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Tumor Targeting with DGEA Peptide Ligands: A New Aromatic Peptide Amphiphile for Imaging Cancers

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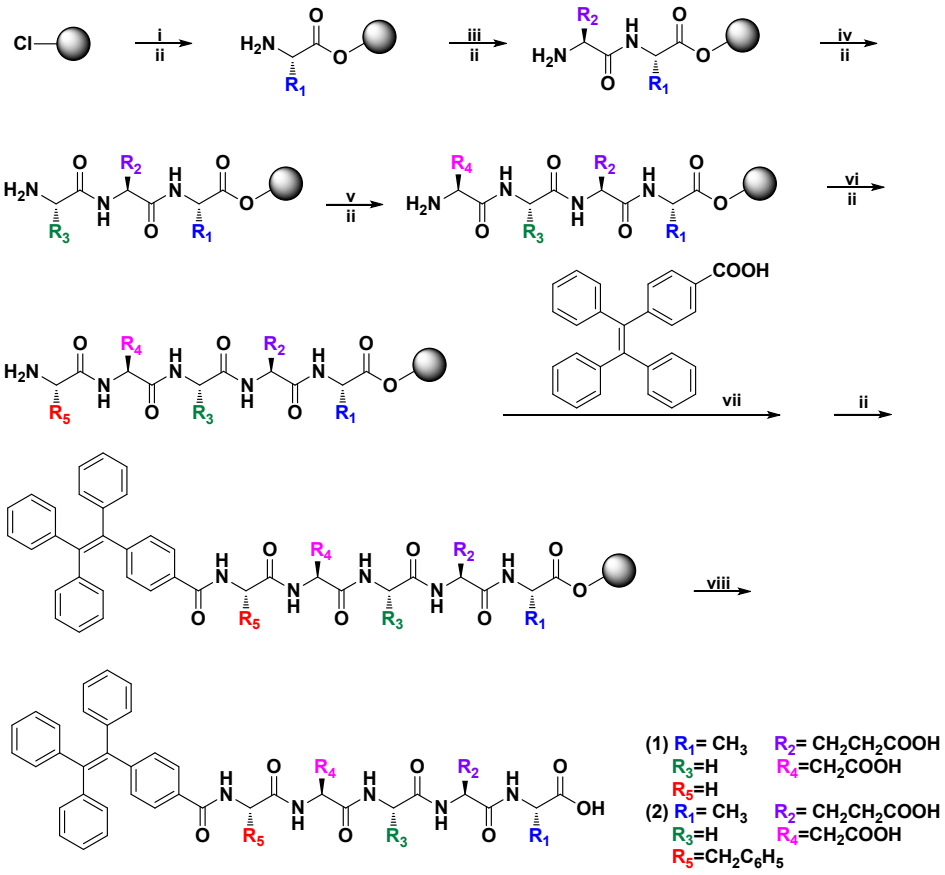

Scheme 1. Synthesis of TPE-GDGEA (**1**) and TPE-FDGEA (**2**).

Synthesis of TPE-GDGEA (1).

TPE-GDGEA was prepared through solid phase peptide synthesis (SPPS). 2-Chlorotrityl chloride resin (0.6 g) was swelled in anhydrous CH_2Cl_2 for 30 min and then Fmoc-$_L$-alanine (0.312 g, 1.00 mmol) was loaded onto the resin in anhydrous DMF and DIEA (0.415 mL, 2.50 mmol) for 1 h. For deprotection of the Fmoc group, piperidine (20% in DMF) was added and the sample left for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-$_L$-glutamic acid (0.425 g, 1.00 mmol) was coupled to the free amino group using HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.500 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-glycine (0.297 g, 1.00 mmol) was coupled to the free amino

group using HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.500 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-L-aspartic acid (0.411 g, 2.00 mmol) was coupled to the free amino group using HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.500 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-glycine (0.595 g, 2.00 mmol) was coupled to the free amino group using (HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.50 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Finally, TPE-COOH (1.13 g, 3.00 mmol) was coupled to the free amino group using HBTU (1.14 g, 3.00 mmol) and DIEA (0.830 mL, 5.00 mmol) as coupling agents. After the mixture had been stirred overnight, the peptide derivative was cleaved through treatment with TFA (90% in DI water) for 3 h. The resulting solution was dried with air and then Et_2O was added to precipitate the target product. The solid was dried under vacuum to remove residual solvent (white solid: 0.111 g). ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 1.30 (d, *J*=7.2 Hz, 3H), 1.72-1.85 (br, 2H), 1.85-2.05 (br, 2H) , 2.20-2.35 (br, 2H) , 3.89-3.96 (m, 2H) , 4.15-4.23 (m, 1H) , 4.25-4.40 (m, 1H) , 4.55-4.65 (m, 1H) ,6.98-7.21 (m, 17H), 7.68 (d, *J*=8.4 Hz, 2H), 7.90 (d, *J*=7.8 Hz, 1H), 8.12-8.20 (m, 1H), 8.25 (d, *J*=7.2 Hz, 1H),8.38 (d, *J*=7.8 Hz, 1H) , 8.70-8.71 (m, 1H);^{13}C NMR (75 MHz, DMSO-d_6): δ=17.4, 28.0, 30.5, 36.7, 42.7, 43.1, 48.0, 50.1, 51.9, 127.2, 127.3, 127.4, 128.3, 128.39, 128.43, 128.5, 131.1, 132.2, 140.3, 142.0, 143.2, 143.3, 146.9, 166.7, 167.5, 168.9, 169.7, 171.2, 171.5, 172.5, 174.4, 174.5; MS[FAB$^-$]: calcd. m/z:805.3，obsvd. 804.0 [M-H]$^-$.

Synthesis of TPE-FDGEA (2). A similar synthetic protocol was used for the

preparation of TPE-FDGEA. 2-Chlorotrityl chloride resin (0.6 g) was swelled in anhydrous CH_2Cl_2 for 30 min and then Fmoc-$_L$-alanine (0.312 g, 1.00 mmol) was loaded onto the resin in anhydrous DMF and DIEA (0.415 mL, 2.50 mmol) for 1 h. For deprotection of the Fmoc group, piperidine (20% in DMF) was added and the sample left for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-$_L$-glutamic acid (0.425 g, 1.00 mmol) was coupled to the free amino group using HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.500 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-glycine (0.297 g, 1.00 mmol) was coupled to the free amino group using HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.500 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-L-aspartic acid (0.411 g, 2.00 mmol) was coupled to the free amino group using HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.500 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Fmoc-L-phenylalanine (0.388 g, 2.000 mmol) was coupled to the free amino group using (HBTU (0.379 g, 1.00 mmol) and DIEA (0.415 mL, 2.50 mmol) as coupling agents for 30 min. Again, the sample was treated with piperidine (20% in DMF) for 20 min; this procedure was repeated twice (each time for 2 min). Finally, TPE-COOH (1.13 g, 3.00 mmol) was coupled to the free amino group using HBTU (1.14 g, 3.00 mmol) and DIEA (0.830 mL, 5.00 mmol) as coupling agents. After the mixture had been stirred overnight, the peptide derivative was cleaved through treatment with TFA (90% in DI water) for 3 h. The resulting solution was dried with air and then Et_2O was added to precipitate the target product. The solid was dried under vacuum to remove residual solvent (white solid: 0.128 g). ^{1}H NMR (300 MHz,

DMSO-d_6, 25 °C): δ= 1.20-1.35 (br, 3H), 1.76-1.85 (br, 2H), 1.85-2.00 (br, 2H) , 2.25-2.34 (br, 2H) , 2.75-2.89 (m, 2H) ,3.74-3.81 (m, 2H) , 4.07-4.25 (m, 1H) , 4.25-4.32 (m, 1H) , 4.51-4.62 (m, 1H) , 4.61-4.80 (m, 1H), 6.95-7.40 (m, 22H), 7.58 (d, *J*=8.1 Hz, 2H), 7.88-8.11 (m, 2H) , 8.23-8.32 (m, 2H) , 8.47-8.60 (m, 1H);^{13}C NMR (75 MHz, DMSO-d_6): δ=17.7, 22.7, 28.1, 30.6, 30.8, 37.4, 44.3, 48.3, 52.3, 55.4, 124.4, 126.8, 127.3, 127.4, 127.6, 128.46, 128.53, 128.6, 129.7, 131.1, 132.3, 138.9, 140.3, 142.0, 143.3, 143.4, 146.9, 166.0, 166.5, 166.7, 169.0, 171.1, 171.3, 171.6, 172.2, 174.8; MS[FAB$^-$]: calcd. m/z:895.4，obsvd. 893.8 $[M-H]^-$.

Hydrogels Preparation. TPE-GDGEA or TPE-FDGEA (4 mg) was dispersed in deionized water, and then an aqueous NaOH solution was added to dissolve the solid. The pH and concentration of the aqueous solution were carefully adjusted by adding HCl_{aq} to obtain a 3 wt% hydrogel (total volume: 300 μL). The gelation was confirmed using the inverted tube method; the stability and characterization of the hydrogels have been described previously.[1,2]

Transmission Electron Microscopy. Each sample was prepared by dropping a small amount of a hydrogel (3 μL) onto a carbon-coated copper grid, followed by adding uranyl acetate. The sample was air-dried for 2 days and then TEM images were recorded using a Hitachi HT7700 transmission electron microscope operated at an accelerating voltage of 100 kV.

Atomic Force Microscopy. For AFM experiments, 3 μL of sample solution (as a gel) was diluted to 30 μL of deionized water and then deposited onto a freshly wafer. Each sample was air dried overnight then freeze dried prior to AFM imaging. The

images were obtained by scanning the wafer surface in air under ambient conditions and using a Bruker, Model : Dimension ICON operated in tapping mode.

Rheological measurements. The rheological measurements of the hydrogels were carried out using TA discovery rheometer (DHR-1) using 40 mm parallel plate geometry. The pH adjusted TPE sample (400 μL, 3 wt%) was sandwiched between stainless parallel plate geometry and a stationary bottom plate. The sample was allowed to stand for 2 h before taking any rheological measurements. The storage modulus (G') and loss modulus (G") were measured at 25 ^{o}C as a function of frequency between the range of 0.1 rad s^{-1} to 100 rad s^{-1} at a constant strain of about 0.01%.

Spectroscopy Analysis. For CD spectroscopy, an aqueous solution was placed in a quartz cell (optical length: 1 mm) and its spectrum recorded using a CD spectrometer JASCO J-715 (wavelength range: 190–800 nm). Emission spectra (concentration-dependent and different water fractions) were recorded using a Hitachi F7000 fluorescence spectrometer.

Titration of Integrin α2β1 protein. For preparing 50 μg/mL protein, 12.5 μL of the protein stock solution was diluted with 35.5 μL of PBS buffer (pH 7.4). 2 μL of TPE-FGDEA (50 μM) was then added and the reaction mixture was incubated at room temperature for 30 min. The reaction mixture was then diluted to a total of 300 μL with deionized water for PL measurement.

Cell Viability. The biocompatibility of the TPE-FDGEA hydrogelator was measured through an MTT cell viability test. PC-3 and 22Rv1 cells were chosen as models. The cells were pre-incubated for 24 h in 24-well plates at a density of 5×10^4 cells/well with Dulbecco's modified Eagle's medium (DMEM, 0.5 mL) containing 10% FBS and 1% penicillin-streptomycin solution. The culture medium was then replaced with fresh medium. Samples prepared at various concentrations (10, 50, 100, 200 μM) were added and then the cells were placed into the solutions and incubated for 24 or 48 h. Fresh medium supplemented with MTT (4 mg/mL, 0.5 mL) was added to each well and then the cells were incubated for another 4 h. The medium containing MTT was removed and DMSO (0.5 mL/well) was added to dissolve the formazan crystals. Each 24-well was transferred to a well in a 96-well plate. The optical densities of the resulting solutions were measured at 595 nm, using an absorbance micro plate reader (Infinite F50, TECAN). Cells that had not been subjected to treatment with the compounds were used as the control. The cell viability percentage was calculated using the formula:

$$\text{cell viability percentage (\%)} = OD_{sample}/OD_{control}.$$

Cell Imaging. The cells were seeded on a 35-mm Petri dish at a density of 5×10^4 cells per well and cultured for 24 h. Samples were prepared at a concentration of 50 μM; the cells were placed into the solutions and incubated for 4 h. After staining for a suitable time, the cells were washed three times with phosphate-buffered saline (pH 7.4) and measured using a laser-confocal microscope (Leica TCS-SP5-X AOBS).

Immunostaining. Approximately 1x10^6 cells/mL of PC-3 were seeded onto a glass slide and incubated at 37 ^oC for one overnight. Next day, 50 μM of TPE-FGDEA were added to the cell culture and incubated at 37 ^oC for 4 h. After the slide was washed with PBS twice, the cells were fixed with 4% paraformaldehyde (diluted in PBS) and incubated at 4 ^oC for one overnight. Subsequently, the fixed cells were permeabilized with 0.1 % PBST at 4 ^oC for 20 minutes, followed by blocking the slide with 1 wt% BSA (diluted in PBS) for one overnight at 4 ^oC. The slide covered with the anti-human integrin $\alpha_2\beta_1$ antibodies (1:500, diluted in PBST) and incubated at 4 ^oC for another overnight. Afterwards, the slide was washed with PBST twice, and covered with FITC conjugated anti-mouse IgG antibodies (1:1000, diluted in PBST) and incubated at room temperature for 1 h. Finally, the slide was washed with PBST three times, followed by imaging with a fluorescence microscope.

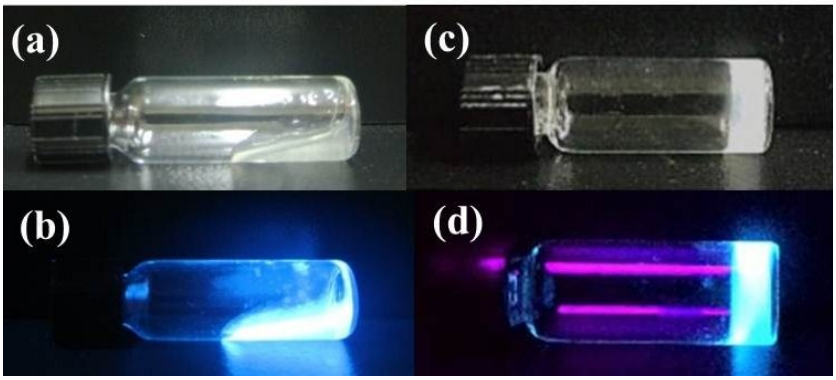

Fig. S1 Optical images of (a,b) **1** at 1 wt% at pH 4.2 and (c,d) **2** at 1 wt% at pH 4.6 (top: under room lighting; bottom: under UV illumination at 365 nm from a handheld UV lamp).

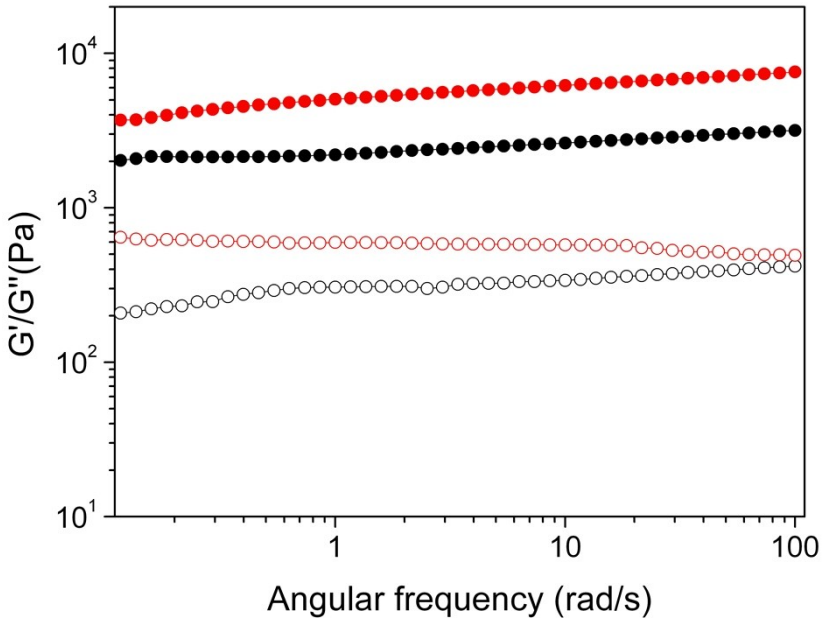

Fig. S2 Frequency sweeps of **1** at 3 wt% at pH 4.2 (black) and **2** at 3 wt% at pH 7.1 (red). (closed-circle for G' and open-circle for G")

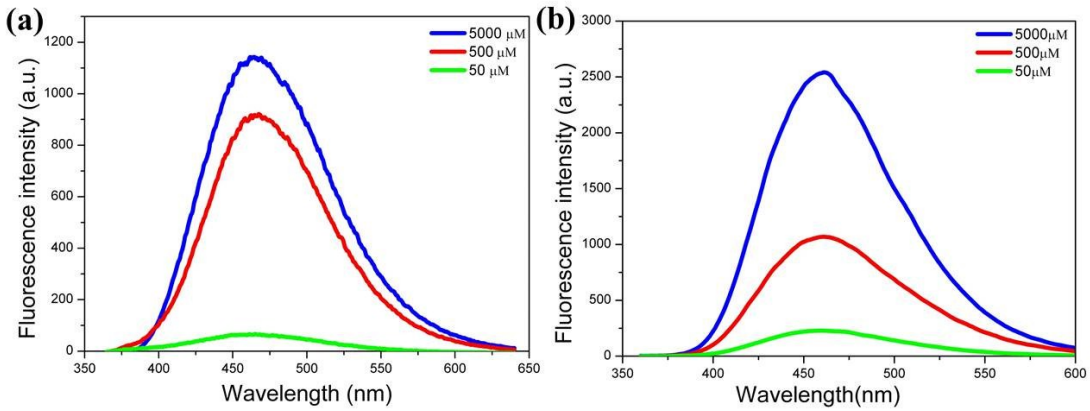

Fig. S3 Concentration-dependent spectra of (a) **1** and (b) **2** at 50, 500, and 5000 μM in water.

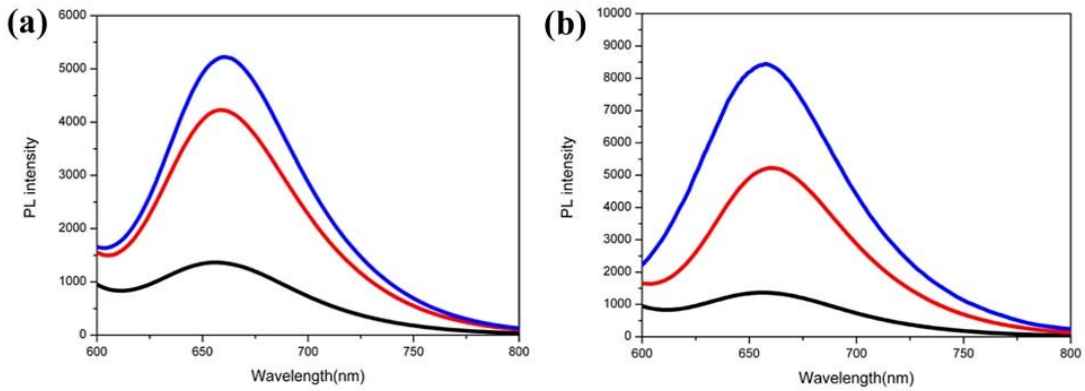

Fig. S4 Emission intensities of Nile Red in solutions containing various concentrations of (a) **1** and (b) **2**. (black for 1000 μM, red for 3000 μM, blue for 5000 μM).

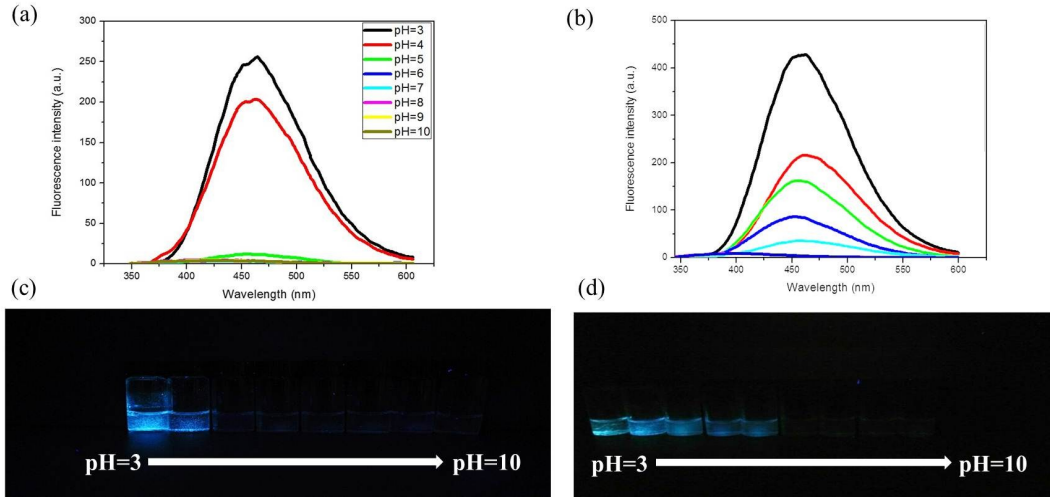

Fig. S5 pH dependence of the fluorescence intensities and optical images of (a,c) **1** and (b,d) **2** at 50 μM.

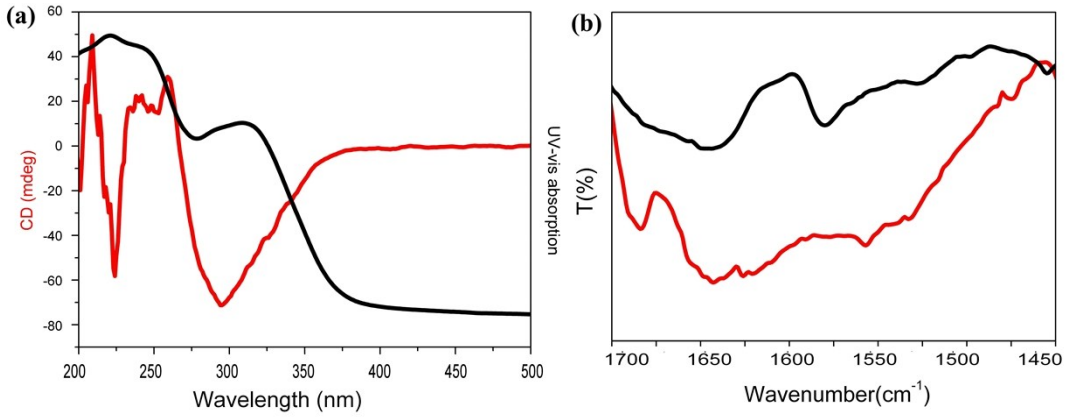

Fig. S6 (a) CD (red line) and UV–Vis (black line) spectra of **1** in water; (b) FTIR spectra of **1** in water (red line) and DMSO (black line).

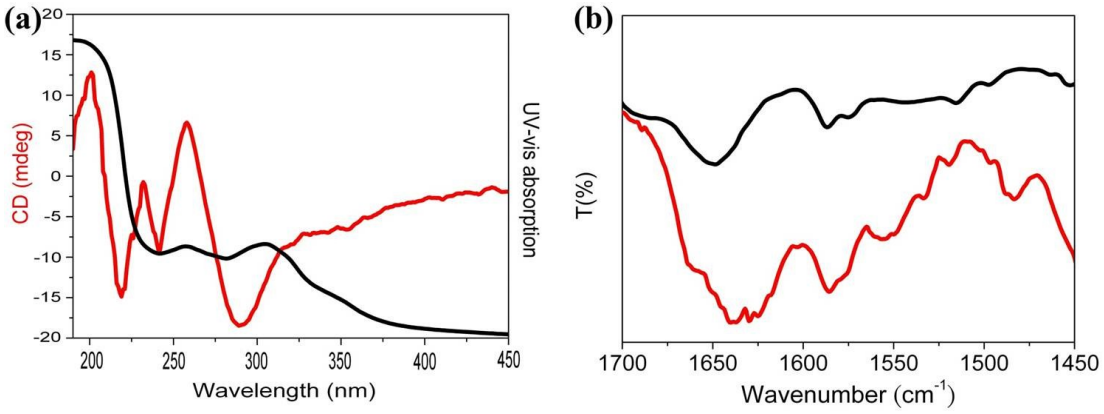

Fig. S7 (a) CD (red line) and UV–Vis (black line) spectra of **2** in water; (b) FTIR spectra of **2** in water (red line) and DMSO (black line).

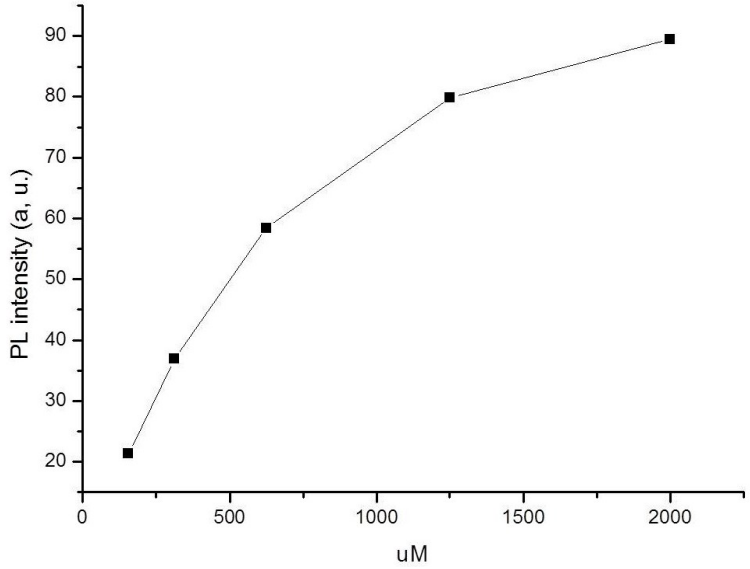

Fig. S8 Emission intensities of ThT (20 μM) at various concentrations of hydrogelators of **2**.

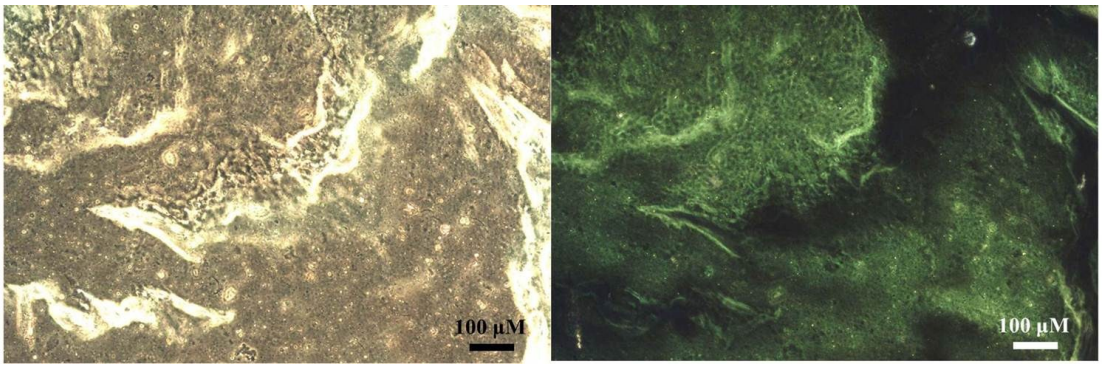

Fig. S9 Bright-field (left) and polarized (right) images of **2** stained by Congo Red.

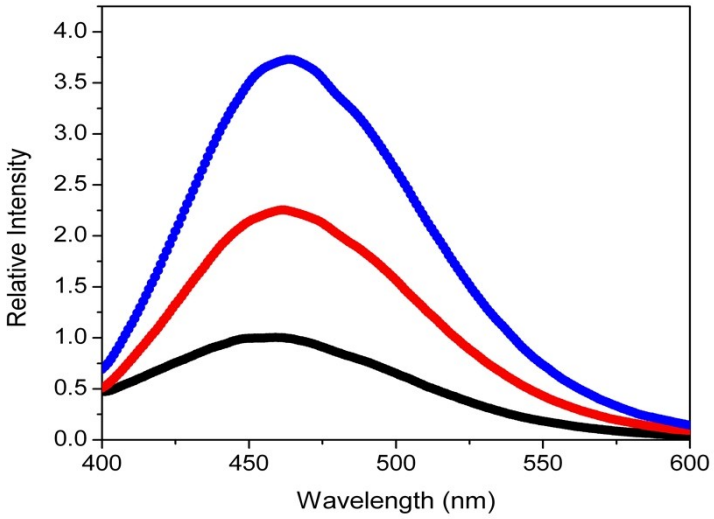

Fig. S10 Emission spectra of **2** in the presence of different amounts of integrin $\alpha_2\beta_1$. ([**2**]= 50 μM, λex= 345 nm, black for integrin = 0 μg/mL, red for integrin = 20 μg/mL and blue for integrin = 50 μg/mL.)

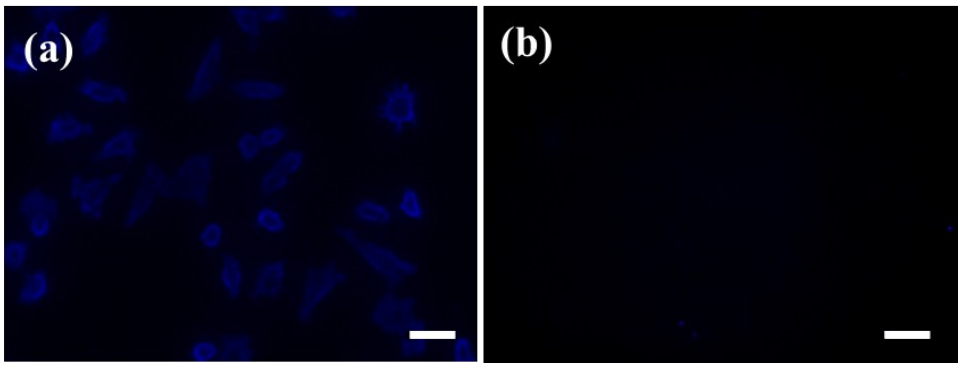

Fig. S11 Fluorescence images of (a) MDA-MB-231 and (b) HEK293 cells incubated for 4 h in the presence of **2** at 50 μM. (scale bar: 20 μm)

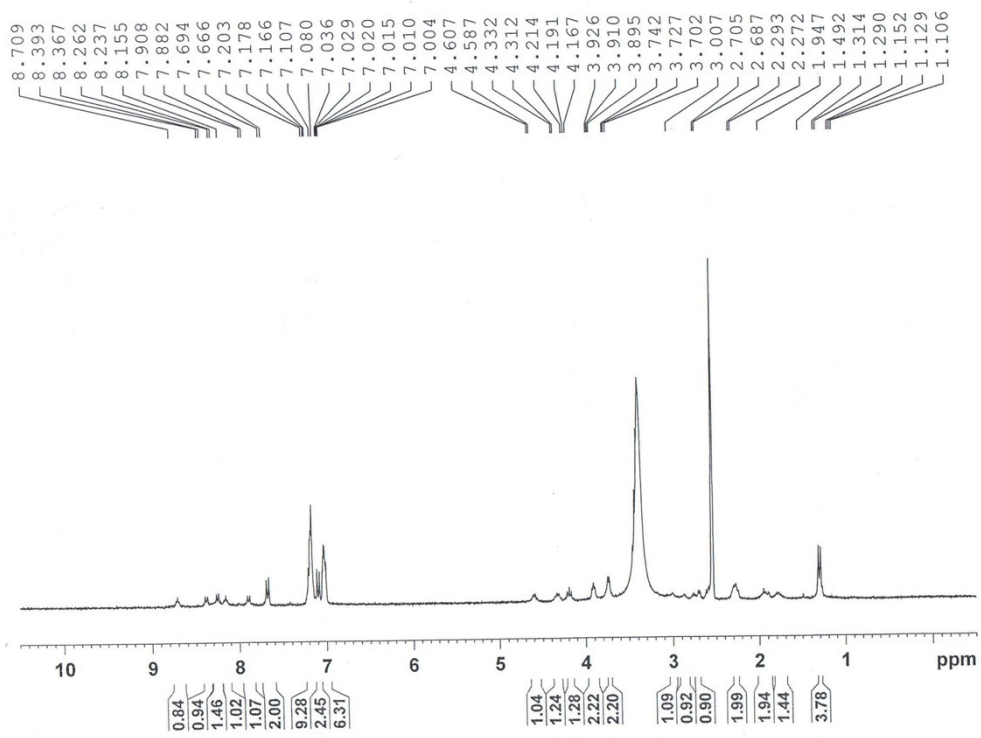

Fig. S12 ^{1}H NMR spectrum for TPE-GDGEA (**1**) in DMSO-d_6.

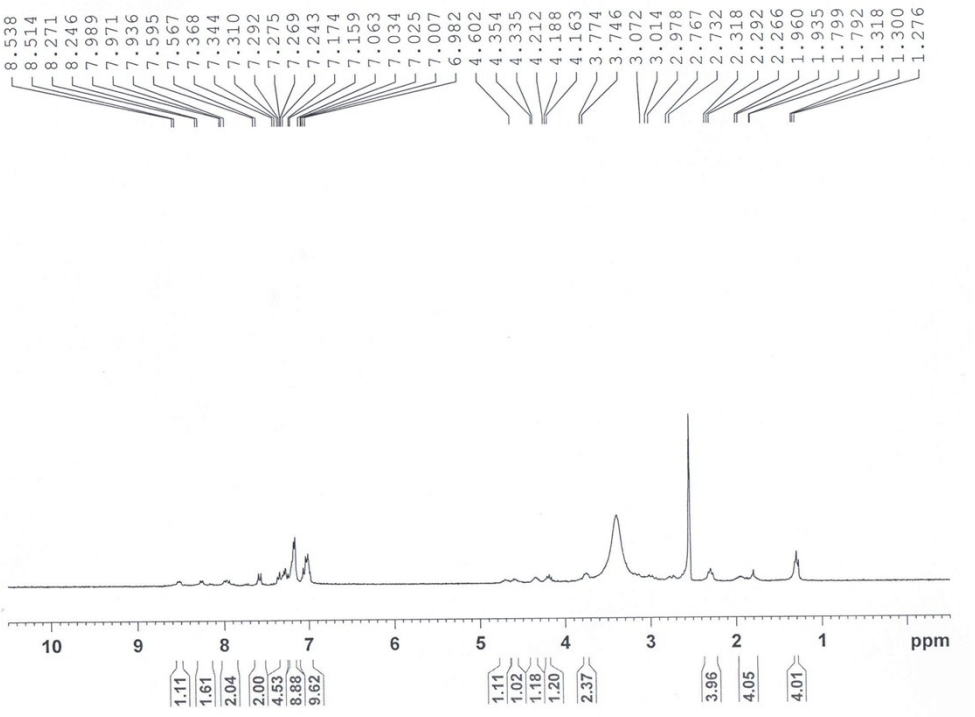

Fig. S13 ^{1}H NMR spectrum for TPE-FDGEA (**2**) in DMSO-d_6.

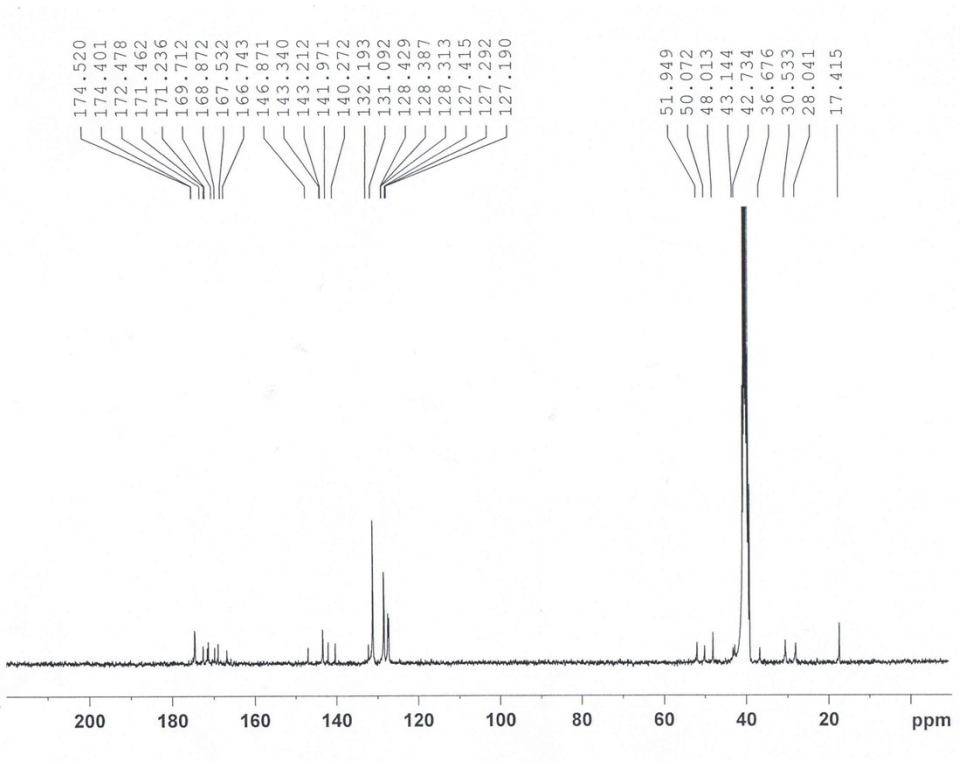

Fig. S14 ^{13}C NMR spectrum for TPE-GDGEA (**1**) in DMSO-d_6.

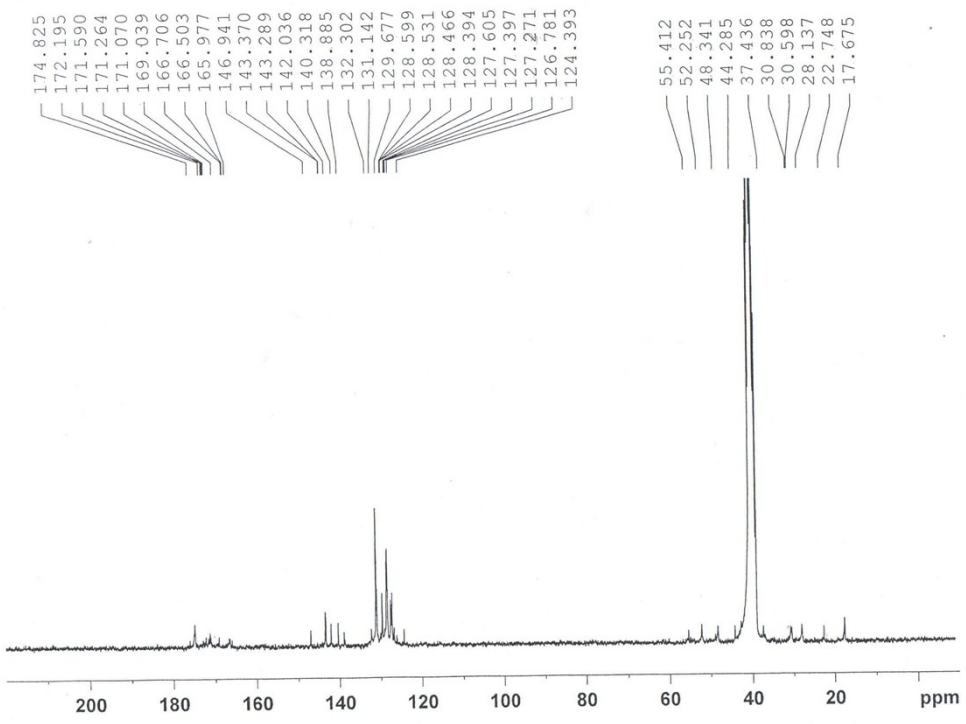

Fig. S15 ^{13}C NMR spectrum for TPE-FDGEA (**2**) in DMSO-d_6.

Reference

1. N. T. Chu, R. D. Chakravarthy, N. C. Shih, Y. H. Lin, Y. C. Liu, J.-H. Lin and H.-C. Lin, *RSC Adv., 2018,* **8**, 20922.

2. M.-Y. Yeh, C.-W. Huang, J.-W. Chang, Y.-T. Huang, J.-H. Lin, S.-M. Hsu, S.-C. Hung and H.-C. Lin, *Soft Matter, 2016,* **12***,* 6347.