in killing both types of bacteria. Compound **2** had the lowest MIC values within 20−50 μg/mL, irrespective of the nature of bacteria. Also, amphiphile **4** exhibited the lowest MIC (10 μg/mL) against Gram-positive *S. aureus*. Interestingly, in these three amphiphiles, L-phenylalanine was attached to the Fmoc moiety. The close proximity of fluorenyl and phenyl moieties is presumably helping to achieve an optimum hydrophobicity known as "threshold hydrophobicity" that is required for the

TABLE 2: Antibacterial Activity (MICs) of the Amphiphiles 1−6 in μg/mL

	Gram-positive		Gram-negative	
amphiphile	*B. subtilis*	*S. aureus*	*E. coli*	*P. aerugenosa*
1	150	200	150	75
2	30	20	50	20
3	30	30	[a]	40
4	25	10	150	40
5	30	20	100	20
6	50	200	200	200

[a] No killing up to 200 μg/mL.

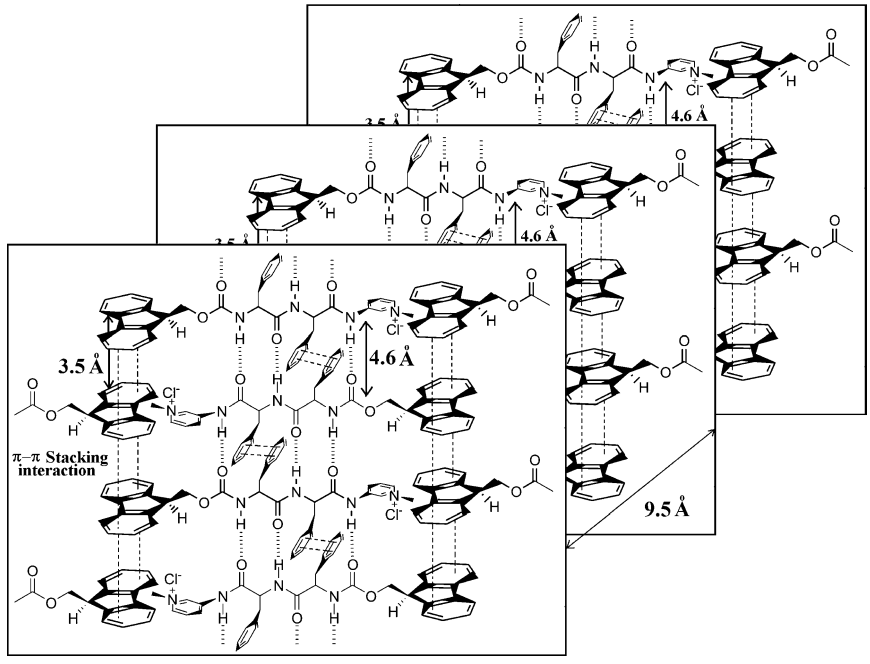

Figure 8. Probable arrangement of **4** in the gel state.

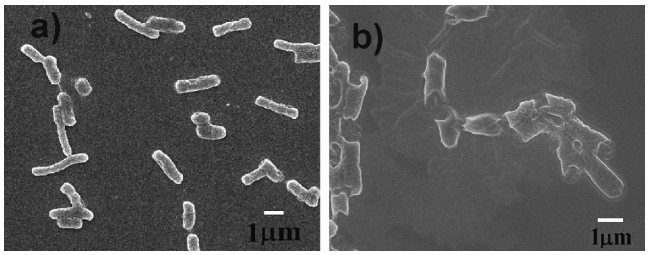

Figure 9. Scanning electron microscopic (SEM) image of (a) control bacterium *E. coli*; (b) *E. coli* after treatment with 200 μg/mL of amphiphile **4**.

effective killing of bacteria.[20] Again, the swapping of L-phenylalanine from *N*-terminal Fmoc unit to *C*-terminal pyridinium group in compound **6** resulted in comparatively poor antibacterial efficacy, possibly because of the loss of said "threshold hydrophobicity". Thus, antibacterial activity of these Fmoc-peptide-based cationic amphiphiles is dependent on each structural motif as well as on their sequence in the molecule.

Although exact mechanism of bactericidal effect remains to be better understood, but in general it seems that the antimicrobial effect is originated from the ability of the cationic compounds to penetrate the cell membrane of the microorganisms.[21] Positively charged peptide/amphiphiles are believed to attack negatively charged cell membrane of microbes, which is also entropically favorable as huge numbers of counterions are released.[22] Next, the hydrophobic moiety helps in the diffusion of the small molecules by "self-promoted" transport across the bacterial membrane resulting in release of cytoplasmic constituents leading to the death of bacteria.[13b,23] In general, it has been observed that it is relatively easier to kill Gram-positive bacteria rather than Gram-negative because the former have a simple cell wall whose major constituent is peptidoglycan, a polysaccharide. In contrast, Gram-negative has an additional outer bilayer membrane composed of lipopolysaccharides and phospholipids.[13c] However, the Fmoc-based cationic amphiphiles used in the present study are almost equally effective in killing both bacteria. To unravel the killing mechanism further in depth investigation is required.

To get the insight into the effect of amphiphiles on the morphology of bacteria,[13b,24] FESEM images of *E. coli* were taken before and after the treatment with amphiphile **4** above MIC at 200 μg/mL (Figure 9). The untreated bacteria *E. coli* (Figure 9a) exhibited integrity of cell membrane. The incubation of *E. coli* with amphiphile **4** for around 30−40 min causes a significant change in their cell morphology where the SEM image showed deformation in the membrane integrity of dead *E. coli* (Figure 9b). The antibacterial activity of the representative amphiphile **4** was also studied by fluorescence microscopy using commercially available Live/Dead BacLight bacterial viability kit.[13a,b] The kit is composed of two nucleic acid binding stains, known as SYTO 9 and propidium iodide. These dyes have different cell penetration properties as well as different spectral characteristics. SYTO 9 binds to the nucleic acid of both living and dead cells and fluoresce green after excitation with BP460−495 nm filter. Propidium iodide can only bind to the nucleic acid of the dead cell and shows red fluorescence. The untreated bacterial cells of *E. coli* and *S. aureus* exhibited green fluorescence (Figures S3a and S3c, Supporting Information), indicating that they were alive. After treating with the 200 and 50 μg/mL of **4** for *E. coli* and *S. aureus* cells, respectively, only red fluorescence (Figures S3b and S3d, Supporting Information) was observed that confirmed the killing efficiency of the amphiphile.

Conclusion

Herein, we have developed Fmoc-peptide functionalized cationic hydrogel. Systematic analysis with special emphasis on the structural aspects as well as the mechanism of the gelation processes reveals that a minute architectural change at the molecular level influences the self-assembling mechanism. Noncovalent interactions including $\pi-\pi$ stacking and intermolecular hydrogen bonding were found to be the major responsible factors for the gelation process. Several spectroscopic and microscopic studies confirm the antiparallel β-sheet arrangement of the self-assembled peptides in the gel phase. These Fmoc-based cationic peptide exhibited efficient antibacterial activity against both Gram-positive and Gram-negative bacteria. Development of such antibacterial soft matters (biomaterials) from Fmoc-protected amino acid/peptide-based cationic gelators comprising efficient antibacterial activity will have immense importance in the material science.

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Supporting Information Available: Generalized synthetic schemes, characterization for all the amphiphiles, temperature dependence CD spectra and Live/Dead fluorescence micrograph of *E. coli* and *S. aureus* before and after treating with **4**, are provided in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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