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Supplementary Information

Title: Hierarchically structured hydrogels utilizing multifunctional assembling peptides for 3D cell culture

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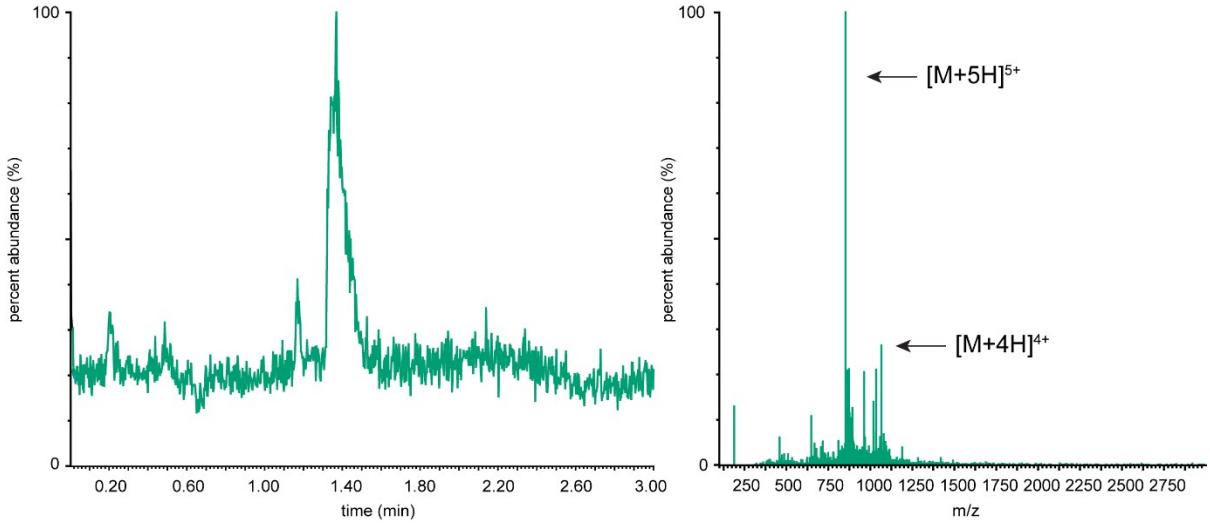

Figure S1: Single Quadruple Detector 2 (SQD2) UPLC trace and ESI+ mass spectrometry of mfCMP-1a. The desired product was observed with expected molecular weight of 4256 g/mol ($[M+4H]^{4+}$ = 1065 g/mol and $[M+5H]^{5+}$ = 852 g/mol).

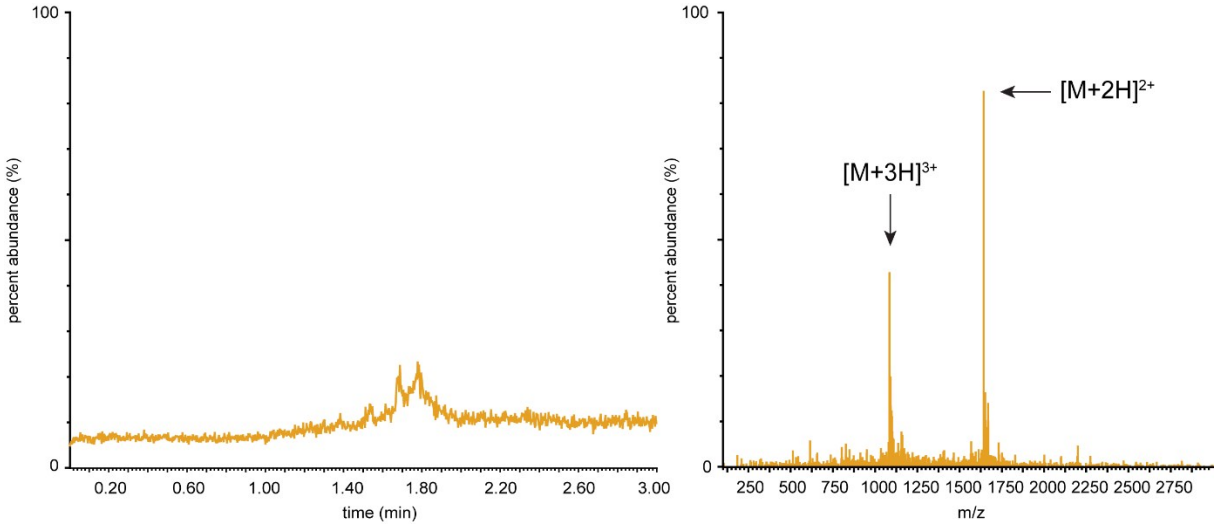

Figure S2: Single Quadruple Detector 2 (SQD2) UPLC trace and ESI+ mass spectrometry of mfCMP-2a. The desired product was observed with expected molecular weight of 3287 g/mol ($[M+2H]^{2+}$ = 1644 g/mol and $[M+3H]^{3+}$ = 1096 g/mol).

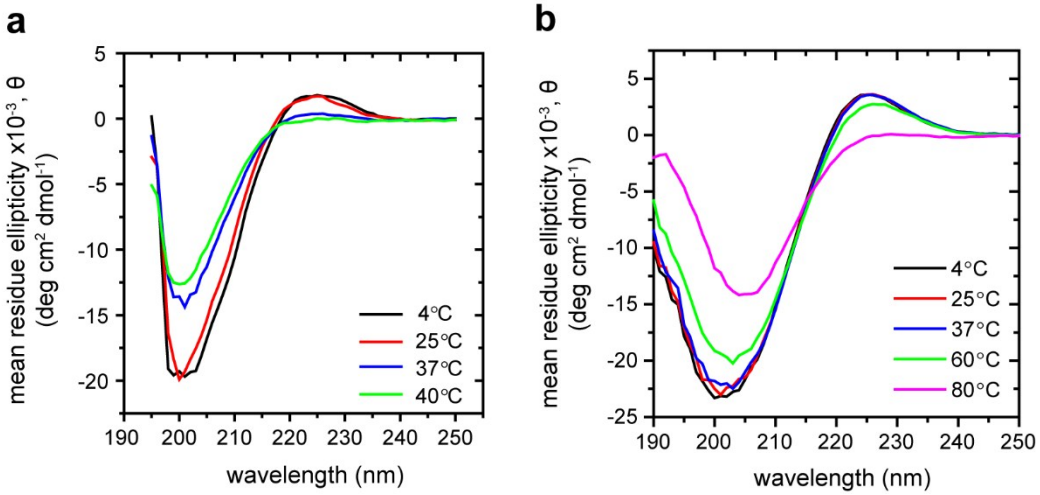

Figure S3: Wavelength scans of **a**) mfCMP-1a (0.1 mM in DPBS) and **b**) mfCMP-2a (0.1 mM in deionized water). Characteristic peak for triple helix at 225 nm was observed in both cases.

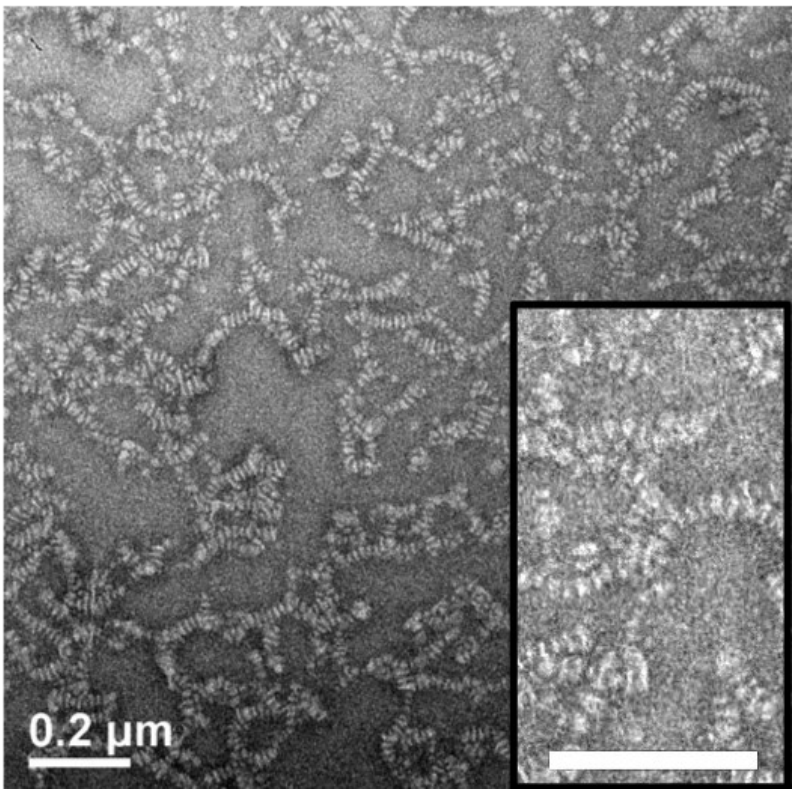

Figure S4: TEM of mfCMP-2a assembled at 0.3 mM (~1 mg/mL). Zoom in shows a striped morphology reminiscent of D-periodic banding structure found in native collagen. Scale bars = 0.2 µm.

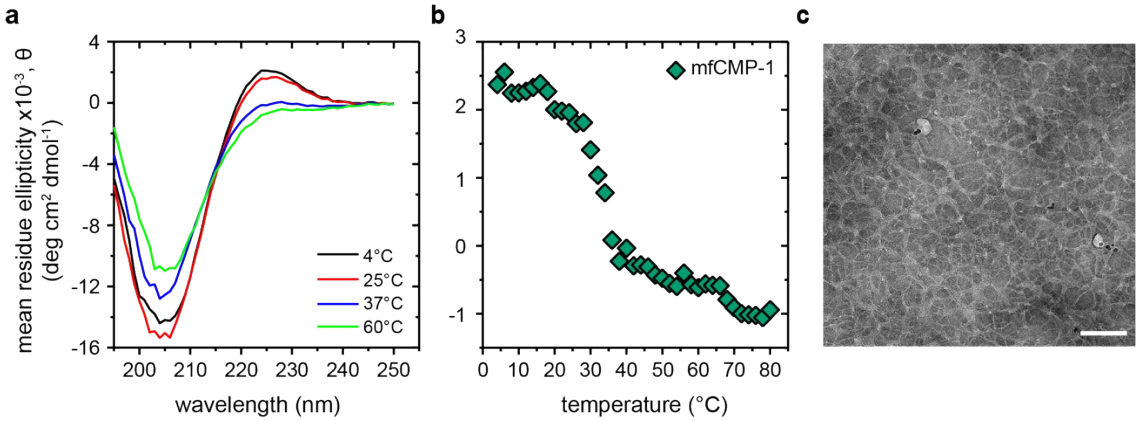

Figure S5: mfCMP-1 assembling control peptide without alloc. **a**) CD wavelength scans for mfCMP-1 in sodium phosphate demonstrated triple helix formation in solution. **b**) Mean residue ellipticity at 225 nm measured over a range of temperatures, where T_m of ~36 °C was observed. **c**) TEM of mfCMP-1 assembled at 0.3 mM in 10 mM sodium phosphate demonstrated the formation of fibrils similar to those observed with mfCMP-1a. Scale bar = 200 nm.

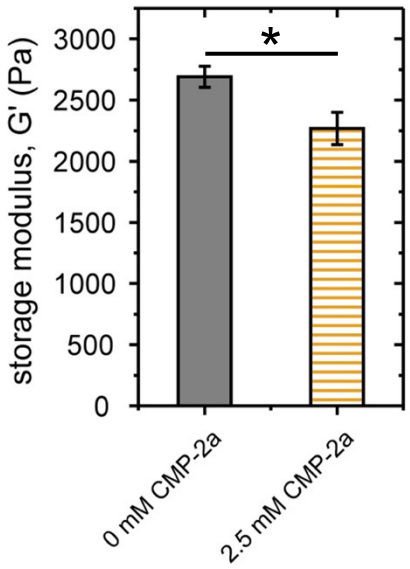

Figure S6: *In situ* oscillatory rheology of hydrogels that contain 2.5 mM mfCMP- 2a. A slight statistical difference in storage modulus was observed when compared to the control (n = 4; *p < 0.05).

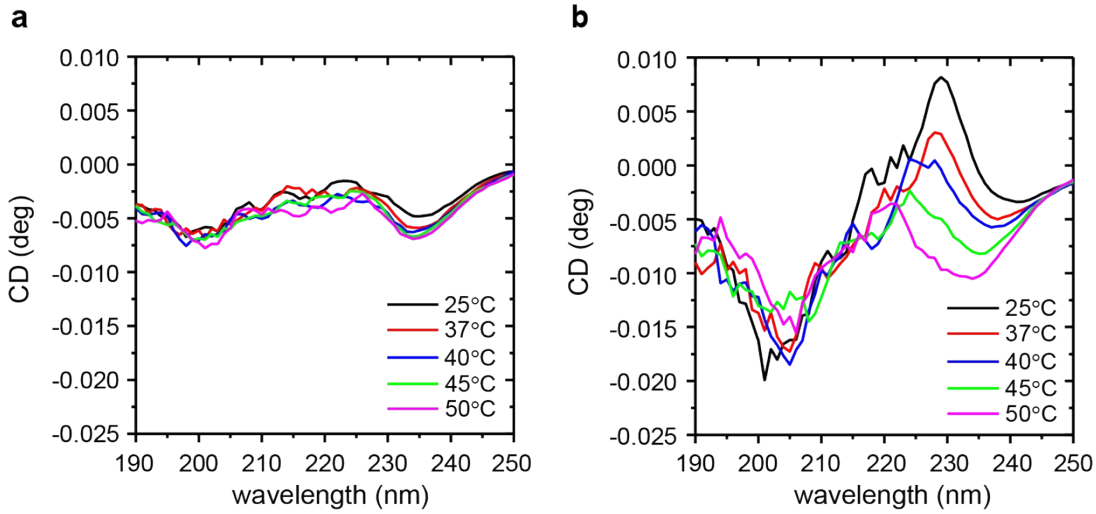

Figure S7: Circular dichroism data on hydrogels with and without 2.5mM mfCMP-1a. **a**) Wavelength scans of 6 wt% PEG-SH hydrogels that contain 0 mM mfCMP-1a at various temperatures. **b**) Wavelength scans of 6 wt% PEG-SH hydrogels that contain 2.5 mM mfCMP-1a, where peak at ~ 231 nm is indicative of a triple helix and decreases with increasing temperature as the triple helices are melted.

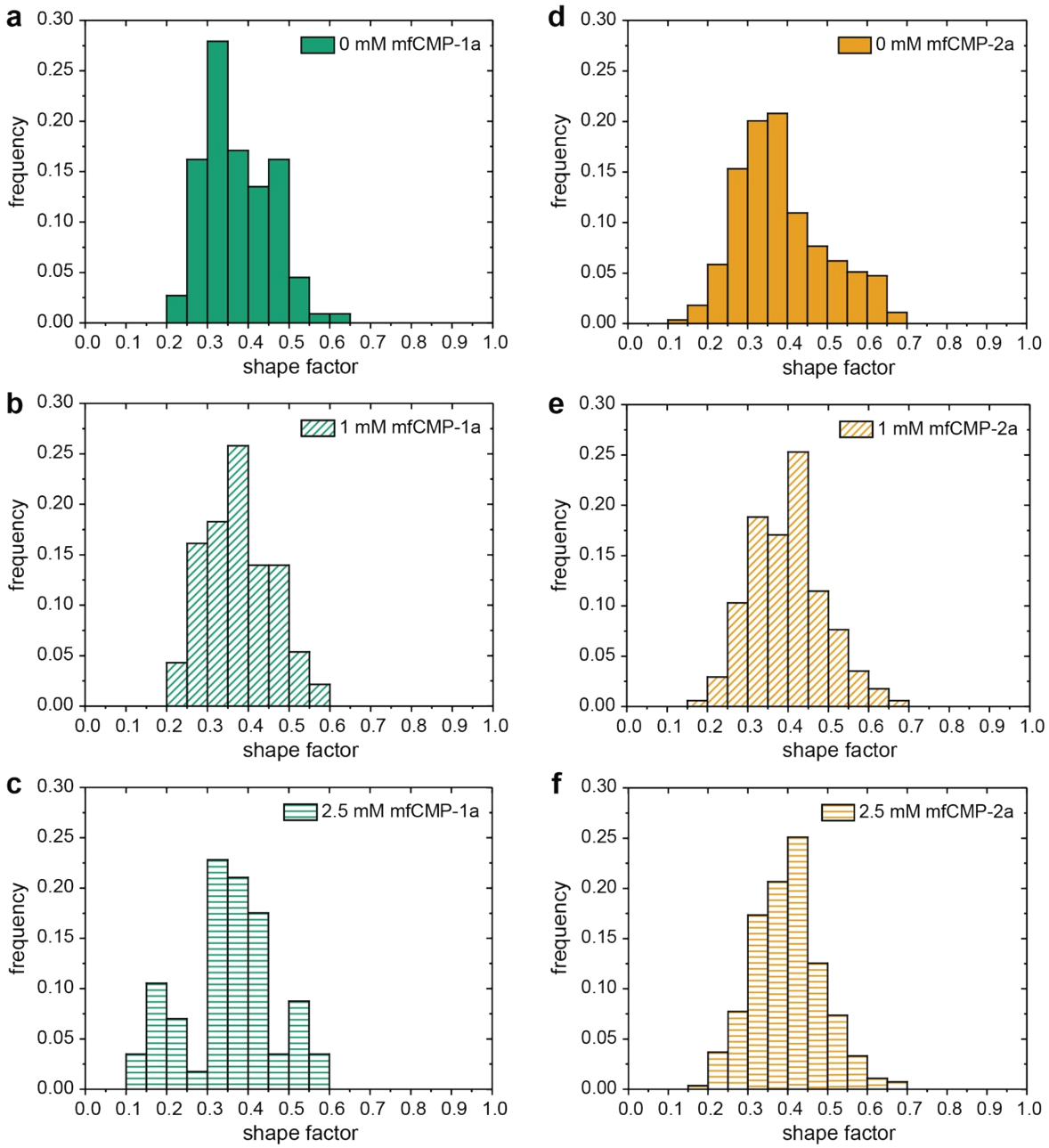

Figure S8: Object shape factor histograms for cells in 3D cultures. Cell morphology was analyzed through quantification of 3D shape factor (Ψ) of objects (individual or clusters of cells) at day 10 for hydrogels containing **a**) 0 mM mfCMP-1a, **b**) 1 mM mfCMP-1a, or **c**) 2.5 mM mfCMP-1a and **d**) 0 mM mfCMP-2a, **e**) 1 mM mfCMP-2a, or **f**) 2.5 mM mfCMP-2a. Shape factor distributions were used to set bins for *elongated* ($0 \leq \Psi \leq 0.25$), *spread* ($0.25 < \Psi \leq 0.5$), and *rounded* ($0.5 < \Psi \leq 1$) objects based on the emergence of multiple modes in conditions with higher mfCMP concentrations, especially in mfCMP-1a (n = 3 hydrogels, > 50 objects or 100 cells counted per condition).

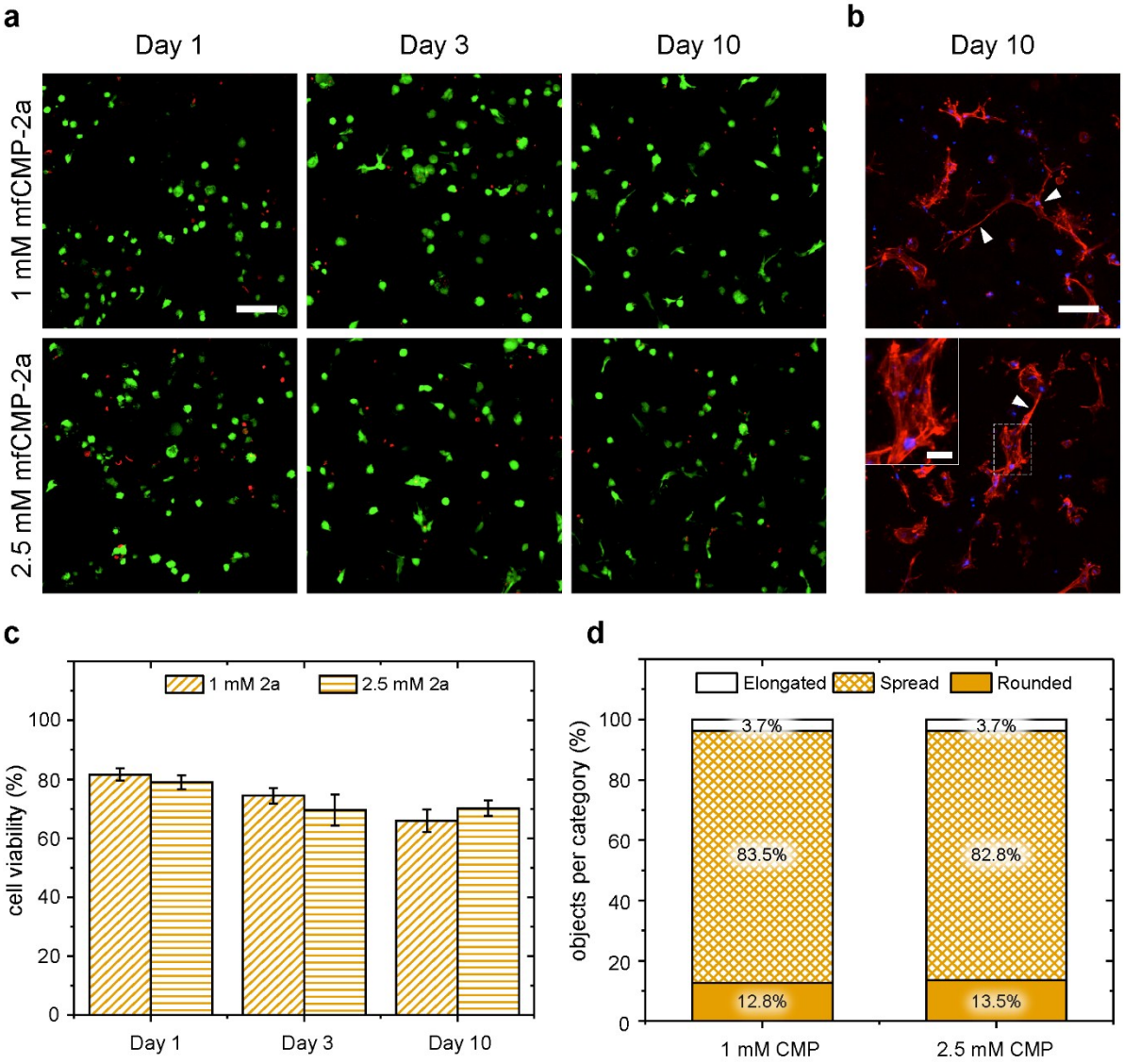

Figure S9: Cell viability and morphology in hydrogels with mfCMP-2a. Representative maximum intensity projections (from z-stacks, 200 μm thick) of hMSCs encapsulated within hydrogels at a low concentration (1 mM) or at a high concentration (2.5 mM) of mfCMP-2a: **a**) cells stained for viability on days 1, 3, and 10, where live cells are shown in green and dead cell nuclei in red and **b**) fixed cells stained for F-actin in red and cell nuclei in blue on day 10 (Scale bars: full image = 100 μm, inset image = 25 μm; arrowheads note long 'chains' of interacting hMSCs). **c**) Cell viability was quantified at days 1, 3, and 10 for hydrogels containing 1 and 2.5 mM mfCMP-2a (error bars represent the standard error around the mean; n = 3 hydrogels, > 700 cells counted per condition; *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). **d**) Cell morphology was analyzed through quantification of 3D shape factor (Ψ) of objects (individual or groups of cells) at day 10 for hydrogels containing 1 and 2.5 mM mfCMP-2a. Results for each hydrogel composition are shown as the percent of *elongated*, *spread*, or *rounded* objects (cells or cell clusters) within the sampled population (n = 3 hydrogels, > 250 objects or 400 cells counted per condition; no statistical differences observed).

Table S1: Viability of hMSCs cultured in hydrogels with 0 mM, 1 mM, or 2.5 mM of mfCMP-1a.

	0 mM CMP		1 mM CMP		2.5 mM CMP	
	Average	Standard Error	Average	Standard Error	Average	Standard Error
Day 1	93%	2.9%	91%	2.1%	84%	4.1%
Day 3	88%	3.0%	86%	1.9%	85%	4.4%
Day 10	85%	3.0%	87%	2.2%	88%	3.8%

Table S2: Results of statistical analysis of CMP-1a viability using a Student's T-test.

	0 mM CMP vs. 1 mM CMP	0 mM CMP vs. 2.5 mM CMP	1 mM CMP vs. 2.5 mM CMP
Day 1	0.291	0.005 **	0.017 *
Day 3	0.338	0.245	0.551
Day 10	0.386	0.298	0.676

*p < 0.05
**p < 0.01

Table S3: Shape factor analysis of hMSCs in hydrogels containing 0 mM, 1 mM, or 2.5 mM mfCMP-1a.

	0 mM CMP		1 mM CMP		2.5 mM CMP	
	Average	Standard Error	Average	Standard Error	Average	Standard Error
Rounded	8.0%	4.8%	6.6%	3.5%	13%	1.5%
Spread	89%	5.1%	89%	5.4%	59%	8.3%
Elongated	2.8%	1.4%	4.0%	2.0%	28%	7.7%

Table S4: Statistical analysis of shape factor distribution of cells cultured in hydrogels containing mfCMP-1a using a Student's T-test.

	0 mM CMP vs. 1 mM CMP	0 mM CMP vs. 2.5 mM CMP	1 mM CMP vs. 2.5 mM CMP
Rounded	0.825	0.420	0.191
Spread	0.971	0.038 *	0.039 *
Elongated	0.669	0.032 *	0.039 *

*p < 0.05

SUPPLEMENTAL FIGURES TO SUPPORT METHODS

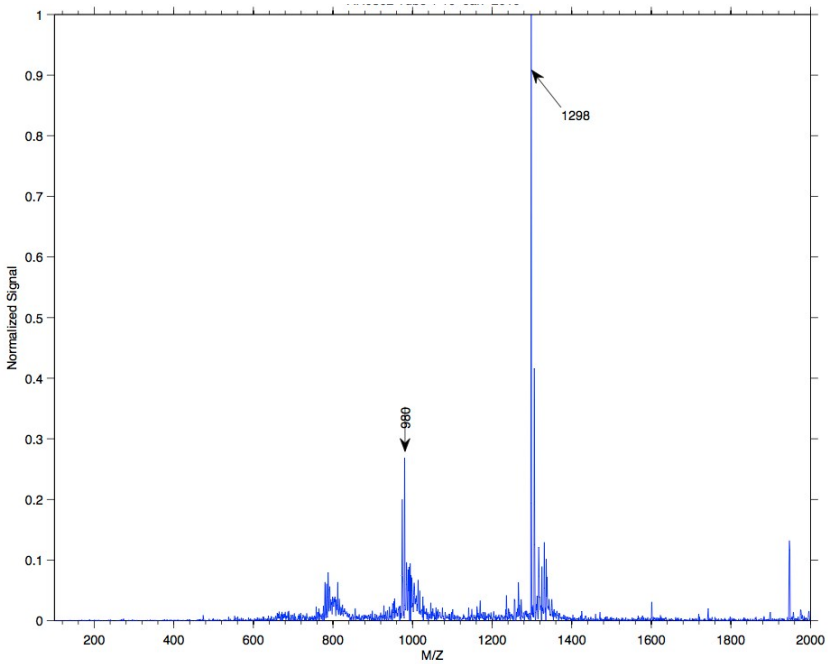

Figure S10: ESI+ spectrum for mfCMP-1 $(PKG)_4(POG)_6(DOG)_4$. ESI+ mass spectrometry confirms the desired product with expected molecular weight of 3891 g/mol ($[M+3H]^{3+}$ = 1298 g/mol and $[M+3H+Na]^{4+}$ = 980 g/mol).

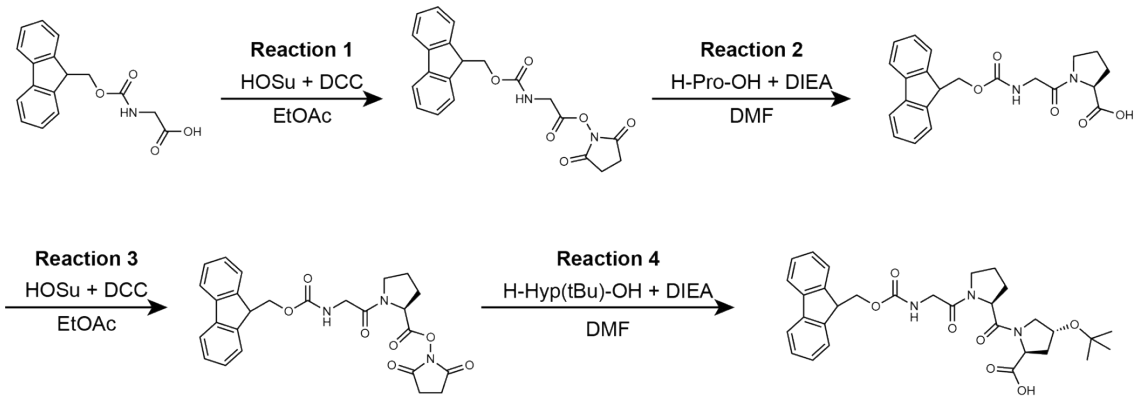

Figure S11: Scheme of Fmoc-GPO(tBu)-OH fragment synthesis.

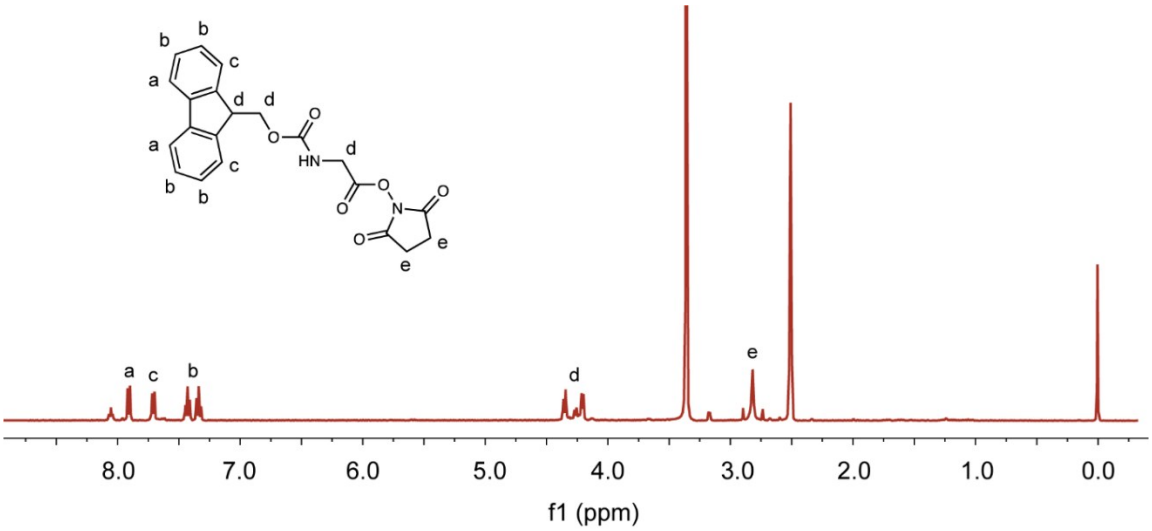

Figure S12: [1]HNMR spectrum of Fmoc-Gly-OSu fragment. [1]H NMR (400 MHz, DMSO-d_6) δ 7.91 (d, J = 7.5 Hz, 2H), 7.71 (d, J = 7.4 Hz, 2H), 7.47 – 7.29 (m, 4H), 4.40 – 4.17 (m, 5H), 2.91 – 2.72 (m, 4H).

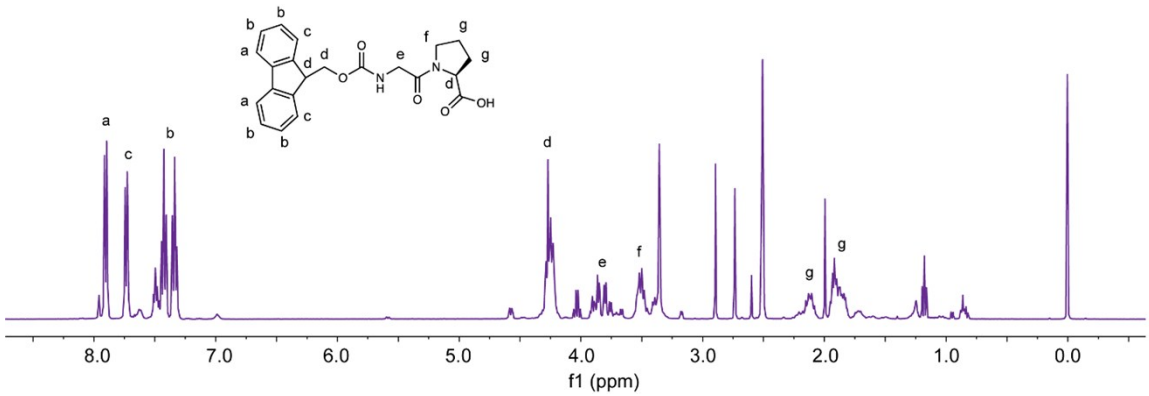

Figure S13: [1]HNMR spectrum of Fmoc-GP-OH. [1]H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2H), 7.73 (d, J = 7.5 Hz, 2H), 7.46 – 7.29 (m, 4H), 4.37 – 4.16 (m, 4H), 3.94 – 3.73 (m, 2H), 3.51 (qd, J = 9.9, 8.2, 2.9 Hz, 2H), 2.17 – 2.04 (m, 1H), 1.96 – 1.77 (m, 3H).

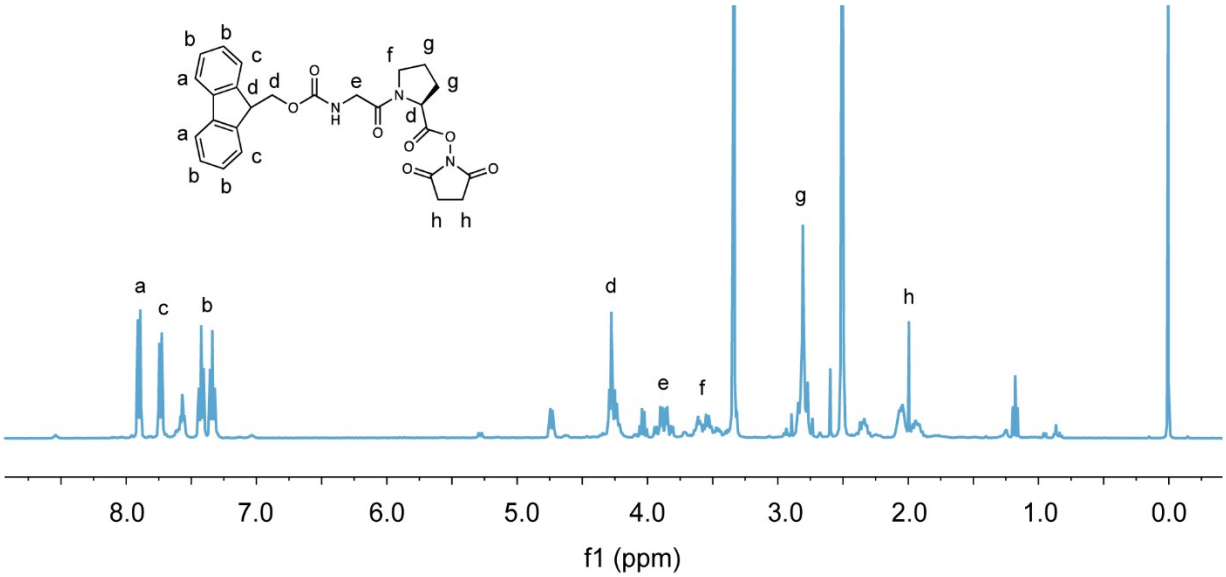

Figure S14: [1]HNMR spectrum of Fmoc-GP-OSu. [1]H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2H), 7.74 (d, J = 7.4 Hz, 2H), 7.48 – 7.28 (m, 4H), 4.34 – 4.17 (m, 3H), 3.87 (qd, J = 17.3, 6.1 Hz, 2H), 3.66 – 3.50 (m, 2H), 2.79 (q, J = 14.6 Hz, 4H), 2.42 – 2.28 (m, 2H), 2.12 – 1.84 (m, 4H).

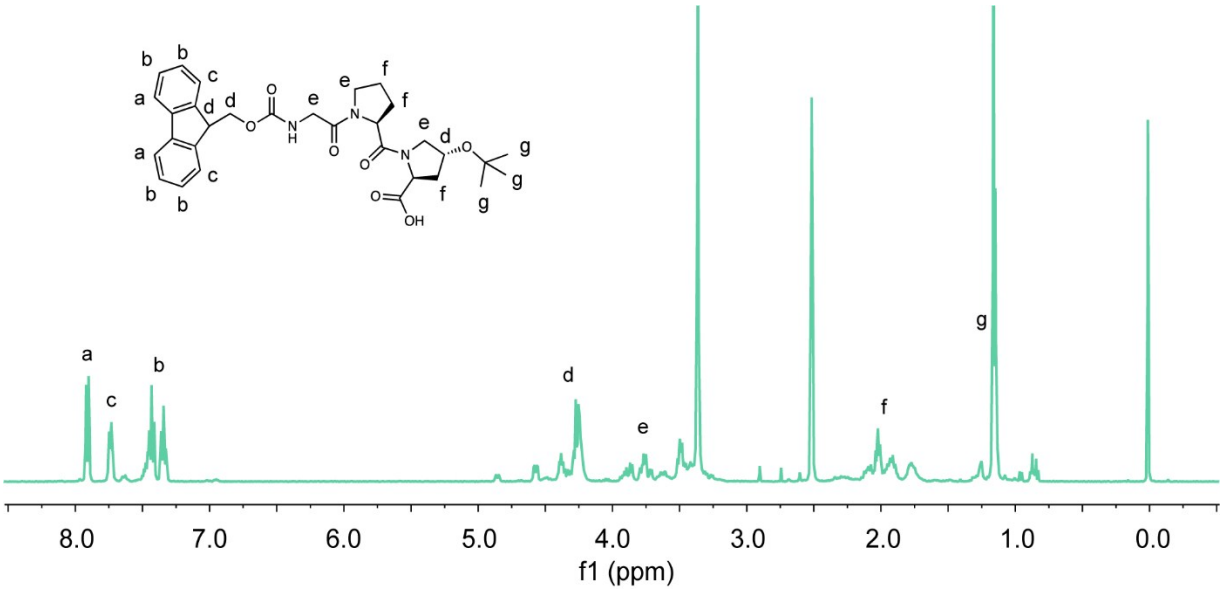

Figure S15: [1]HNMR spectrum of Fmoc-GPO(tBu)-OH. [1]H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2H), 7.73 (t, J = 6.0 Hz, 2H), 7.46 – 7.30 (m, 4H), 4.43 – 4.15 (m, 6H), 3.98 – 3.55 (m, 6H), 2.05 – 1.84 (m, 4H), 1.83 – 1.68 (m, 2H), 1.15 (d, J = 6.4 Hz, 9H).

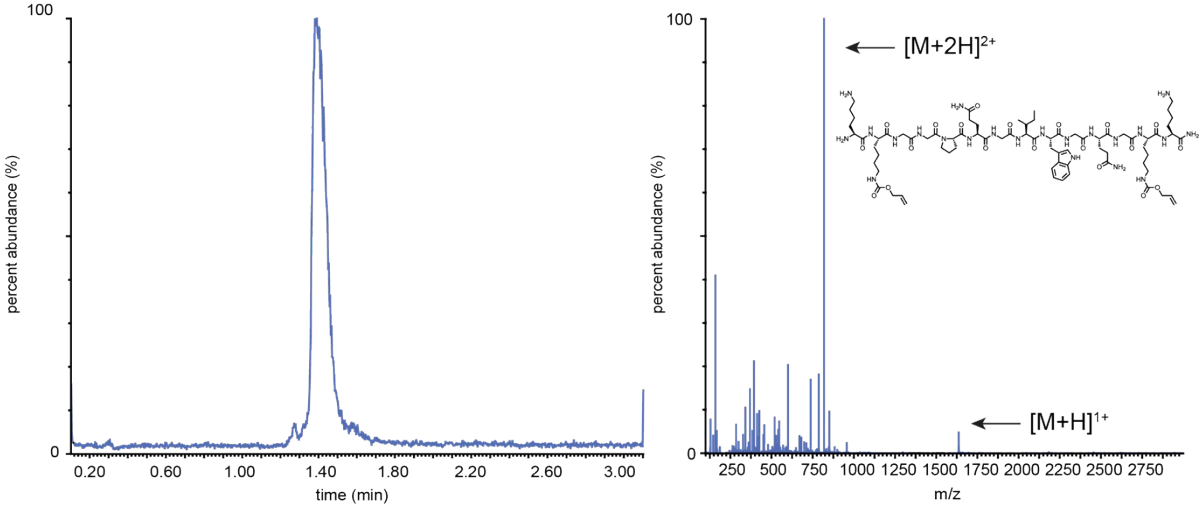

Figure S16: UPLC trace and ESI+ spectrum for linker peptide KK(alloc)GGPQGIWGQGK(alloc)K. Single Quadruple Detector 2 (SQD2) UPLC trace and ESI+ mass spectrometry confirmed the desired product with expected molecular weight of 1635 g/mol ($[M+H]^+$ = 1636 g/mol and $[M+2H]^{2+}$ = 818 g/mol).

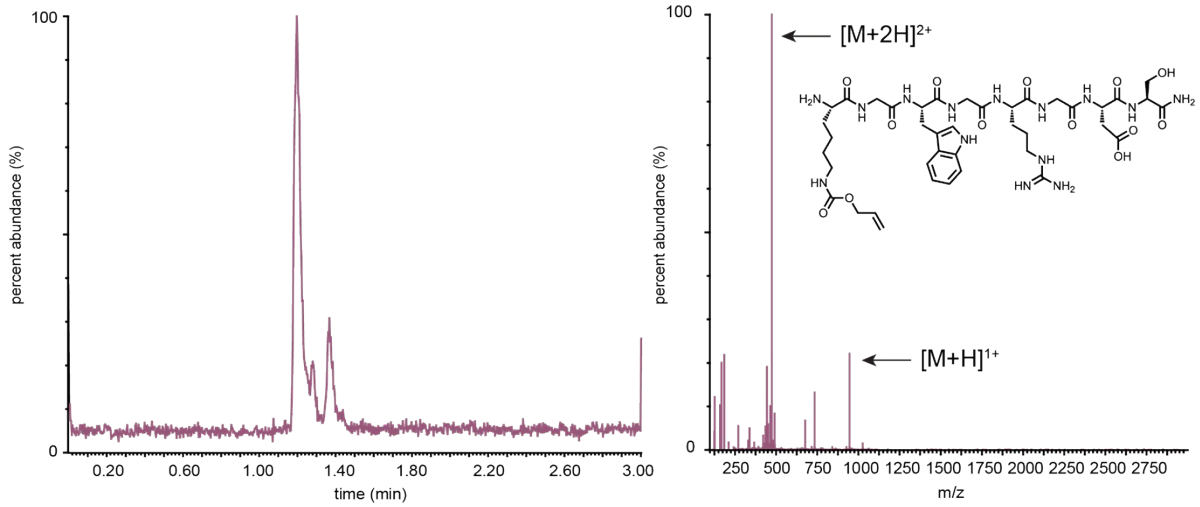

Figure S17: UPLC trace and ESI+ spectrum for integrin binding peptide K(alloc)GWGRGDS. Single Quadruple Detector 2 (SQD2) UPLC trace and ESI+ mass spectrometry confirmed the desired product with expected molecular weight of 945 g/mol ($[M+H]^+$ = 946 g/mol and $[M+2H]^{2+}$ = 473 g/mol).

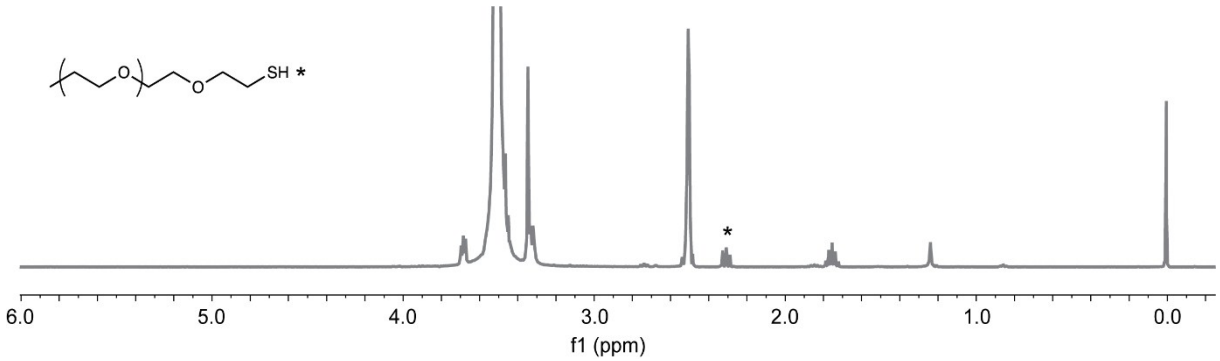

Figure S18: ^{1}HNMR spectrum of PEG-4-SH. ^{1}HNMR (400 MHz, DMSO-d_6): = 3.69 (s, 454H), 2.51 (d, 13H), 2.36-2.25 (m, 1H), 1.75 (p, 2H). Peak at 3.69 ppm corresponds to the repeat units within the 4-arm PEG backbone and was assigned an integration value of 454H. Integration of the thiol proton (starred peak) was normalized to the integration of the PEG backbone protons to determine functionality (typically ~95% as shown here).

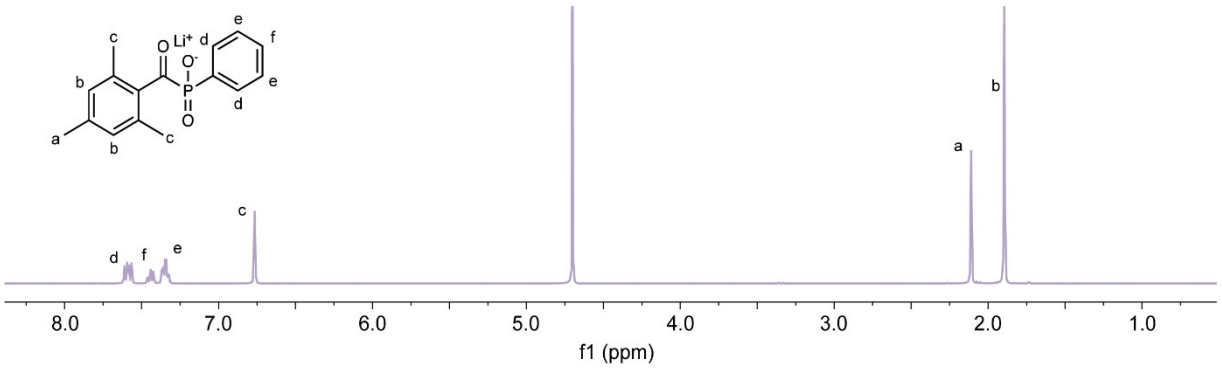

Figure S19: [1]HNMR spectrum of LAP photoinitiator. [1]H NMR (400 MHz, Deuterium Oxide) δ 7.63 – 7.54 (m, 2H), 7.48 – 7.41 (m, 1H), 7.34 (tdd, *J* = 6.8, 3.1, 1.3 Hz, 2H), 6.77 (s, 2H), 2.11 (s, 3H), 1.89 (s, 6H).

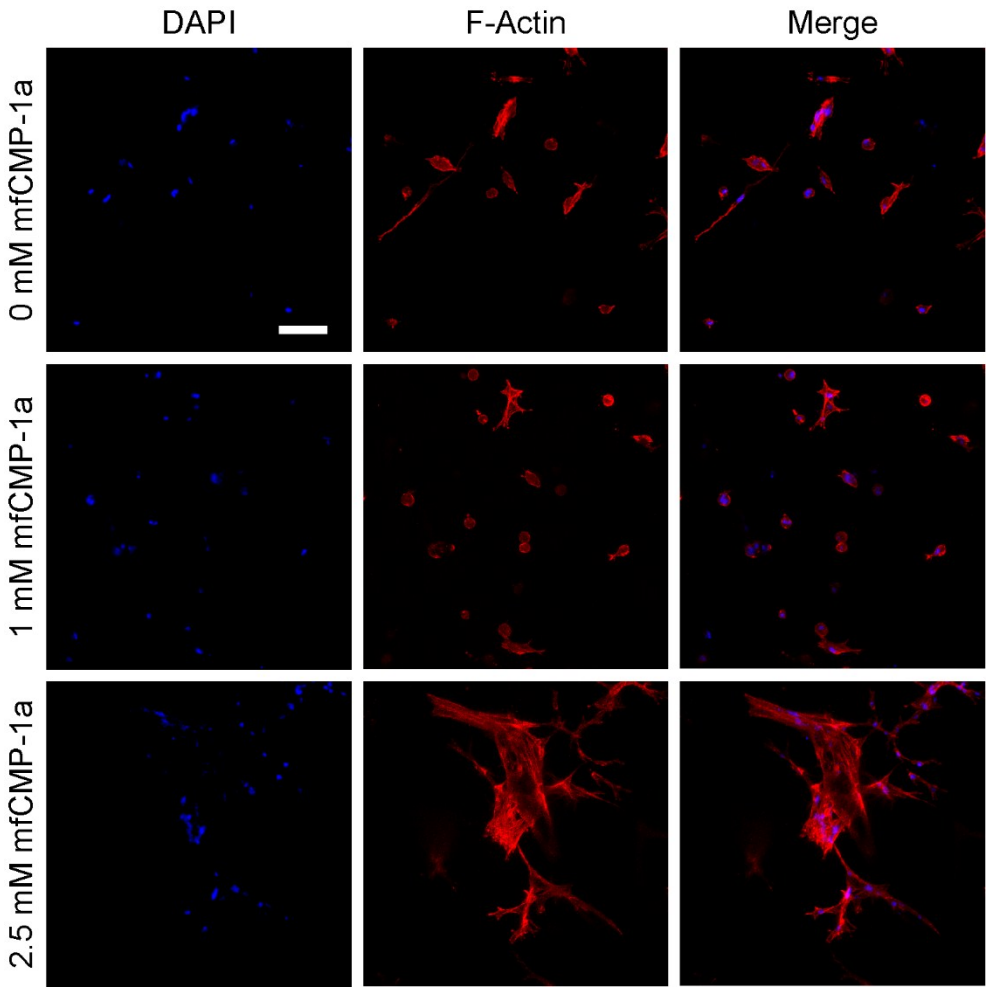

Figure S20: Day 10 image splits of cells cultured within hydrogels with mfCMP-1a. Representative maximum intensity projections (from z-stacks, 200 μm thick) of hMSCs encapsulated within hydrogels without mfCMP-1a (0 mM), at a low concentration (1 mM), or at a high concentration (2.5 mM) of mfCMP-1a. Cell nuclei are blue (left, DAPI), cell bodies are red (center, F-Actin), and the merged images are shown on the right. Scale bar = 100 μm.

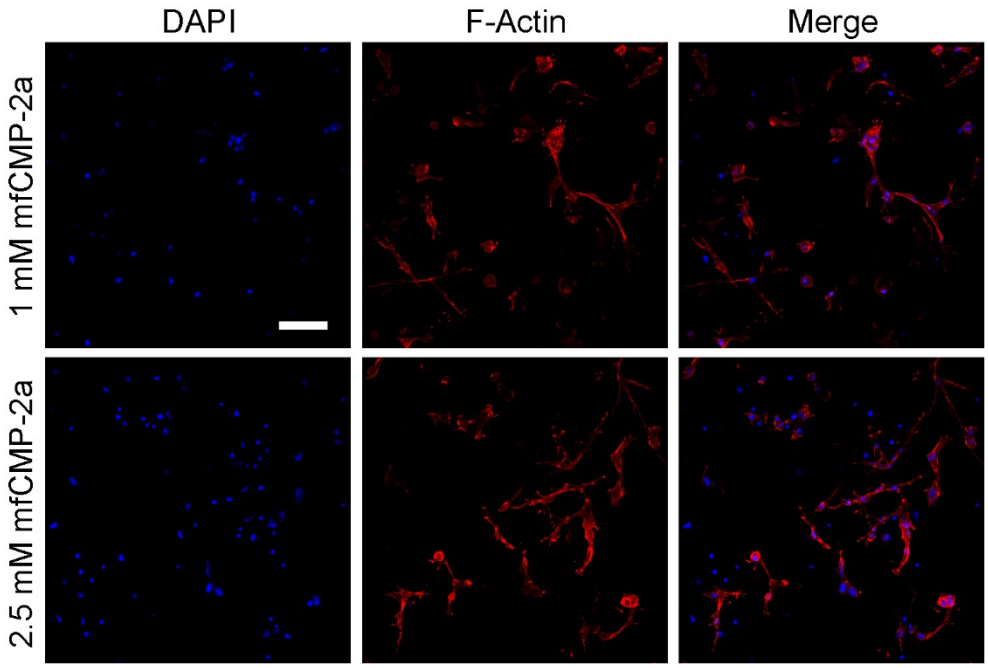

Figure S21: Day 10 image splits of cells cultured within hydrogels with mfCMP-2a. Representative maximum intensity projections (from z-stacks, 200 μm thick) of hMSCs encapsulated within hydrogels at a low concentration (1 mM) or at a high concentration (2.5 mM) of mfCMP-2a. Cell nuclei are blue (left, DAPI), cell bodies are red (center, F-Actin), and the merged images are shown on the right. Scale bar = 100 μm.

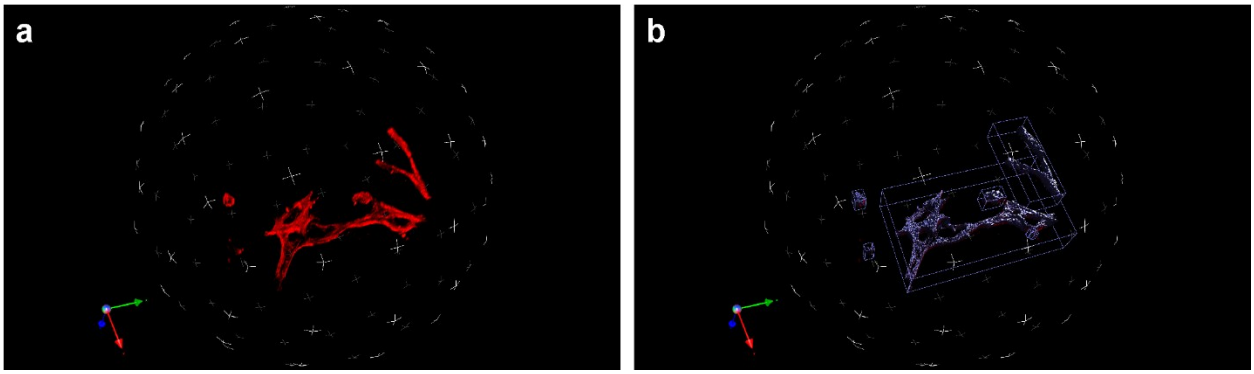

Figure S22: Volocity processing for shape factor analysis. **a**) An example 3D rendering of a confocal z-stack (200 μm thick) allows for visualization of cell interactions in the z direction in addition to the traditional x and y directions. **b**) Through Volocity processing (as described in the Methods section) we are able to isolate objects (either single cells or multiple cells in contact) and calculate the object surface area and volume in order to determine the shape factor.

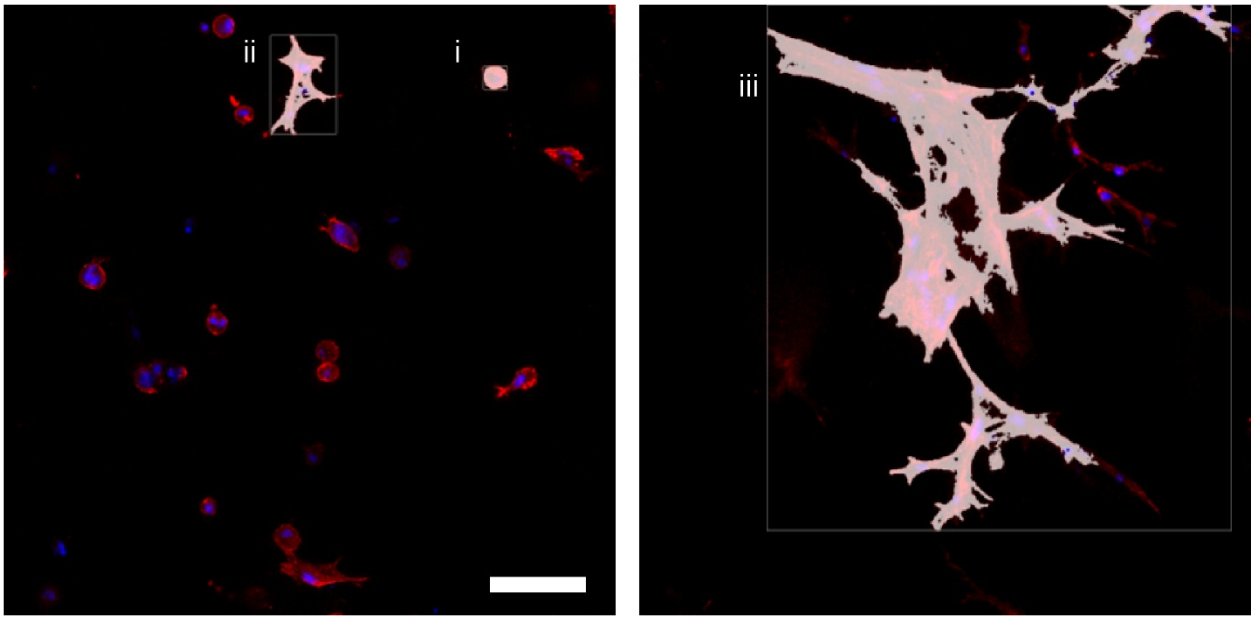

Figure S23: Volocity shape factor analysis and binning examples. Example images of cells in hydrogels with 1 mM mfCMP-1a (left) or 2.5 mM mfCMP-1a (right) demonstrating classification of objects (single cells or groups of cells in contact) as *rounded* (i), *spread* (ii), and *elongated* (iii). Scale bar = 100 μm.