Supporting Information

Self-Healing Self-Assembled β-sheet Peptide Poly(γ-glutamic acid) Hybrid Hydrogels

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Supplementary Figures

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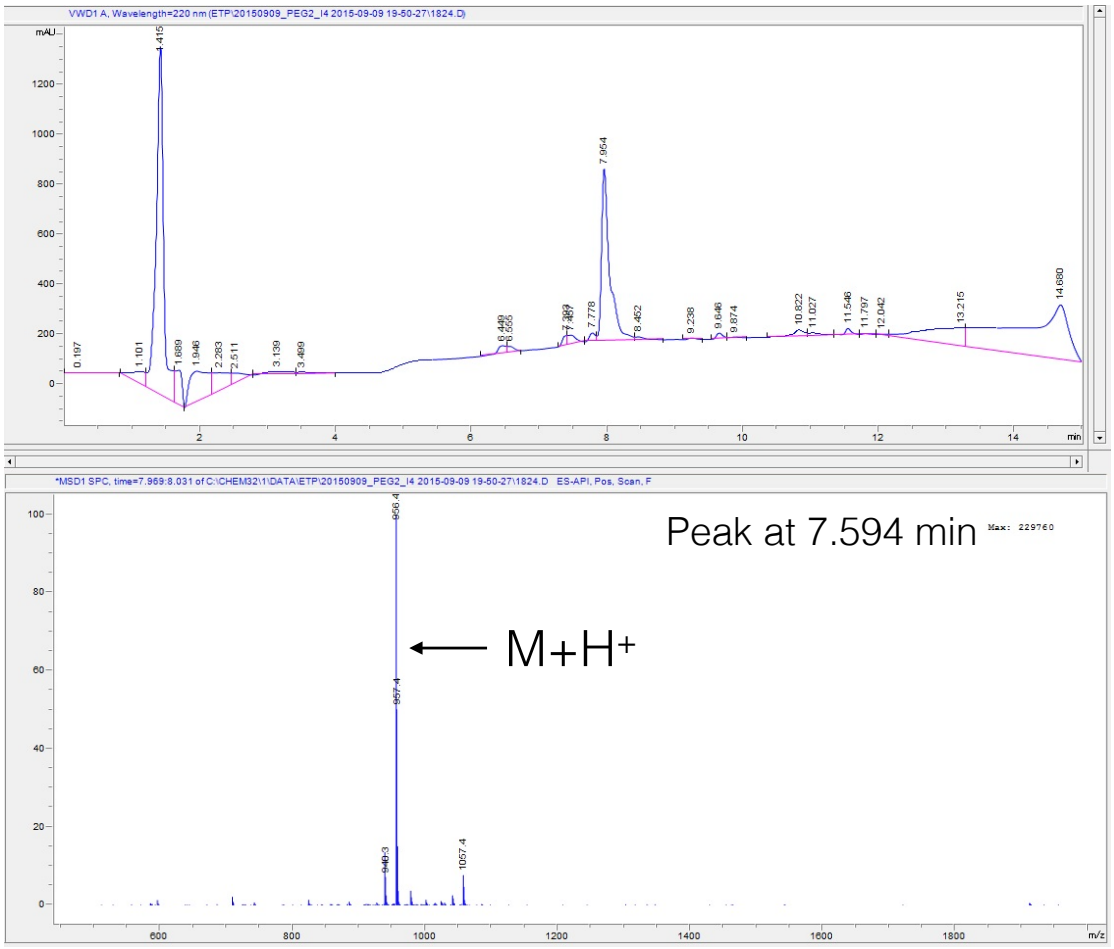


Figure S1. Analytical high performance liquid chromatography (HPLC) trace of the I4-azide peptide, N_3-$D_2I_4D_2$ peptide (I4-azide peptide) (top) and corresponding electrospray ionization (ESI) spectra (bottom).

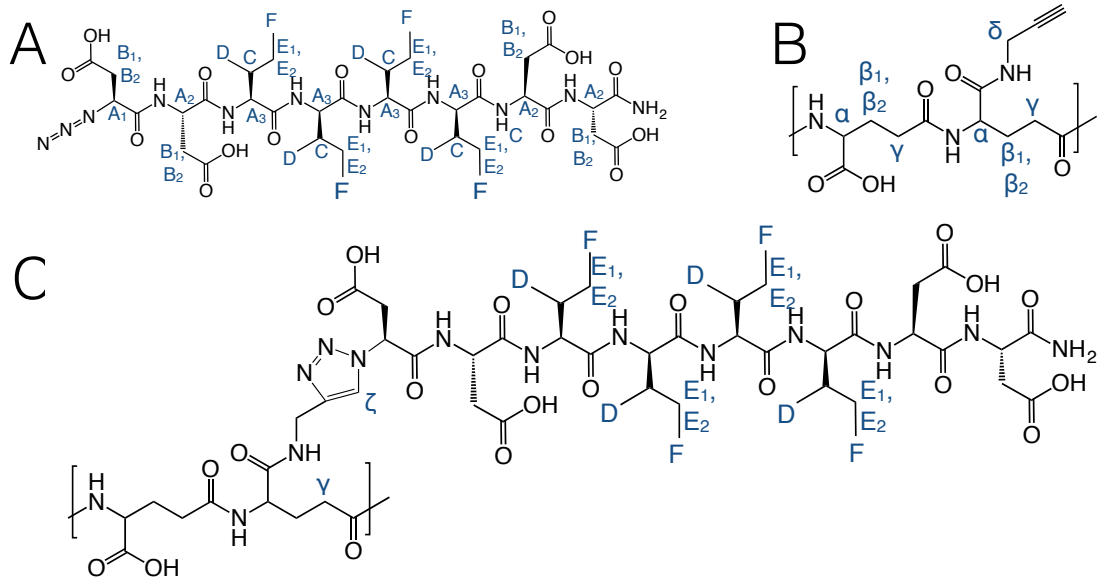
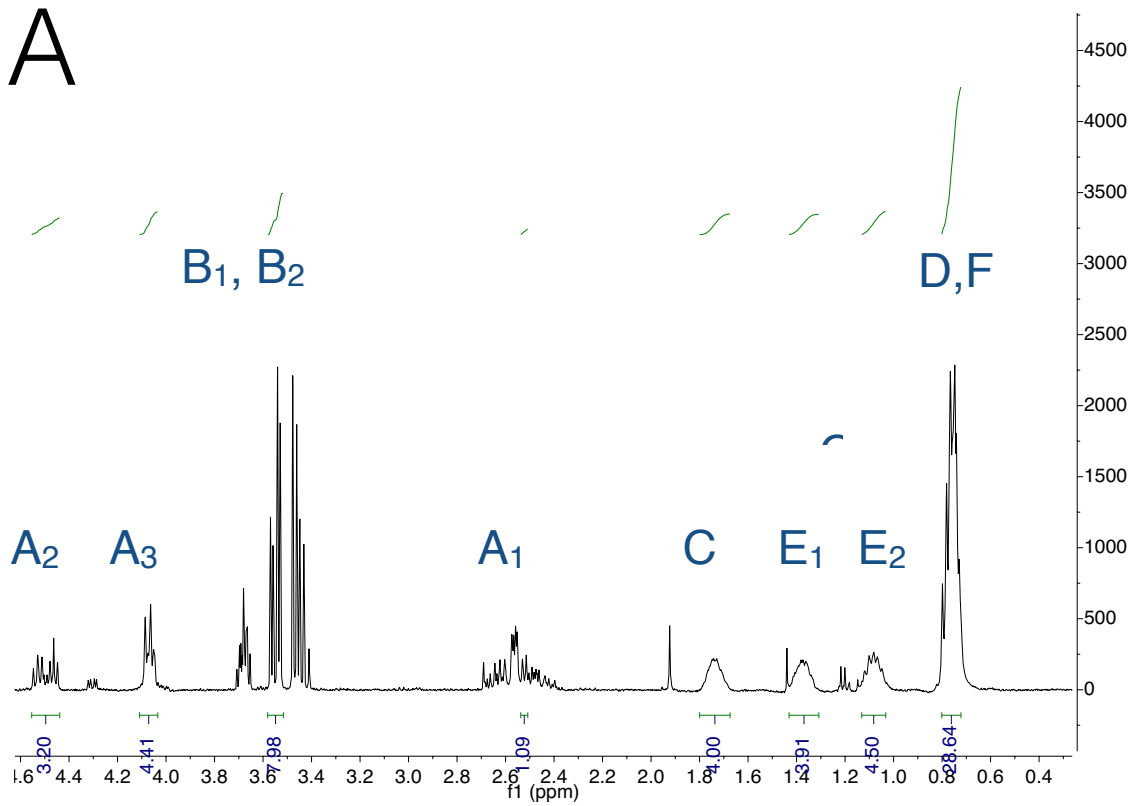
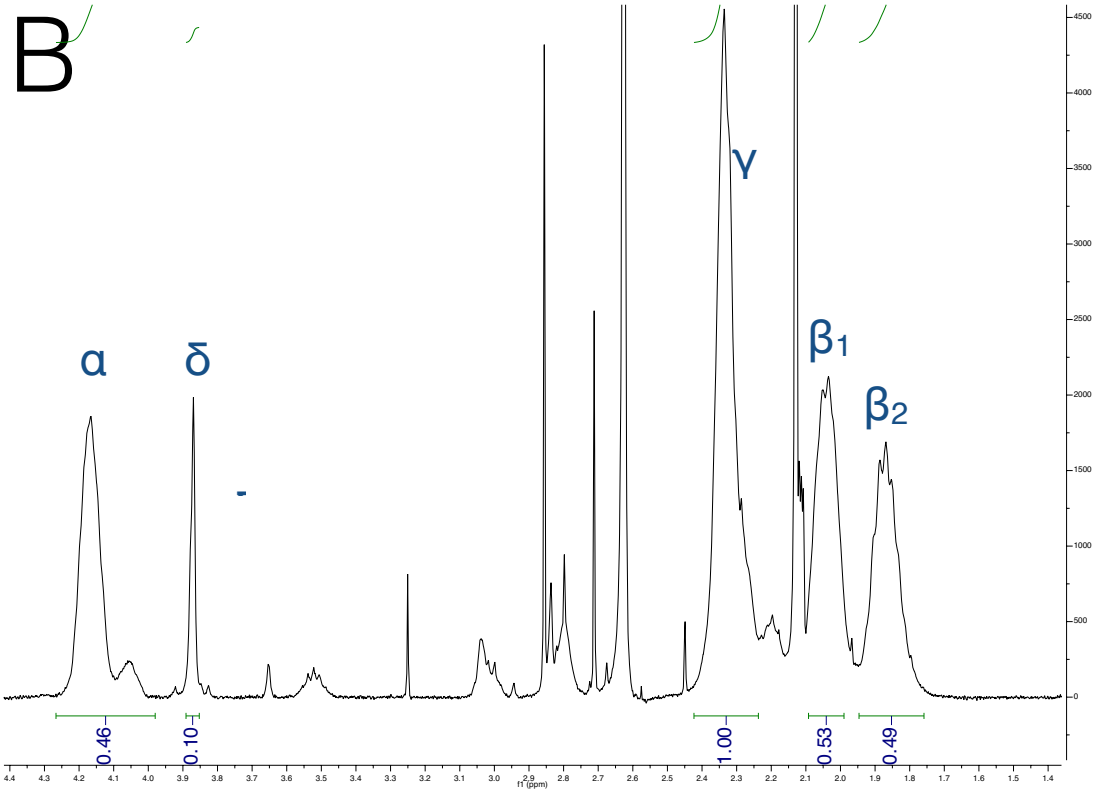
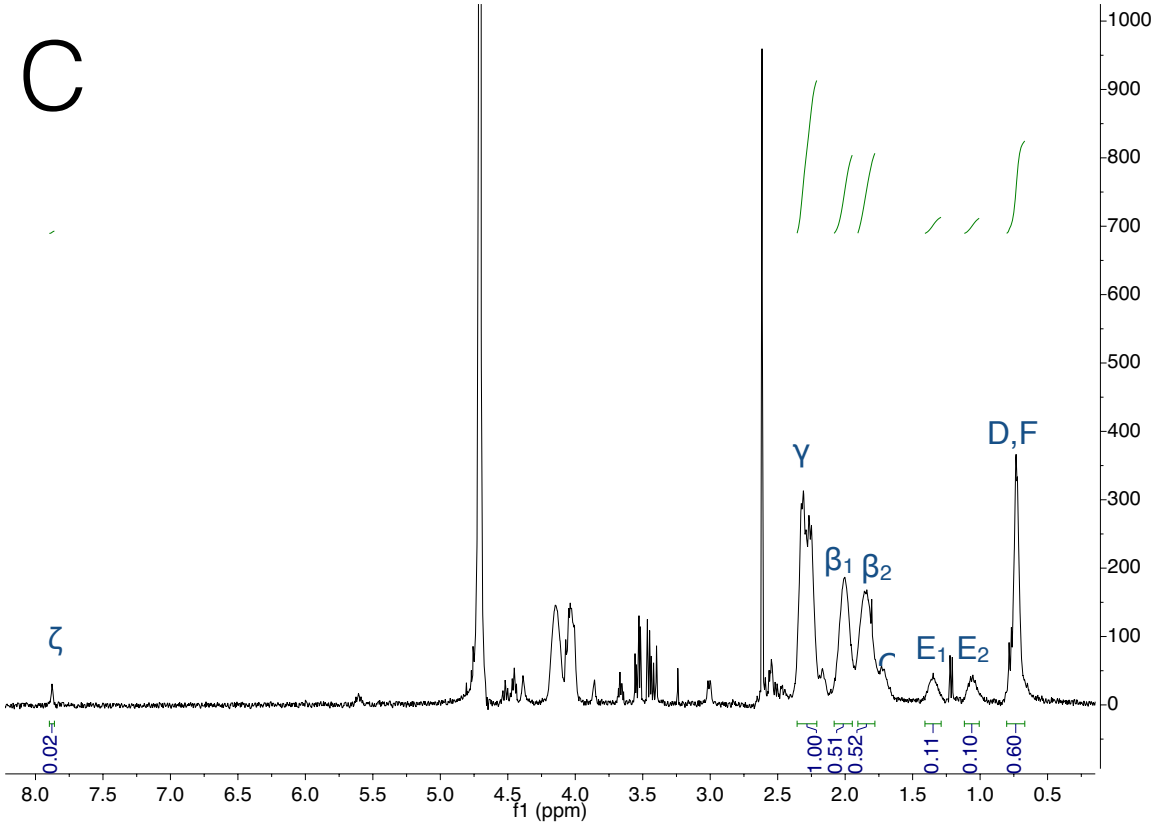
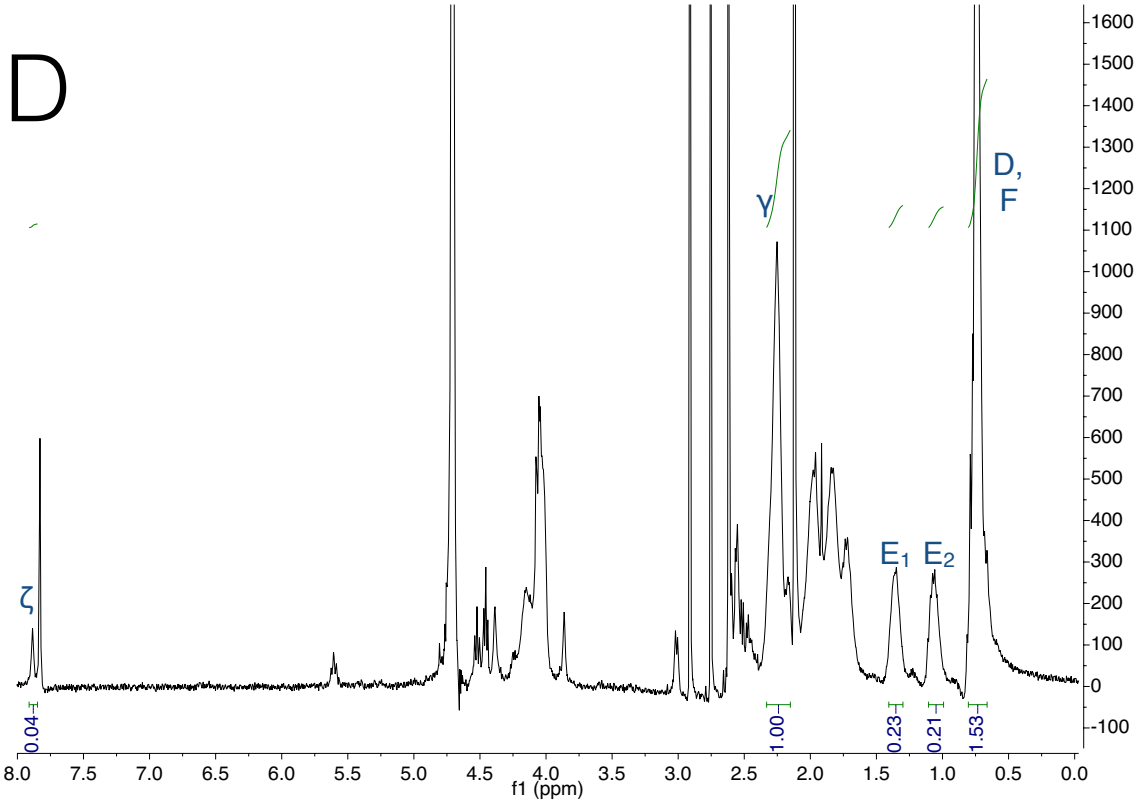


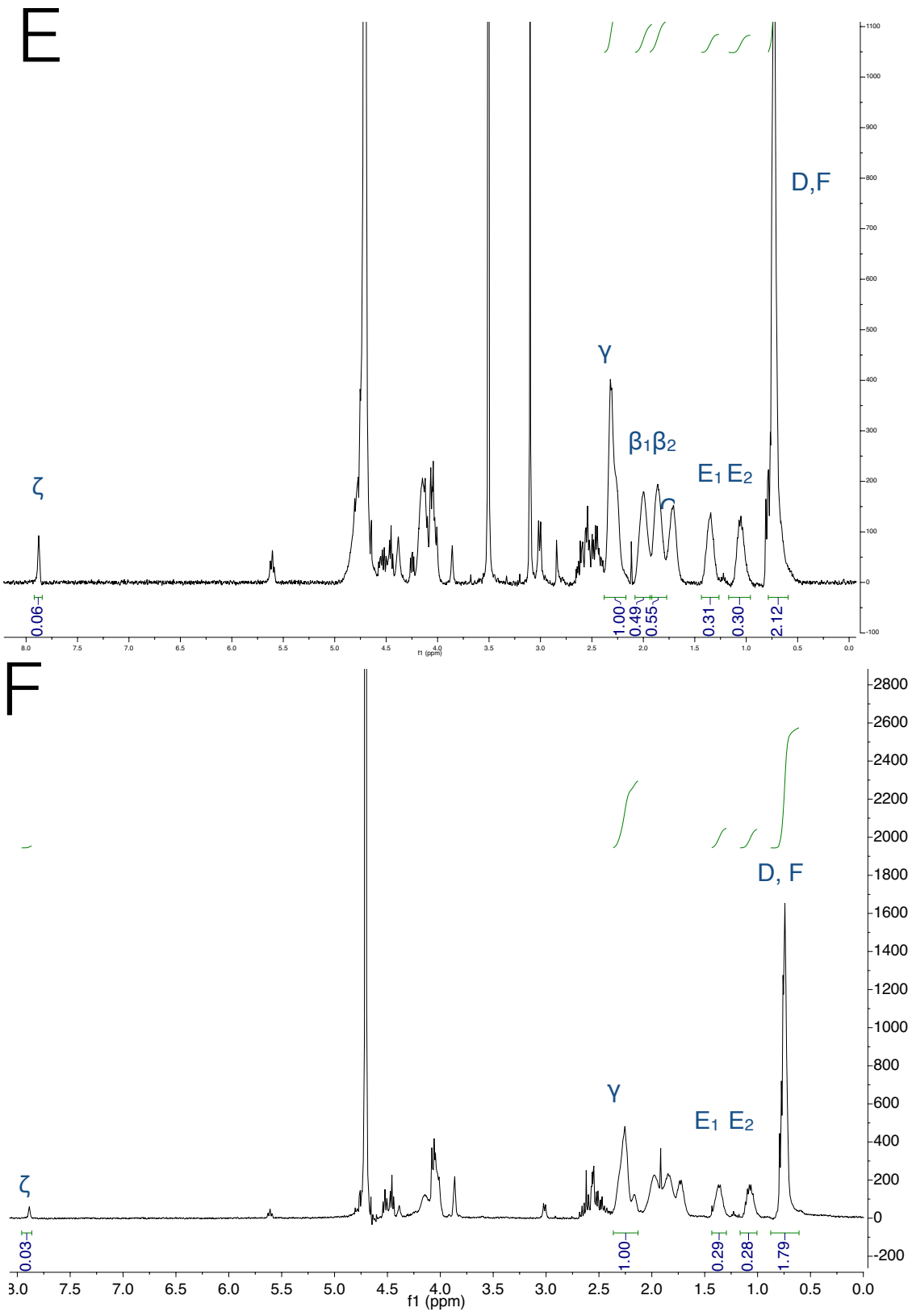
Figure S2. ^{1}H-NMR nomenclature assignment of A) I4-azide peptide, B) γ-PGA-alkyne and C) γ-PGA-β-sheet conjugate.


A
B1, B2
D,F
A2
A3
A1
C
E1
E2
3.20
4.41
7.98
1.09
4.00
3.91
4.50
28.64
f1 (ppm)


B
α
δ
γ
β1
β2
0.46
0.10
1.00
0.53
0.49


C
ζ
γ
β1 β2
ς
E1 E2
D,F
0.02
1.00
0.51
0.52
0.11
0.10
0.60
f1 (ppm)


D
ζ
γ
E1 E2
D,
F
0.04
1.00
0.23
0.21
1.53
f1 (ppm)


E
D,F
γ
β1β2
E1 E2
ζ
0.06
1.00
0.49
0.55
0.31
0.30
2.12
F
D, F
γ
E1 E2
ζ
0.03
1.00
0.29
0.28
1.79
f1 (ppm)

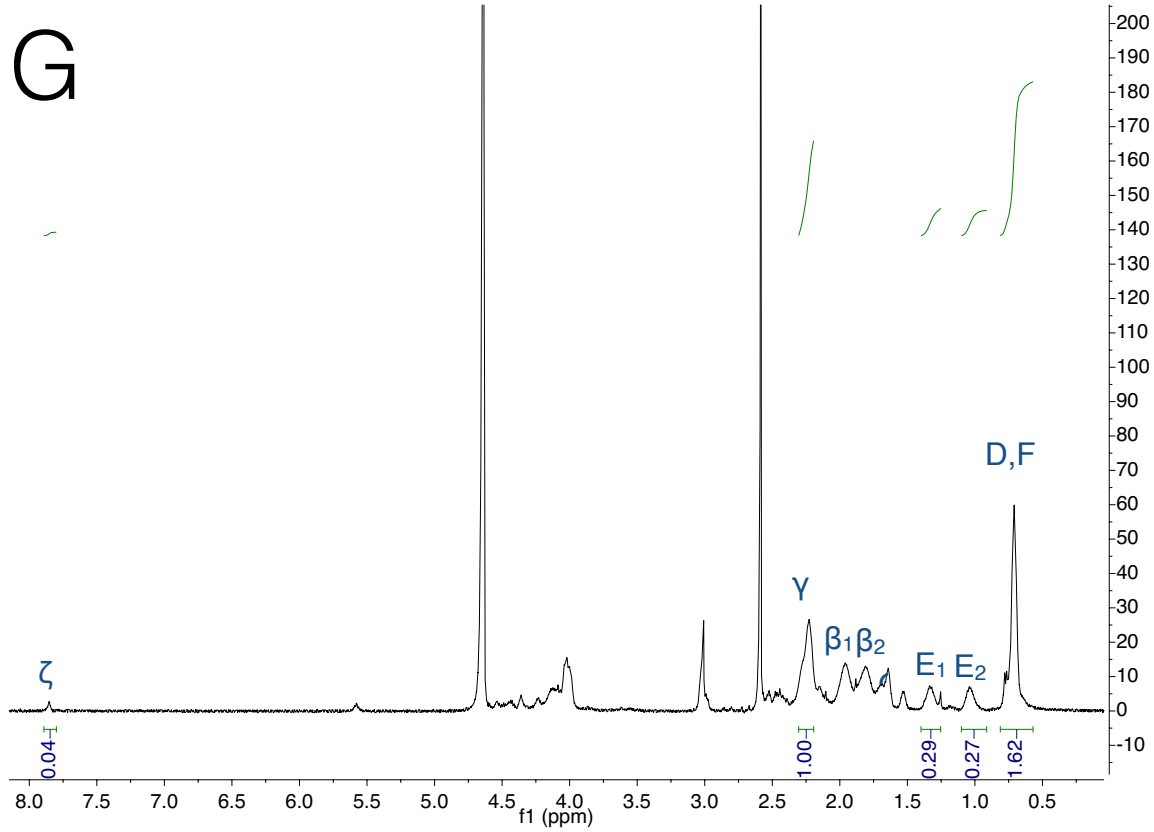
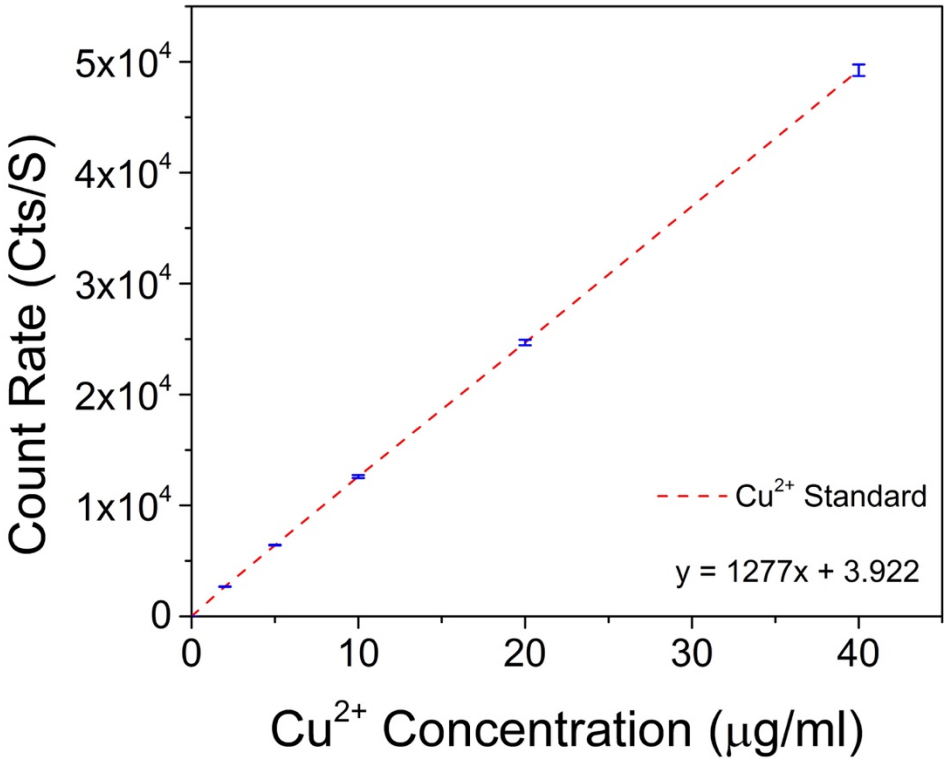


Figure S3. ^{1}H-NMR spectra and corresponding peak assignment of A) I4-azide peptide, B) γ-PGA-alkyne, C) γ-PGA 5%βC, D) γ-PGA 10%βC, E) γ-PGA 15%βC, F) γ-PGA 5%βC+10%βF and G) γ-PGA 10%βC+5%βF performed in D_2O.

A



B

Copper Standard (µg/ml)	Count Rate (Cts/S)
0	1.958 ± 2.688
2	2683 ± 21
5	6416 ± 38
10	12600 ± 133
20	24690 ± 242
40	49240 ± 518
Ultrapure H_2O	2.4400 ± 1.4100
γ-PGA-10%βC - 1	1.7702 ± 0.5833
γ-PGA-10%βC - 2	2.4382 ± 0.5678

Figure S4. Inductively coupled plasma optical emission spectroscopy (ICP-OES) on the hybrid hydrogels. (A) Standard solutions of known copper concentrations gave a linear count rate curve with respect to copper concentration. (B) The count rate for copper standard solutions indicates that the γ-PGA-10%βC has a copper concentration that is not statistically different from ultrapure water.

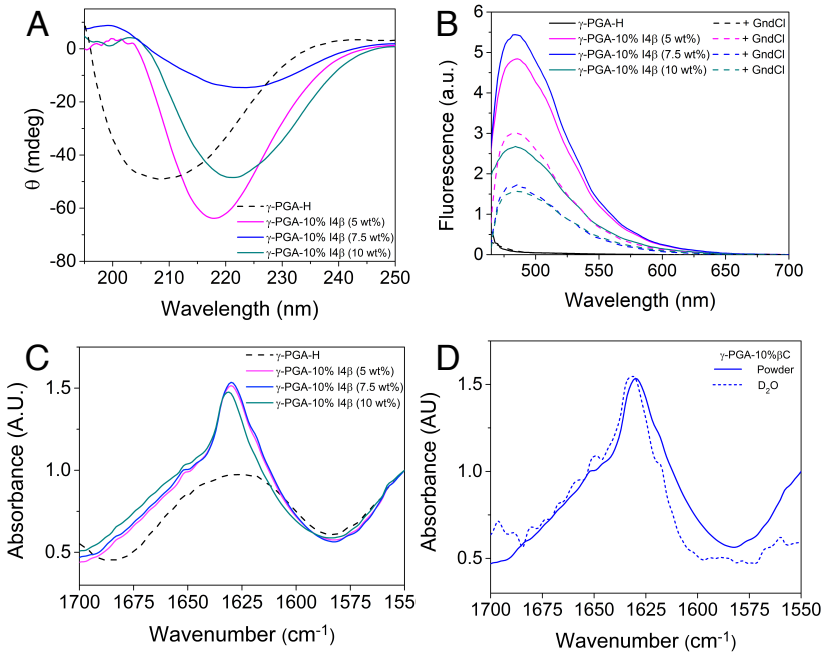


Figure S5. Spectroscopic studies of hydrogels at different concentrations. A) Circular dichroism showing minima for hybrid hydrogels between 220 nm and 230 nm, typically indicative of β-sheet formation, B) thioflavin T (ThT) assay indicating the presence of β-sheets in the hydrogels that is greatly reduced in the presence of guanidinium chloride (GndCl), C) FTIR spectra of the amide I region, with a peak at 1630 cm^{-1} indicating β-sheet formation and D) FTIR of γ-PGA 10%βC as a powder and in D_2O.

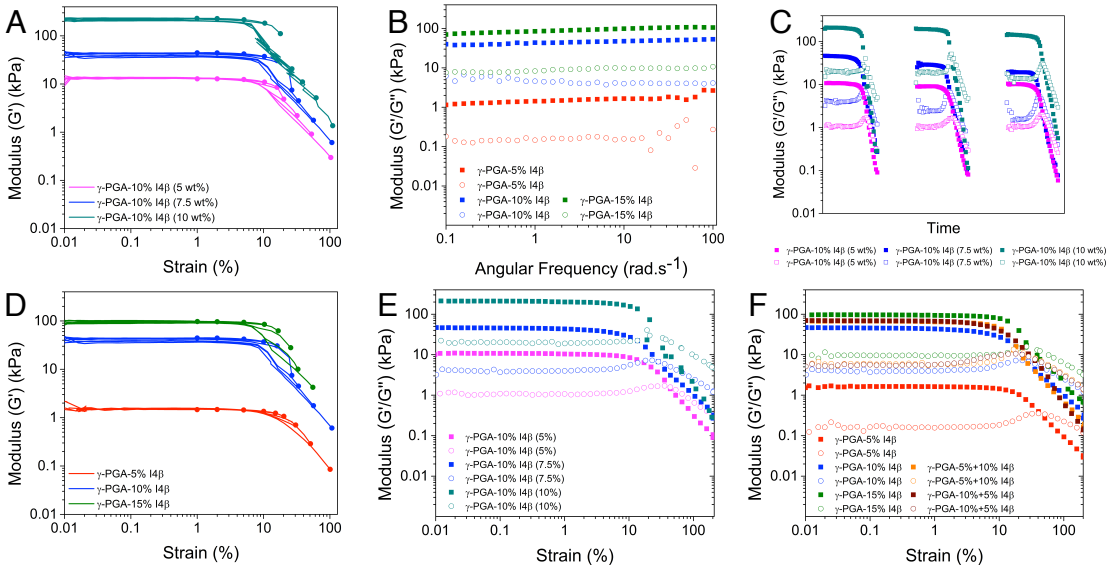


Figure S6. A) Strain sweep at an oscillation frequency of 6.283 rad s^{-1} for the hybrid hydrogels at different concentrations. B) Frequency sweep at 1% strain hydrogels with varying peptide grafting densities. C) Recovery from 200% failure strains of hydrogels at different concentrations with 30-minute recovery periods in between the sweeps. D) Increasing strain series on hydrogels with different peptide grafting densities. E) Strain sweeps of hybrid hydrogels at different concentrations. F) Strain sweeps of hydrogels with varying amounts of coupled and free peptide.

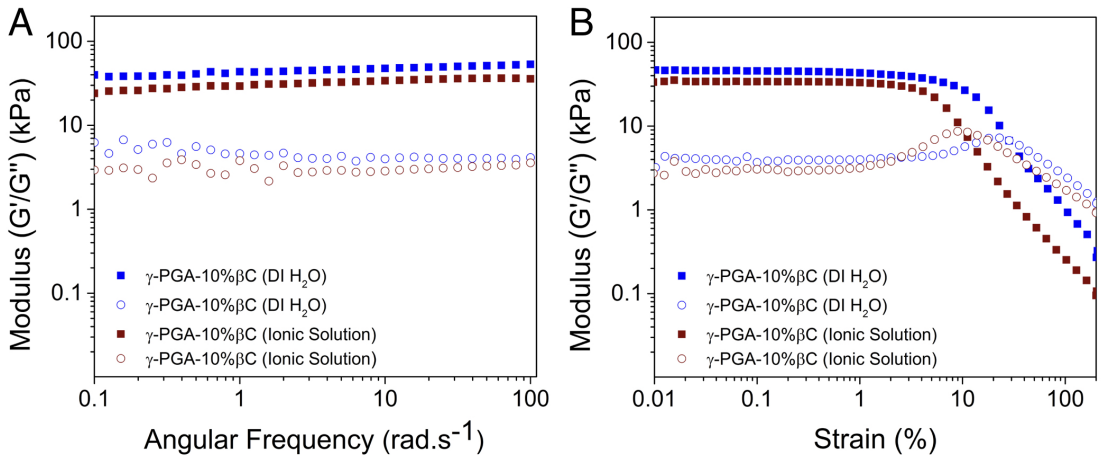


Figure S7. Frequency sweep (A) and strain sweep (B) of of γ-PGA 10%βC (7.5 wt%) hydrogel in ultrapure water and in physiological saline solution containing 135 mM NaCl, 10.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$.

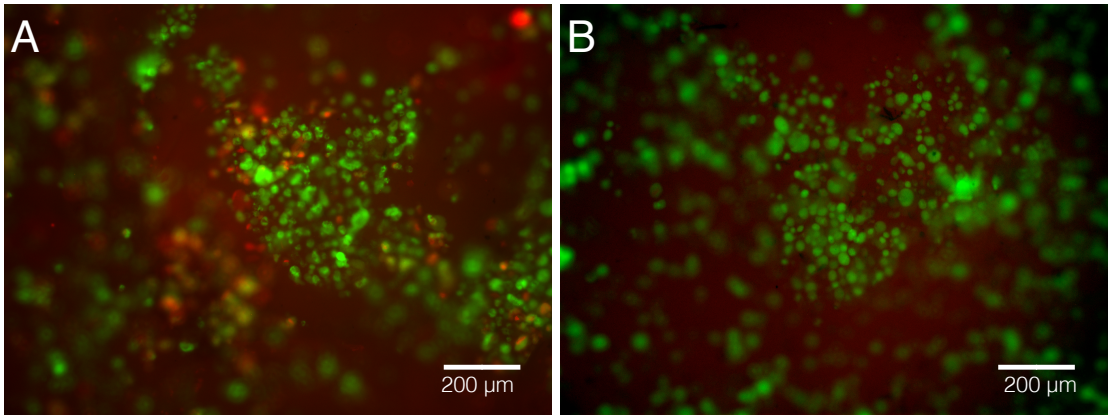


Figure S8 Hybrid hydrogels support cell attachment and viability. Human dermal fibroblasts (HDFs) were seeded on top of the hybrid γ-PGA 10%βC (7.5 wt%) hydrogel and cultured for 1 day (A) or 3 days (B). Cells were stained with a green calcein AM "live" stain and red ethidium homodimer "dead" stain to label living and dead cells, respectively.

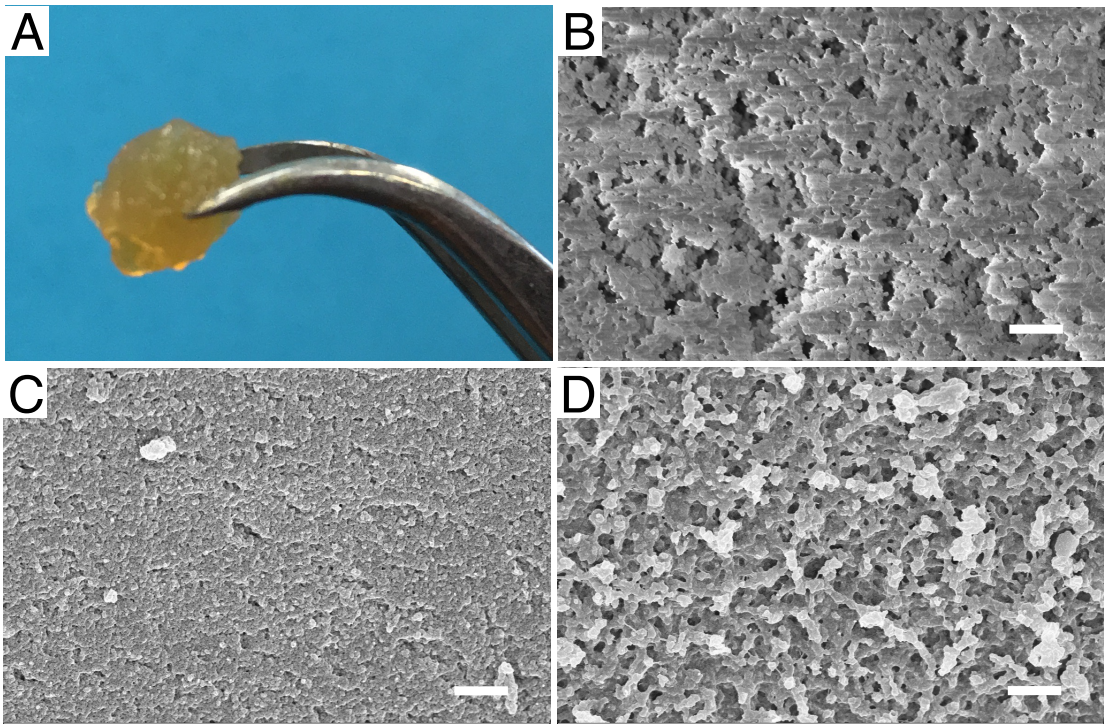


Figure S9. Microscopy of hybrid hydrogels. A) Photograph of γ-PGA 10%βC. SEM images of B) γ-PGA 10%βC (5 wt%), C) γ-PGA 10%βC (10 wt%), and D) γ-PGA 5%βC+10%βF (7.5 wt%). Scale bar is 1 μm.

	Mass Swollen (mg)	**Mass Dried (mg)**	**Mass Fraction (wt%)**
γ-PGA-10%βC - 1	23.9	1.8	7.54
γ-PGA-10%βC - 2	20.7	1.5	7.24

Table S1. Hydrogel swelling study. γ-PGA 10%βC was dissolved in DMSO at 7.5 weight percent and pipetted into a dialysis tube to induce gelation. The hydrogel was weighed after dialysis was complete, and the lyophilized to determine the mass of the) γ-PGA 10%βC remaining. As can be seen in the right column, the final mass is close to 7.5% by weight, suggesting that the hydrogels do not swell significantly.

Experimental

Materials

γ-PGA was purchased in its sodium salt form (γ-PGA-Na^+ produced by Bacillus Subtilis; Natto Science\varpi Ltd, Japan). To verify the molecular weight distribution of γ-PGA, size exclusion chromatography was performed courtesy of Smithers Rapra (UK) (Mw = 282 kDa, Mn = 117 kDa and PDI = 2.4). All Fmoc-protected amino acids, solvents and rink amide 4-methyl-benzhydrylamine (MBHA) resin used for peptide synthesis were purchased from AGTC Bioproducts (UK). Thioflavin T (ThT) was purchased from Novabiochem (UK). All other solvents and reagents for the γ-PGA-H and γ-PGA-g-β-sheet synthesis were purchased from Sigma-Aldrich (UK) and used as received.

γ-PGA-alkyne Synthesis

One gram of γ-PGA-Na^+ was first dissolved in 50 mL of distilled water (DI H_2O). The solution was acidified with 6M HCl to a pH of 1.5 and subsequently lyophilized until dry. Next, the lyophilized γ-PGA-H was suspended in DMSO at 5 wt%. The N-terminal amines on the polypeptide were capped by adding 190 μLs of acetic acid dissolved into 10 mLs of DMSO follow by the addition of 1376 milligrams of HCTU (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3–tetramethylaminium hexafluorophosphate) and 866 μL of diisoproylethylamine (DIPEA). This was then added to 20 mL (1 gram) of γ-PGA in a round bottom flask and reacted for 4 hours at RT under stirring. The solution was then precipitated in acetone, re-dissolved in basic DI H_2O, acidified to pH 2, and re-precipitated in acetone. The precipitate was then dialyzed overnight (SpectraPor 3,500 Da MWCO), acidified to pH 2 with HCl and lyophilized. 320 milligrams of HCTU was added to 100 milligrams of γ-PGA (2 mL) followed by the addition of 200 μL of DIPEA to activate all of the carboxylic acids on the γ-PGA. Propargylamine was added to DMSO at 10 μL per mL of DMSO and this solution was added to the γ-PGA in according to the desired functionalization (247 μL propargylamine/DMSO for 100 milligrams of the 5% γ-PGA, 495 μL for the 10% and 743 μL for the 15%). This was stirred in a round bottom flask for 4 hours, precipitated

in acetone, re-dissolved in basic DI H_2O, re-precipitated in acetone. The precipitate was dialyzed overnight and lyophilized yielding the final alkyne functionalized γ-PGA.

Peptide Synthesis and Characterization

All peptide sequences were manually synthesized using solid-phase peptide synthesis and Fmoc/tBu strategy on rink amine MBHA resin following previously published protocols.[1] After the deprotection of the final amine the N-terminus was modified into an azide using an imidazole-1-sulfonyl azide transfer reagent.[2,3] To synthesize the azide transfer reagent, 6.5 grams of sodium azide was suspended in ice-cold acetonitrile followed by the dropwise addition of 8 mL of sulfuryl chloride and overnight stirring. It was then chilled on ice and 13 grams of imidazole was added over 20 minutes and stirred for 3 hours. The solution was diluted with 200 mL of ethyl acetate, washed twice with 200 mL of DI H_2O, twice with 200 mLs of saturated sodium bicarbonate and dried with $MgSO_4$ and cooled on ice. 10 mLs of acetyl chloride was slowly added to 40 mLs of ethanol was cooled on dry ice to generate HCl in ethanol. The HCl/ethanol solution was added dropwise to the ethyl acetate solution to generate colorless imidazole-1-sulfonyl azide hydrochloride needles. These were filtered out of the solution, washed twice with ethyl acetate and dried under vacuum.

Once the complete peptide had been synthesized, the resin was placed into a round bottom flask and three equivalents of the azide transfer reagent was dissolved in 10 mLs of DMSO with 9 equivalents of DIPEA. This was stirred overnight at 45°C and conversion was monitored by using a ninhydrin assay. For incomplete conversions the azide transfer was repeated with the addition of 0.1% Triton-X and 20 mM LiBr. After the azide transfer the resin was place back into a peptide synthesis vessel, washed twice with dimethylformamide (DMF) and twice with dichloromethane (DCM) before being cleaved with 95% trifluoroacetic acid (TFA) with 2.5% triisopropylsilane and 2.5% DI H_2O.

The crude peptides were precipitated and washed with cold diethyl ether (DEE), then left to dry under vacuum. I4β peptide was purified by high pressure liquid chromatography (HPLC) using a Phenomenex C18 Gemini NX column with a 5 micron pore size, a 110 Å particle size and with the dimensions 150 x 30 mm. A gradient from 5% acetonitrile 95% DI H_2O to 100% acetonitrile was run with 0.1% ammonium

hydroxide and 20 mM ammonium formate added to the aqueous phase to prevent aggregation.

Synthesis of γ-PGA-β-sheet Conjugate

Lyophilized γ-PGA was dissolved at 5 wt% in DMSO while stirring until a completely clear solution was obtained. The β-sheet peptide sequences were dissolved in DMSO at 5 wt% and added to the γ-PGA at the desired ratio. Five milligrams of the γ-PGA/peptide solution was precipitated in acetone, dried and dissolved in D_2O with K_2CO_3 to ensure the correct peptide:γ-PGA by NMR. Then one equivalent of $CuSO_4$ and two equivalents of sodium ascorbate (relative to the peptide) were added and the solution was stirred overnight at 50°C to perform the click reaction.

After coupling, the DMSO solutions were dialyzed with a 8,000 Da MWCO regenerated cellulose membrane (Spectrum Laboratories, Inc.) against DI H_2O for 3 days, with the DI H_2O changed twice daily. The hybrid hydrogels formed gels in the dialysis bags in the first few hours of dialysis.

Nuclear Magnetic Resonance Spectroscopy

NMR spectra were recorded on a Bruker Biospin GmbH AV400 operating at 400 MHz for ^1H using DMSO-*d*6 as a deuterated solvent. Following dialysis, 50 μl gel samples were lyophilized and the re-dissolved in D_2O with K_2CO_3. ^1H-NMR spectra were referenced to the residual protons in the deuterated solvent. The number of β-sheet peptides present in the different hybrid hydrogels was then determined by ^1H-NMR (Figure S2 & 3). We used the integral of the methyl groups present in the isoleucine residues (F, H) of the β-sheet peptide sequences, the methylene protons on the propargylamine and proton on the triazole and normalized to the γ-protons on the γ-PGA to determine the relative ratios of peptide, γ-PGA, propargylamine and clicked peptide.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

The efficiency of the dialysis process and the removal of the $CuSO_4$ catalyst was evaluated using inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo Scientific ICap 6000series). The hybrid hydrogel samples were first diluted in 2 M HNO_3 and then analysed for Cu concentration in solution. To determine the final

Cu concentration of the sample solutions, a calibration curve was generated using a commercial Cu ICP-OES standard (Sigma-Aldrich). All measurements were performed in triplicate and averaged.

Scanning Electron Microscopy

Hybrid hydrogels were dried via critical point drying using a Samdri-795 critical point drier, mounted on aluminum Scanning Electron Microscopy (SEM) slides using carbon tape and then sputter coated with gold for 2 minutes. The morphology of the hydrogels was then examined using SEM (Leo1525 Gemini) at an accelerating voltage of 5 kV.

Thioflavin T Binding Studies

Fluorescence spectroscopy was used to study the β-sheet structures in the hybrid hydrogels. The hydrogels were further hydrated with either DI H_2O or with the addition of 6M guanidinium chloride (GndCl), normalizing to the γ-PGA polymer concentration across the gels. As a control γ-PGA-H was also dissolved in both DI H_2O and 6M GndCl at the same polymer concentration of the hydrogels. ThT was dissolved at 1 mg/ml and filtered through a 0.2 μm syringe filter to make a 0.1 wt% stock solution. The ThT stock solution was then diluted 1:50 in DI H_2O, γ-PGA-H or the hybrid hydrogels and incubated for 2 hours. Spectroscopic studies were completed by exciting at 440 nm and recording the emission spectra from 450 nm to 700 nm at 2 nm intervals with using a SpectraMax M5 microplate reader (Molecular Devices, Wokingham, UK).

Fourier Transform Infrared Spectroscopy

To further assay the formation of β-sheet structures in the hybrid hydrogels, Fourier transform infrared spectroscopy (FT-IR) was carried out using a Perkin-Elmer Spectrum 100 FT-IR Spectrometer in the amide I region (1550-1700 cm-1) at a resolution of 1 cm^{-1} and averaged from 52 consecutive scans. The hybrid hydogels were lyophilized, and the resultant dried material used for analysis. The hybrid hydrogels were either lyophilized or solvent exchanged with D_2O, and the resultant dried material or D_2O swollen hydrogel used for analysis. All the spectra were normalized to the absorbance at 1550 cm^{-1}, which is considered to be relatively insensitive to peptide secondary structure.[4]

Circular Dichroism

Circular Dichroism (CD) measurements were carried out on a JASCO J-810 CD spectrometer. The gels were place in a 0.1mm path length quartz plate cuvette with roughly 50 μL of gel per sample. All scans were performed at 25°C from 195-250 nm at 0.1 nm intervals with averaging time of 3 seconds and spectra were averaged across three scans.

Rheology

All rheological studies were performed on a TA-AR200 rheometer using an 8 mm parallel plate and a 0.5 mm gap distance at 25 °C. 200 μL of DMSO-hybrid hydrogel solution was placed into the 10 mm diameter dialysis tubing and the sample was dialyzed for 24 hours with three exchanges of deionized water or a physiological salt solution (135 mM NaCl, 10.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$). The gels were gently removed from the dialysis tubes and a 1 mm slice was taken from the gel immediately before being placed on the rheometer. Frequency sweeps were performed at 1% strain and at oscillation frequencies from 0.1 – 200 rad s^{-1}. Strain sweeps had a fixed oscillation frequency of 6.283 rad s^{-1} and variable applied strain of 0.01 – 200%. For the recovery experiments, three identical strain sweeps were performed in series with a 30-minute rest period between each strain sweep. For the cyclic strain measurements, the hydrogels were subjected to an immediate series of strain sweeps with incrementally increasing strains from (0.01% - 1, 2, 5, 10, 15, 20, 30, 50 and 100%) at a fixed oscillation frequency of 6.283 rad s^{-1}.

Cell Studies

Human dermal fibrobasts (ATCC) were cultured in Dulbecco's modified eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and 1X penicillin/streptomycin. Cells were cultured until 90% confluency and then subcultured. γ-PGA-10%βC was dissolved in DMSO at 75 mg/mL and dialyzed for 24 hours in ultrapure water. Approximately 50 μL of hydrogel was added to the bottom of a 96 well plate and it was centrifuged at 4,000 RPM for 5 minutes. HDFs were trypsinized for 3 minutes, centrifuged at 1,000 RPM and resuspended at 1 million cells/mL. 50 μL of cell

suspension was added on top of each hydrogel, followed by 250 μL of media. At Days 1 and 3 the samples were washed once with phosphate buffered saline (PBS) and then incubated with PBS and Live/Dead^{TM} stain (Thermofisher Scientific) for 30 minutes before imaging.

Reference

(1) Greenfield, M. A.; Hoffman, J. R.; la Cruz, de, M. O. Langmuir. **2010,** 26(5), 3641–3647.
(2) Castro, V.; Blanco-Canosa, J. B.; Rodriguez, H.; Albericio, F. *ACS Comb. Sci.* **2013**, *15* (7), 331–334.
(3) Chapman, R.; Jolliffe, K. A.; Perrier, S. B. *Aust. J. Chem.* **2010**, *63* (8), 1169.
(4) Kubelka, J.; Keiderling, T. A. *J. Am. Chem. Soc.* **2001**, *123* (48), 12048–12058.