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Supporting Information

Engineering bioorthogonal protein-polymer hybrid hydrogel as a functional protein immobilization platform

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Materials and Methods

1. Protein expression and purification

The genes encoding SpyTag-γPFD-Cys, mCerulean3 and mVenus with and without SpyCatcher were synthesized as gBlocks gene fragments (Integrated DNA Technologies) and were inserted into the multiple cloning site of the pET-19b plasmid (Novagen) using the Gibson assembly (New England Biolabs). The assembled plasmids were transformed into T7 Express competent cells, which were grown at 37°C in Terrific Broth (IBI Scientific) at 100 μg mL^{-1} ampicillin up to OD 600 = 0.6. Protein expression was induced by adding IPTG to final concentration of 0.1 mM and cells were grown for an additional 15 hours at 25°C. The cells were harvested by centrifugation at 6000 g for 10 minutes, lysed by French press, and centrifuged at 22,000 x g for 50 minutes.

Since γPFD proteins are thermostable, the soluble protein lysate containing SpyTag-γPFD-Cys was placed in an 80°C oven for 30 minutes, and centrifuged at 22,000 x g for 50 minutes again to eliminate aggregated impurities. The resulting supernatant was then purified by anion exchange (Hitrap Q column, GE healthcare) using AKTA FPLC. The proteins were loaded on the column with the equilibrium buffer (100 mM NaCl, 50 mM NaH_2PO4, 5 mM DTT, pH 7.4) and eluted using a gradient of 100 mM to 1 M of NaCl in 50 mM NaH_2PO4, 5 mM DTT at pH 7.4. Each elution fraction was inspected using SDS-PAGE and SimplyBlue staining (Invitrogen), and the fractions containing the pure target protein were dialyzed overnight against dialysis buffer (100 mM NaCl, 50

mM NaH_2PO4, pH 7.4). Finally, the purified proteins were concentrated using Amicon Ultra 15-mL centrifugal columns (10 kDa MWCO, Millipore) and lyophilized for storage at -20°C.

The fluorescent proteins (both with and without SpyCatcher) containing the N-terminal 6xHis tag were purified by binding to Ni-NTA resin (Life Technologies), washed with the equilibration buffer (20 mM imidazole, 50 mM NaH_2PO4, 1 M NaCl, pH 8.0), and eluted using a gradient of 20 mM to 1 M imidazole in 50 mM NaH_2PO4, 1 M NaCl at pH 8.0. Fractions containing the pure proteins were dialyzed, concentrated and lyophilized under conditions the same as above.

2. Hydrogel formation and swelling

Lyophilized SpyTag-γPFD-Cys was resuspended in phosphate buffer (100 mM NaCl, 50 mM NaH_2PO4, pH 7.4; "phosphate buffer" will refer to this buffer composition in subsequent sections), and the concentration was measured using the Bradford reagent (Bio-Rad). Proteins were then concentrated to twice the desired final concentration using Amicon Ultra 0.5-mL centrifugal columns (10 kDa MWCO, Millipore). PEG-4MAL of 20,000 Da Mw (JenKem Technology) was dissolved in phosphate buffer to twice the desired final concentration. Each component (50 µL) was then mixed by pipetting several times to form the hydrogel with total volume of 100 µL, which was allowed to cure at room temperature for 6 hours. The molar stoichiometry between protein and polymer was always fixed at 4:1.

To measure swelling, formed and cured hydrogels were first weighed (W_i) and then incubated overnight in 900 µL phosphate buffer at room temperature. After removing the buffer, swelled hydrogels were weighed again (W_f). The swelling ratio was calculated as below.[1]

$$\text{Swelling ratio (\%)} = (W_f - W_i) / W_i \times 100$$

3. Hydrogel erosion

Hydrogels of 100-µL volume were formed and cured as described above. Phosphate buffer (900 µL) was added and incubated at room temperature or at 37°C. At each time point of measurement, a small aliquot of buffer was removed and its protein concentration was measured using the Bradford method to calculate the amount of protein released.

4. Rheology tests

Oscillatory shear rheology tests were performed with a Physica MCR 301 parallel plate rheometer (Anton Paar), using an 8-mm diameter top plate. Hydrogels of total volume 40 µL were formed in situ between the parallel plates at a gap width of 0.8 mm and were allowed to cure in the humidified chamber. Time sweep experiments were conducted at the fixed frequency and applied strain of 1 Hz and 1%, respectively. Amplitude sweep tests were performed at 1 Hz frequency, while frequency sweep runs were performed at 1% applied strain;

for all hydrogel samples tested in this study, 1% applied strain was within the linear viscoelastic range.

5. Fluorescent gel formation and leaching measurements

SpyTag-γPFD-Cys solution at twice the desired final concentration was prepared in phosphate buffer as described above. PEG-4MAL and fluorescent protein (both with and without SpyCatcher) solutions were prepared in phosphate buffer at 4 times the desired final concentration. 25 µL of the PEG-4MAL and fluorescent protein solutions were mixed to yield 50 µL solution; this mixture was then blended with 50 µL SpyTag-γPFD-Cys solution by pipetting several times to form 100 µL of the fluorescent protein-incorporated hydrogel, which was allowed to cure at room temperature for 6 hours. Hydrogel erosion studies were performed as described above.

To confirm the conjugation between SpyTag-γPFD-Cys and SpyCatcher-FPs, mixed samples were loaded onto Bolt 4-12% Bis-Tris SDS-PAGE gels (Invitrogen) containing Spectra BR protein ladder (Thermo Scientific). Separated protein bands were visualized by SimplyBlue staining (Invitrogen).

In order to study leaching of the fluorescent proteins, gels of 100-µL volume were formed and cured as described above, followed by addition of 900 µL phosphate buffer and incubation at room temperature. At each time point of measurement, a small aliquot of buffer was removed and the fluorescence was measured at the maximum emission peaks of mCerulean3 and mVenus (475 nm

and 528 nm) using a Spectramax M2 plate reader (Molecular Devices) to determine the amount of leached proteins.

6. FRET measurements

Hydrogels containing the same amounts of mCerulean3 and mVenus were formed in situ on a black 96-well plate. Control hydrogels containing only one of the fluorescent proteins were also formed. The fluorescence was measured with the Spectramax M2 plate reader (Molecular Devices) using a 412-nm excitation, 430-nm cutoff filter and emission scan of 450 – 600 nm. A buffer blank was subtracted from all samples. FRET spectra were deconvoluted using the control spectra as references. The isolated mCerulean3 components of the FRET spectra as well as the control mCerulean3 spectra were integrated to calculate the fluorescence intensities. FRET efficiencies were calculated from the equation below:

$$\text{FRET efficiency (\%)} = (1 - I_{DA} / I_D) \times 100$$

where I_{DA} is the integrated fluorescence intensity from the decomposed FRET spectrum, and I_D is that calculated from the mCerulean3 spectrum. Spectral decomposition and integration were performed using a|e UV-Vis-IR Spectral Software 2.2 (Fluortools). Measurements were only made for the concentration range in which the fluorescence intensity increased linearly with the protein concentration.

7. Human Pluripotent Stem Cell Culture

Human embryonic stem cells (H9s, NIH Stem Cell Registry #0062) were subcultured in monolayer format on a layer of 1% Matrigel (354277, Corning) and maintained in Essential 8 medium (A1517001, ThermoFisher) during expansion. At 80% confluency, H9s were passaged using Versene solution (15040066, Thermo Fisher) and re-plated at a 1:8 split.

8. Viability Assays

H9s were dissociated into single cells using Accutase solution (A6964, Sigma-Aldrich) and resuspended in Essential 8 medium containing 10 µM Y-27632 (Rock Inhibitor, 1254, Tocris). H9s were counted and resuspended at defined densities and mixed 1:1 with PEG-4MAL solution. Suspended H9s were mixed 1:1 with SpyTag-γPFD-Cys in a µClear 96-well plate (655090, Greiner Bio-One). At specific time points, wells with H9s in hydrogels were incubated in 250 µL of Calcein AM solution (C3100MP, Thermo Fisher) diluted 2,000 times from the stock solution using sterile PBS for 20 minutes. After staining, cells were imaged using a confocal microscope.

9. Confocal Microscopy and Image Processing

Stained cultures were imaged with a 5x objective using an Opera Phenix automated confocal fluorescence microscope (Perkin Elmer) available in the High-Throughput Screening Facility at UC Berkeley. Laser exposure time and power were kept constant for a fluorescence channel within an imaging set. Fluorescent images were processed in ImageJ where background fluorescence

was removed using a rolling bar radius algorithm and z-stacks were imported into 3D viewer for visualization.[2]

10. MTT assay

Cells encapsulated in hydrogel were first cultured in 96-well plates as described above. At particular time points, 100 µL culture medium was replaced with new medium containing 1.1mM MTT (Thermo Fisher Scientific) and incubated for 4 hours at 37°C. Subsequently, 100 µL DMSO was added to each well, incubated for 10 min at 37°C, and the absorbance at 540 nm was measured using a Spectramax M2 plate reader.

Supplementary Figures

SpyTag-γPFD-Cys:

MAHIVMVDAYKPTKGSVNEVIDINEAVRAYIAQIEGLRAEIGRLDATIATLRQSLATLKSLKTLGEGKT

VLVPVGSIAQVEMKVEKMDKVVVAAAAAAAAELEYEEALKYIEDEIKKLLTFRLVLEQAIAELYAKIED

LIAEAQQTSEEEKAEEEENEEKAEC

mCerulean3:

MHHHHHHGGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPW

PTLVTTLSWGVQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI

ELKGIDFKEDGNILGHKLEYNAIHGNVYITADKQKNGIKANFGLNCNIEDGSVQLADHYQQNTPIGD

GPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGS

mVenus:

MHHHHHHGGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPW

PTLVTTLGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI

ELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGD

GPVLLPDNHYLSYQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGS

SpyCatcher-mCerulean3:

MGHHHHHHGSAMVDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGK
TISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGSSKGEELFTG
VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLSWGVQCFARYPDH
MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN
AIHGNVYITADKQKNGIKANFGLNCNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDP
NEKRDHMVLLEFVTAAGITLGMDELYK

SpyCatcher-mVenus:

MGHHHHHHGSAMVDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGK
TISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGSSKGEELFTG
VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCFARYPDH
MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN
YNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDP
NEKRDHMVLLEFVTAAGITLGMDELYK

Fig. S1 Amino acid sequences of the recombinantly expressed proteins used in this study.

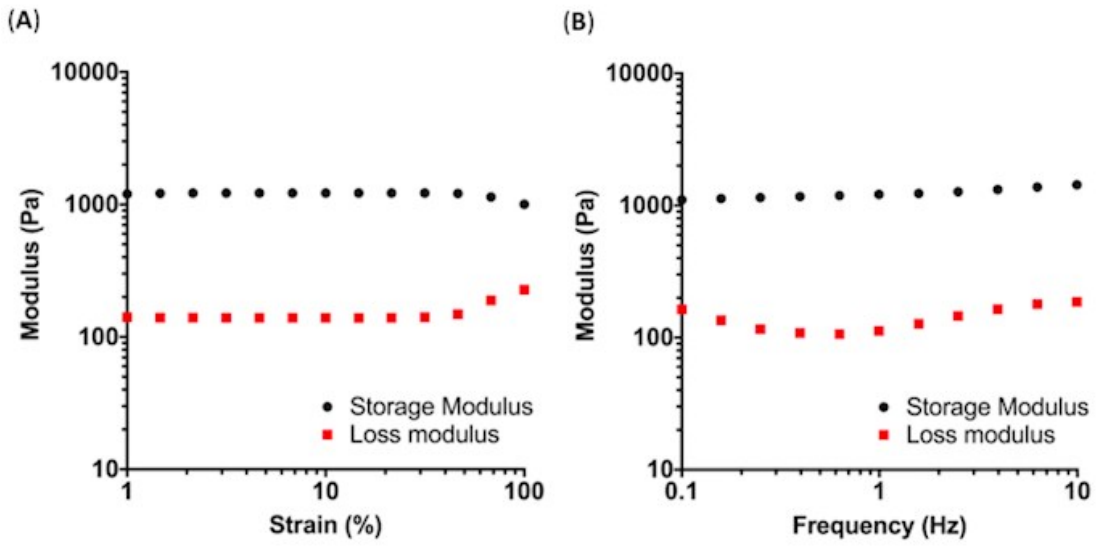

Fig. S2 (A) Frequency sweep of the hydrogel formed at 2.5 mM PEG and 10 mM γPFD, measured at fixed strain of 1%. (B) Amplitude sweep of the hydrogel formed at 2.5 mM PEG and 10 mM γPFD, measured at fixed frequency of 1 Hz.

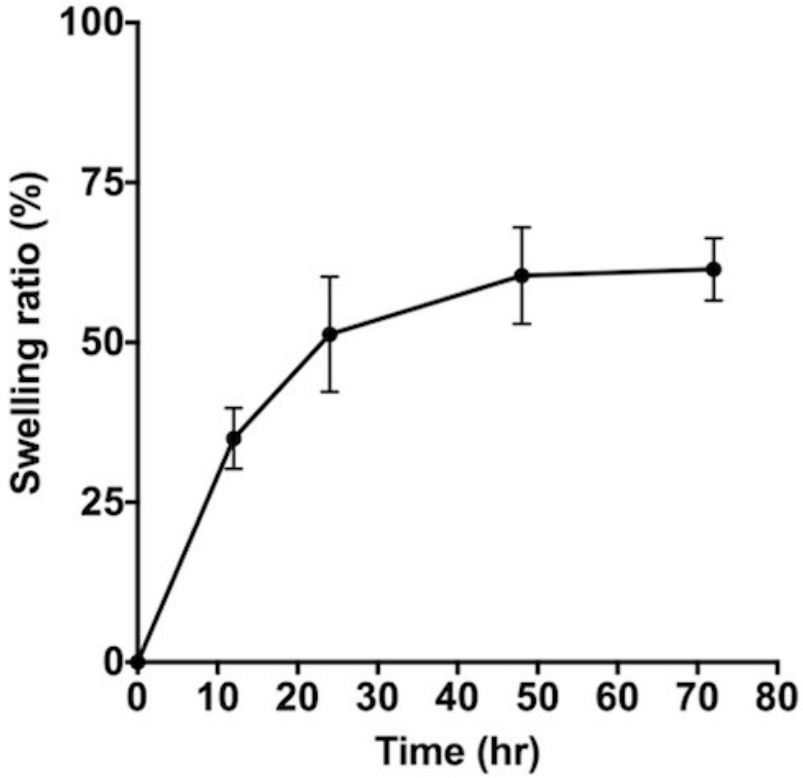

Fig. S3 Swelling of the hybrid hydrogels formed at 1.25 mM PEG and 5 mM γPFD, after submerging the formed hydrogel in phosphate buffer. Experiments were performed at least twice, and the error bars represent the standard deviation (SD).

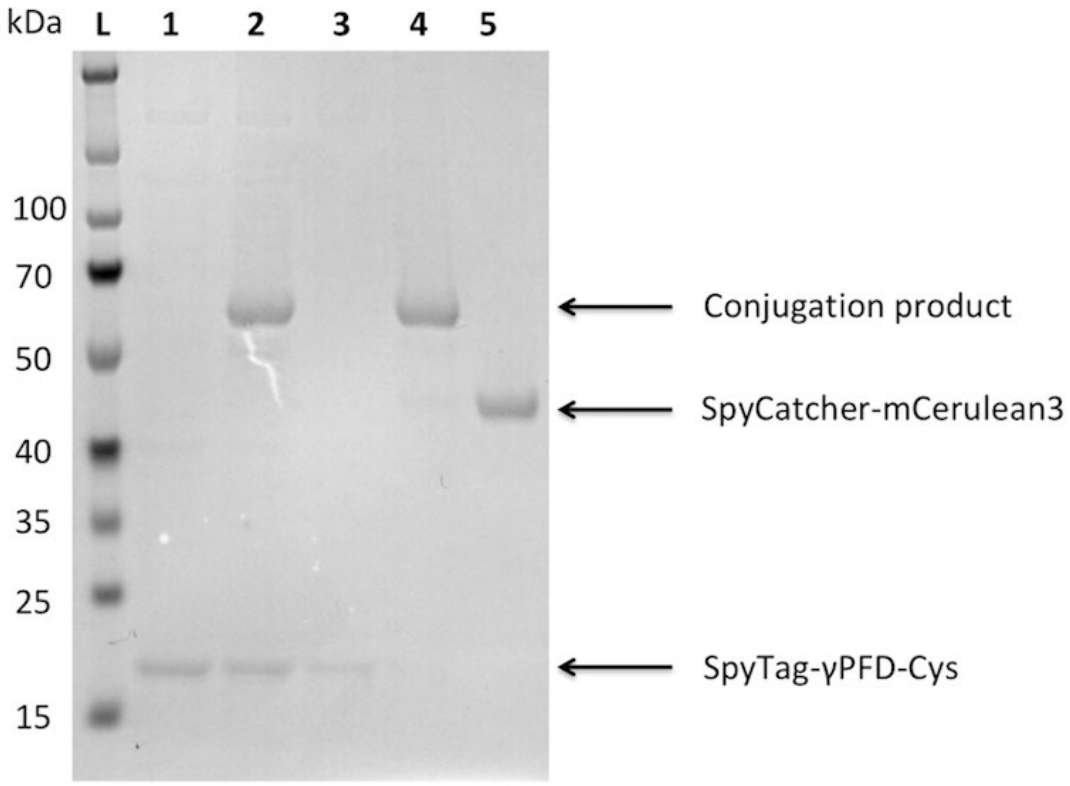

Fig. S4 SDS-PAGE results showing the conjugation between SpyTag-γPFD-Cys (18 kDa) and SpyCatcher-mCerulean3 (40 kDa). Loaded samples were 8 µM γPFD (lane 1), 2 µM SpyCatcher-mCerulean3 mixed with 8 µM γPFD (lane 2), 2 µM γPFD (lane 3), 2 µM SpyCatcher-mCerulean3 mixed with 2 µM γPFD (lane 4) and 2 µM SpyCatcher-mCerulean3 (lane 5). Hydrogel samples could not be loaded onto SDS-PAGE, and thus diluted samples were used to confirm the conjugation.

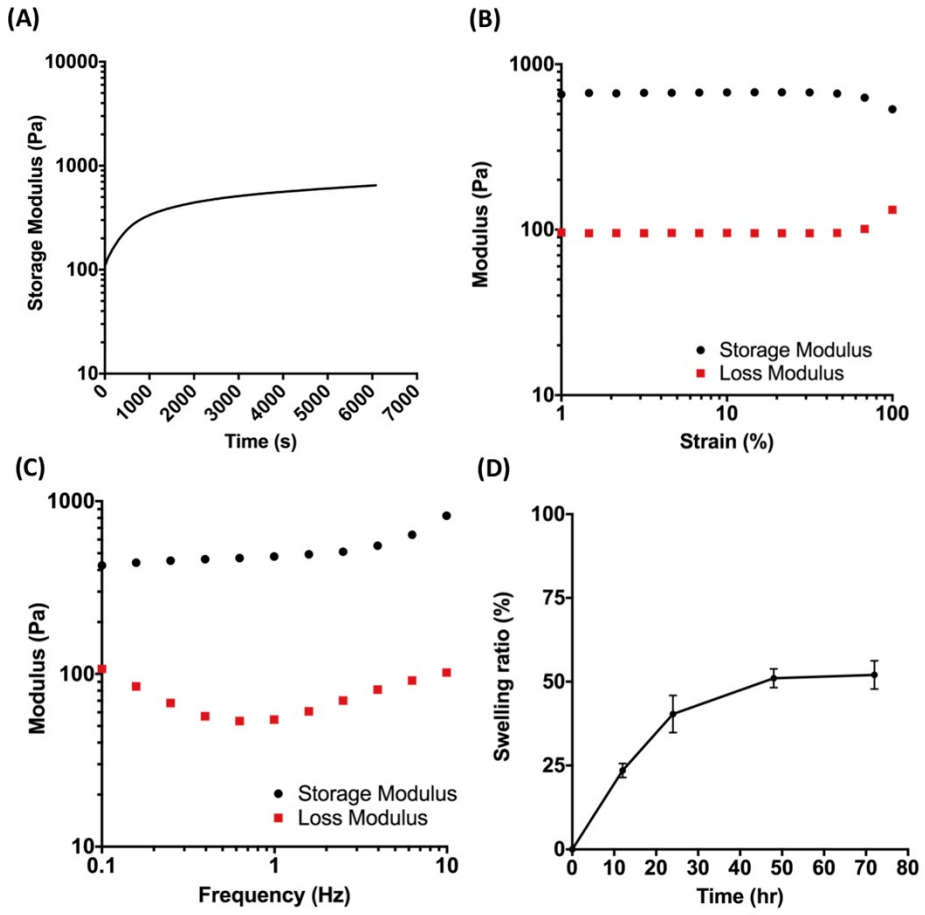

Fig. S5 Properties of the hybrid hydrogels formed with 1.25 mM PEG and 5 mM γPFD containing 1.25 mM SpyCatcher-mCerulean3. (A) Change in storage modulus as a function of time after mixing the protein and polymer components. Measurements were performed at fixed frequency of 1 Hz and strain of 1%. (B) Amplitude sweep of the hydrogel, measured at fixed frequency of 1 Hz. (C) Frequency sweep of the hydrogel, measured at fixed strain of 1%. (D) Swelling of the hydrogel after submerging in phosphate buffer. Experiments were performed at least twice, and the error bars represent the SD.

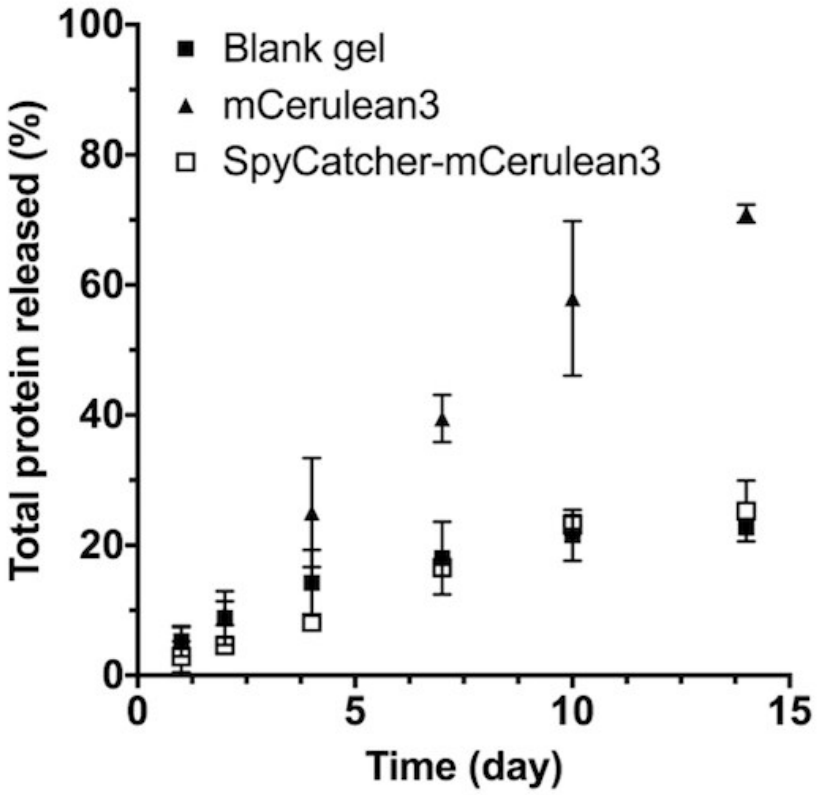

Fig. S6 Total protein erosion profile of the hybrid hydrogels formed at 1.25 mM PEG and 5 mM γPFD without any fluorescent protein, with 1.25 mM native mCerulean3, and with 1.25 mM SpyCatcher-mCerulean3. Experiments were performed at least twice, and the error bars represent the SD.

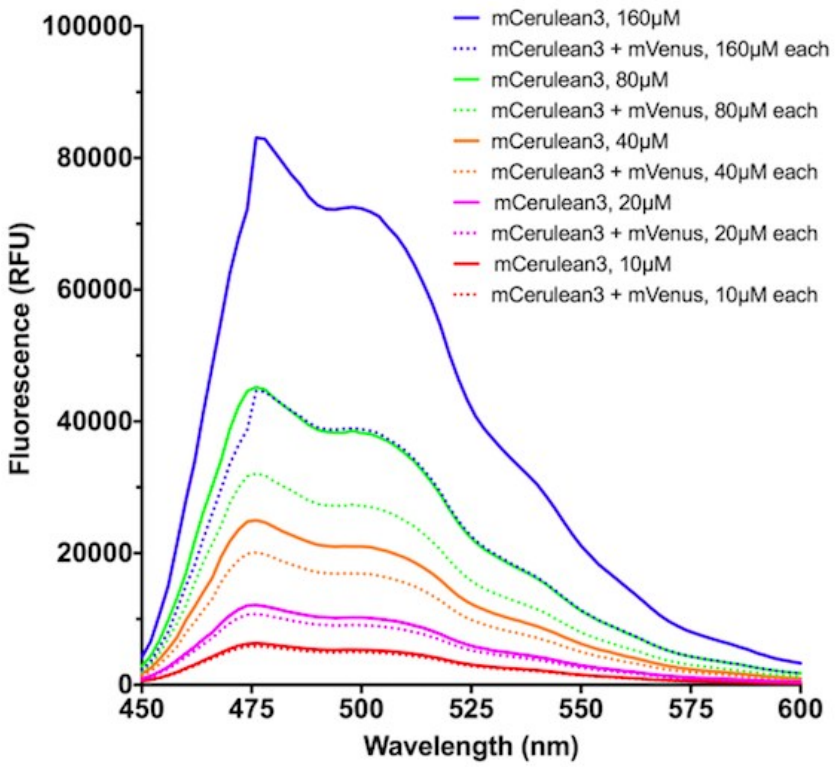

Fig. S7 Emission spectra of the hydrogels containing both mCerulean3 and mVenus, compared to those containing only mCerulean3. Total fluorescent protein concentrations were varied within a linear concentration-fluorescence range. The samples were excited at 412-nm, using a 430-nm cutoff filter. Solid lines represent the emission spectra from the gels containing only mCerulean3. Dotted lines represent the mCerulean3 component of the decomposed emission spectra from the gels containing equal amounts of mCerulean3 and mVenus.

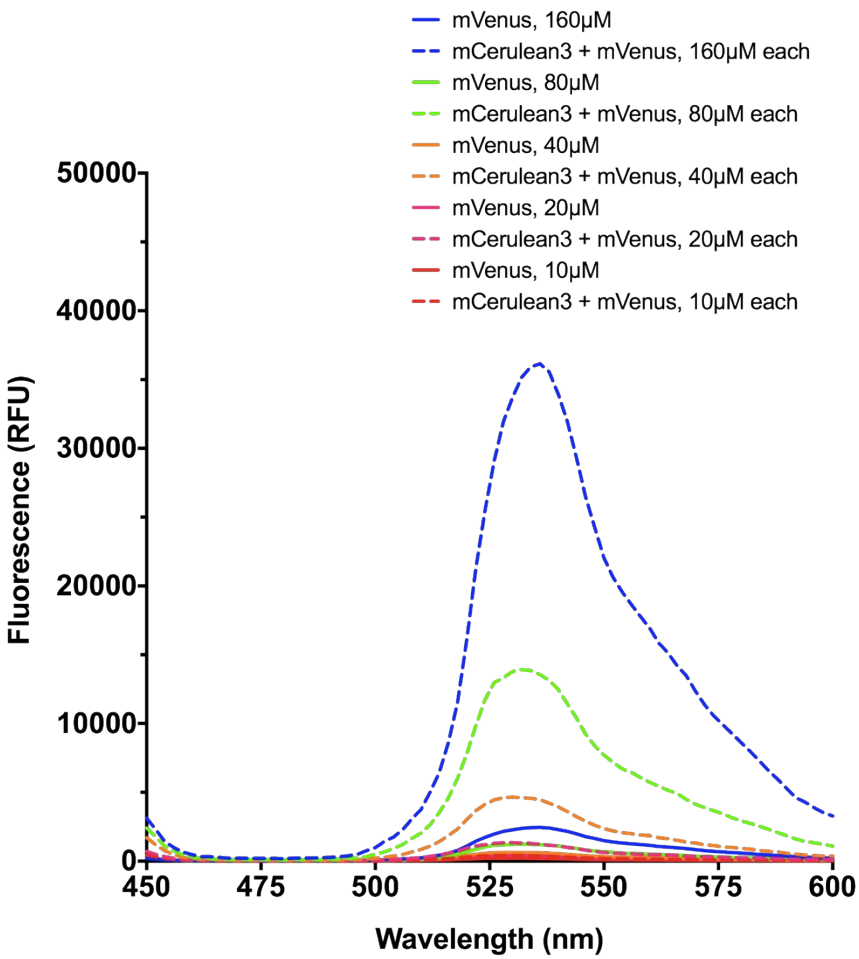

Fig. S8 Emission spectra of the hydrogels containing both mCerulean3 and mVenus, compared to those containing only mVenus. Total fluorescent protein concentrations were varied within a linear concentration-fluorescence range. The samples were excited at 412-nm, using a 430-nm cutoff filter. Solid lines represent the emission spectra from the gels containing only mVenus; note that the signals are very low because mVenus without a donor (mCerulean3) cannot be excited at this wavelength. Dotted lines represent the mVenus component of the decomposed emission spectra from the gels containing equal amounts of mCerulean3 and mVenus.

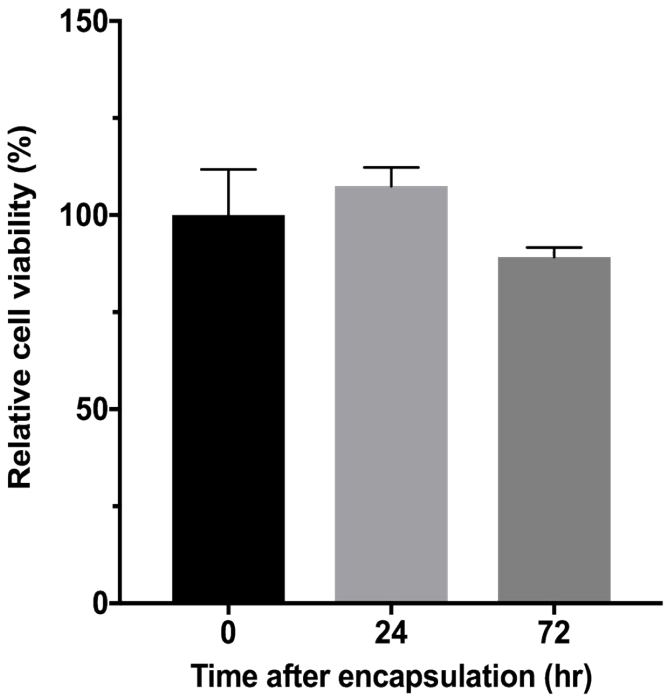

Fig. S9 Viability of the hPSCs encapsulated in hydrogel over 72-hour culture period. Absorbance values at 540 nm were normalized to the value at time zero, which was measured immediately after gel formation. The error bars represent the SD from triplicate measurements. At 24 and 72 hours after encapsulation in hydrogel, relative cell viabilities were ~100% and ~90%, respectively. The results indicate that most of the seeded cells survived both the initial processing condition and subsequent culturing.

References

1. X. Gao, J. Fang, B. Xue, L. Fu and H. Li, Biomacromolecules, 2016, **17**, 2812.

2. P. Bankhead, *ImageJ*, 2014, **1**, 195.