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Water Gelation of an Amino Acid-Based Amphiphile

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Abstract: The water immobilization by a simple amino acid-containing cationic surfactant was investigated. A variety of techniques, such as ^{1}H NMR spectroscopy, circular dichroism (CD), steady-state fluorescence spectroscopy, and field-emission scanning electron microscopy (FESEM) were applied to determine the formation and architecture of the hydrogel. The new gelator with a minimum gelation concentration (MGC) of 0.3% w/v shows prolonged stability and a low melting temperature (39 °C). ^{1}H NMR experiments revealed that intermolecular hydrogen bonding between the amide groups and π–π stacking of the indole rings are the two regulating parameters for gelation. Furthermore, fluorescence studies with 8-anilino-1-naphthalenesulfonic acid (ANS) as the probe indicate the participation of hydrophobicity during gelation. The luminescence study using both ANS and pyrene, along with FESEM results, indicate a critical concentration, well below the MGC, at which fibres begin to form. These cross-link further to give thicker fibers, leading to the formation of a hydrogel (0.3% w/v). This new hydrogelator expresses high supramolecular chirality, as evidenced by the CD spectra. In addition, the gelator molecule was found to be nontoxic up to a concentration of 4 mM (0.2% w/v). The high supramolecular chirality, prolonged stability, low melting point, and biocompatibility of the molecule make it a focus of chemical and biological interest.

Keywords: amino acids · amphiphiles · fluorescence · hydrogels · supramolecular chirality

Introduction

The group of soft materials known as gels has attracted the attention of modern-day chemists, owing to its versatile application in many areas, such as photography, cosmetics, drug delivery, tissue engineering, sensors, and food processing.[1] These supramolecular aggregates neither flow freely like a liquid nor take a definite shape like a rigid solid. Owing to their diverse applications, water-gelating compounds (hydrogelators) have been studied extensively, although most reports have dealt with the gels formed by polymeric molecules.[1] Nevertheless, low-molecular-weight-hydrogelators (LMWH) are emerging as the preferred choice over their polymeric counterparts, primarily because of their rapid response to external stimuli and their thermoreversible nature, due to the noncovalent intermolecular associations within the three-dimensional network of the self-assembly.[1,2] Although gels derived from low-molecular-weight compounds ("supramolecular gels" or "physical gels") have been known for a long time,[3] it is only within the last couple of decades that they have been actively investigated.[1,2]

The coexistence of extremely ordered fibers with fluids, the well-defined structure, quick responsiveness, high interfacial area and, most importantly, noncovalent interactions, make low-molecular-weight gelators very attractive alternatives to the polymeric gelators.[1,2] For the biological application of such physical/molecular gels, their biocompatibility and supramolecular responsiveness are indispensable.[4] The possibility to entrap solutes within the fibrillar network of a biocompatible hydrogel provides the opportunity to find a suitable carrier for drug delivery.[1,4,5] Although quite a few low-mass organogelators[6] and their applications have been reported, the study of LMWH has been very limited. Furthermore, hydrogelators formed in the presence of 1–10% of an additional solvent or acid limit their application in biological systems.[7] The search for molecules that gelate in simple water, an essential criteria for biological use, is intensifying.[4c,8]

To this end, amphiphilic molecules, namely surfactants, have shown the potential to form well-characterized, supra-

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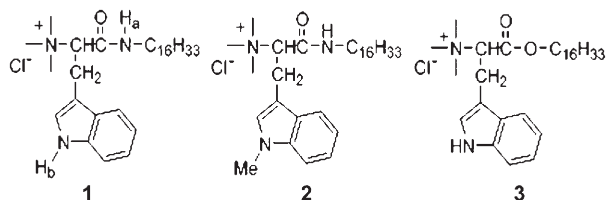
molecular structures, such as micelles, microemulsions, and bilayers (membrane mimetic systems), which have been utilized in several chemical and biological applications.[9] The presence of both hydrophilic and hydrophobic groups within the same molecule also allows them to aggregate in an ordered fashion to induce immobilization of solvents.[1a,10] Here, we report the hydrogelation of a simple amino acid-based amphiphile (**1**, Scheme 1) in plain water (minimum



Scheme 1. Structures of the synthesized compounds.

gelation concentration (MGC), 0.3% w/v). The molecular arrangement of the hydrogel formed by the L-tryptophan-containing surfactant molecule (**1**) was investigated by spectroscopic and microscopic techniques. Intermolecular hydrogen bonding, π–π stacking, and hydrophobic interactions were responsible for its gelation. The hydrogelator **1** also showed high supramolecular chirality and biocompatibility, which make it an appropriate material for both chemical and biomedicinal applications.

Results and Discussion

In our recent study,[11] the aqueous self-assembly of the L-tryptophan-based cationic surfactant, **1** (Scheme 1, M_r = 505), was utilized at 5 mM (0.25% w/v) as a template for the asymmetric reduction of ketones/esters in the presence of $NaBH_4$. We found that at a slightly higher concentration (6 mM, 0.3% w/v), amphiphile **1** forms a visible and transparent hydrogel (Figure 1) in a glass vial of inner diameter

Figure 1. Transparent hydrogel of **1** (0.3% w/v).

10 mm. No such gelation was observed during the asymmetric reduction of esters and ketones[11] in aqueous solution of **1** (5 mM, 0.25% w/v), possibly because the vigorous stirring and vortexing of the reaction mixture led to the rupture of the weak three-dimensional network within the physical gel.

The thermoreversible hydrogelation of **1** was observed at 0.3% w/v (6 mM, Figure 1), which upon slow heating to 39 °C melts and on cooling turned again to gel. Each gelator molecule has the ability to immobilize ~9300 molecules of water at room temperature. The sol-gel transition temperature T_g increased as gelator concentration increased, which is in agreement with previous reports (Figure 2).[7a,b] This ob-

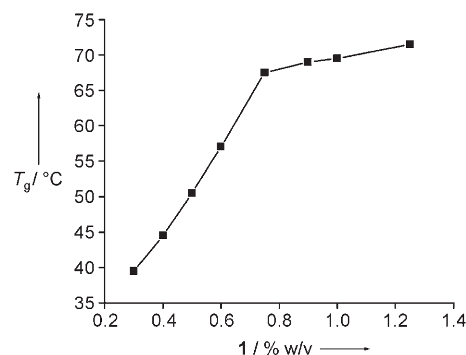


Figure 2. Plot of T_g against gelator concentration.

servation and also the low gelation concentration indicate that self-assembly in the gel state is driven by strong intermolecular, noncovalent interactions. Furthermore, the hydrogel formed by **1** (0.3% w/v) was observed to be stable at room temperature over a period of one year. Because some biological applications require a stable gel with low sol-gel transition temperature,[1e] amphiphile **1** might have applications in the area of biomedicine.

The structural requirements for a molecule to immobilize water are still poorly understood. One of the key parameters for gelation is the balance between hydrophobicity and hydrophilicity (hydrogen bonding). To elucidate the role of hydrogen bonding in the hydrogelation of **1**, related compounds **2** and **3** were synthesized (Scheme 1). Compound **2**, in which indole N−H was replaced by N−Me, hydrogelated at 0.45% w/v, whereas **3** (amide bond was replaced by an ester linkage), at concentrations greater than 0.05% w/v (below this concentration it was soluble in water) precipitated from the warm solution upon cooling to room temperature. This indicates the essential participation of the amide N−H (H_a, Scheme 1), possibly through intermolecular hydrogen bonding, and a less-significant influence of the indole N−H (H_b) in hydrogelation.

^{1}H NMR experiment: In general, NMR techniques provide a great deal of information about the supramolecular arrangement of gelators, and in particular, they give a qualitative idea of the possible orientation of a single molecule in an aggregated system. To elucidate the possible supramolec-

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ular architecture of the hydrogel formed by compound **1**, a thorough NMR study was performed by using 1% w/v **1** in [D$_6$]DMSO with an increasing amount of water. As shown in Figure 3a, intermolecular hydrogen-bonding of H_a and H_b

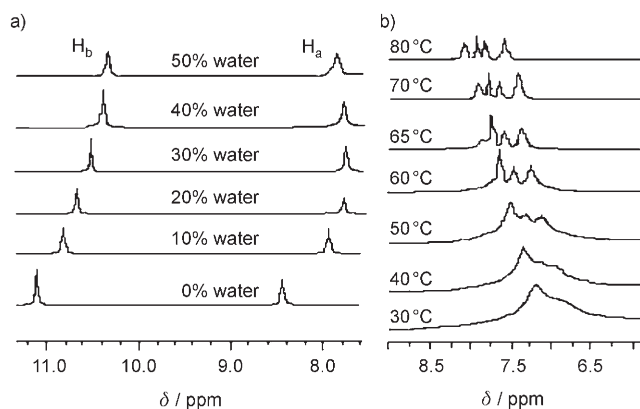

Figure 3. a) ^{1}H NMR spectra of **1** in [D$_6$]DMSO with increasing H_2O content. b) ^{1}H NMR spectra of **1** in D_2O at increasing temperature.

was found to be one of the regulating factors for the self-assembly of the surfactant molecules. The amide N–H shifted upfield (from 8.55 to 7.79 ppm) as the water content increased up to 30%, and then moved downfield (to 7.99 ppm) as the proportion of water increased further. In contrast, the indole N–H showed a continuous upfield shift (from 11.09 to 10.29 ppm) as the water content increased. Upon initiation of the self-assembly process, the bulky indole group possibly twists toward the hydrophobic domain of the self-aggregate, thereby exposing the carbonyl group in the aqueous phase.[12] Thus, also conformational changes presumably forced the H_a to move towards the hydrophobic region, resulting in upfield shift of both H_a and H_b.[12] Above 30% water content, intermolecular hydrogen bonding might have taken place between the H_a and the carbonyl oxygen, which deshielded the amide N–H. Moreover, as the water content increases, the ammonium segment (headgroup) of the molecule becomes hydrated, leading to the upfield shift of the neighboring protons of H_a and H_b.[10c] Above 30% water content, the amide proton possibly starts participating in the intermolecular hydrogen bonding, leading to the dehydration and initiation of fiber formation and resulting in the downfield shift of H_a. In case of H_b, the upfield shift continued, due presumably to the increasing π–π interaction of the parallel indole moieties, as well as hydrogen bonding with the H_2O molecules.[10c,12]

^{1}H NMR spectra were also recorded for 1% w/v **1** in D_2O at different temperatures (Figure 3b). In the gel state at 30 °C, a broad peak was observed in the aromatic region, and this showed splitting with a downfield shift (7.22 to 8.08 ppm) as the temperature increased from 30–80 °C. In the gelated state, these protons cannot produce individual sharp signals as they are in aggregated form.[13] In addition, the aromatic protons were very much shielded, due possibly to the π–π interaction of the indole rings in the self-assembled structure.[10c] As the temperature increases, the intermolecular hydrogen bonding is destroyed, leading to the transition from gel to sol (non-self-assembled species), in which the spinning nuclei showed their characteristic in-solution signals. The rise in temperature also eliminates the hydrophobic stacking of indole moieties, resulting in the downfield shift of the aromatic protons. Interestingly, the protons signals at ~70 °C are comparable to those in the sol state, indicating that the T_g of 1% w/v **1** is similar to that deduced from the plot of T_g vs gelator concentration (Figure 2).

Luminiscence studies: Because hydrophobic interaction is also one of the major driving forces in the self-assembly process, its crucial role in the present gelation was deciphered by recording luminescence spectra with 8-anilino-1-naphthalenesulfonic acid (ANS), a popular probe for hydrophobic domains. Figure 4a shows the emission spectra of ANS obtained by varying the concentration of **1** in water.[7b,c,8a]

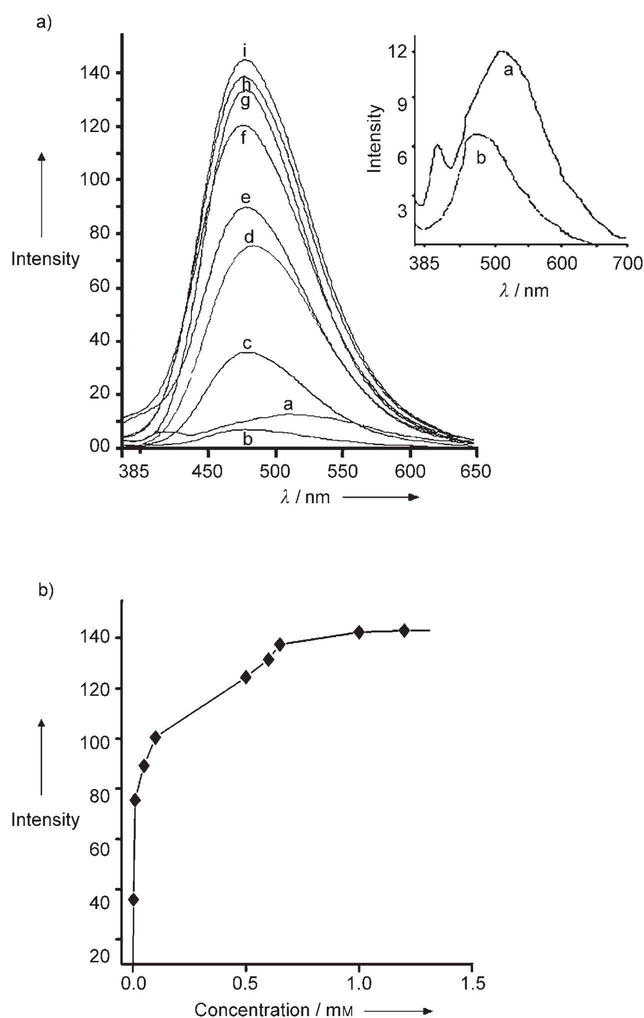

Figure 4. a) Luminescence spectra of ANS (1×10^{-5} M) with increasing concentration of **1** in water at RT. [**1**] (×10^3% w/v): a: 0; b: 0.05; c: 0.15; d: 0.5; e: 2.5; f: 5; g: 25; h: 50; i: 300. Inset: enlargement of the low concentration range. b) Dependence of luminescence intensity of ANS on gelator concentration.

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As the gelator concentration increases to 5×10^{-5}% w/v (1×10^{-3} mM), initially a blue-shift in the λ_{max} (511 to 477 nm) accompanied by a decrease in the intensity was observed. Thereafter, only a moderate blue-shift (472 nm) was seen, however, the intensity increased steadily as the concentration of **1** increased. Such luminescence behavior of ANS indicates the existence of a hydrophobic environment,[8a] thereby confirming its participation in hydrogelation. Furthermore, the dependence of the emission intensity of ANS on gelator concentration (Figure 4b) suggested two inflection points at ~5.5×10^{-3}% w/v (0.11 mM) and 0.03% w/v (0.6 mM). The first inflection point probably indicates the critical micelle concentration (cmc) of **1**, which was observed to be ~0.14 mM from the plot of concentration vs first-to-third vibronic band ratio (I_1/I_3) in pyrene fluorescence experiments.[11b] At 0.6 mM (0.03% w/v), after which no change in intensity was noted, the gelator molecules probably show the propensity to aggregate further into fibers, which become cross-linked (noncovalent) at 6.0 mM (0.3% w/v) to form the hydrogel.

The overlayed spectra of pyrene (Figure 5) show a red-shift in λ_{max} to 408 nm, along with a steady increase in the intensity above the gelator concentration of 0.025% w/v

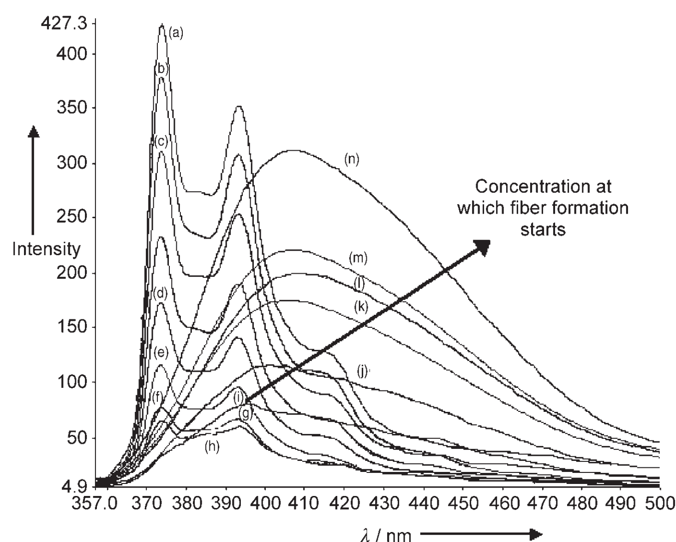

Figure 5. Luminescence spectra of pyrene (1×10^{-7}M) in aqueous solutions of various concentration of **1** at RT. [**1**] (% w/v): a) 0.0003; b) 0.0005; c) 0.001; d) 0.0025; e) 0.005; f) 0.01; g) 0.02; h) 0.025; i) 0.03; j) 0.1; k) 0.15; l) 0.2; m) 0.25; n) 0.4.

(0.5 mM). The formation of exciplex of pyrene with the solvent or any other molecule present in the solution is a well-known phenomena.[14] The appearance of the red-shifted peak may be explained in terms of the formation of exciplex. As the concentration of **1** increased, micellar aggregate was formed initially (cmc was ~0.14 mM (0.007% w/v), as obtained from the I_1/I_3 vs surfactant concentration plot (not shown), as well as from the first inflection point in Figure 4b). A further increase in concentration leads possibly to the formation of fibers, in which the pyrene molecules become incorporated, and consequently, the local concentration of the probe is increased. The increased concentration presumably allows the formation of exciplex[14] through the interaction with the indole moiety of the gelator. The possibility of the red-shifted peak due to the excimer formation is very remote. The excimer peak of pyrene is known to appear from the dimeric form of the probe; however, the concentration of pyrene in the 0.03% w/v solution of **1** at which the red-shifted peak was observed is ~6000 times lower than that of the gelator. Thus, the formation of pyrene dimer is very unlikely. Furthermore, the typical λ_{max} for the pyrene excimer fluorescence is 454 nm and the red-shifted peak appeared in the present case at 408 nm. Similar observations have been reported previously,[14c,d] which, along with the explanations discussed above, support the appearance of exciplex and not the excimer in the pyrene fluorescence spectra (Figure 5). From the observations described and the results obtained from the ANS fluorescence study mentioned above, we can conclude that the surfactant molecules start aggregating to form the fibers at this stage (0.03% w/v, 0.6 mM), and a further increase in concentration leads to formation of the gel network (at 0.3% w/v, 6 mM, MGC).

Circular dichroism: The circular dichroism (CD) spectra of **1** in water were recorded to show the supramolecular chirality expected to originate from chiral monomer during self-aggregation (Figure 6). A positive cotton effect in the

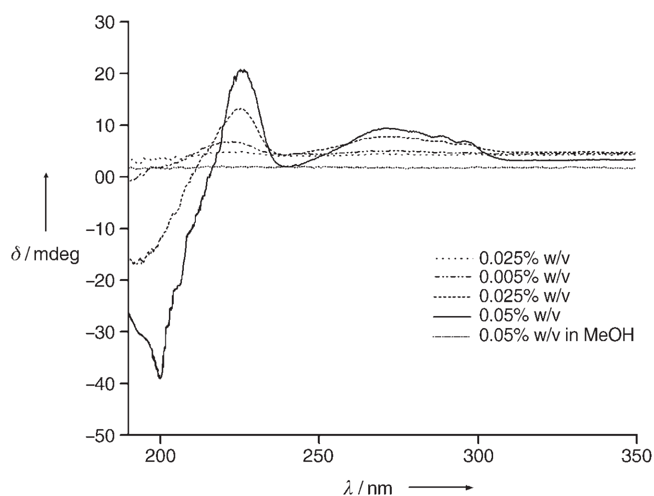

Figure 6. CD spectra of **1** at increasing concentrations in water, and in 1:1 (v/v) MeOH/H_2O at RT.

amide-absorption region, as well as an increase in the molar ellipticity as gelator concentration increases, confirms an ordered arrangement of the chiral planes that lead to the high supramolecular chirality. The peak at 220–225 nm could be attributed to the π–π^* transition of the amide bond, and the shoulder at longer wavelength originates from the n–π^* transition of the same.[15] These transitions are extremely sensitive to coupling with neighboring amides. The increased in-

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tensity at 225 nm suggests a helical arrangement[4a] at the supramolecular level induced by the L-tryptophan residue, whereas in the presence of an organic protic solvent, such as methanol, all the CD peaks disappeared, due to the disintegration of the self-assembly. This is also supported by the field-emission scanning electron microscopy (FESEM) image of the xerogel (0.3% w/v), in which each helical fiber of regular thickness (~40 nm) combines to form thicker fibers of ~200 nm (Figure 7b). The FESEM images show a

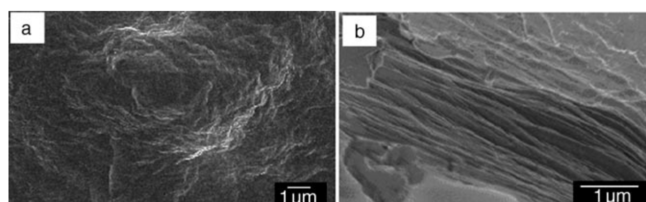

Figure 7. FESEM images of the dried samples of a) 0.03% w/v and b) 0.3% w/v of **1** in water.

very thin fibrous morphology in the sample of 0.03% w/v **1** (0.6 mM, Figure 7a). As discussed above, these thin fibers probably become aggregated at high concentration and form bundles of thicker fibers through cross-linking, as seen in the FESEM image of the xerogel at 0.3% w/v (6 mM, Figure 7b). Thus, the second inflection point in Figure 4b, the appearance of the exciplex peak in the pyrene fluorescence spectra (Figure 5), and the presence of the thin fibers in Figure 7a allows us to conclude that the fiber formation starts at a much lower concentration (0.03% w/v; 0.6 mM), and this turned to visible gel through a cross-linking network at a concentration of 0.3% w/v (6 mM, Figure 7b).

Results of the spectroscopic and microscopic studies suggest an ordered arrangement within a three-dimensional network of the hydrogel of **1** through hydrogen bonding assisted by hydrophobic packing (Figure 8). The intermolecular hydrogen bonds between amide N−H and carbonyl oxygen, and π–π stacking of the aromatic planes leads to the helical-fiber structure within the self-assembly.

Cytotoxicity: Cytotoxicity of the gelator **1** was tested in HELA cells (Figure 9) by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[16] Cationic surfactants are generally toxic in nature as they lyse[17] the cells. Thus, the normal concentration range of cationic lipid used for biomedicinal studies, such as transfection and drug delivery, is from micromolar to a few millimolar.[18] In this respect, the single-chain amphiphile **1** may have potential for biomedical applications, as it was shown to be nontoxic; the cell viabilities were >75% for concentrations of **1** up to 4 mM (0.2% w/v, below MGC), although this reduced to 25% at MGC (0.3% w/v, Figure 9).

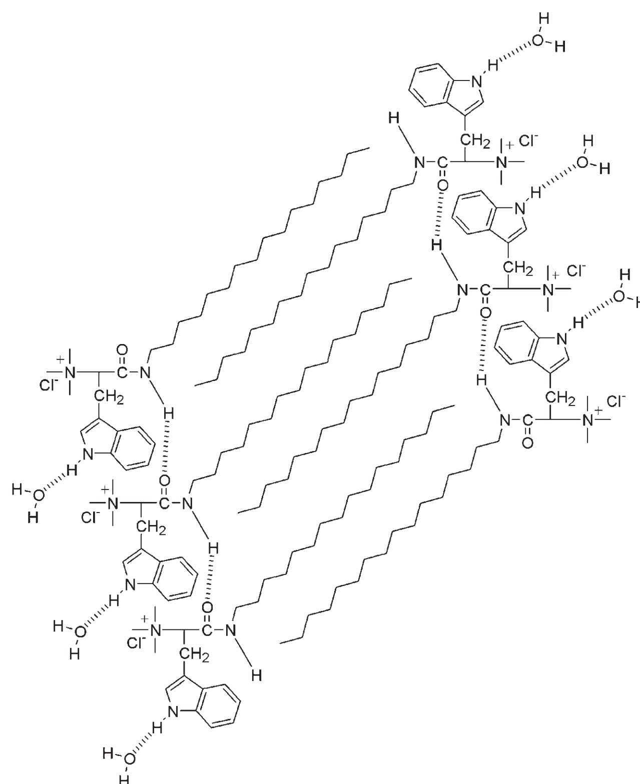

Figure 8. Schematic representation of the possible network in the hydrogel of **1**.

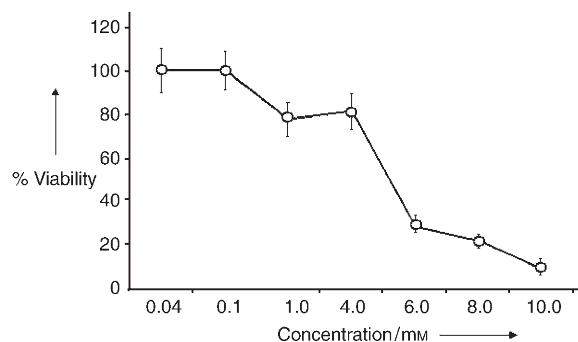

Figure 9. Viability of HELA cells in increasing concentrations of **1**.

Conclusion

We have demonstrated the hydrogelation properties of an L-tryptophan-based simple amphiphile. Structural elucidation of the hydrogel shows that intermolecular hydrogen bonding, π–π stacking, and the hydrophobic–lipophilic balance of the molecule leads to the formation of helical fibers, which in turn produce the gel network. The high supramolecular chirality in the gel state makes this hydrogel useful as a template, especially in asymmetric transformations. Owing to its unique characteristics of nontoxicity, high stability, and low sol-gel transition temperature, this simple molecule may also find important biochemical and biomedicinal applications.

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Experimental Section

Materials: HPLC-grade water was purchased from Qualigens (India). L-Tryptophan, *n*-hexadecylamine, *n*-hexadecanol, *N,N'*-dicyclohexylcarbodiimide (DCC), 4-*N,N*-dimethylaminopyridine (DMAP), iodomethane, sodium hydride, solvents, and all other reagents were procured from SRL (India). All the deuteriated solvents for NMR experiments and 8-anilino-1-naphthalenesulfonic acid (ANS) were obtained from Aldrich. Amberlyst A-26 chloride ion-exchange resin was obtained from BDH (UK), and pyrene was procured from Fluka. ^{1}H NMR spectra were recorded by using an AVANCE 300 MHz (Bruker) spectrometer. ESI-MS was performed by using a Q-tof-Micro Quadruple mass spectrophotometer (Micromass). FESEM images were taken by using a JEOL-6700F microscope. Emission spectra were recorded by using a Perkin–Elmer LS55 luminescence spectrometer. CD experiments were performed by using a Jasco J-600C spectropolarimeter.

Synthesis of [2-(1*H*-indole-3-yl)-1-hexadecylcarbamoylethyl]trimethylammonium chloride (1): *Tert*-butyloxycarbonyl (Boc)-protected L-tryptophan was coupled with *n*-hexadecylamine by using DCC (1 equiv) and a catalytic amount of DMAP in the presence of 1 equivalent of *N*-hydroxybenztriazole in dry dichloromethane (DCM). The Boc-protected amide obtained was then subjected to deprotection by trifluoroacetic acid (TFA, 4 equiv) in dry DCM. After 2 h of stirring, solvents were removed by using a rotary evaporator and the mixture was taken up in ethyl acetate. The EtOAc phase was washed thoroughly with aqueous 10% sodium carbonate solution followed by brine to neutrality. The organic phase was dried over anhydrous sodium sulfate and concentrated to get the corresponding amine. The primary amine (1 equiv) obtained was quaternized with excess iodomethane by using 2.2 equivalents of anhydrous potassium carbonate and a catalytic amount of [18]crown-6 ether in dry DMF for 2 h. The reaction mixture was taken up in ethyl acetate and washed with aqueous thiosulfate solution and water, respectively. The concentrated ethyl acetate phase was crystallized from methanol/ether to obtain solid quaternized iodide salt, which was subjected to ion exchange on an Amberlyst A-26 chloride ion-exchange resin column to yield the pure chloride (**1**, ~60%).

Data for 1: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.83$ (t, 3H), 0.97–0.99 (br, 2H), 1.11–1.32 (br, 24H), 1.66–1.73 (br, 2H), 2.82–2.91 (m, 2H), 3.20–3.24 (m, 2H), 3.31 (s, 9H), 5.60 (br, 1H), 7.01–7.06 (br, 1H), 7.29–7.31 (d, 2H), 7.41 (d, 1H), 7.48–7.51 ppm (d, 1H); MS (ESI): *m/z* calcd for $C_{30}H_{52}N_3O$ (the 4° ammonium ion, 100%): 470.41; found: 470.5699 [M^+]; elemental analysis calcd (%) for $C_{30}H_{52}N_3OCl$: C 71.18, H 10.35, N, 8.30; found: C 71.28, H 10.43, N 8.35.

Synthesis of [2-(1-methyl-indole-3-yl)-1-hexadecylcarbamoylethyl]trimethylammonium chloride (2): The iodide salt of compound **1** was treated with 1.2 equivalents of sodium hydride and excess iodomethane in dry dimethyl sulfoxide (DMSO) under inert condition at 55°C for 4 h. Water was added and the material was extracted with chloroform and dried over anhydrous sodium sulfate. The organic phase was concentrated to give a gummy material, which was loaded onto a 230–400 mesh silica-gel column. The column was eluted with MeOH/$CHCl_3$ and the desired material was obtained with 4% MeOH/$CHCl_3$. It was then converted to its chloride form by passing it through an Amberlyst A-26 chloride ion-exchange column (overall yield ~30%).

Data for 2: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.85$ (t, 3H), 1.07–1.33 (br, 26H), 1.81 (br, 2H), 2.82–2.91 (m, 2H), 3.06–3.11 (m, 2H), 3.37 (s, 9H), 3.76 (s, 3H), 5.77–5.82 (br, 1H), 7.105–7.109 (br, 1H), 7.153–7.158 (d, 2H), 7.35 (d, 1H), 7.59–7.61 ppm (d, 1H); MS (ESI): *m/z* calcd for $C_{31}H_{54}N_3O$ (the 4° ammonium ion, 100%): 484.43; found: 484.5528 [M^+]; elemental analysis calcd (%) for $C_{31}H_{54}N_3OCl$: C 71.57, H 10.46, N 8.08; found: C 71.48, H 10.52, N 8.15.

Synthesis of [2-(1*H*-indole-3-yl)-1-hexadecyloxycarbonylethyl]trimethylammonium chloride (3): Compound **3** was prepared by following the same procedure as that used for compound **1**, except that *n*-hexadecanol was used instead of *n*-hexadecylamine.

Data for 3: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.83$ (t, 3H), 1.06–1.35 (br, 26H), 1.87 (br, 2H), 3.29 (br, 2H), 3.48 (s, 9H), 3.73 (br, 2H), 4.60 (br, 1H), 7.05–7.07 (br, 1H), 7.09–7.11 (d, 2H), 7.26–7.27 (br, 1H), 7.50 ppm (br, 1H); MS (ESI): *m/z* calcd for $C_{30}H_{51}N_2O_2$ (the 4° ammonium ion, 100%): 471.40; found: 471.4618 [M^+]; elemental analysis calcd (%) for $C_{30}H_{51}N_2O_2Cl$: C 71.04, H 10.14, N 5.52; found: C 71.23, H 10.06, N 5.47.

Preparation of the hydrogel: In a typical gelation experiment, **1** (3 mg) was dissolved in 1 mL of HPLC-grade water by slow heating up to ~40°C in a glass vial with i.d. of 10 mm. The vial was then allowed to stand without any disturbance at RT. After 30 min a colorless and transparent gel was obtained, which on inversion of the glass vial did not flow downward.

Determination of sol-gel transition temperature (T_g): T_g was determined by placing an inverted screw-capped glass vial with i.d. of 10 mm in a thermocontrolled oil bath and then increasing the temperature at 1°C min^{-1}. Here, T_g is defined as the temperature (±0.5°C) at which the hydrogel melts and begins to flow out of the gel.

Fluorescence spectroscopy: The emission spectra of ANS and pyrene were recorded by adding the probe molecules to aqueous solutions of the hydrogelator **1** at different concentrations at RT. For ANS, $\lambda_{ex} = 365$ nm and for pyrene, $\lambda_{ex} = 337$ nm. ANS was initially dissolved in MeOH and from this superstock the required amount of ANS solution was added to the experimental solutions (5 µL of superstock (0.01 M) was added to a 5 mL aqueous solution of the gelator to reach a probe concentration of 1×10^{-5} M). In the case of pyrene, the probe was dissolved in water by overnight stirring, then sonicated for 3 h followed by filtration. All the experimental solutions were prepared by using this aqueous solution of pyrene.

Circular dichroism (CD): The CD spectra of aqueous solutions of **1** at various concentrations were recorded by using a 1.2-mL quartz cuvette of 0.5-mm path length with a Jasco J-600C spectropolarimeter at RT.

Cytotoxicity assay: Cytotoxicity of the hydrogelator **1** was assessed by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay by using HELA cells, as described elsewhere.[16] The assay was performed in 96-well plates by varying the hydrogelator concentration. MTT was added 3 h after addition of cationic amphiphile to the cells. Results were expressed as percent viability, which was defined as [A_{540}(treated cells)−background/A_{540}(untreated cells)−background]×100. Experiments were performed in triplicate.

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