Supplementary Information

Design of supramolecular hybrid nanomaterials comprising peptide-based supramolecular nanofibers and *in situ* generated DNA nanoflowers through rolling circle amplification

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Contents:

1. Experimental

Generals Unless stated otherwise, all commercial reagents were used as received. EquiPhi29 DNA polymerase, Proteostat® aggregation detection kit and proteinase K were obtained from Thermo Fisher (USA), Enzo Life Sciences (USA) and Kanto Chemical Co. (Japan), respectively. Oligonucleotides including Cy5-ON (5′-[Cy5]-GCCGCAGCAGCA-3′) were purchased from FASMSC (Japan). 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). ^{1}H and ^{13}C NMR spectra were obtained on a JEOL JNM ECS-400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) with tetramethylsilane (TMS) or residual non-deuterated solvents as the internal references. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, br = broad. ESI mass spectrometry was performed on a JEOL JMS-T100LP AccuTOF LC-plus mass spectrometer.

Conventional hydrogelation ability test: Gelation ability was evaluated by an inverted tube test. Typically, a DMSO stock solution of **Z-AF-BPS** (500 mg/mL, 4.0 μL) was mixed with an aqueous buffer [50 mM MES-NaOH (pH 7.0), 196 μL] to obtain an aqueous dispersion (1.0 wt%, 15 mM) 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 and the hydrogel formation was evaluated by inverting the glass vial at designated time (10 min, 3 h, and 24 h). Hydrogel formation of **Z-AF-BPS** was observed within 10 min at 1.0 wt% while 3 h at 0.40 wt%.

Circular dichroism: CD spectra were recorded in a 1-mm quartz cell or a 0.1-mm demountable quartz cell 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 400 to 220 nm. Each spectrum was an average of 4 scans with the solvent background subtracted.

FTIR: FTIR spectra were acquired using a Shimadzu IRAffinity-1 spectrometer with a spectral resolution of 4 cm^{-1}. The spectra were obtained by averaging 128 scans for each sample. Hydrogels prepared with D_2O were sandwiched directly between CaF_2 windows (32 × 3 mm) separated by a 0.1-μm PTFE spacer.

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 1500 μm. A freshly prepared solution (800 μL) of

Z-AF-BPS as described above in a glass vial was immediately transferred into a mold, which was prepared by cutting the top off a 20 mL (2 cm diameter) plastic syringe [S1] and set up vertically with its plunger. The top of the syringe mold was covered with Parafilm and left to stand still for 24 h at room temperature. The hydrogel was ejected by carefully pushing the plunger and transferred onto the bottom plate of the rheometer. 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.

AFM observation: The hydrogel (approx. 10 μL) prepared as described above was dropped on freshly cleaved mica and the excess hydrogel was removed by blotting with a filter paper. The AFM observations (512 × 512 pixels resolution) were performed using a Shimadzu SPM-9700HT microscope in air at ambient temperature with standard silicon cantilevers (AR5-NCHR, Nanosensors) in a tapping mode.

TEM observation: Sample (approx. 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 without staining. TEM images were acquired using a Hitachi H7000 (accelerating voltage: 100 kV) equipped with a CCD camera and analyzed with ImageJ on a Windows PC.

SEM observation: Sample (approx. 10 μL) was dropped on a silicon wafer and the excess solution was blotted with the filter paper immediately. The specimen was dried under a reduced pressure for at least 6 h prior to SEM observation. SEM images were acquired using a Hitachi S-4800 (accelerating voltage: 15 kV) equipped with an Oxford Instruments Ultim Max 100 detector for EDS analysis (Aztec software, Oxford instruments). Osmium coating (10 s) was carried out using a Neoc-Pro/p (Meiwafosis Co., Ltd.) for specimens containing **Z-AF-BPS** nanofibers.

XRD measurement: Powder X-ray diffraction (XRD) analysis using CuKα radiation (45 kV, 45 mA) was carried out for **Z-AF-BPS** freeze-dried xerogel filled inside a glass capillary (outside ϕ = 1.0 mm) with a Rigaku R-AXIS IV X-ray diffractometer. The diffracted radiation was recorded by an imaging plate with a sample-to-detector distance of 165 mm and exposure time of 30 min.

Construction of circular DNA for the RCA template: According to the previous report [S2], two oligonucleotides [template (0.60 μM) and primer (1.2 μM), see also **Fig. S12**] were mixed and annealed in a DNA ligation buffer [5 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM ATP, 1 mM dithiothreitol] by

heating at 95 °C for 5 min, followed by slow cooling (1 ºC/min) to 25 ºC using a T-100™ thermal cycler (Bio-Rad). The obtained mixture was incubated with T4 DNA ligase (10 U/μL, New England Biolabs) at 25 ºC for 5 h. For RCA, an aqueous mixture of the resultant circular DNA template (approx. 0.3 μM), EquiPhi29 DNA polymerase (1000 U/mL) and dNTPs (1.0 mM) in a reaction buffer solution [5 mM Tris-HCl (pH 7.5), 1.1 mM DTT, 10 mM KCl, 10 μM EDTA, 0.60 mg/mL BSA, 0.05% (v/v) nonidet P40, 0.05% (v/v) Tween 20, 5% (v/v) glycerol] was incubated at rt for 4 h. For CLSM observations, Cy5-ON (16 μM) was added to the reaction mixture.

CLSM observation: The aqueous mixture (typically, approx. 10 μL) for imaging 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, and was subjected to observations using an inverted confocal laser scanning microscope (FV1000-D, Olympus) equipped with an Ar laser (488 nm) and LED laser (635 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.

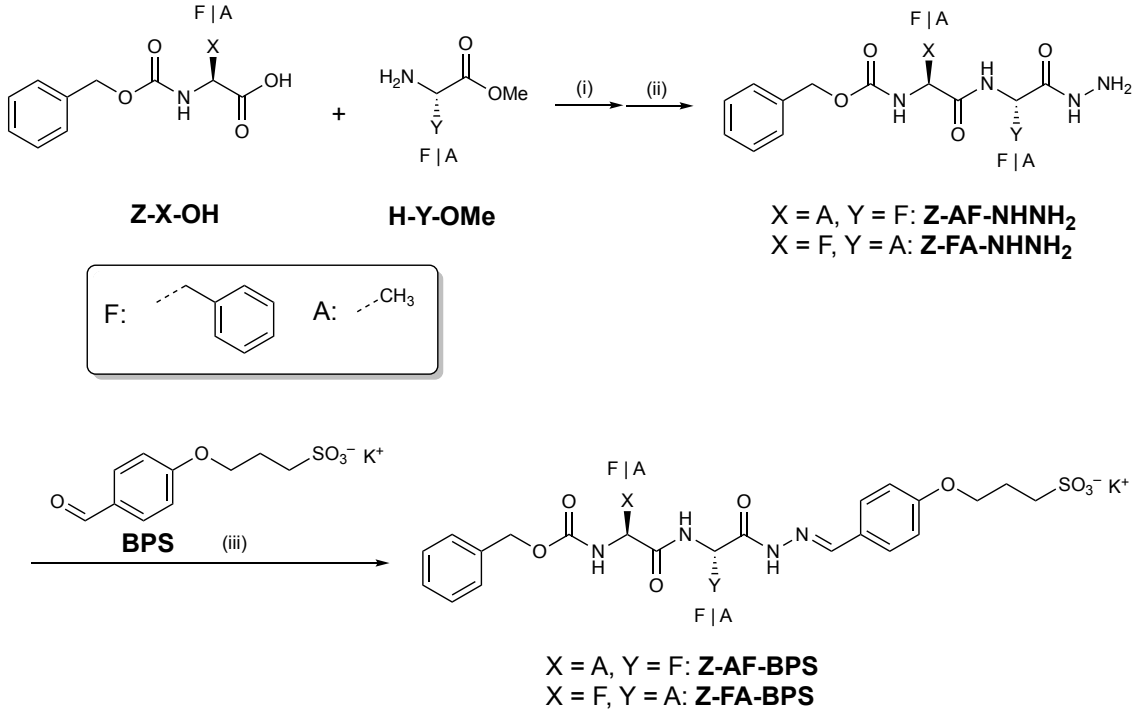
Construction of hybrid materials according to (i) post-RCA mixing protocol: An aqueous mixture of the circular DNA template (approx. 0.3 μM), EquiPhi29 DNA polymerase (1000 U/mL) and dNTPs (1.0 mM) in a reaction buffer solution [5 mM Tris-HCl (pH 7.5), 1.1 mM DTT, 10 mM KCl, 10 μM EDTA, 0.60 mg/mL BSA, 0.05% (v/v) nonidet P40, 0.05% (v/v) Tween 20, 5% (v/v) glycerol] was incubated at rt for 4 h. Then, to the mixture (18 μL) was added a DMSO stock solution of **Z-AF-BPS** (100 mg/mL, 1.0 μL) and an aqueous solution of Cy5-ON (16 μM, 1.0 μL) and the resultant mixture was incubated at room temperature for 10 min. After spotting the mixture (10 μL) on a glass coverslip as described above (*CLSM observation*), ProteoStat stock solution (1/500 diluted with an assay buffer supplied by manufacture, 2.0 μL) was added and the resultant mixture was subjected to CLSM observations. Separately, to an aqueous mixture (10 μL) before the addition of ProteoStat stock solution was added a solution of proteinase K [11 mg/mL in 50 mM Tris-HCl (pH 8.0), 3.0 mM $CaCl_2$, 50% (v/v) glycerol, 1.0 μL] and the resultant mixture was incubated at 40 ºC for 16 h (overnight). After spotting the mixture (10 μL) on a glass coverslip, the ProteoStat stock solution (2.0 μL) was added and the resultant mixture was also subjected to CLSM observations.

Construction of hybrid materials according to (ii) pre-RCA mixing protocol: An aqueous mixture of the circular DNA template (approx. 0.3 μM), **Z-AF-BPS** (8.2 mM), EquiPhi29 DNA polymerase (1000 U/mL) and dNTPs (1.0 mM) in a reaction buffer solution [5 mM Tris-HCl (pH 7.5), 1.1 mM DTT, 10 mM KCl, 10 μM EDTA, 0.60 mg/mL BSA, 0.05% (v/v) nonidet P40, 0.05% (v/v) Tween 20, 5% (v/v) glycerol] was incubated at rt for 4 h. Then, to the mixture (19 μL) was added an aqueous solution of Cy5-ON (16 μM, 1.0 μL) and the resultant mixture was incubated at room temperature for 10 min. After spotting the mixture (10 μL) on a glass coverslip as described above (*CLSM observation*), ProteoStat stock solution (1/500 diluted, 2.0 μL) was added and the resultant mixture was subjected to CLSM observations. Separately, to an aqueous mixture (10 μL) before the addition of ProteoStat stock solution was added a solution of proteinase K [11 mg/mL in 50 mM Tris-HCl (pH 8.0), 3.0 mM $CaCl_2$, 50% (v/v) glycerol, 1.0 μL] and the resultant mixture was incubated at 40 ºC for 16 h (overnight). After spotting the mixture (10 μL) on a glass coverslip, ProteoStat stock solution (1/500 diluted, 2.0 μL) was added and the resultant mixture was also subjected to CLSM observations.

2. Synthesis

2.1. Synthesis of Z-XY-BPS (XY = AF or FA)

Synthesis of **Z-XY-BPS** (**XY** = **AF** or **FA**) was carried out according to slightly modified method from our previous reports [S3,S4] as shown in **Scheme S1**.



Scheme S1. Synthesis of dipeptides bearing hydrazide group (**Z-XY-NHNH$_2$**) and their hydrazone derivatives (**Z-XY-BPS**). (i) HBTU, HOBt•H_2O, DIEA, dry CH_2Cl_2, (ii) Hydrazine hydrate, MeOH: 44% (2 steps, 58% and 75%) for **Z-AF-NHNH$_2$**, 23% (2 steps, 48% and 48%) for **Z-FA-NHNH$_2$**, (iii) TFA, MeOH: 93% for **Z-AF-BPS**, quant. for **Z-FA-BPS**.

Synthesis of Z-AF-NHNH$_2$: To a mixture of **Z-A-OH** (1.1 g, 5.1 mmol) and **H-F-OMe•HCl** (1.1 g, 5.0 mmol) in dry dichloromethane (40 mL) were added HOBt•H_2O (0.92 g, 6.0 mmol), HBTU (2.3 g, 5.9 mmol), and DIEA (1.7 mL) and the resultant mixture was stirred at rt for 23 hours. After the concentration of the reaction mixture, the residue was dissolved in ethyl acetate (40 mL) and the solution was washed with 5% aqueous citrate (40 mL). The organic layer was collected and dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness to afford **Z-AF-OMe** (1.1 g, 58%) as a white solid. To a solution of the obtained **Z-AF-OMe** (0.39 g, 1.0 mmol) in MeOH (12 mL) was added hydrazine hydrate (0.49 mL). The reaction mixture was refluxed under Ar atmosphere for 2.5 h. After the mixture was cooled, H_2O (100 mL) was added and the resultant precipitate was collected by filtration, washed with H_2O, and dried to yield **Z-AF-NHNH$_2$** (0.29 g, 75%) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm)

1.06 (d, J = 7.3 Hz, 3H), 2.72–2.92 (m, 2H), 3.97 (m, 1H), 4.18 (br s, 2H), 4.35–4.46 (m, 1H), 4.92–5.02 (m, 2H), 7.05–7.25 (m, 5H), 7.25–7.40 (m, 6H), 7.88 (d, J = 8.2 Hz, 1H), 9.10 (br s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 18.7, 38.6, 50.7, 53.0, 66.0, 126.8, 128.3, 128.4, 128.6, 128.9, 129.8, 137.5, 138.1, 156.1, 170.6, 172.5; HRMS (ESI, positive): Calcd. for $[M(C_{20}H_{24}N_4O_4) + Na]^+$: m/z = 407.1695; Found: 407.1725.

Synthesis of Z-FA-NHNH$_2$: The titled compound was prepared from **Z-F-OH** and **H-A-OMe•HCl** in the similar way for **Z-FA-NHNH$_2$** and was obtained in 23% yield (over 2 steps) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 1.09–1.21 (m, 3H), 2.60–3.02 (m, 2H), 4.18–4.27 (m, 4H), 4.89 (s, 2H), 7.10–7.32 (m, 10H), 7.45 (d, J = 8.7 Hz, 1H), 8.08 (d, J = 7.8 Hz, 1H), 9.03 (br s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 19.2, 37.9, 47.4, 56.6, 65.7, 126.8, 127.9, 128.2, 128.6, 128.8, 129.7, 137.6, 138.7, 156.4, 171.6, 172.0; HRMS (ESI, positive): Calcd. for $[M(C_{20}H_{24}N_4O_4) + Na]^+$: m/z = 407.1695; Found: 407.1740.

Synthesis of Z-AF-BPS: To a solution of **Z-AF-NHNH$_2$** (0.26 g, 0.66 mmol) in methanol (50 mL) and TFA (0.92 mL) was added **BPS** (0.17 g, 0.60 mmol). The resultant mixture was stirred at rt under Ar atmosphere for 3 days. After the concentration of the reaction mixture, diethyl ether (50 mL) was added and the precipitate was collected by filtration, washed with diethyl ether, and dried to yield **Z-AF-BPS** (0.35 g, 93%) as a pale yellow solid: ^{1}H NMR (400 MHz, DMSO-d_6, assigned as the mixture of isomers [S3]) δ (ppm) 1.07–1.13 (m, 3H), 1.93–2.00 (m, 2H), 2.49–2.53 (m, 2H (overlapped with DMSO)), 2.81–3.02 (m, 2H), 3.96–4.11 (m, 3H), 4.42–4.53 (m, 0.5H), 4.92–5.05 (m, 2H), 5.29–5.34 (m, 0.5H), 6.93–6.97 (m, 2H), 7.12–7.41 (m, 11H), 7.54–7.57 (m, 2H), 7.87 (s, 0.5H), 7.97 (d, J = 8.2 Hz, 0.5H), 8.05 (s, 0.5H), 8.12 (d, J = 8.2 Hz, 0.5H), 11.26 (s, 0.5H), 11.31 (s, 0.5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 18.7, 18.8, 25.8, 48.4, 65.9, 66.0, 67.4, 115.3, 115.4, 126.9, 126.9, 127.0, 128.2, 128.3, 128.6, 128.9, 129.2, 129.8, 138.2, 160.7, 160.8, 172.5; HRMS (ESI, negative): Calcd. for $[M(C_{30}H_{33}N_4O_8S)]^-$: m/z = 609.2026; Found: 609.2031.

Synthesis of Z-FA-BPS: The titled compound was prepared from **Z-FA-NHNH$_2$** (0.17 g, 0.44 mmol) and **BPS** (0.11 g, 0.40 mmol) in the similar way for **Z-FA-BPS** and was obtained quantitatively (0.25 g) as a pale yellow solid: ^{1}H NMR (400 MHz, DMSO-d_6, assigned as the mixture of isomers [S3]) δ (ppm) 1.19–1.30 (m, 3H), 1.92–2.01 (m, 2H), 2.49–2.53 (m, 2H (overlapped with DMSO)), 2.63–2.71 (m, 1H), 2.98–3.01 (m, 1H), 4.07 (t, J = 6.2 Hz, 2H), 4.24–4.32 (m, 1H), 4.82–4.92 (m, 2H), 5.08 (t, J = 7.1 Hz, 0.5H), 6.95 (d, J = 8.2 Hz, 2H), 7.14–7.30 (m, 10H), 7.47 (dd, J = 8.0, 4.4 Hz, 1H), 7.57 (d, J = 6.9 Hz, 2H), 7.91

(s, 0.5H), 8.09 (s, 0.5H), 8.21 (d, J = 7.3 Hz, 0.5H), 8.12 (d, J = 7.3 Hz, 0.5H), 11.21 (s, 0.5H), 11.32 (s, 0.5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 17.8, 18.7, 25.8, 48.4, 65.7, 65.7, 67.4, 115.3, 115.4, 127.1, 127.9, 128.2, 128.6, 128.8, 128.9, 129.2, 129.8, 137.6, 156.4, 156.4, 160.8, 169.0, 173.8; HRMS (ESI, negative): Calcd. for $[M(C_{30}H_{33}N_4O_8S)]^-$: m/z = 609.2026; Found: 609.2026.

3. Characterization of compounds

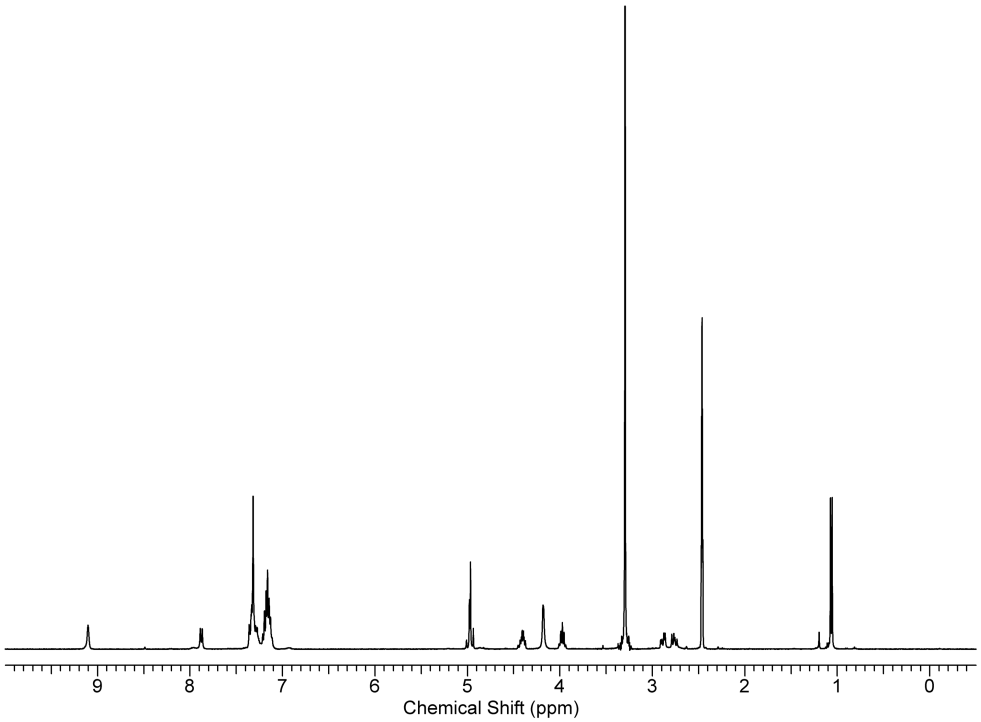


Fig. S1. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **Z-AF-NHNH$_2$**.

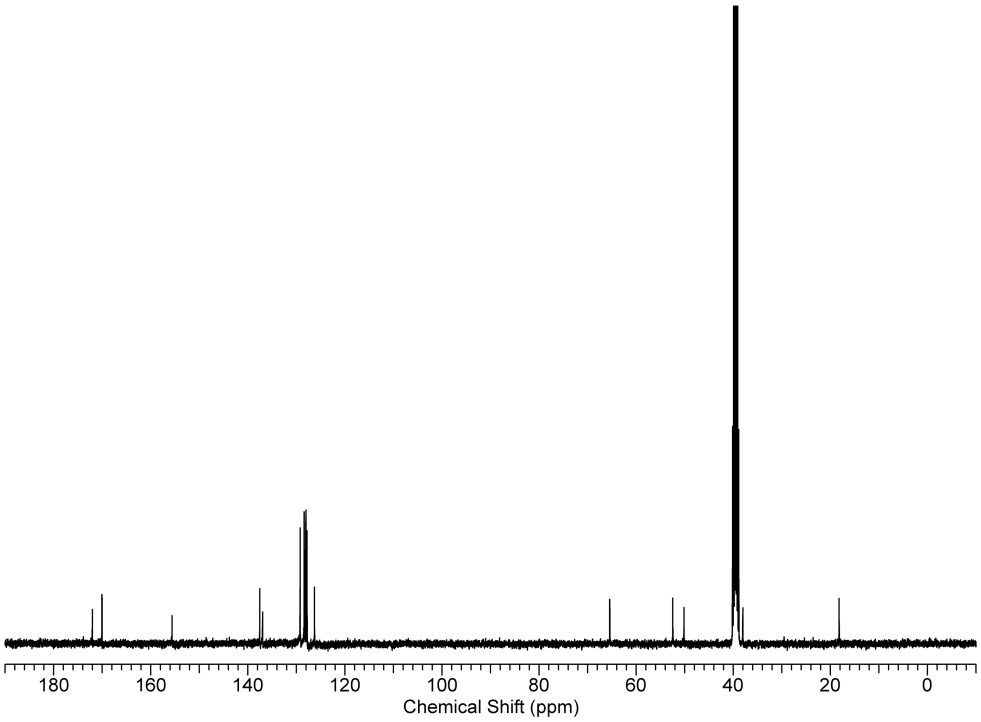


Fig. S2. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of **Z-AF-NHNH$_2$**.

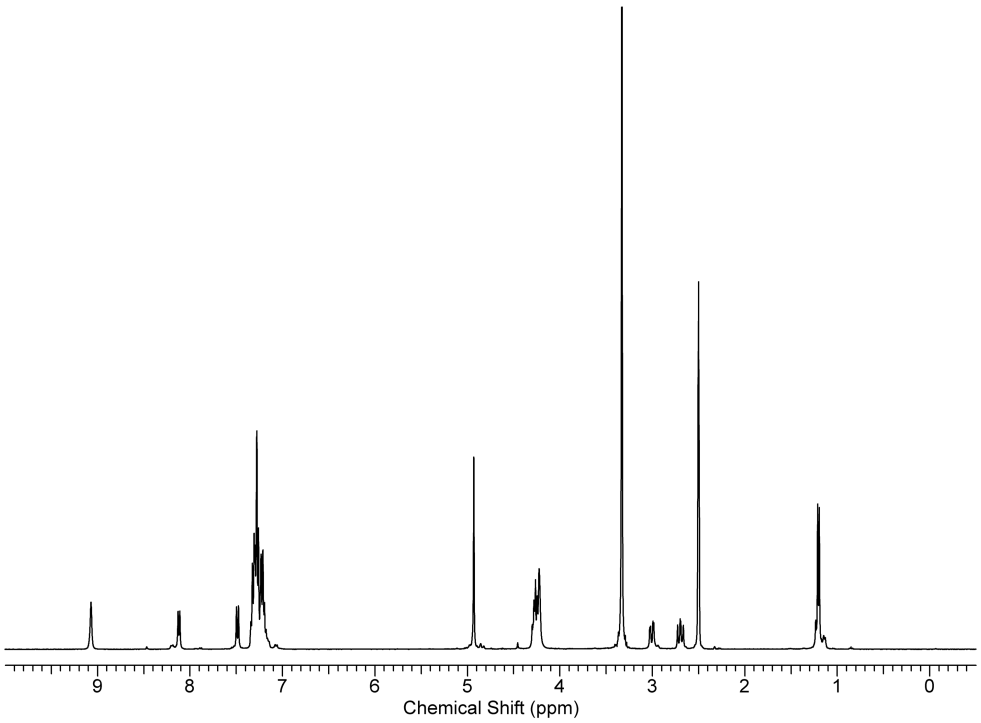


Fig. S3. ^{1}H NMR spectrum (400 MHz, DMSO-*d*$_6$) of **Z-FA-NHNH$_2$**.

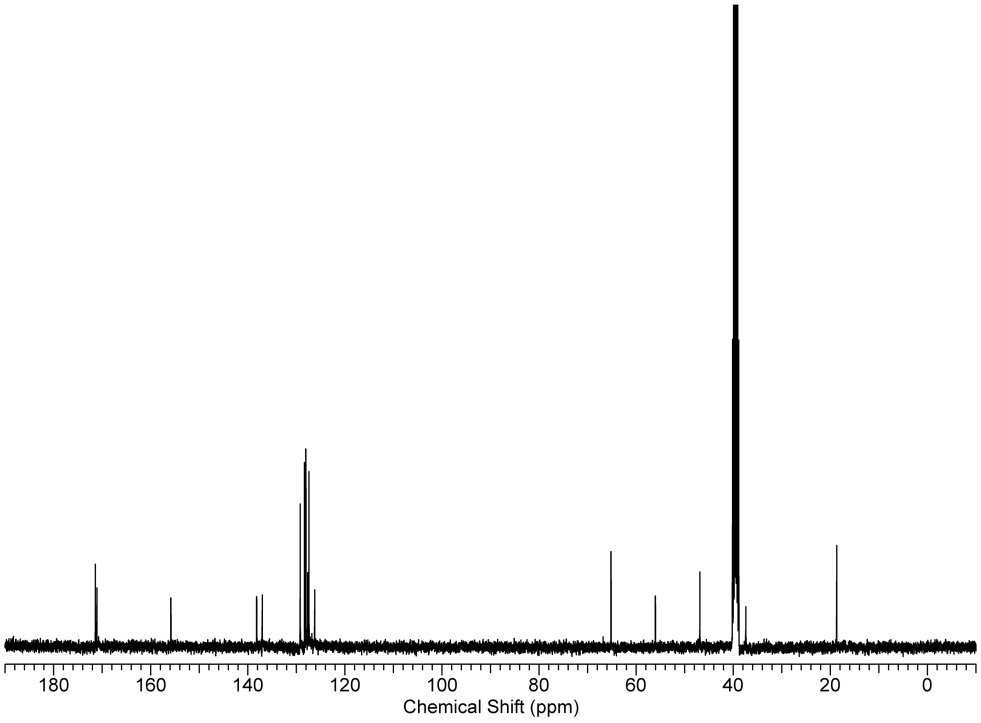


Fig. S4. ^{13}C NMR spectrum (100 MHz, DMSO-*d*$_6$) of **Z-FA-NHNH$_2$**.

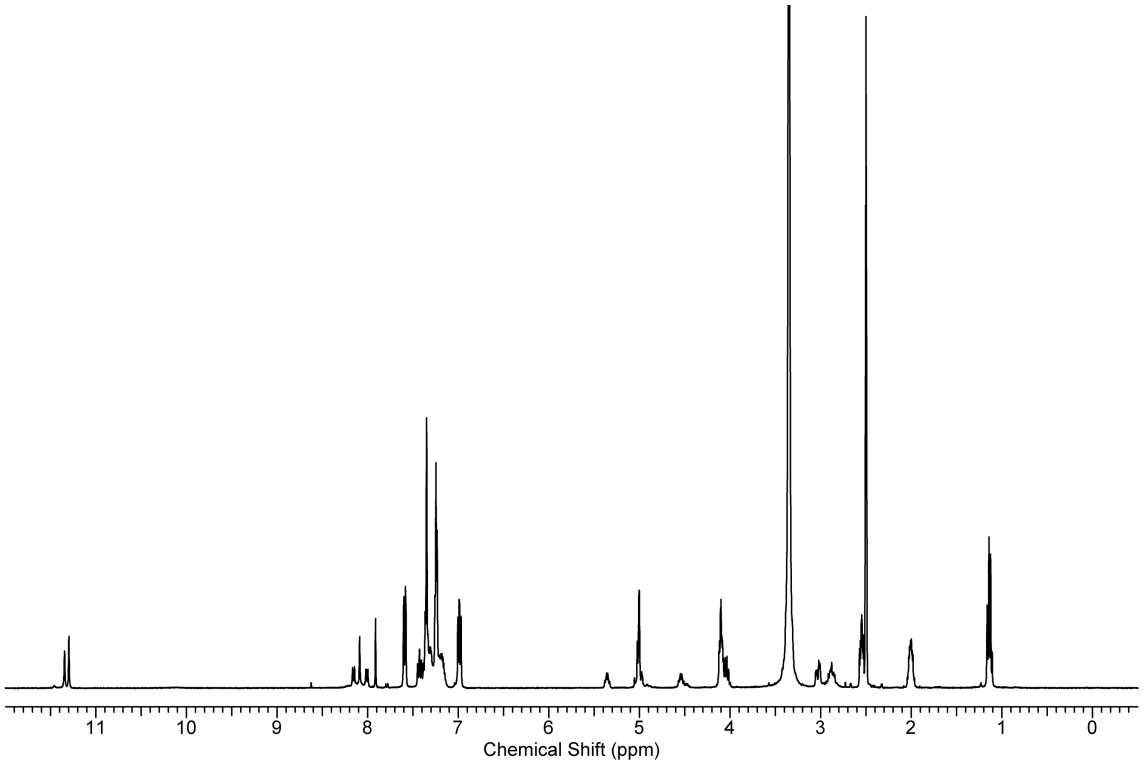


Fig. S5. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **Z-AF-BPS**.

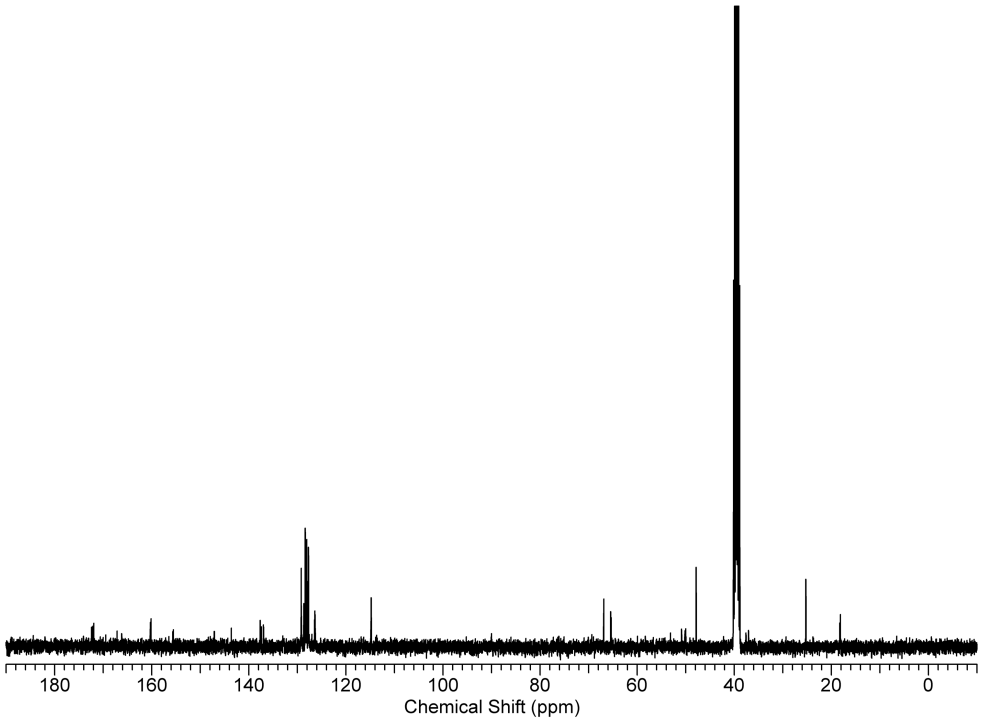


Fig. S6. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of **Z-AF-BPS**.

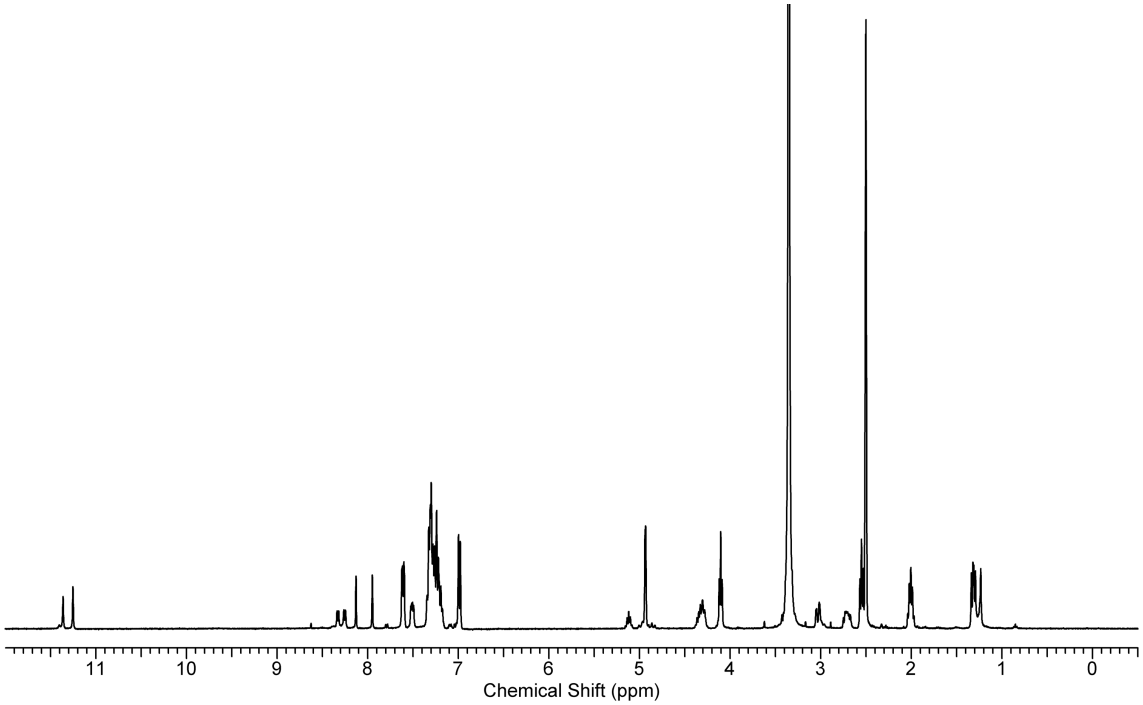


Fig. S7. ^{1}H NMR spectrum (400 MHz, DMSO-*d*$_6$) of **Z-FA-BPS**.

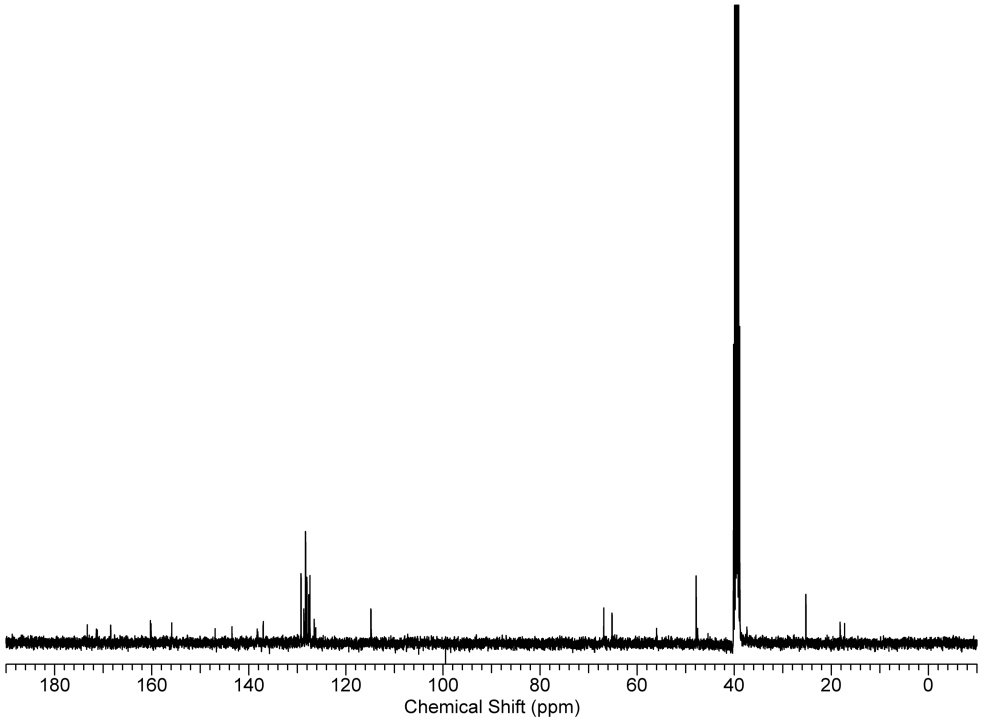


Fig. S8. ^{13}C NMR spectrum (100 MHz, DMSO-*d*$_6$) of **Z-FA-BPS**.

4. Characterization of Z-AF-BPS hydrogels

■Gelation ability of Z-AF-BPS and Z-FA-BPS

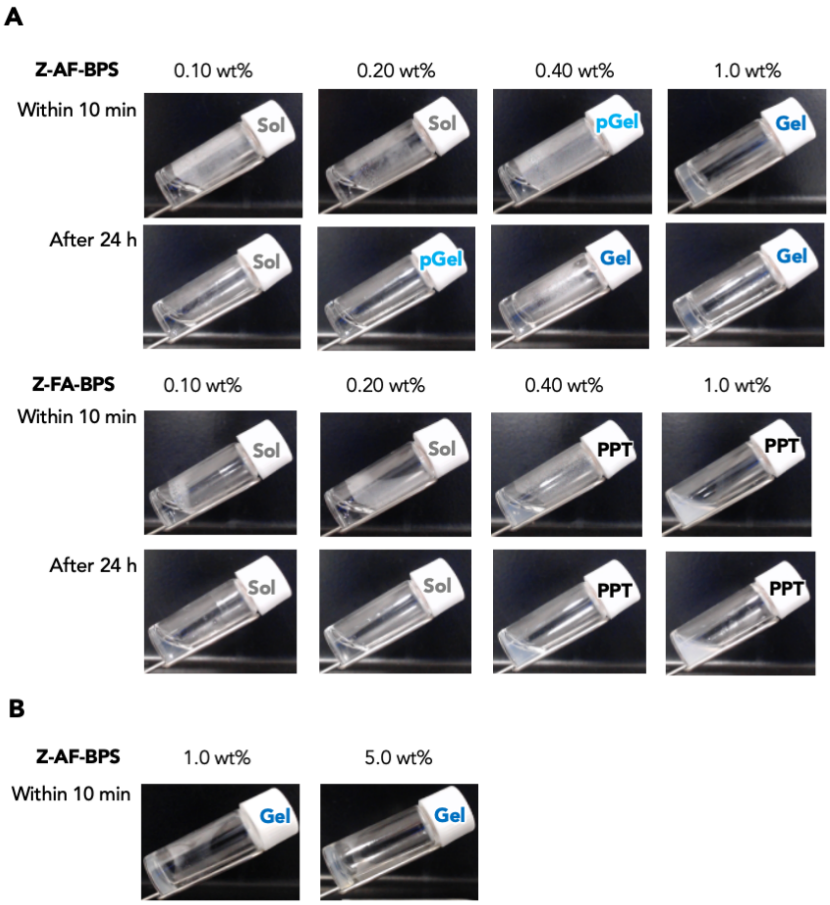


Fig. S9. Photographs showing gelation ability of **Z-AF-BPS** and **Z-FA-BPS** dependent on the concentrations [**A**: 50 mM MES-NaOH (pH 7.0) containing DMSO (2.0 vol%), **B**: H_2O].

■Rheological property of Z-AF-BPS hydrogel

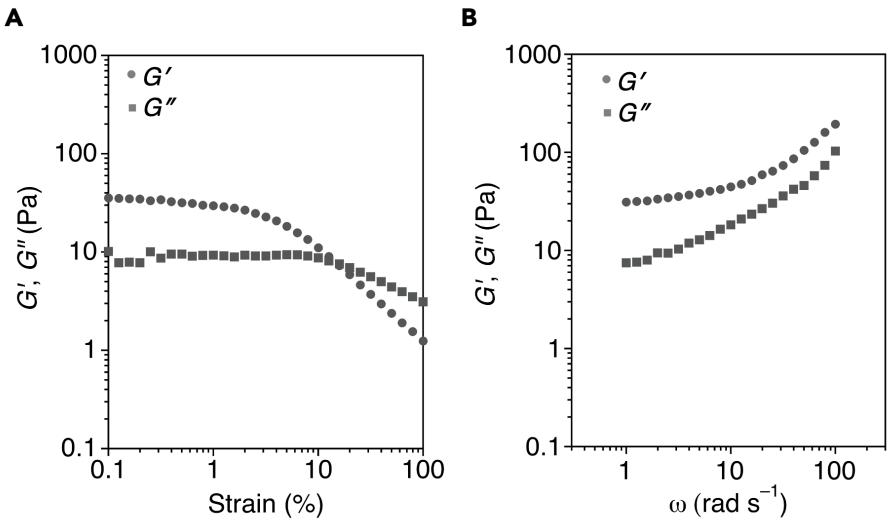


Fig. S10. (**A**) Strain (1.0 rad/s) and (**B**) frequency (0.2% strain) sweep rheological properties of **Z-AF-BPS** hydrogel ([**Z-AF-BPS**] = 1.0 wt%: *G′*, storage modulus; *G″*, loss modulus). *Conditions*: 50 mM MES-NaOH (pH 7.0) containing DMSO (2.0 vol%).

■TEM and SEM observations of Z-AF-BPS hydrogel

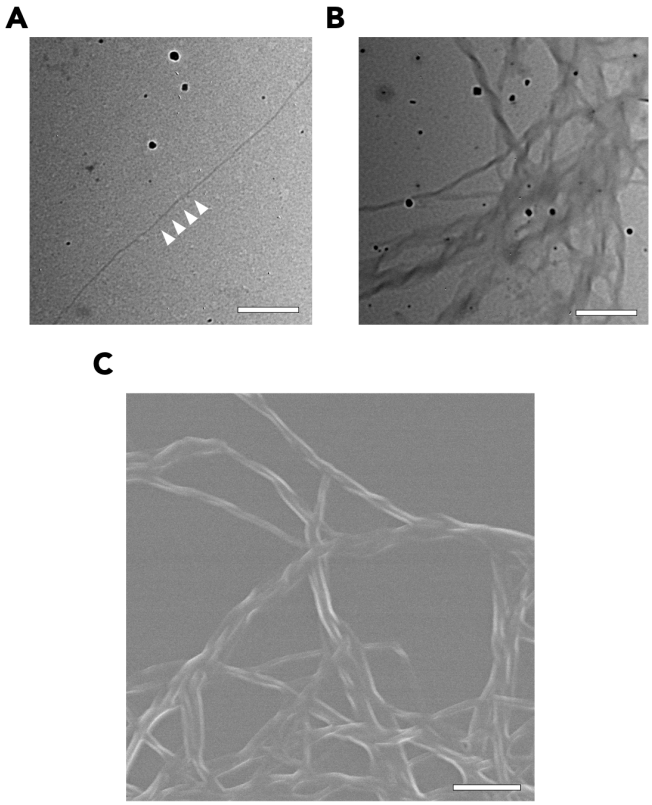


Fig. S11. Representative TEM (**A**, **B**) and SEM (**C**) images of **Z-AF-BPS** hydrogels prepared by (**A**) thermal treatment and (**B**,**C**) only solvent mixing without thermal treatment. Fibrous architecture is highlighted using white arrows in panel (**A**). Scale bar: 500 nm

■*CAC* evaluation for Z-AF-BPS by ThT assay

Based on the thioflavin T (ThT) assay, critical aggregation concentration (*CAC*) of **Z-AF-BPS** was estimated to be 0.73 ± 0.03 mM (n = 3; **Fig. S12B**). Moreover, the enhanced fluorescence of ThT at 495 nm (**Fig. S12A**) suggests the presence of β-sheet structure of **Z-AF-BPS** through it self-assembly [S5].

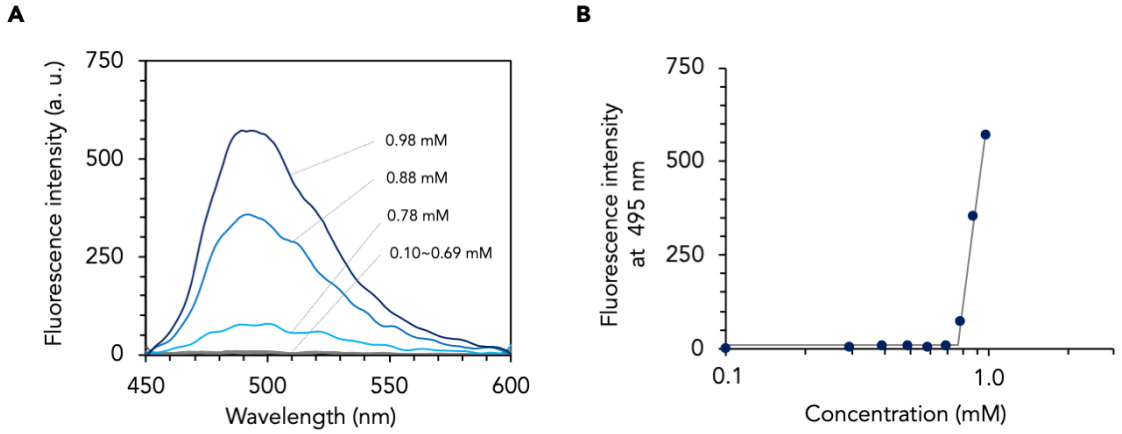


Fig. S12. (**A**) Fluorescence spectral change of ThT (λ_{ex} = 425 nm) upon the addition of **Z-AF-BPS** (0.10~0.98 mM). (**B**) Representative plot of fluorescence intensity at 495 nm against the **Z-AF-BPS** concentrations to evaluate critical aggregation concentration (*CAC*). The gray line is a guide for the eyes. *Conditions*: [ThT] = 10 μM, 50 mM MES-NaOH (pH 7.0) containing DMSO (up to 2.0 vol%).

■CD and IR spectrum of Z-AF-BPS hydrogel

Fig. S13 shows a circular dichroism (CD) spectrum of the **Z-AF-BPS** hydrogel (0.40 wt%, 6.4 mM) exhibited a negative CD signal at 310 nm, assignable to a chiral arrangement of BPS moiety in the self-assembled fibrous structures that can be originated from chiral information transfer from peptide moiety to BPS moiety enhanced by self-assembly.[S3] Furthermore, a positive CD signal at 240 nm and a negative CD signal shorter than 235 nm were observed. In this wavelength region (200–250 nm), the presence of phenylalanine (F) as well as the Z and BPS moieties in **Z-AF-BPS** could lead to a mixed spectra ascribable to the electronic transitions of its peptide backbone and aromatic moieties.[S3,S4] The CD signals became almost silent at a lower concentration (0.040 wt%, 0.64 mM), indicating the absence of self-assembled structures at the lower concentration, which is consistent with CAC of **Z-AF-BPS** (0.73 ± 0.03 mM). Fourier-transform infrared (FTIR) spectra of **Z-AF-BPS** hydrogel exhibited two major bands centered at 1639 and 1686 cm^{-1} with shoulder peaks as displayed in **Fig. S13B**, suggesting that hydrogen bonding of an amide backbone and (Z-related) carbonate moieties at the N-terminal via β-sheet formation, consistent with the proposed self-assembled structure depicted in **Fig. 3B**.

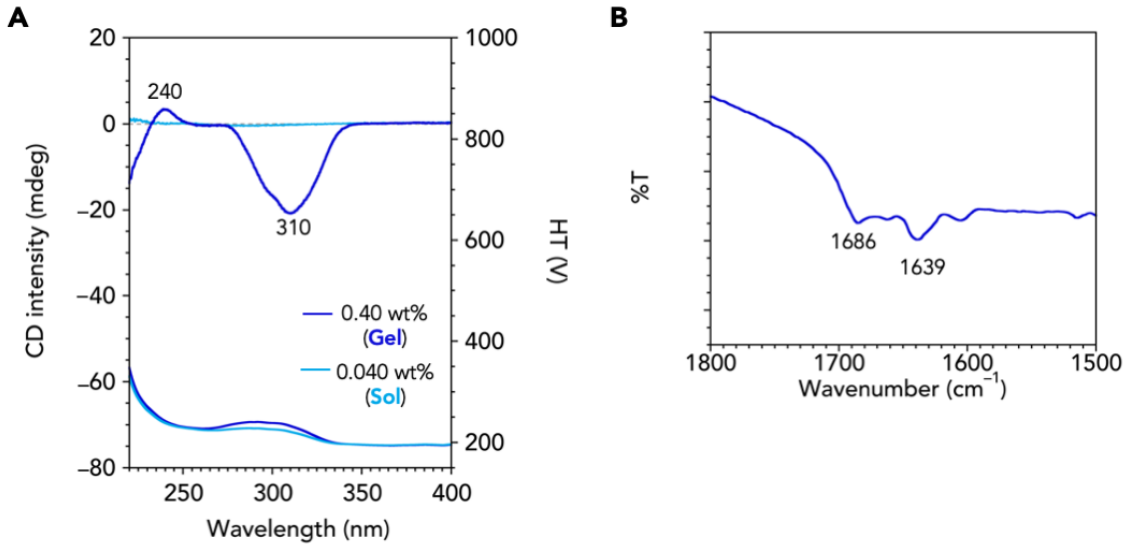
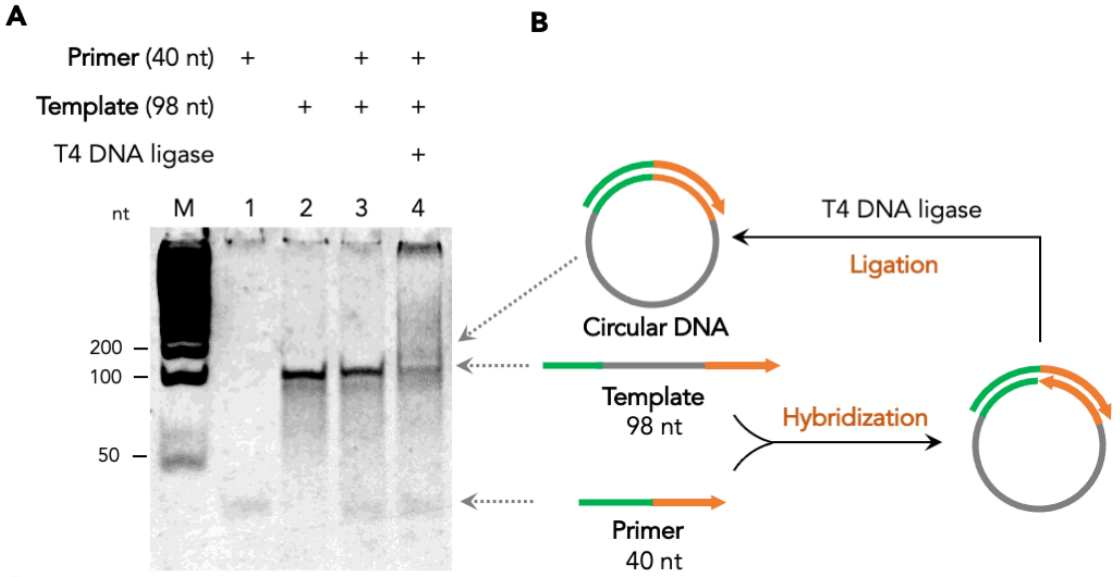


Fig. S13 (**A**) CD spectra of the **Z-AF-BPS** hydrogel (0.40 wt%, 0.1-mm cell) and sol (0.040 wt%, 1.0-mm cell) and the corresponding HT voltage data (bottom). *Conditions*: 50 mM MES-NaOH (pH 7.0) containing DMSO (2.0 vol%). (**B**) IR spectrum of the **Z-AF-BPS** hydrogel [5.0 wt%, prepared with D_2O (**Fig. S9B**) to detect the bands in the amide I region].

5. PAGE analysis for the construction of circular DNA



C

DNA	Sequence (5′→3′)
Template (98 nt)	5′-PTTCCCGGCGGCGCAGCAGTTAGA*TGCTGCTGCAGCGATACGCGTATCG CTATGGCATATCGTACGATATGCCGCAGCAGCA*TCTAACCGTACAGTATT-3′
Primer (40 nt)	5′-TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA-3′

Fig. S14. (**A**) PAGE (10%, denatured, SYBR Green II) analysis to evaluate the construction of circular DNA from template (98 nt) and primer (40 nt). (**B**) Scheme showing the construction of circular DNA from template and primer through hybridization and subsequent ligation catalyzed by T4 DNA ligase. (**C**) Sequences of ssDNAs (template and primer) used in this study [S2].

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