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Maintaining homogeneity during a sol-gel transition by an autocatalytic enzyme reaction

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Supporting Information

Experimental

Materials All Fmoc-based compounds were purchased from Sigma Aldrich and used as received. Urease (J61455 Urease, Jack Beans, minimum 45.0 units/mg solid) and urea (ultrapure 99%) were obtained from Alfa Aesar. Deionised water was used throughout all experiments. d_6-DMSO used in NMR experiments was obtained from Aldrich.

Preparation of solutions Aqueous solutions of the gelators **Fmoc-2**–**Fmoc-6** were prepared by dissolving the required amount of the compounds in H_2O by stirring. An aqueous solution of **Fmoc-Hz** was prepared by adding 1 equivalent amount of 0.1M HCl to the weighed amount of gelator followed by stirring for 24 hours. The pH of all the gelator solutions was acidic (<pH 5.5). The enzyme and urea were highly soluble in H_2O and therefore did not require stirring. The enzyme concentration in the stock solution was determined from the mass (in mg) dissolved in known volume of H_2O. Solutions of gelators and urease were prepared freshly before each experiment.
For the enzyme catalyzed reactions, initially a stock solution of **Fmoc-2** and urease was prepared of which 2 mL was transferred to the vial containing pre-weighed amount of urea and was gently swirled for few seconds. The aqueous solution of **Fmoc-3** was pH 3.0-3.2. Therefore, the initial pH of the solution was adjusted to pH 6-7 before addition of urease as at ~pH 3 the enzyme lost its activity. Then, the pH of the mixture was adjusted to pH 6.4. Finally, 2 mL of this solution was used for further studies. Throughout all enzymatic experiments, the concentration of urea was 0.1M and the concentration of urease was 0.1 mg/mL and 0.03 mg/mL as required. The final concentration of **Fmoc-2** and **Fmoc-3** was 2 and 5 mg/mL, respectively. The experimental temperature was 25 ^{o}C.

pH measurements A FC200 pH probe from HANNA instruments with a 6 mm x 10 mm conical tip was used for pH measurements. The stated accuracy of the pH measurements is ±0.1. For the urea-urease reaction, in the absence of gelator, the change in pH with time was recorded by adding 1 mL of urea solution to the vial containing 4 mL of urease.
To examine the change in pH with time in presence of the gelators, 2 mL of mixture of respective gelator and urease solutions (as described above) was transfer to the vial containing pre-weighed amount of urea and the pH change was monitored with time. The temperature was maintained at 25 °C during the titration by using a circulating water bath. Initial pH of the respective solutions was adjusted by using dilute HCl (0.1M) and AcOH (0.1M) as required. pK_a determination was carried out by recording the pH values after each addition of NaOH (0.1M) to the aqueous solution of the gelators. To prevent any gel formation, the solutions were stirred continuously.

Hydrogel Formation A pH switch method was used to form the hydrogels. Firstly, the gelation test was performed by adding equivalent amount of 0.1 M NaOH solution to the gelator solutions (final volume 2 mL). The mixtures were left undisturbed for 30 mins. Then, the vials were inverted and the minimum gelation concentration (mgc) was determined as the lowest concentration at which a self-supporting gel was formed. Only **Fmoc-2** and **Fmoc-3** formed a gel using this method and the mgc values were calculated to be 2 and 5 mg/mL for **Fmoc-2** and **Fmoc-3**, respectively.
For the enzyme catalyzed reactions, initially a stock solution of respective gelator and urease was prepared by the method as described above, of which 2 mL was transferred to the vial containing pre-weighed amount of urea and was gently swirled for few seconds. The samples were then left to stand overnight to allow gelation.

Rheological measurements
All rheological measurements were performed using an Anton Paar Physica MCR101 and MCR301 rheometer using a cup and vane system. Strain sweeps were performed at 10 rad/s from 0.1 % to 1000 % strain. Frequency sweeps were carried out from 1 rad/s to 100 rad/s at 0.5 % strain. All gels were left ~16 hours before being measured. Time sweeps were performed at an angular frequency of 10 rad/s and with a strain of 0.5%. For all experiments, gels were prepared as mentioned earlier in 2 mL volume in a 7 mL Sterilin vials. All measurements were performed at 25 ^{o}C.

Confocal microscopy
A Zeiss LSM510 on a Zeiss Observer Z1 (Zeiss, Jena, Germany) was used for imaging. The gel samples were prepared as mentioned above containing Nile blue (2 µL/mL of a 0.1 wt% solution) in CELLview Culture dishes (35 mm diameter) and were excited at 633 nm and detected with a Zeiss Meta detector. Data were captured using Zeiss Zen software (Zeiss, Jena, Germany) and analysed using Zeiss LSM image browser (version 4.2.0.121).

UV-Vis measurements
Absorption spectra of the gelators in solution and gel states were recorded on Agilent Technologies Cary 60 UV-Vis spectrophotometer using a 0.1 mm path length quartz cuvette. Samples were allowed gel overnight inside the cuvettes before they were measured.

Fluorescence spectroscopy
Emission spectra of the gelators in solution and gel states were recorded on Agilent Technologies fluorescence spectrofluorophotometer. Samples were prepared in a PMMA cuvette with a path length of 1 cm. The top was covered with parafilm and the samples allowed to gel overnight. In all cases, the excitation wavelength was 265 nm. Both the excitation and emission slit widths were 5 nm.

Table S1. Summary of results of gelation test in presence of 1 molar equivalent of NaOH

Fmoc-HZ	Fmoc-2	Fmoc-3	Fmoc-4	Fmoc-5	Fmoc-6
Insoluble (no clear solution was obtained even at concentration of 2 mg/mL after stirring for 24 hours)	Gel	Gel	No gel (gelation test was performed up to a concentration of 10 mg/mL)	No gel (Gelation test was performed up to a concentration of 10 mg/mL)	No gel (soluble in water up to the concentration of 3 mg/mL on stirring for 1 hour). For higher gelator concentrations, no clear solution was obtained even after stirring for 24 hours

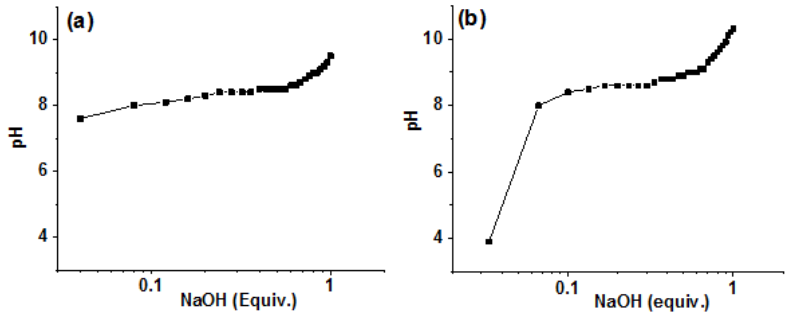


Fig. S1. Determination of apparent pK_a of gelators **Fmoc-2** (a) and **Fmoc-3** (b). The plateaus in the data were taken to represent the apparent pK_a values.

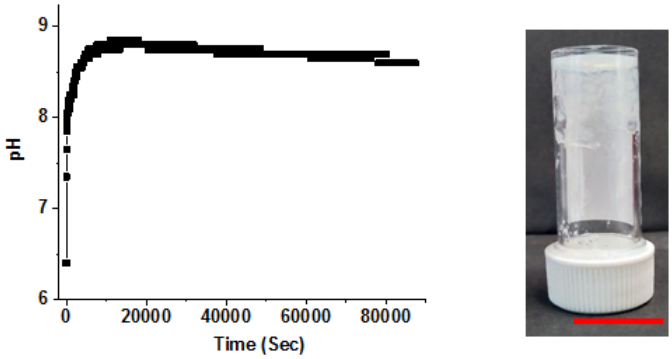
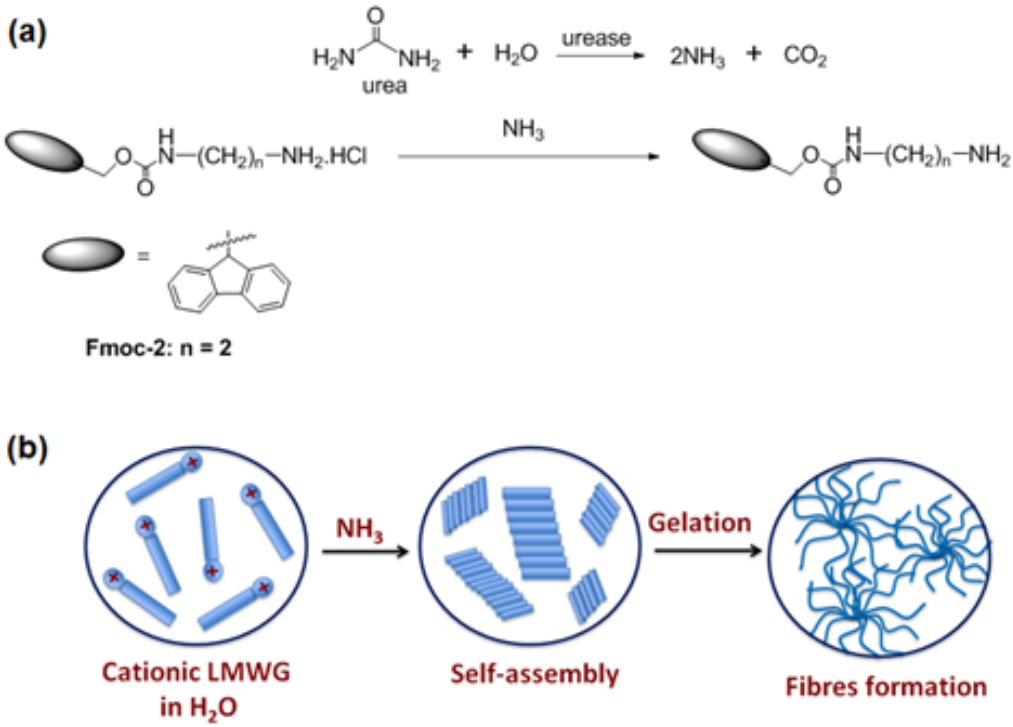


Fig. S2. (a) Change in pH with time for the urease-urea reaction in presence of **Fmoc-3** (concentration of urease = 0.1 mg/mL, concentration of urea = 0.01M, concentration of **Fmoc-3** = 5 mg/mL). (b) Photograph (scale bar is 1.7 cm) represents the phase change of **Fmoc-3** solution after 24 hours.

Table S2: Summary of pH-time profile for **Fmoc-2** and **Fmoc-3** under different conditions

Gelator	Method of gelation	Initial pH of solutions	Final pH of the gels	Approximate time requires to reach the final pH plateau (mins)	Appearance of the gels
Fmoc-2	NaOH	5.1-5.3	9.2-9.4	Instantaneous	Turbid and inhomogeneous
	Urea-urease reaction, [urease] = 0.10 mg/mL	6.2-6.4 (no acid)	9.1	15	Opaque/quite transparent
		3.9 (HCl)	9.1	75	Opaque/quite transparent
		3.9 (AcOH)	9.1	50	Opaque/quite transparent
	Urea-urease reaction, [urease] = 0.03 mg/mL	3.9 (HCl)	9.0	340	Opaque/quite transparent
		3.9 (AcOH)	9.0	240	Opaque/quite transparent
Fmoc-3	NaOH	3.0-3.2	10.0-10.3	Instantaneous	Turbid and inhomogeneous
Remark: [**Fmoc-2**] = 2 mg/mL, [urea] = 0.01M, [**Fmoc-3**] = 5 mg/mL					



Scheme S1. Schematic representation of the change in chemical structure of cationic amphiphile **Fmoc-2** during the enzymatic reaction (a) along with the self-assembly process leading to a fibrous network formation during gelation (b).

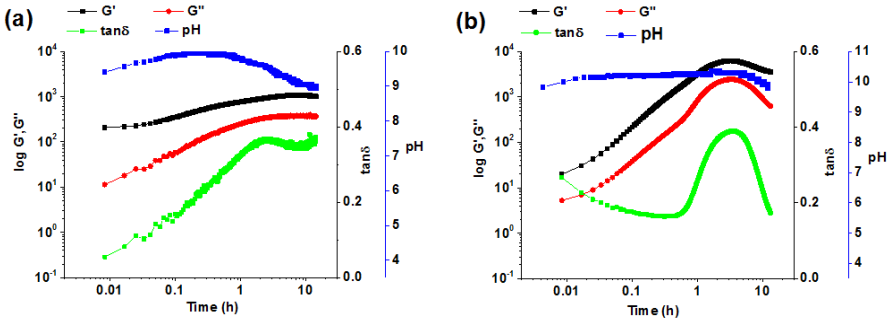


Fig. S3. Variation of G′, G″ and pH with time for (a) **Fmoc-2** (2 mg/mL) and (b) **Fmoc-3** (5 mg/mL) in presence of NaOH (1 equiv.). As the pH change is diffusion controlled, after certain time the pH around the pH-meter tip slowly decreases and the pH time profile showed a lowering of pH values when the time is longer. Moreover, Aggregate formation reduces the diffusion rate. Such diffusion controlled pH change produces inhomogeneous distribution of hydroxide ions in the systems, which resulted in unpredictable and uncontrollable variation of G′ and G″ with time.

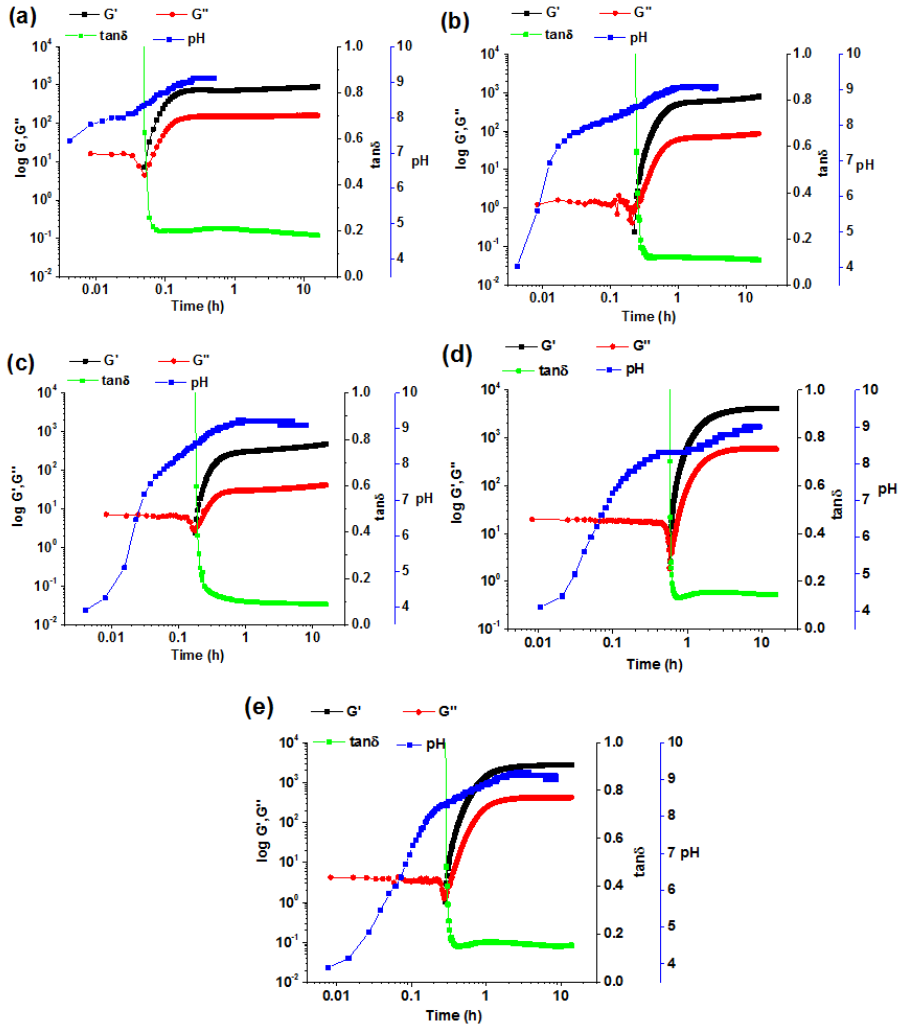


Fig. S4. Variation of G′, G″ and pH with time for **Fmoc-2** in presence of urea-urease reaction involving initial conditions (a) no acid, [urease] = 0.1 mg/mL; (b) pH 3.9 (HCl, [urease] = 0.1 mg/mL); (c) pH 3.9 (AcOH, [urease] = 0.1 mg/mL); (d) pH 3.9, (HCl, [urease] = 0.03 mg/mL); (e) pH 3.9 (AcOH, [urease] = 0.03 mg/mL). In all cases, concentration of **Fmoc-2** is 2 mg/mL, initial concentration of urea is 0.01M.

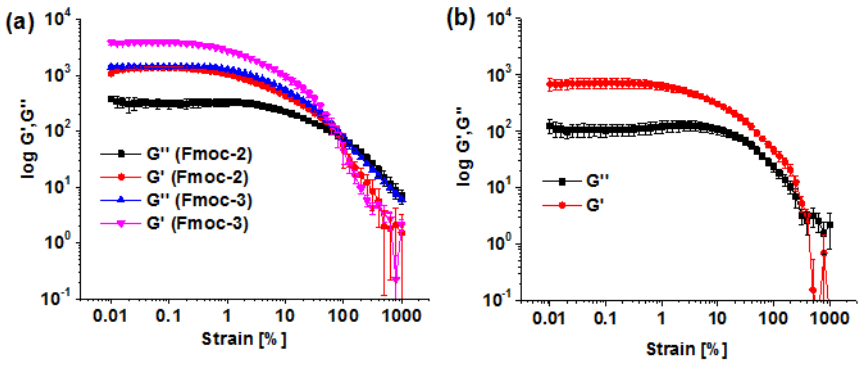


Fig. S5. (a) Strain sweep experiments of the hydrogels of **Fmoc-2** and **Fmoc-3** obtained from NaOH. (b) Strain sweep experiment of the hydrogel of **Fmoc-2** prepared by enzyme catalyzed reaction in absence of any acid ([urea] = 0.01M and [urease] = 0.1 mg/mL,).

Table S3: Crossover points of different gels of **Fmoc-2** and **Fmoc-3** from the rheological strain sweeps.

Gelator	Method of gelation	Initial pH of the solutions	G′ (Pa) [at 0.5% strain]	%Strain at crossover point
Fmoc-2	Bulk addition of NaOH	5.1-5.3	1180	90
	Addition of NaOH in portion over 1h	5.1-5.3	1230	65
	Addition of NH_4OH in portion over 1h	5.1-5.3	650	105
	Urea-urease reaction, [urease] = 0.10 mg/mL	6.2-6.4 (no acid)	700	395
		3.9 (HCl)	1160	415
		3.9 (AcOH)	610	690
	Urea-urease reaction, [urease] = 0.03 mg/mL	3.9 (HCl)	2500	500
		3.9 (AcOH)	1725	No crossover
Fmoc-3	Bulk addition of NaOH	3.0-3.2	3300	60

Remark: [Fmoc-2] = 2 mg/mL, [urea] = 0.01M, [Fmoc-3] = 5 mg/mL. 1 molar equiv. amount of NaOH and NH_4OH are used to prepare gels.

The storage modulus (G′) indicates whether a gel material is stiff or soft. A higher G′ value indicates a stiffer gel. The gel strength refers to the strain at which the gel breaks i.e. the critical strain. Thus, a high gel strength is indicated by a higher value of critical strain i.e. more capacity to resist an elastic deformation. In our case, all the enzyme triggered gels showed higher critical strain (0.7-0.9% strain) compare to the NaOH triggered gels (0.5% strain). The crossover point or the yield point represents maximum capacity of a material to maintain its viscoelastic nature before complete destruction. In equating crossover points, independent of the rate of gelation, the enzyme-triggered gels always exhibits superiority over the NaOH-triggered gels. Moreover, among the enzyme-triggered gels the yield/crossover point increases when the gelation rate is slow.

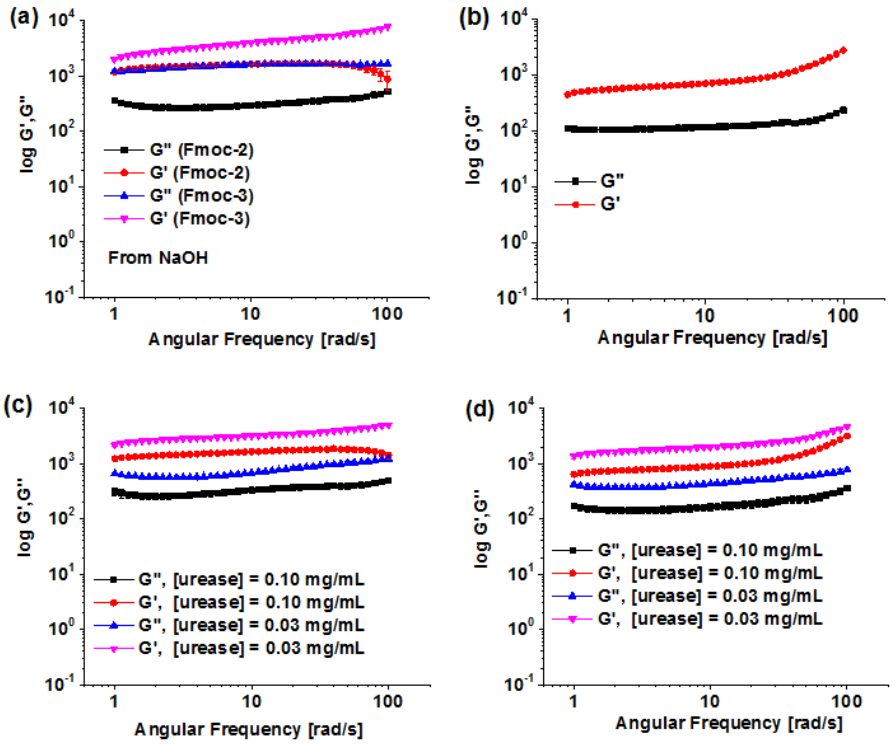


Fig. S6. (a) Frequency sweep experiments of the hydrogels of **Fmoc-2** and **Fmoc-3** obtained from NaOH. (b-d) Frequency sweep experiments of the hydrogels of **Fmoc-2** obtained from enzyme catalyzed reaction carried out in absence (b) and presence (initial pH of solution was 3.9) of HCl (c) and AcOH (d). In all cases, concentration of urea = 0.01M.

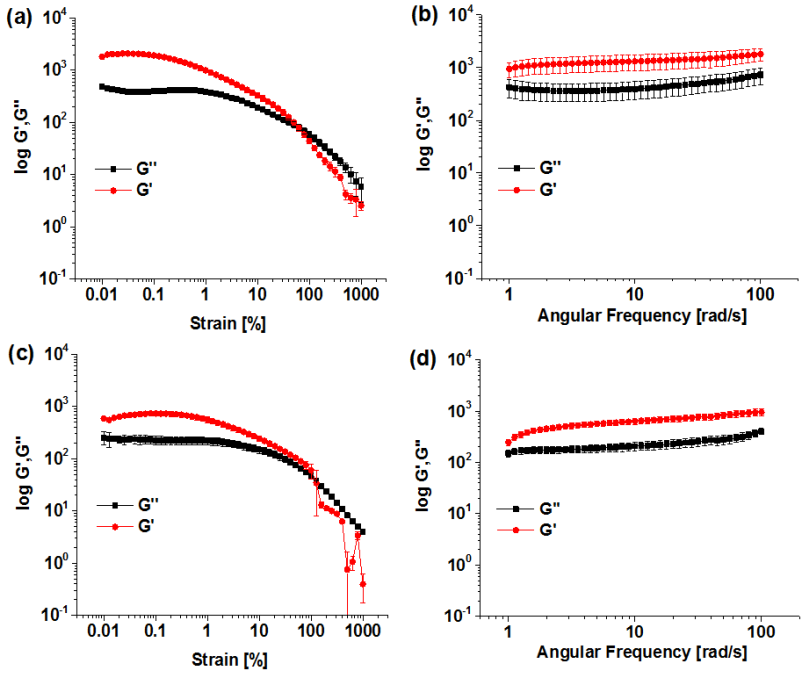


Fig. S7. (a) Strain and (b) frequency sweep experiments of the hydrogels of **Fmoc-2** obtained from slow addition (over 1h) of NaOH. (c) Strain and (d) frequency sweep experiments of the hydrogels of **Fmoc-2** obtained from slow addition (over 1h) of NH_4OH.

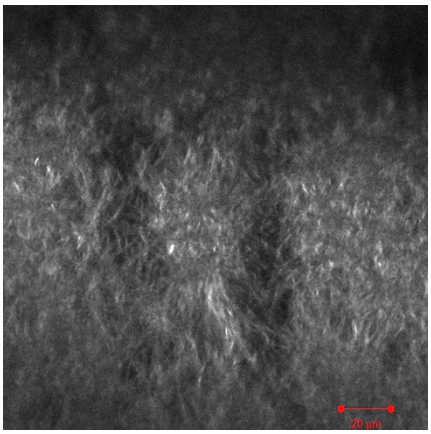


Fig. S8. Confocal fluorescence microscopy images of the NaOH-triggered hydrogel of **Fmoc-3** (concentration = 5 mg/mL). Scale bar: 20 μm.

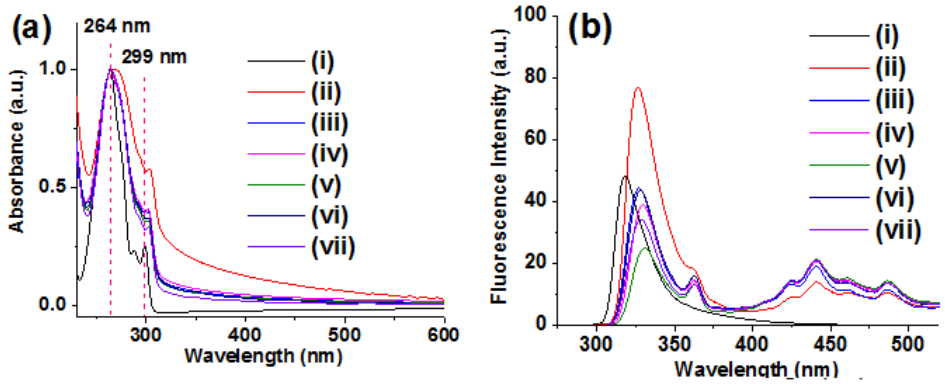


Fig. S9. Comparison of (a) normalized UV-vis and (b) emission spectra of **Fmoc-2** (Conc. = 2 mg/mL, λ_{ex} = 265 nm) in (i) solution and gel states triggered by NaOH (ii) and urea-urease reaction having initial conditions (iii) no acid; (iv) pH 3.9 (HCl), [urease] = 0.1 mg/mL; (v) pH 3.9 (HCl), [urease] = 0.03 mg/mL; (vi) pH 3.9 (AcOH), [urease] = 0.1 mg/mL; (vii) pH 3.9 (AcOH), [urease] = 0.03 mg/mL. In all cases, concentration of urea = 0.01M.

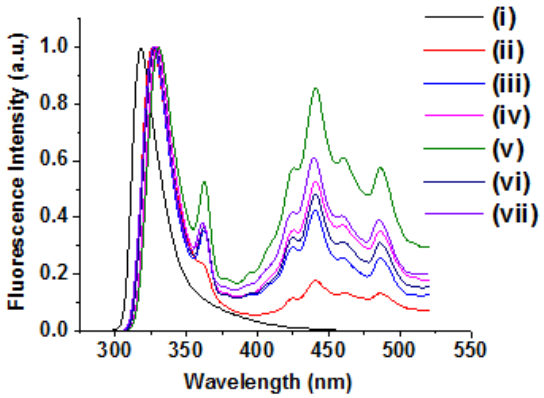


Fig. S10. Comparison of normalized emission spectra of **Fnoc-2** in (i) solution and gel states triggered by (ii) NaOH and urea-urease reaction reaction having initial conditions (iii) no acid; (iv) pH 3.9 (HCl), [urease] = 0.1 mg/mL; (v) pH 3.9 (HCl), [urease] = 0.03 mg/mL; (vi) pH 3.9 (AcOH), [urease] = 0.1 mg/mL; (vii) pH 3.9 (AcOH), [urease] = 0.03 mg/mL after 18 hours. In all cases, concentration of urea = 0.01M.

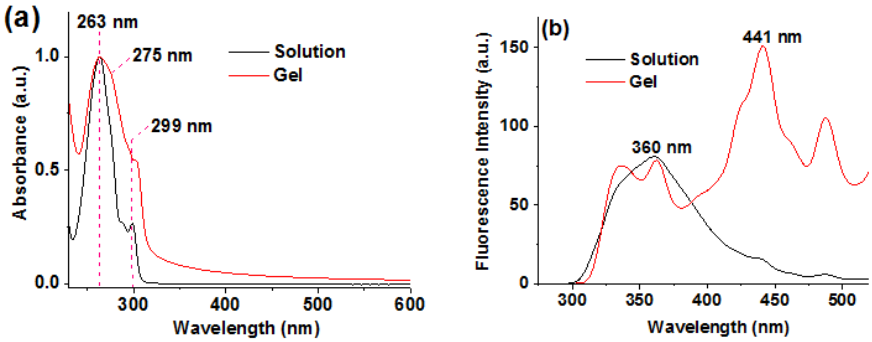


Fig. S11. Comparison of normalized UV-vis (a) and emission (b) spectra of **Fmoc-3** (λ_{ex} = 265 nm, concentration = 5 mg/mL) in solution and gel states triggered by NaOH.

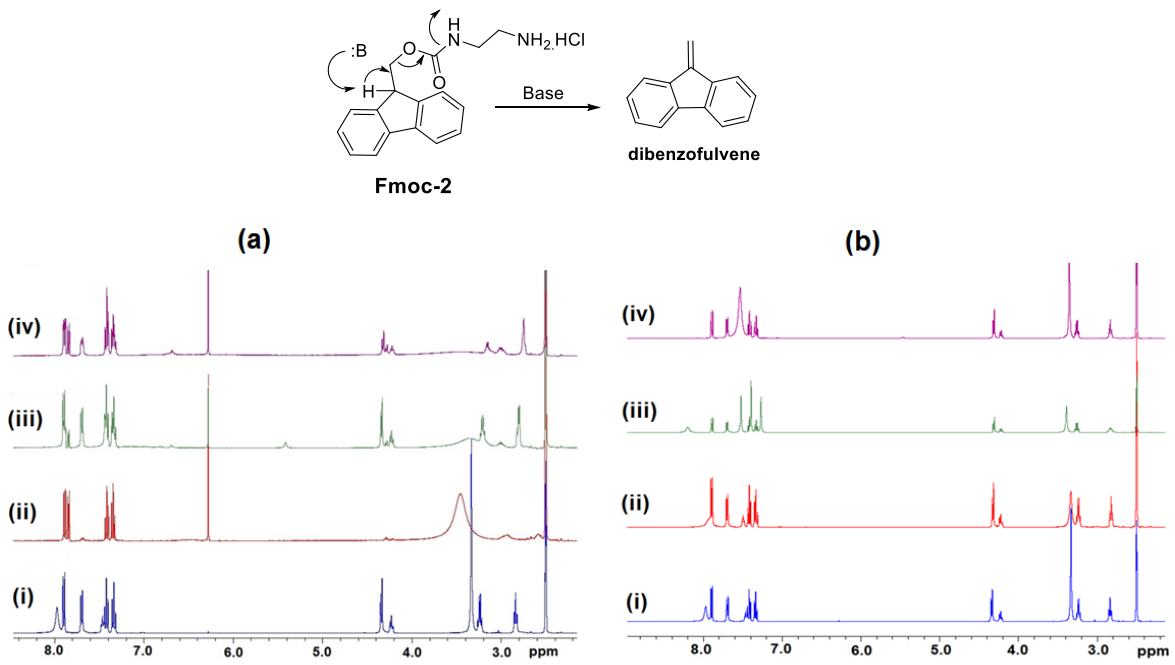


Fig. S12. Comparison of ^{1}H NMR (500 MHz, d_6-DMSO) of freeze-dried gels (frozen after 2-3 hours) of **Fmoc-2** (concentration is 4 mg in 0.5 mL) under different conditions: (a) gels were freeze-dried without acidification and (b) gels were freeze-dried after acidification; (i) **Fmoc-2**, (ii) gel from NaOH, (iii) enzyme triggered gel in absence of any acid and (iv) enzyme triggered gel from pH 3.9 (HCl). For the enzyme triggered gels: Concentration of **Fmoc-2** = 2 mg/mL, concentration of urea = 0.01M, concentration of urease = 0.1 mg/mL.

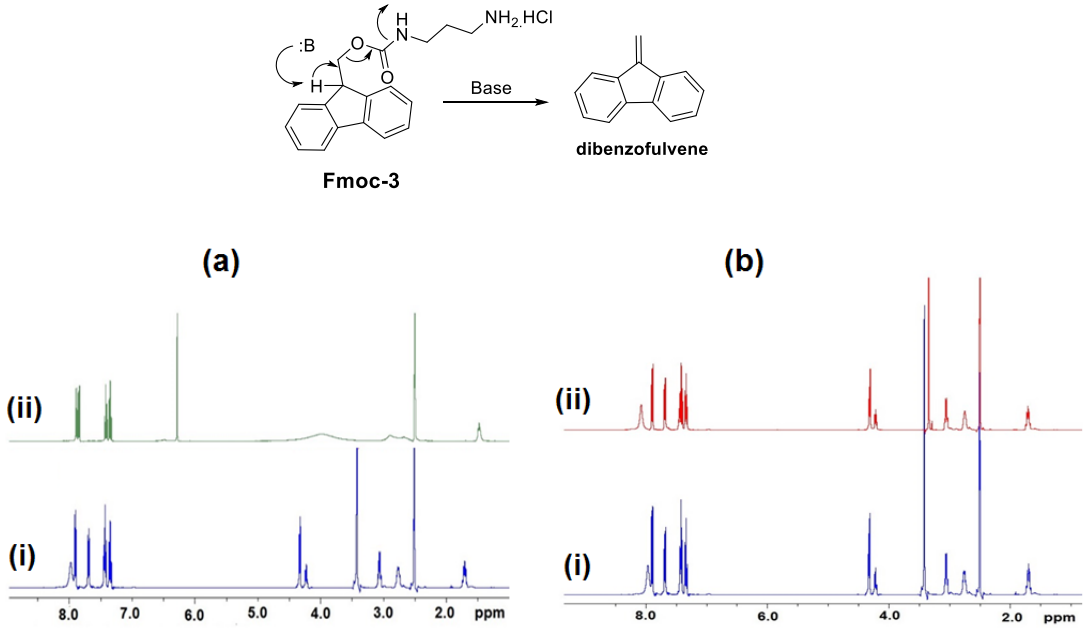


Fig. S13: Comparison of ^{1}H NMR (500 MHz, d_6-DMSO) of freeze-dried gels (frozen after 2-3 hours) of **Fmoc-3** (concentration is 10 mg in 0.5 mL)under different conditions: (a) gels were freeze-dried without acidification and (b) gels were freeze-dried after acidification; (i) **Fmoc-3**, (ii) gel from NaOH.

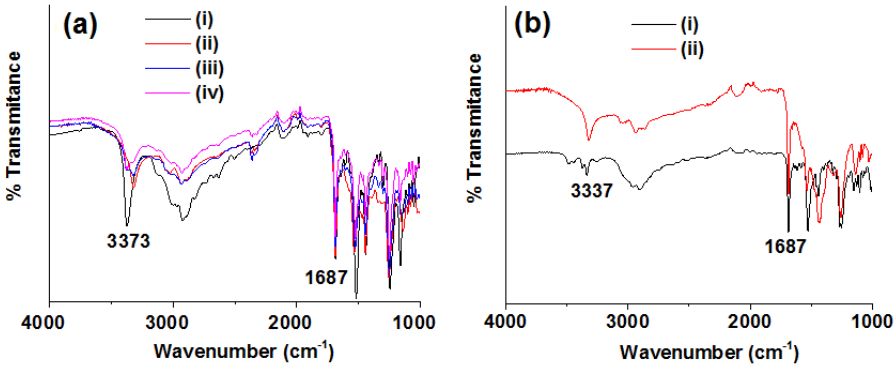


Fig. S14: (a) Comparison of FTIR spectra of **Fmoc-2** in its (i) amorphous and (ii-iv) gel states after freeze-drying; (ii) gel from NaOH, (iii) enzyme triggered gel in absence of any acid and (iv) enzyme triggered gel from pH 3.9 (HCl); For the gels: concentration of **Fmoc-2** = 2 mg/mL, concentration of urea = 0.01M, concentration of urease = 0.1 mg/mL. (b) Comparison of FTIR spectra of **Fmoc-3** in (i) amorphous and (ii) NaOH triggered gel state after freeze-drying.