Supplementary Information

Chemical Stimulus-Responsive Supramolecular Hydrogel Formation and Shrinkage of a Hydrazone-containing Short Peptide Derivative

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Contents:

1. Experimental

Generals Unless stated otherwise, all commercial reagents were used as received. All water used in the experiments refers to ultra-pure water obtained from a Millipore system having a specific resistance of 18 MΩ•cm. Thin layer chromatography (TLC) was performed on silica gel $60F_{254}$ (Merck). Column chromatography was performed on silica gel PSQ-100B (Fuji Silysia Chemical, 100 μm) or DispoPackAT ODS (YMC, 50 μm). Reverse phase HPLC (RP-HPLC) was conducted with a Shimadzu Prominence instrument LC-20AT and SPD-20A equipped with a YMC Triart C18 column (150 mm × 4.6 mm I. D., 5 μm). ^{1}H and ^{13}C NMR spectra were obtained on a JEOL JNM ECS-400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) with tetramethylsilane (TMS) or residual non-deuterated solvents (internal 1,4-dioxane for D_2O) as the internal references. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, br = broad. MALDI-TOF mass spectra were recorded using a Shimadzu AXIMA-CFR plus mass spectrometer. Other mass spectrometry was performed on a JEOL JMS-T100LP AccuTOF LC-plus (ESI and DART) or a Waters Xevo QTof (ESI) mass spectrometer.

Conventional hydrogelation ability test: Gelation ability was evaluated by an inverted tube test. Typically, DMSO stock solution of **Z-F$_2$-BPS$_n$** (5.0 wt%, 4.0 μL) was mixed with aqueous buffer (196 μL, 50 mM MES-NaOH (pH 7.0 and 5.5)) to obtain an aqueous dispersion (0.10 wt%, 1.2 mM, the final DMSO concentration was kept constant at 2% for the other concentrations of **Z-F$_2$-BPS$_n$** (0.20, 0.40, 1.0 wt%)) in a glass vial. The resultant solution was applied with sonication and heated by a heat gun. When a transparent aqueous solution was obtained, the solution was cooled down at room temperature for designated time and the hydrogel formation was evaluated by inverting the glass vial.

Hydroxylamine-induced hydrogel formation and HPLC analysis: Typically, to an aqueous solution (100 μL) of **Z-F$_2$-BPS$_2$** (0.10 wt%, 1.2 mM) prepared according to the procedure described above was added an aqueous solution of hydroxylamine (300 mM hydroxylamine•HCl in water, 4.0 μL) and the resultant solution was incubated at room temperature. After designated time, acetonitrile (100 μL) and a DMSO stock solution of **Z-F-OH** (50 mM, 4.5 μL, internal stand) was added to dissolve the hydrogel for HPLC analysis. An aliquot (10 μL) of the obtained solution was subjected to RP-HPLC analysis (YMC Triart C18 column (150 mm × 4.6 mm I. D., 5 μm), Wavelength of detection: 260 nm, Eluent: **A**:**B** = 80:20 to 40:60 (**A**: Acetonitrile, **B**: H_2O/0.1%TEA), linear gradient over 30 min, flow rate = 1.0 mL/min). Internal standard (**Z-F-OH**) was used to estimate the concentrations of **Z-F$_2$-BPS$_2$** and **Z-F$_2$-NHNH$_2$**.

TEM observation: Sample (ca. 10 μL) was dropped on a copper TEM grid covered by an elastic carbon-support film (20–25 nm) with a filter paper underneath and the excess solution was blotted with the filter paper immediately. The TEM grid was dried under a reduced pressure for at least 6 h prior to TEM observation. TEM images were acquired using a JEOL JEM-1025 (accelerating voltage: 100 kV) equipped with a CCD camera and analyzed with ImageJ on a Windows PC.

CLSM observation: A freshly prepared homogeneous solution (0.10 wt%, 50 mM MES-NaOH (pH 5.5, 200 μL) of **Z-F_2-BPS_2** obtained according to the procedure described above was mixed with a DMSO solution of Nile-blue (5 mM (final concentration is 25 μM), 1.0 μL) and aniline (2.2 μL). The solution was left to cool down at room temperature. The solution (20 μL) was spotted on a glass coverslip (diameter: 25 mm, thickness: 0.13–0.17 mm, Fisher Scientific) placed in an Attofluor cell chamber (Thermo Fisher Scientific) with water drops (50 μL) around the sample drop to avoid dryness. The sample before and after the addition of hydroxylamine (300 mM, 0.8 μL, 10 eq.) was subjected to observations using an inverted confocal laser scanning microscope (FV1000-D, Olympus) equipped with an Ar laser (488 nm) and LED laser (559 nm) and a Gallium Arsenide Phosphide (GaAsP) detector. A 60× (numerical aperture (NA) = 1.49) oil objective was employed to obtain images. The images were obtained and analyzed by the acquisition software FV10-ASW4.2 equipped with the microscope.

Circular dichroism: CD spectra (with HT voltage data) were recorded in a 0.1-mm quartz cell unless otherwise noted on a Jasco J-820 spectropolarimeter equipped with a programmable temperature-control unit (Julabo HP-4). The spectra were obtained by using a 2-nm slit width and a scanning step of 0.1 nm from 300 to 210 nm. Each spectrum was an average of 4 scans with the buffer background subtracted.

Rheological measurement: Dynamic frequency and strain sweep experiments were carried out on a TA instruments AR-G2 rheometer using a 20-mm stainless steel parallel plate (The temperature of the plate was controlled at 25 ºC by peltier system) at the gap of 1000 μm. Hydrogel samples were placed on the plate. All the gels showed almost linear viscoelastic regime up to 1.0% strain (frequency: 1.0 rad/s). Therefore, frequency sweep (0.1–100 rad/s) was performed under 0.2 % strain.

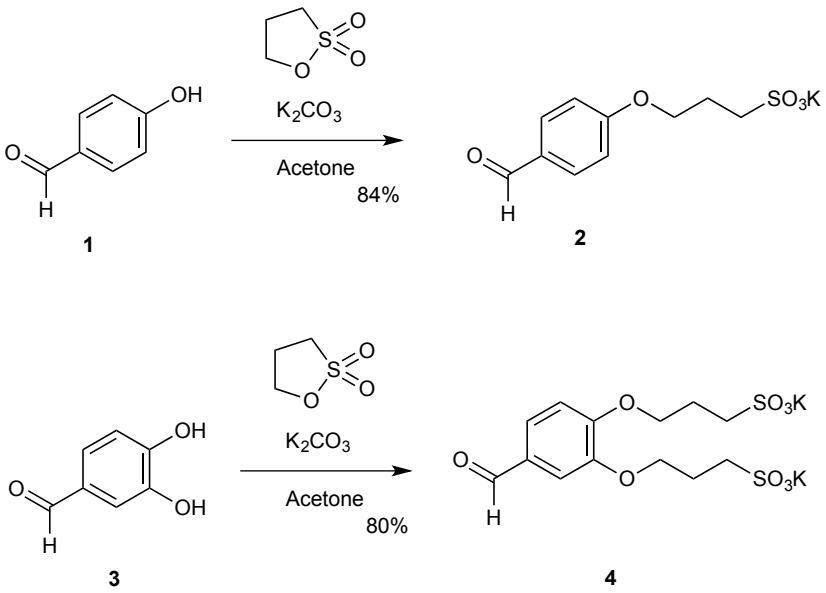
DLS measurements: DLS measurements were performed on a Malvern Zetasizer Nano equipped with a He-Ne laser (633 nm) and a detection angle of 173º. Aqueous solutions were filtered using a Millipore

membrane filter (0.45 μm pore size) before measurements. The particle size distribution was derived from a deconvolution of the measured intensity autocorrelation function of the sample by the "General Purpose Mode" (non-negative least-squares) algorithm included in the DTS software.

2. Synthesis

2.1. Synthesis of water-soluble aldehyde derivatives (BPS_n) bearing propylsulfonate anions

Compound **2** (**BPS**)[S1] was synthesized according to the slightly modified method reported previously. Compound **4** (**BPS_2**) was synthesized according to the similar method (**Scheme S1**).



Scheme S1. Synthesis of anionic aldehyde derivatives (**2** (**BPS**) and **4** (**BPS_2**)).

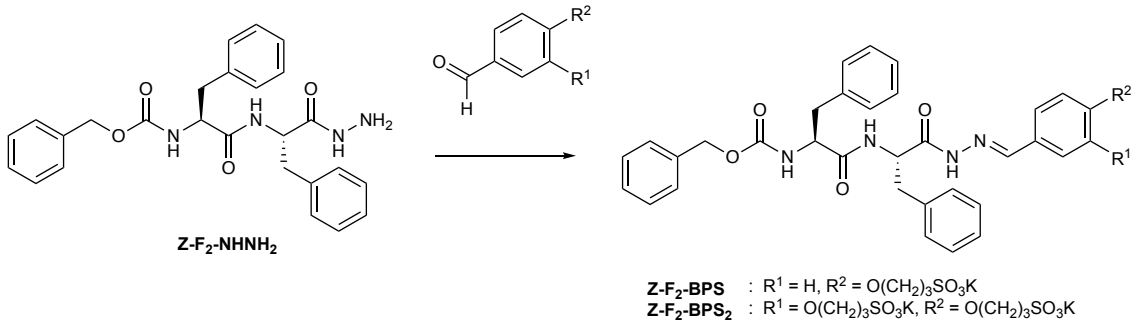
Synthesis of compound 2[S1]: To a mixture of 4-hydroxybenzladehyde (**1**) (123 mg, 1.0 mmol), K_2CO_3 (205 mg, 1.5 mmol), and acetone (5 mL) was added 1,3-propanesultone (125 mg, 1.0 mmol) and the resultant mixture was stirred at 40 ºC for 5 h. After the mixture was cooled, 5% aqueous HCl was added to neutralize and precipitate was removed by filtration. The filtrate was concentrated to dryness and the residue was purified by column chromatography (SiO_2, chloroform: methanol = 2:1) to afford compound **2** (**BPS**)[S1] (206 mg, 84%) as a white solid: ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ (ppm) 2.02 (quin, 2H), 2.51−2.60 (m, 2H), 4.19 (t, *J* = 6.6 Hz, 2H), 7.11 (d, *J* = 8.7 Hz, 2H), 7.85 (d, *J* = 8.5 Hz, 2H), 9.86 (s, 1H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ (ppm) 25.09, 47.68, 67.26, 114.94, 129.50, 131.86, 163.69, 191.339.

Synthesis of compound 4: To a mixture of 3,4-dihydroxybenzladehyde (**3**) (61 mg, 0.5 mmol), K_2CO_3 (912 mg, 6.0 mmol), and acetone (10 mL) was added 1,3-propanesultone (484 mg, 4.0 mmol) and the resultant mixture was refluxed for 3 days. After the mixture was cooled, precipitate was removed by filtration. The filtrate was concentrated to dryness and the residue was purified by re-precipitation with

H_2O/acetone to afford compound **4** (**BPS_2**) (151 mg, 80%) as a yellow solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.91–2.14 (m, 4H), 2.52–2.61 (m, 4H), 4.13 (t, J = 6.6 Hz, 2H), 4.21 (t, J = 6.8 Hz, 2H), 7.16 (d, J = 8.2 Hz, 1H), 7.37 (d, J = 1.8 Hz, 1H), 7.51 (dd, J = 8.2, 1.8 Hz, 1H), 9.81 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 25.06, 38.16, 25.21, 47.66, 47.84, 67.57, 67.63, 111.32, 125.79, 129.44, 148.58, 153.88, 191.38; HRMS (ESI, negative): Calcd. for [M($C_{13}H_{16}K_2O_9S_2$) –2 K]$^{2-}$: m/z = 190.0118; Found: 190.0104.

2.2. Synthesis of precursors (Z-F_2-BPS and Z-F_2-BPS_2)

Precursors (**Z-F_2-BPS and Z-F_2-BPS_2**) were synthesized from **Z-F_2-NHNH_2**[S2] as shown in Scheme S2.



Scheme S2. Synthesis of precursors **Z-F_2-BPS** and **Z-F_2-BPS_2**.

Synthesis of Z-F_2-BPS_2: To a solution of **Z-F_2-NHNH_2** (48 mg, 0.10 mmol) in ethanol (8 mL), H_2O (2 mL), and TFA (0.15 mL) was added compound **4**(**BPS_2**) (39 mg, 0.10 mmol). The resultant mixture was stirred at 40 ºC under Ar atmosphere for 2 days. After the mixture was cooled and concentrated, diethyl ether (50 mL) was added and the precipitate was collected by filtration, washed with diethyl ether, and dried to yield **Z-F_2-BPS_2** (37 mg, 45%) as a pale yellow solid: ^{1}H NMR (400 MHz, DMSO-d_6, assigned as the mixture of two amide rotamers (**Fig. S6,S7**), 25 ºC) δ (ppm) 1.92–2.15 (m, 4H), 2.55–2.75 (m, 4H (overlapped with DMSO)), 2.80–3.20 (m, 4H), 3.94–4.18 (m, 4H), 4.20–4.35 (m, 1H), 4.50–4.68 (m, 0.5H), 4.92 (m, 2H), 5.28–5.48 (m, 0.5H), 7.01 (dd, J = 8.2, 4.1 Hz, 1H), 7.09–7.39 (m, 17H), 7.45 (d, J = 9.4 Hz, 1H), 8.00 (s, 0.5H), 8.06 (s, 0.5H), 8.27 (d, J = 7.8 Hz, 0.5H), 8.38 (d, J = 7.8 Hz, 0.5H), 11.32 (s, 0.5H), 11.44 (s, 0.5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 25.24, 25.40, 47.88, 47.97, 48.05, 56.08, 65.17, 65.20, 67.47, 67.51, 67.55, 67.63, 126.20, 126.22, 126.42, 126.71, 126.75, 127.39, 127.42, 127.66, 128.00, 128.03, 128.18, 128.19, 128.29, 128.32, 129.06, 129.18, 129.22, 129.27, 137.00, 137.02, 137.38, 137.84, 137.99, 138.15, 148.59, 148.63, 150.23, 150.40, 155.71, 155.72, 167.13, 171.49, 172.05; HRMS (ESI, negative): Calcd. for [M($C_{39}H_{42}N_4O_{12}S_2K_2$) – 2K + H]$^-$: m/z = 823.2319;

Found: 823.2338.

Synthesis of Z-F_2-BPS: The titled compound was prepared from **Z-F_2-$NHNH_2$** (23 mg, 50 μmol) and compound **2**(**BPS**) (14 mg, 50 μmol) in the similar way for **Z-F_2-BPS** and was obtained in 84% yield (29 mg) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6, assigned as the mixture of two amide rotamers, 25 ºC) δ (ppm) 1.93–2.09 (m, 2H), 2.52–2.59 (m, 2H (overlapped with DMSO)), 2.62–3.14 (m, 4H), 4.05–4.16 (m, 2H), 4.21–4.35 (m, 1H), 4.52–4.66 (m, 0.5H), 4.93 (m, 2H), 5.34–5.47 (m, 0.5H), 6.99 (m, 1H), 7.14–7.37 (m, 15H), 7.46 (d, J = 8.5 Hz, 2H), 7.60 (d, J = 8.2 Hz, 2H), 7.92 (s, 0.5H), 8.09 (s, 0.5H), 8.23 (d, J = 7.8 Hz, 0.5H), 8.36 (d, J = 7.8 Hz, 0.5H), 11.33 (s, 0.5H), 11.42 (s, 0.5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 25.11, 25.24, 47.69, 47.85, 51.88, 53.66, 56.11, 65.19, 66.91, 67.27, 114.80, 114.85, 114.95, 126.24, 126.26, 126.41, 126.46, 126.49, 126.61, 127.68, 128.04, 128.17, 128.31, 128.42, 128.73, 129.12, 129.21, 129.26, 131.85, 137.00, 137.01, 137.03, 137.37, 137.68, 137.98, 138.12, 155.73, 160.16, 160.32, 167.14, 171.35, 171.46, 171.76, 171.81, 171.98, 191.32; HRMS (ESI, positive): Calcd. for $[M(C_{36}H_{37}N_4O_8SK) - K]^-$: m/z = 685.2332; Found: 685.2355.

3. Characterization of compounds

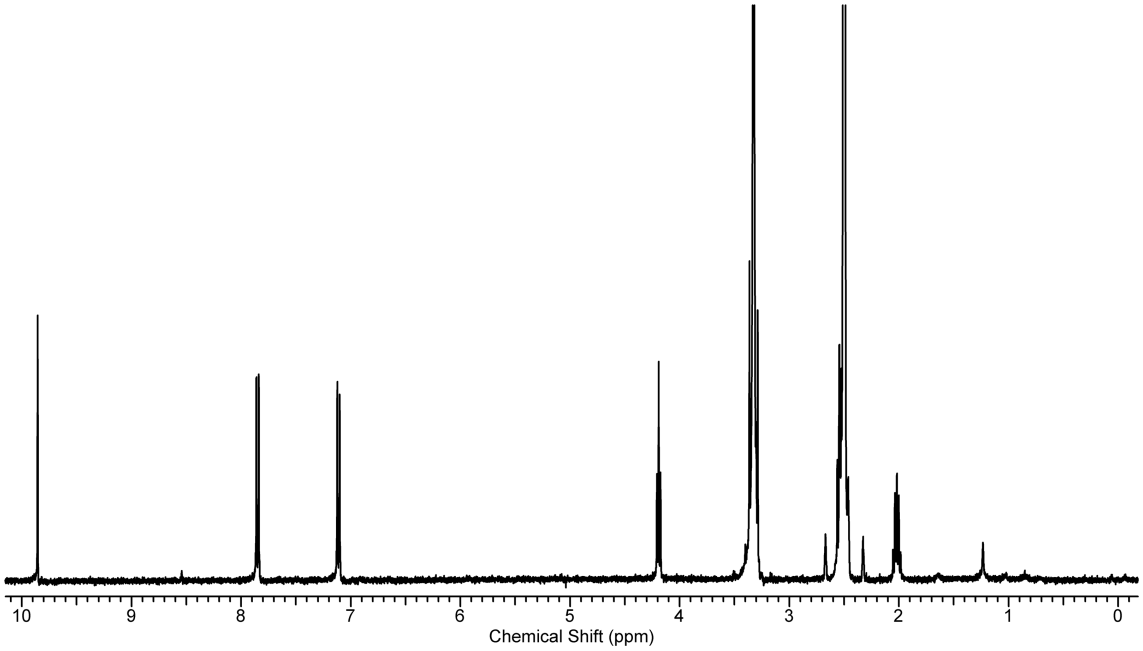


Fig. S1. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **BPS**.

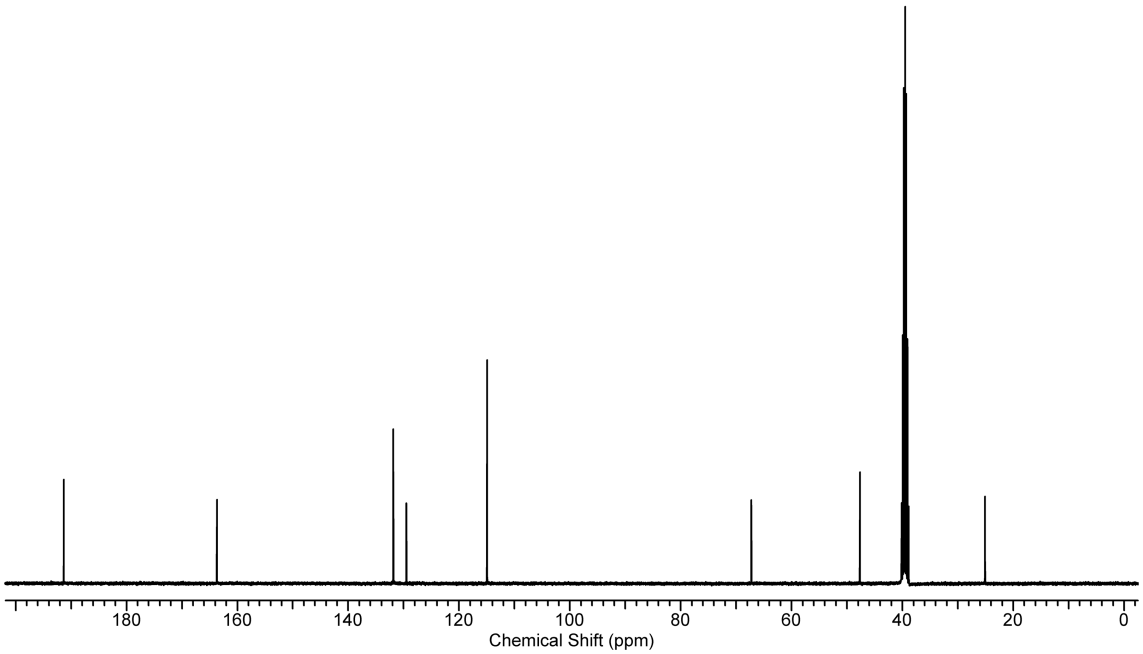


Fig. S2. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of **BPS**.

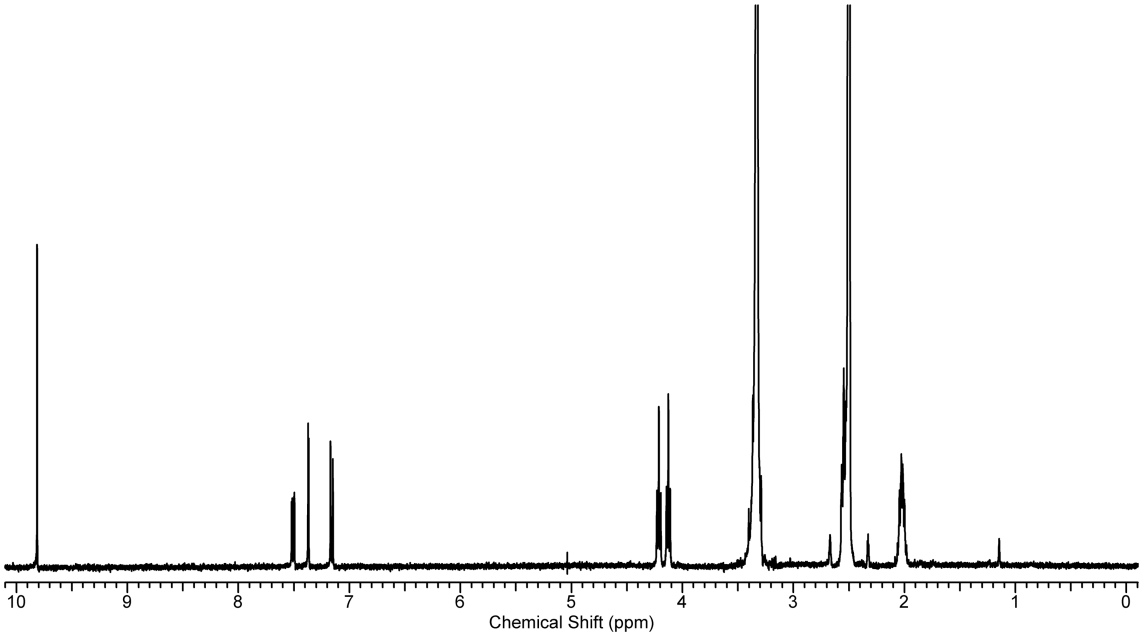


Fig. S3. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **BPS_2**.

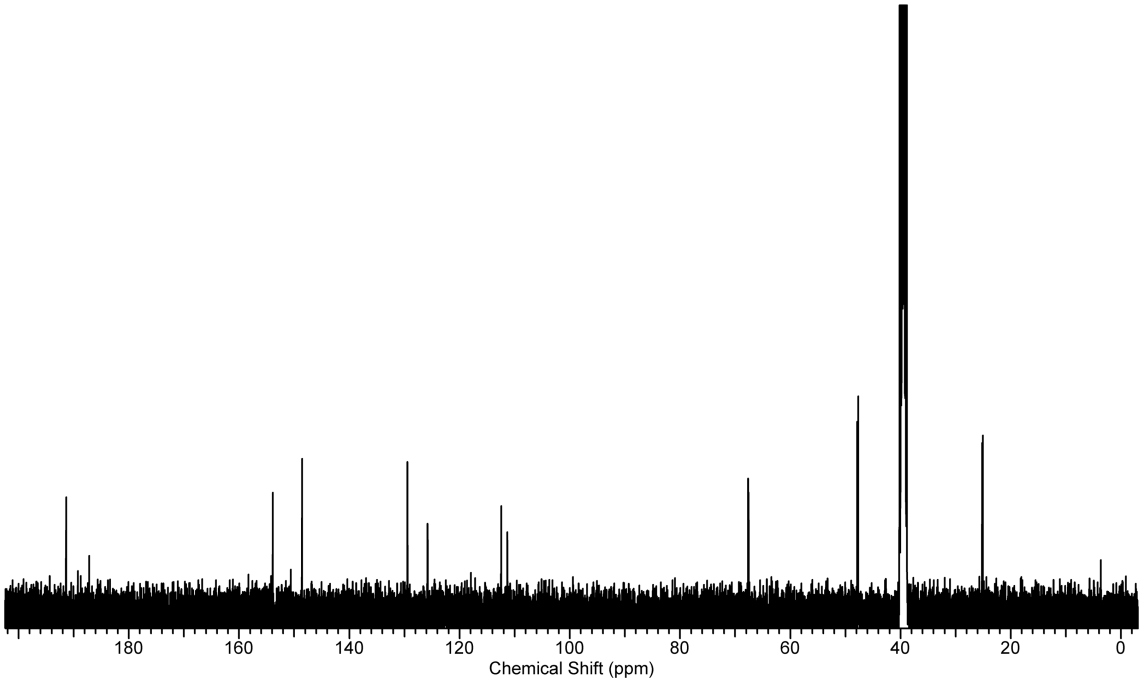


Fig. S4. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of **BPS_2**.

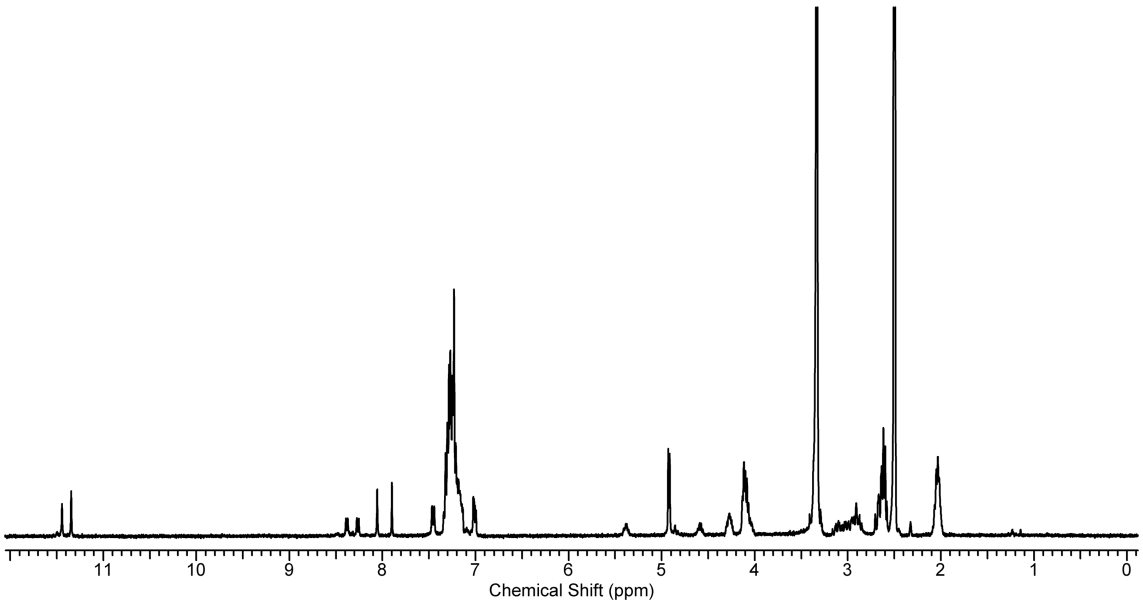


Fig. S5. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **Z-F$_2$-BPS$_2$**.

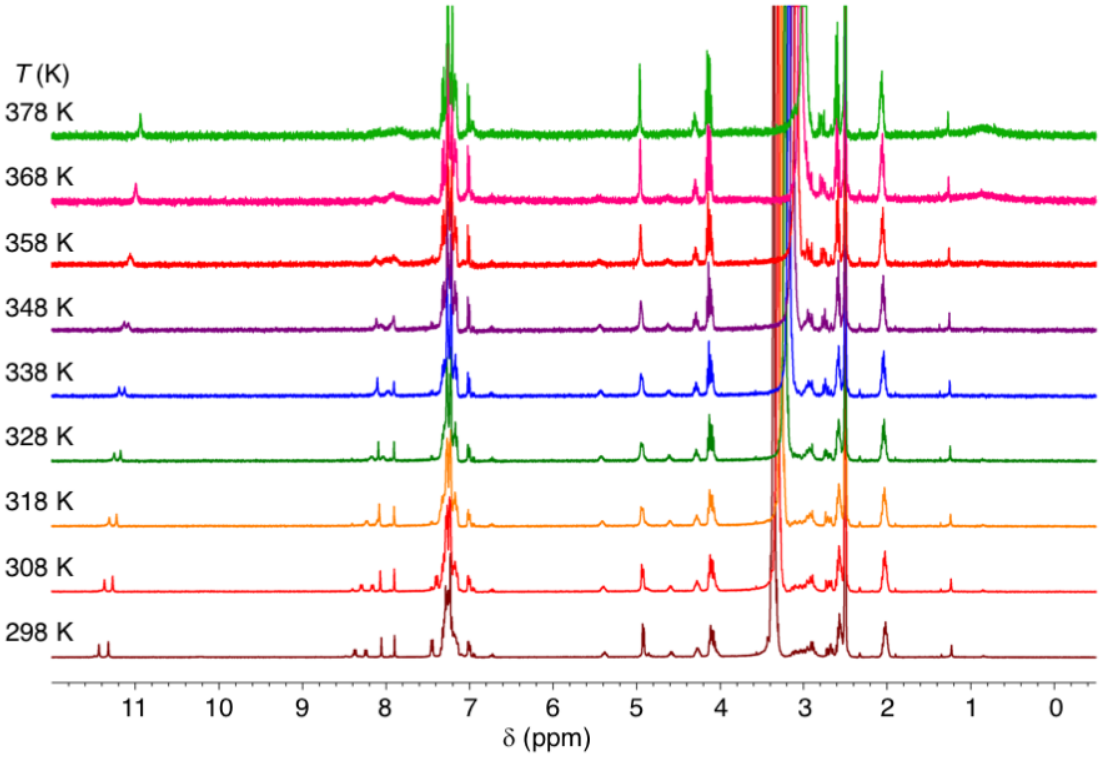


Fig. S6. Variable-temperature (VT) ^{1}H NMR spectra (400 MHz, DMSO-d_6, 1 mM) of **Z-F$_2$-BPS$_2$**.

Duplication of signals was observed especially in the low-field region, indicating that the exchange between the two amide rotamers (*anti* and *syn*, see **Fig. S7A** for the chemical structures) is slow on NMR time scale, according to previous reports for similar compounds.[S3] Variable-temperature (VT) ^{1}H NMR

measurements allow for the estimation of the free-energy barrier for the amide-bond rotation. From the coalescence temperature (T_C) and Gutowsky equation (equation (2)), $\Delta G^{\ddagger}$ and k value was evaluated on the basis of

$$k = \pi\Delta\nu / 2^{1/2} \qquad (1)$$

$$\Delta G^{\ddagger} = RT_C \ln[2^{1/2}k_B T_C / \pi h(\Delta\nu)] \qquad (2)$$

where Δν is difference in the chemical shifts (Hz). From **Fig. S6** (from a set of signals assignable to NH {"a_a for *anti* and a_s for *syn*"} as shown in **Fig. S7A**), T_C was estimated to be 85 °C (358 K), then, $\Delta G^{\ddagger}$ and k was evaluated to be 74.5 kJ mol^{-1} and 101 s^{-1}, respectively, according to the equations (1) and (2). These values are well comparable to the similar compounds.[S3] In addition, this result indicates that the two rotamers can be exchanged rapidly at ambient temperature, which is consistent with the fact that a single peak was observed in the HPLC analysis of **Z-F$_2$-BPS$_2$** (**Fig. 4**).

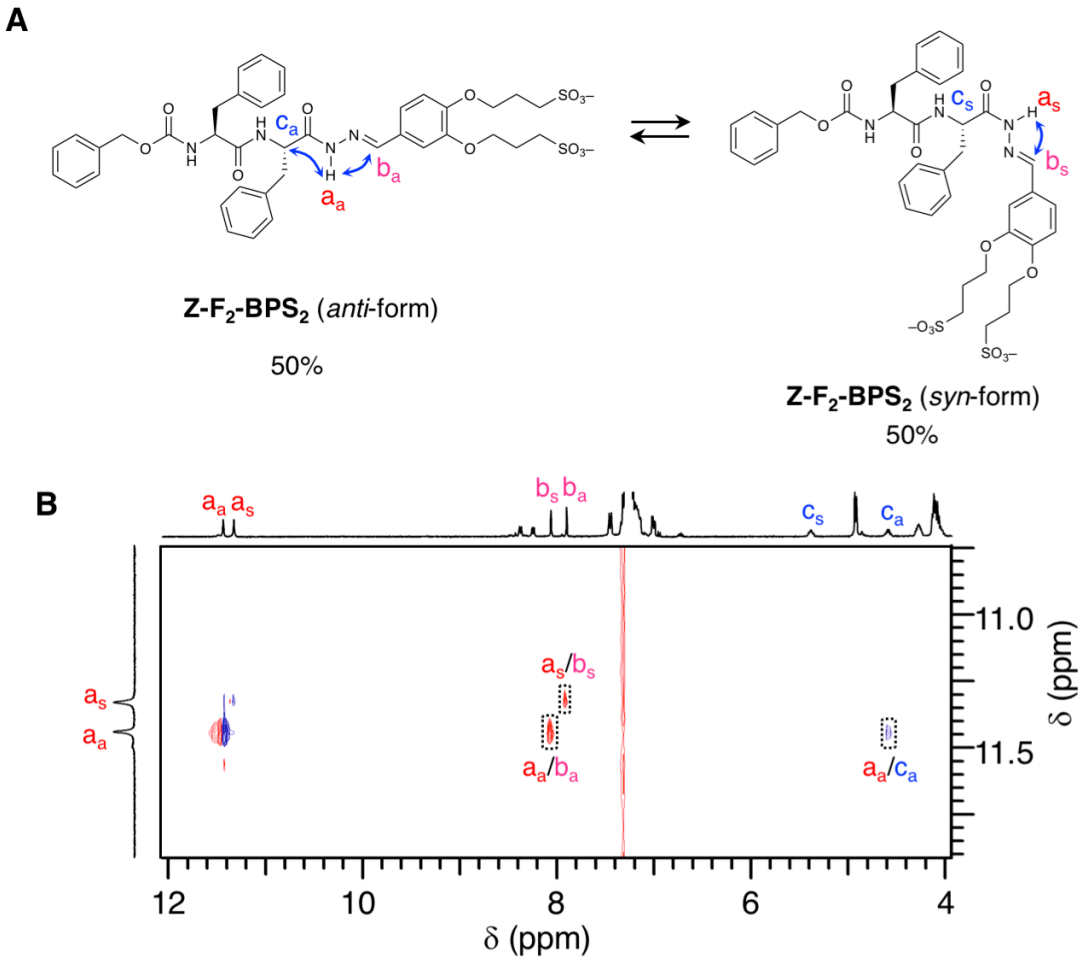


Fig. S7. (**A**) Proposed exchange between the two amide rotamers of **Z-F$_2$-BPS$_2$**. The blue arrows indicate the assignments of the observed NOE correlations (a_a/b_a, a_a/b_a, and a_s/b_s, shown in panel (**B**)).

Each composition (%) at room temperature estimated from the ^{1}H NMR spectrum is shown. (**B**) Partial NOESY spectrum (400 MHz, DMSO-d_6, 25 ºC, 2 mM, mixing time = 500 ms) of **Z-F$_2$-BPS$_2$**.

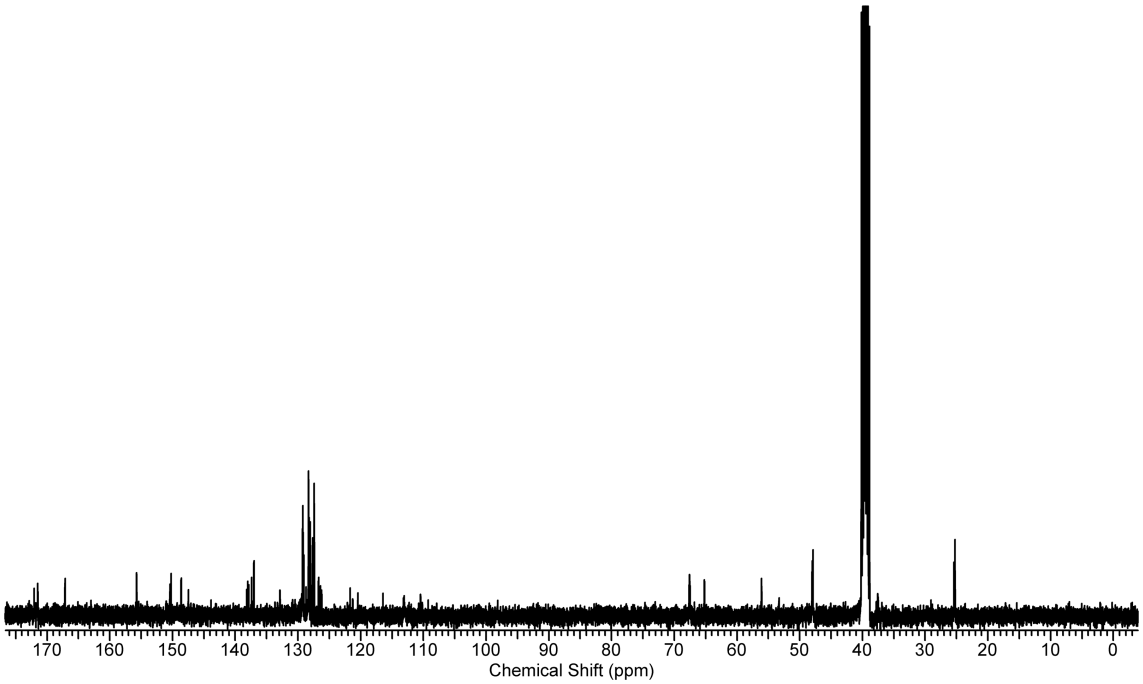


Fig. S8. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of **Z-F$_2$-BPS$_2$**.

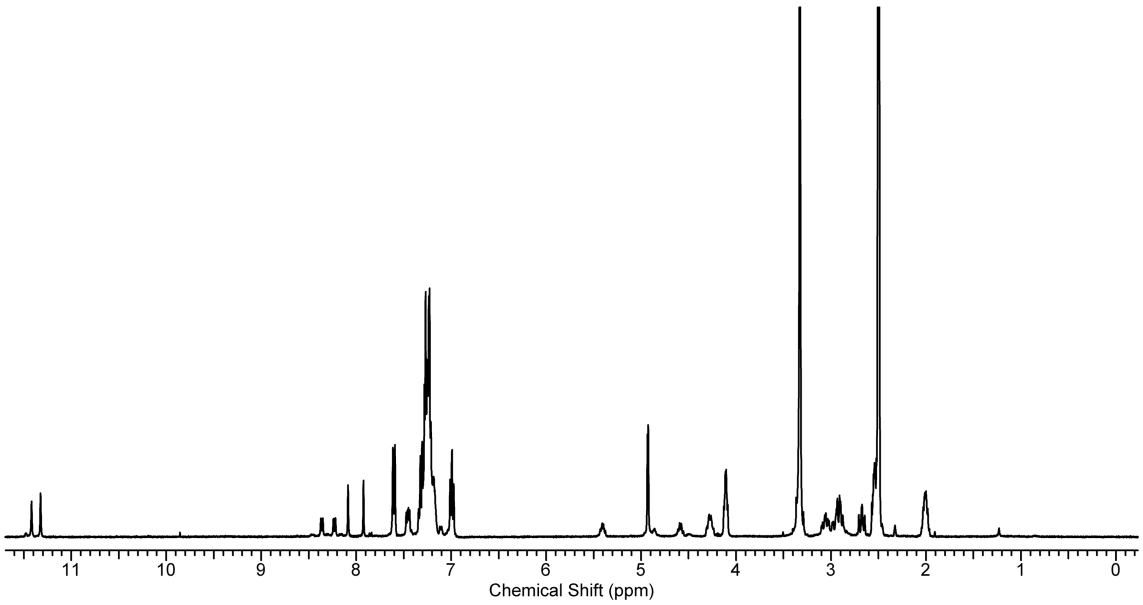


Fig. S9. ^{1}H NMR spectrum (400 MHz, DMSO-*d*$_6$) of **Z-F$_2$-BPS**.

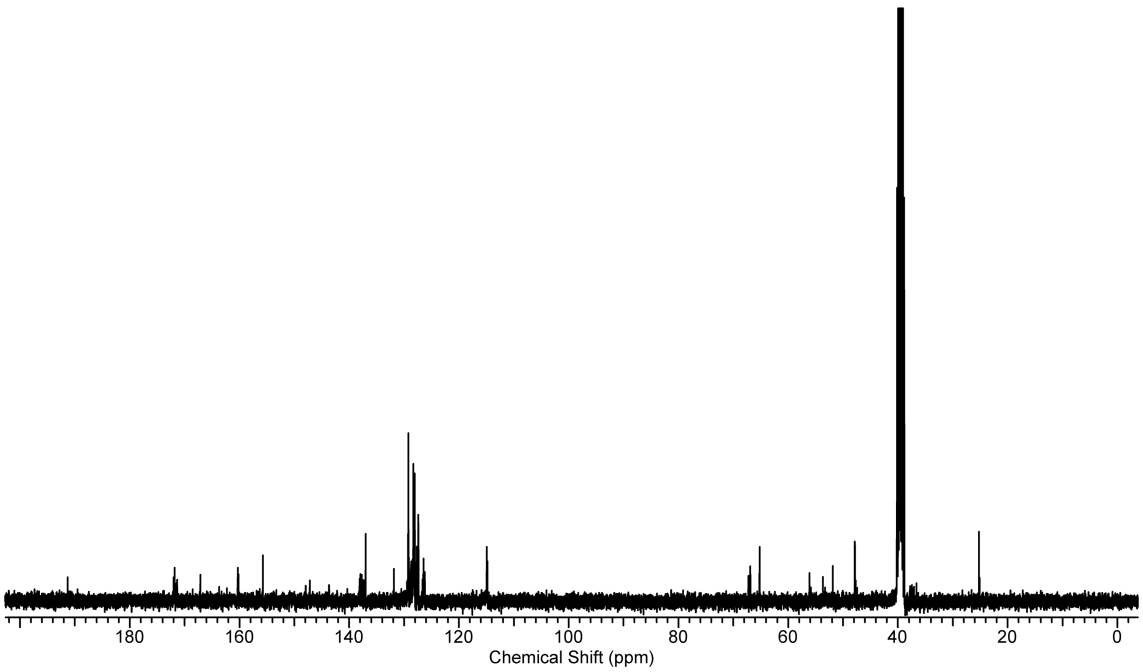


Fig. S10. ^{13}C NMR spectrum (100 MHz, DMSO-*d*$_6$) of **Z-F$_2$-BPS**.

4. Characterization of supramolecular hydrogels

■Gelation ability of Z-F_2-BPS_n (n = 1, 2)

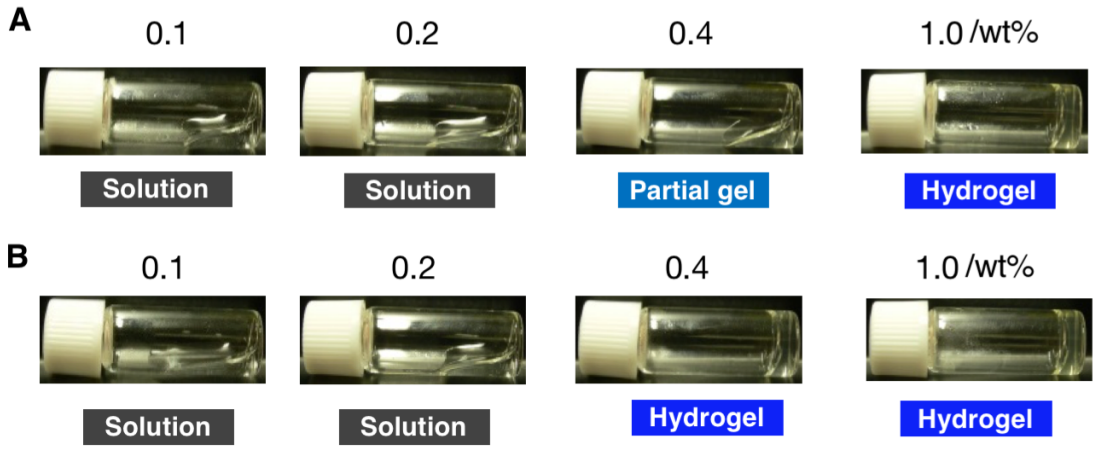


Fig. S11. Photographs showing gelation ability of **Z-F_2-BPS_2** (tube-inversion experiments [Insoluble: Insoluble residue remained even after heating, Hydrogel: No flow was observed when inverting the vial, Partial gel: weak partial gel (Flow was observed when inverting the vial while part of the solution was gelled), Solution: solution or clear dispersion]) dependent on the concentration (**A**: 50 mM MES-NaOH (pH 7.0) containing DMSO (2.0 vol%), **B**: 50 mM MES-NaOH (pH 5.5) containing DMSO (2.0 vol%)).

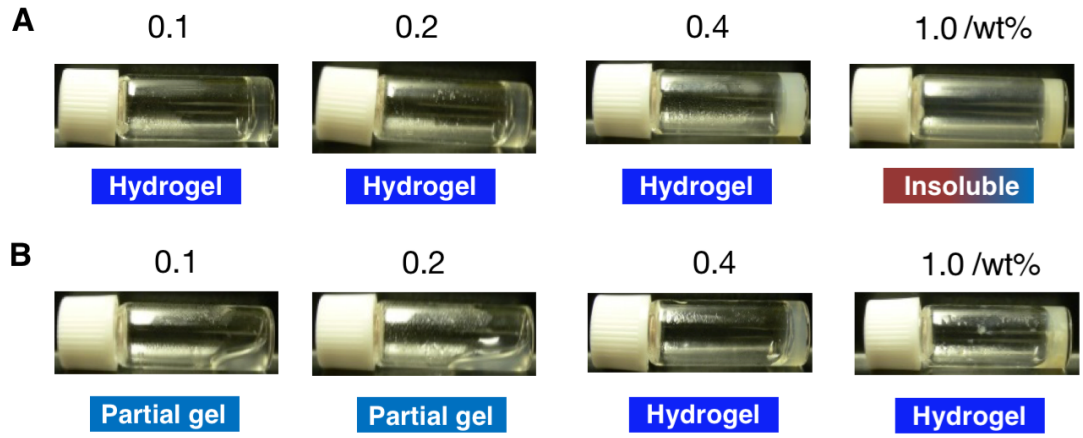


Fig. S12. Photographs showing gelation ability of **Z-F_2-BPS** (tube-inversion experiments [Insoluble: Insoluble residue remained even after heating, Hydrogel: No flow was observed when inverting the vial, Partial gel: weak partial gel (Flow was observed when inverting the vial while part of the solution was gelled), Solution: solution or clear dispersion]) dependent on the concentration (**A**: 50 mM MES-NaOH (pH 7.0) containing DMSO (2.0 vol%), **B**: 50 mM MES-NaOH (pH 5.5) containing DMSO (2.0 vol%)).

■**Rheological property of the hydrogels**

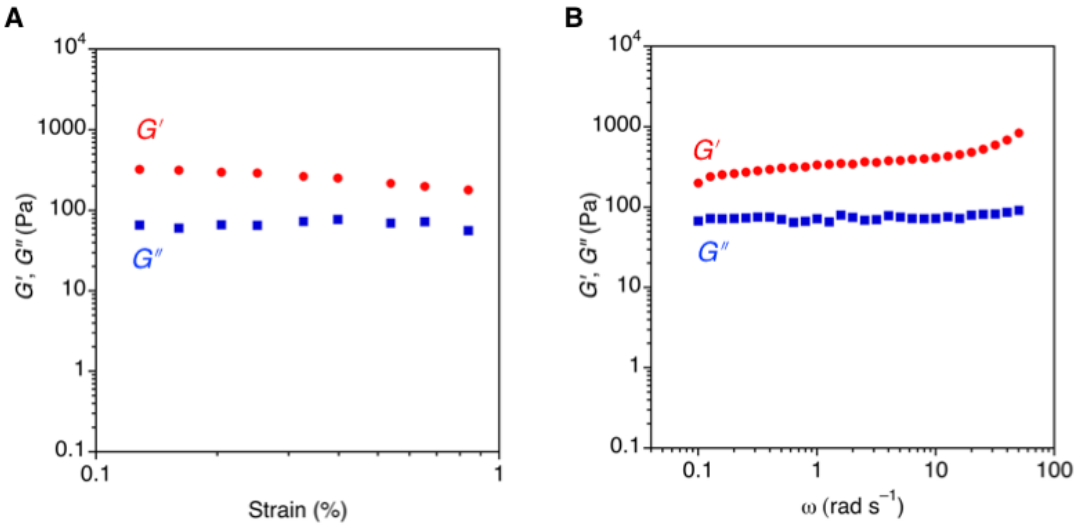


Fig. S13. (**A**) Strain sweep (1.0 rad/s) and (**B**) frequency sweep (0.2% strain) rheological properties of the hydrogel prepared from **Z-F_2-BPS_2** at 4.5 h after the addition of hydroxylamine (*G′*: storage shear modulus, *G″*: loss shear modulus). *Conditions*: **Z-F_2-BPS_2** (0.10 wt%, 1.2 mM), 50 mM MES-NaOH (pH 5.5) containing DMSO (2.0 vol%), [NH_2OH] = 40 eq.

■**CD spectral change (with HT voltage data) of Z-F$_2$-BPS$_2$**

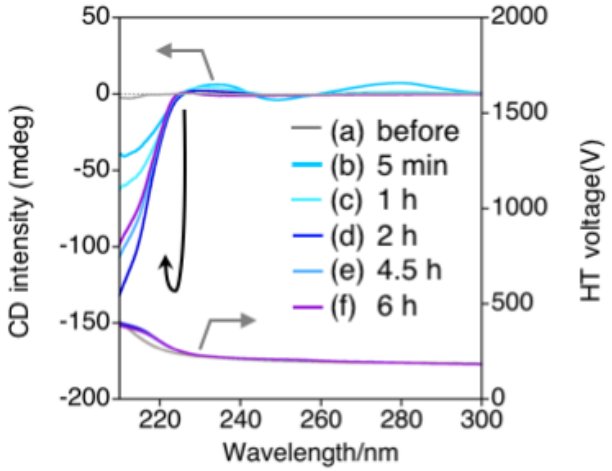


Fig. S14. (**A**) CD spectral change (the same as **Fig. 5A**) and HT voltage data during the hydrogel formation and subsequent shrinkage of **Z-F$_2$-BPS$_2$** upon the addition of NH_2OH. *Conditions*: **Z-F$_2$-BPS$_2$** (0.10 wt%, 1.2 mM), 50 mM MES-NaOH (pH 5.5) containing DMSO (2.0 vol%), [NH_2OH] = 40 eq.

■**DLS analysis of Z-F$_2$-BPS$_2$**

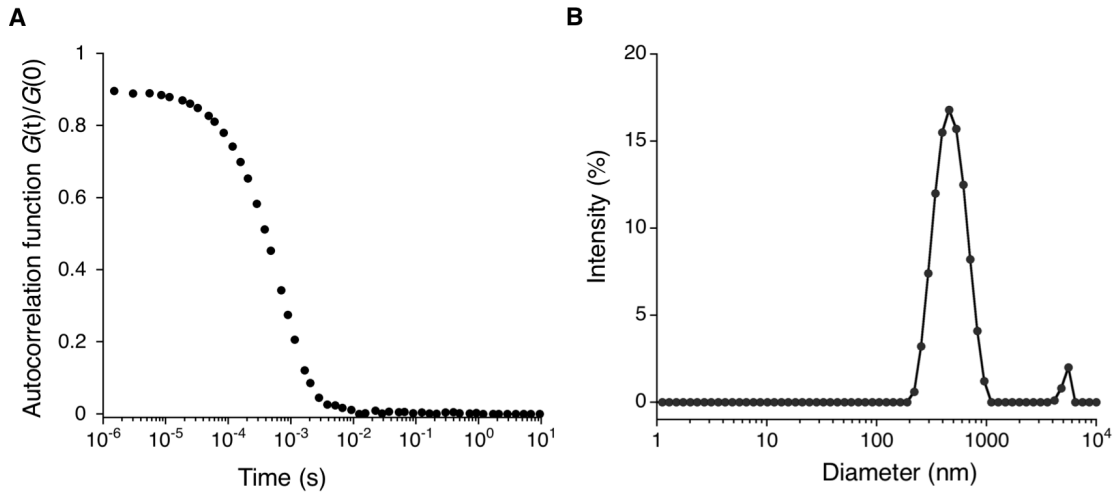


Fig. S15. (**A**) Intensity correlation functions of the aqueous solution of **Z-F$_2$-BPS$_2$**. (**B**) Size distribution of **Z-F$_2$-BPS$_2$**. *Conditions*: **Z-F$_2$-BPS$_2$** (0.10 wt%, 1.2 mM), 50 mM MES-NaOH (pH 5.5) containing DMSO (2.0 vol%), 25 ºC.

References

[S1] H. Li and S. Valiyaveettil, Water-soluble multifunctional cross-conjugated poly(*p*-phenylenes) as stimuli-responsive materials: design, synthesis, and characterization, *Macromolecules*, 2007, **40**, 6057–6066.

[S2] T. Tsuzuki, M. Kabumoto, H. Arakawa and M. Ikeda, Effect of carbohydrate structures on the hydrogelation ability and morphology of self-assembled structures of peptide-carbohydrate conjugates in water, *Org. Biomol. Chem.,* 2017, **15**, 4595–4600.

[S3] B. Levrand, W. Fieber, J.-M. Lehn and A. Herrmann, Controlled release of volatile aldehydes and ketones from dynamic mixtures generated by reversible hydrazone formation, *Helv. Chim. Acta,* 2007, **90**, 2281–2313.