Multicomponent dipeptide hydrogels as extracellular matrix-mimetic scaffolds for cell culture applications

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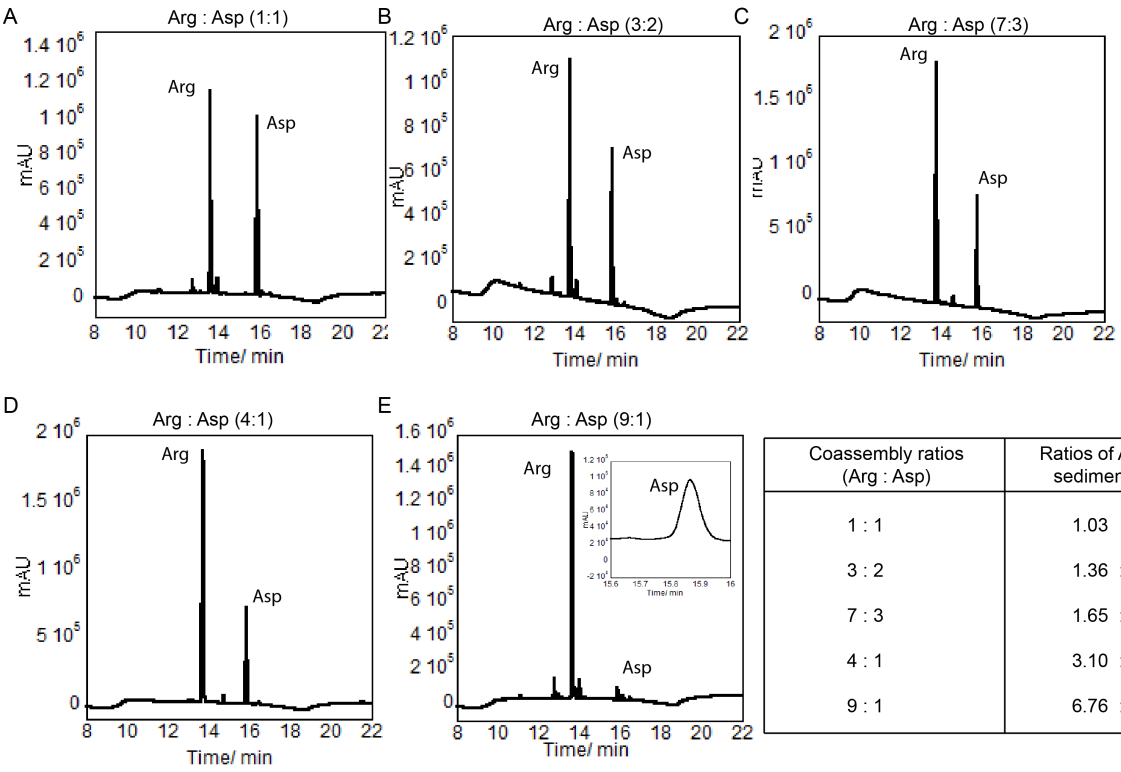
Table S1. Coassembly behavior of ionic-complementary dipeptides in water/DMSO[a].

Fmoc-3F-Phe-Arg-NH_2 : Fmoc-3F-Phe-Asp-OH	Hydrogel pH	Clarification time (min)[b]	Hydrogel appearance[c]	G′ (Pa)	G″ (Pa)	Fibril diameter (nm)
0:10	3.27	~2	OG	*NA*	*NA*	21±2
1:1	3.32	~1	TG	2481±132	197±30	10±1
3:2	3.48	~1	TG	2029±114	102±20	10±1
7:3	4.00	~5	TG	1826±117	94±20	14±2
4:1	4.06	~5	TG	1435±97	71±12	11±2
9:1	3.65	~5	TG	1540±125	92±11	16±2
10:0	NA	*NA*	TS	*NA*	*NA*	*NA*

[a]9.8 mM total dipeptide in 98% H_2O/2%DMSO, [b]Observed time required for optical transition from a opaque solution to transparent solution/hydrogel. [c]TG, transparent gel (stable to vial inversion); TS, transparent solution; OG, opaque gel. Errors are reported as the standard deviation about the mean for at least three replicate measurements of different gel samples for rheological experiments and the standard deviation about the mean for at least 100 measurements of unique fibril diameters in TEM images for fibril dimensions.

Table S2. Concentration of dipeptides in sedimented fibrils from coassembled Fmoc-3F-Phe-Arg-NH_2 (**2**):Fmoc-3F-Phe-Asp-OH (**1**) hydrogels.

Coassembly ratio (**2**:**1**)	Concentration of each component in sedimented fibrils (mM)	
	2	**1**
1:1	~4.94	4.94
3:2	3.22	3.95
7:3	4.89	2.96
4:1	4.90	1.98
9:1	6.01	~1.00



Coassembly ratios (Arg : Asp)	Ratios of Arg : Asp in sedimented fibrils
1 : 1	1.03 : 1.00
3 : 2	1.36 : 1.00
7 : 3	1.65 : 1.00
4 : 1	3.10 : 1.00
9 : 1	6.76 : 1.00

Figure S1. HPLC analyses of Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH ratios in sedimented fibrils from the hydrogel networks: A. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1), B. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (3:2), C. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (7:3), D. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (4:1), E. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (9:1).

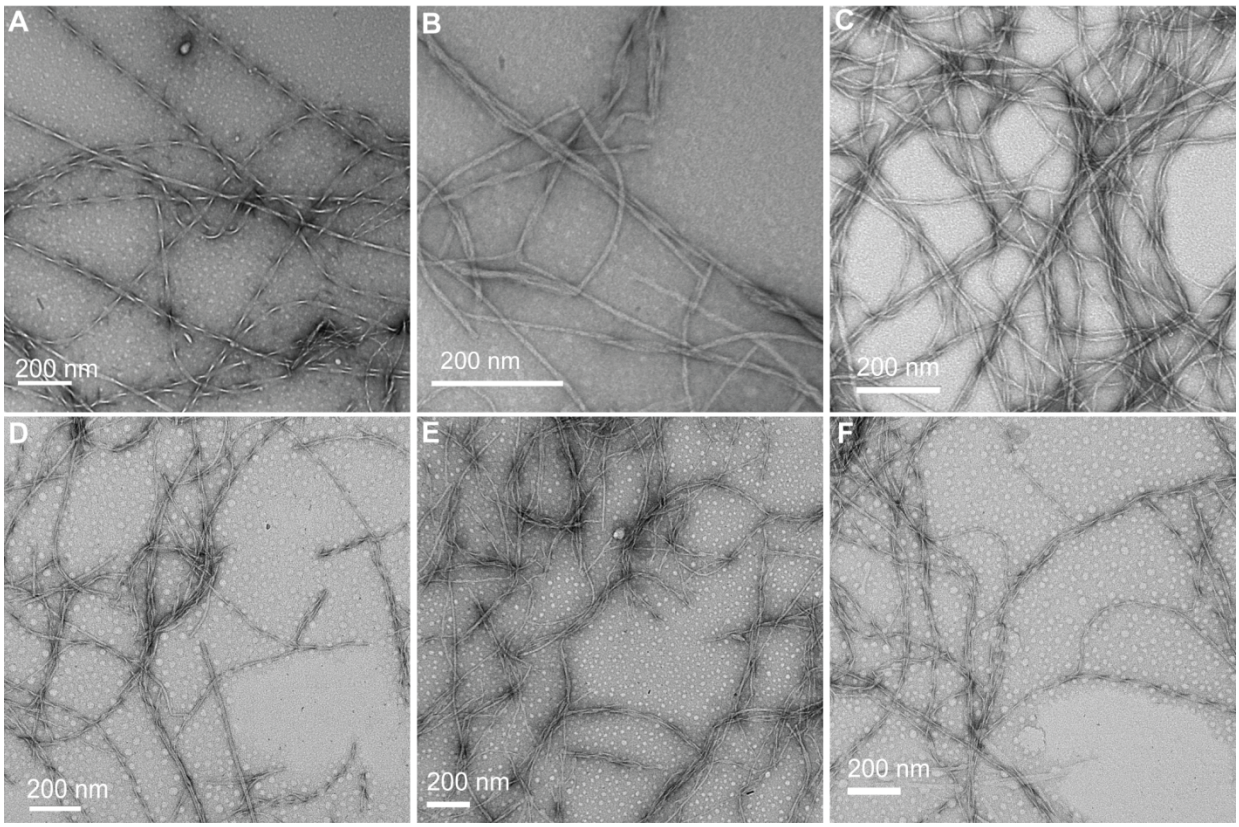


Figure S2. TEM images of fibrils formed by A. Fmoc-3F-Phe-Asp-OH, B. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1), C. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (3:2), D. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (7:3), E. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (4:1), F. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (9:1).

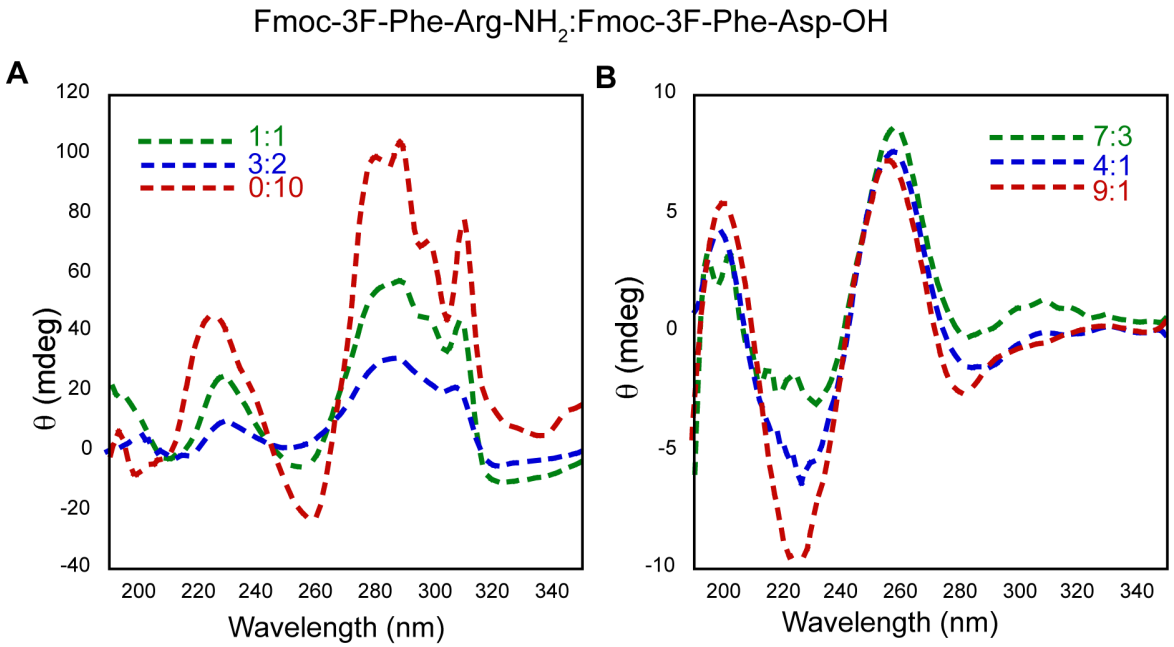


Figure S3. A. CD spectra of Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH coassembled hydrogels (1:1, green; 3:2, blue; 0:10, red). B.CD spectra of Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH coassembled hydrogels (7:3, green; 4:1, blue; 9:1, red).

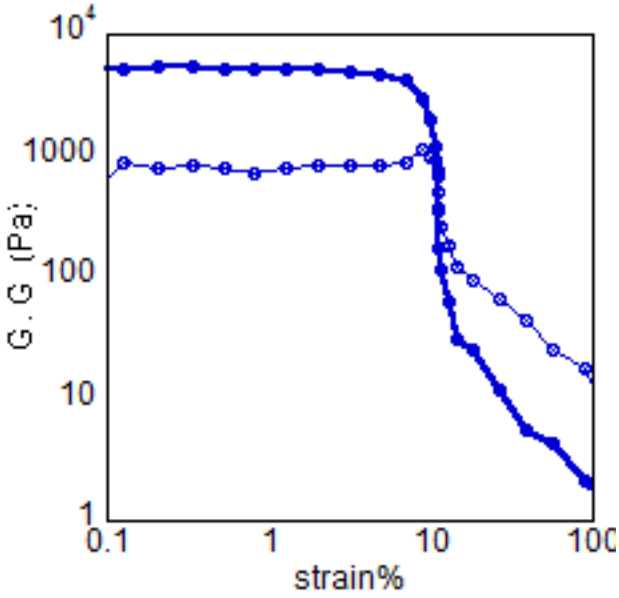


Figure S4. Representative strain sweep experiment for the 1:1 Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH coassembled hydrogel.

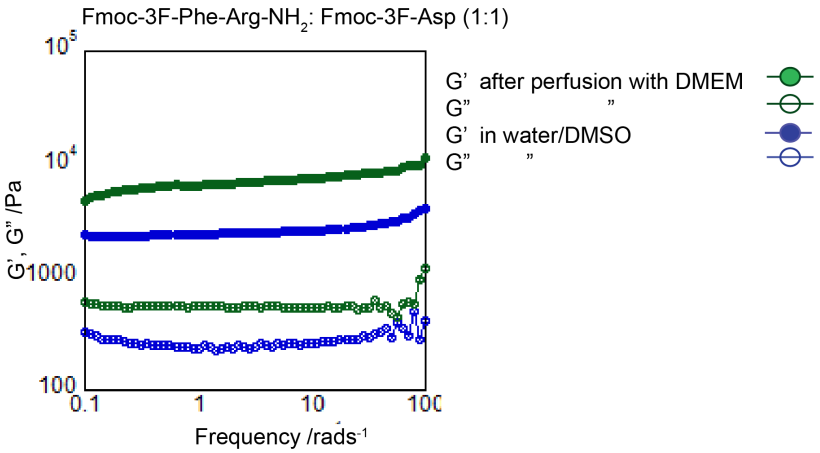


Figure S5. Representative rheological frequency sweep of the 1:1 Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH coassembled hydrogel before and after perfusion of the hydrogel with DMEM.

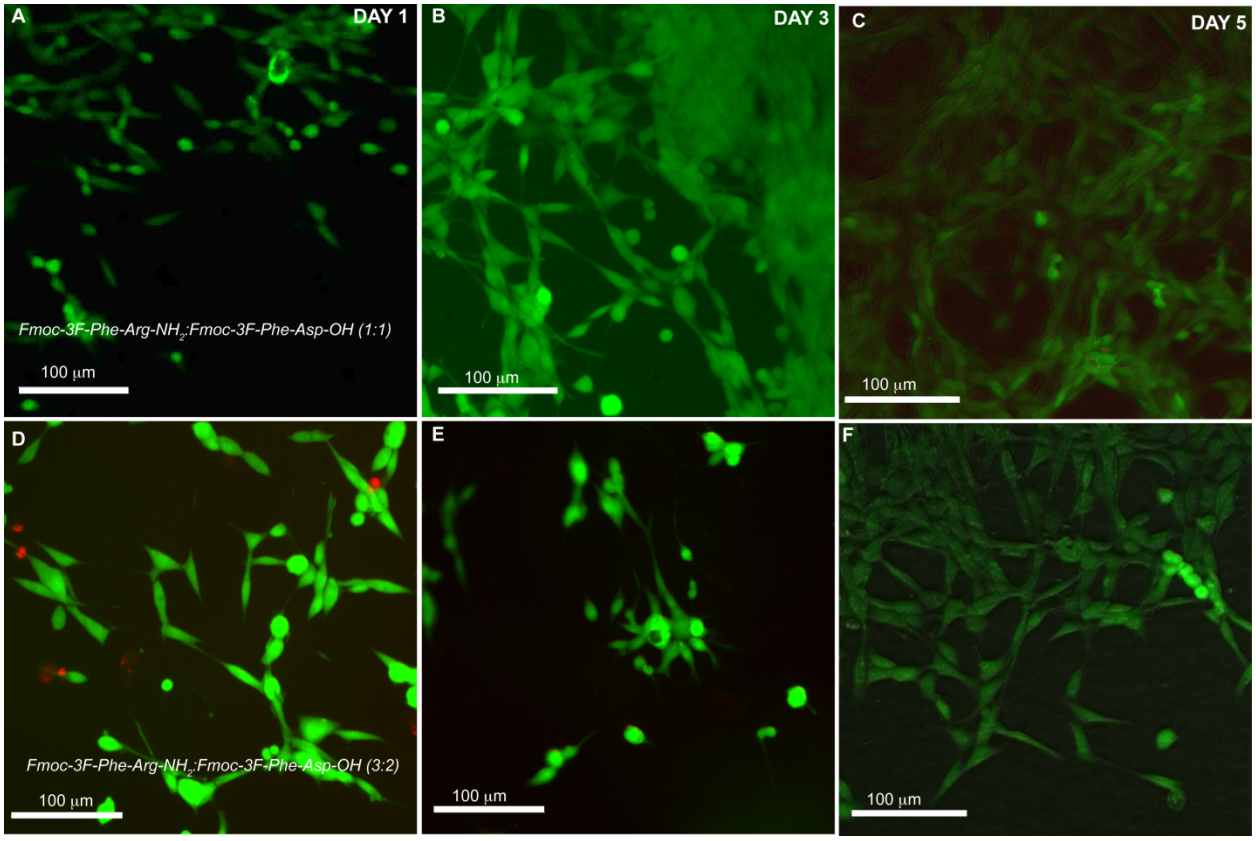


Figure S6. Fluorescent microscopy images of NIH 3T3 cells 1, 3, 5 days after cell seeding A–C. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH(1:1) coassembled hydrogel; D–F. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (3:2) coassembled hydrogel. Bright field image overlaid with images of dead cells stained red and live cells stained green.

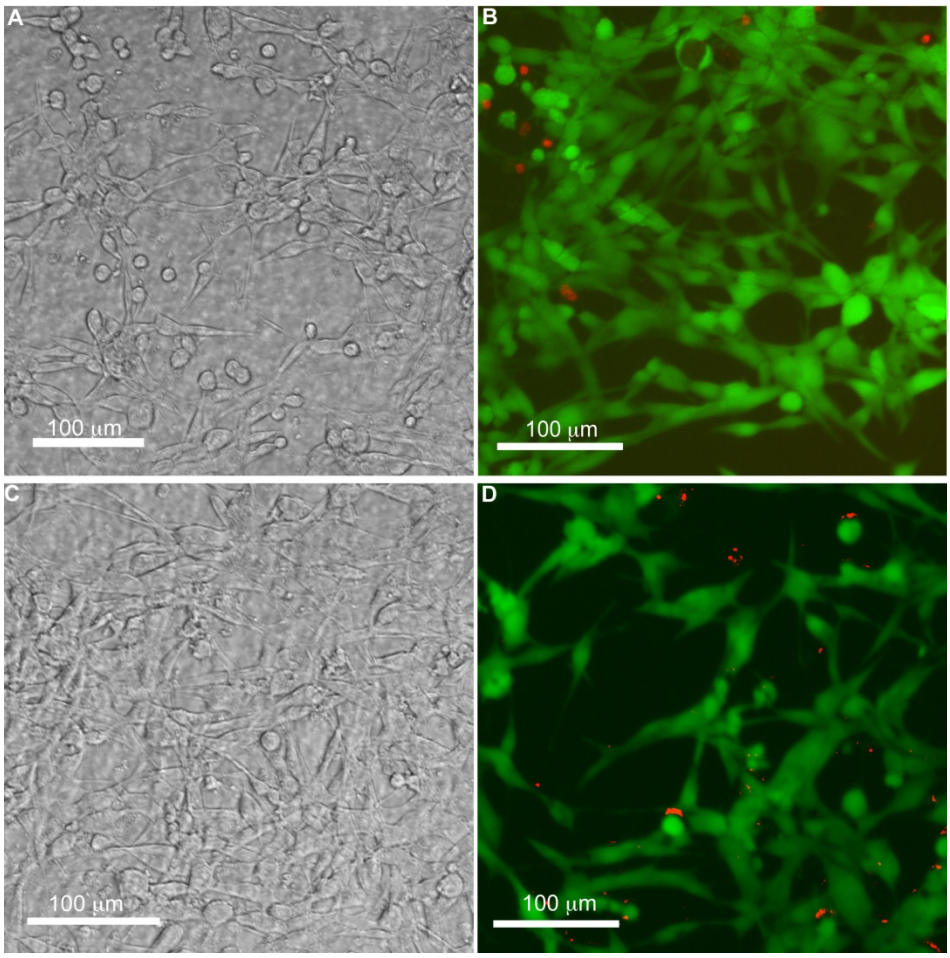


Figure S7. Fluorescent microscopy images of NIH 3T3 cells 2 days after cell seeding. A. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (7:3) hydrogel brightfield image with seeded cells; B. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (7:3) hydrogel image of LIVE/DEAD stained cells (live cells = green, dead cells = red); C. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (4:1) hydrogel brightfield image with seeded cells; D. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (4:1) hydrogel image of LIVE/DEAD stained cells (live cells = green, dead cells = red).

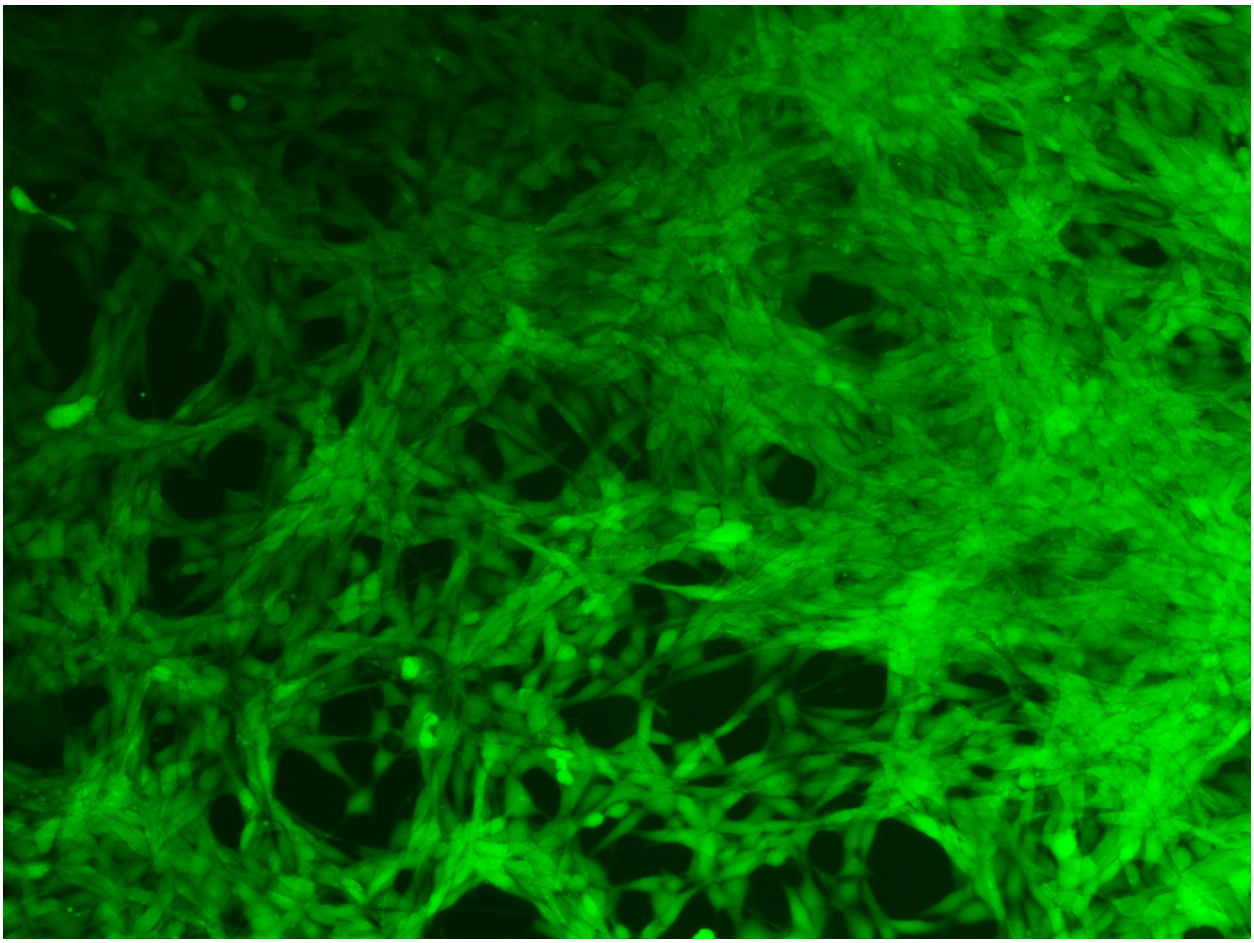


Figure S8. Fluorescent microscopy images of NIH 3T3 cells 5 days after cell seeding on Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1) coassembled hydrogels. This image indicates high cell density.

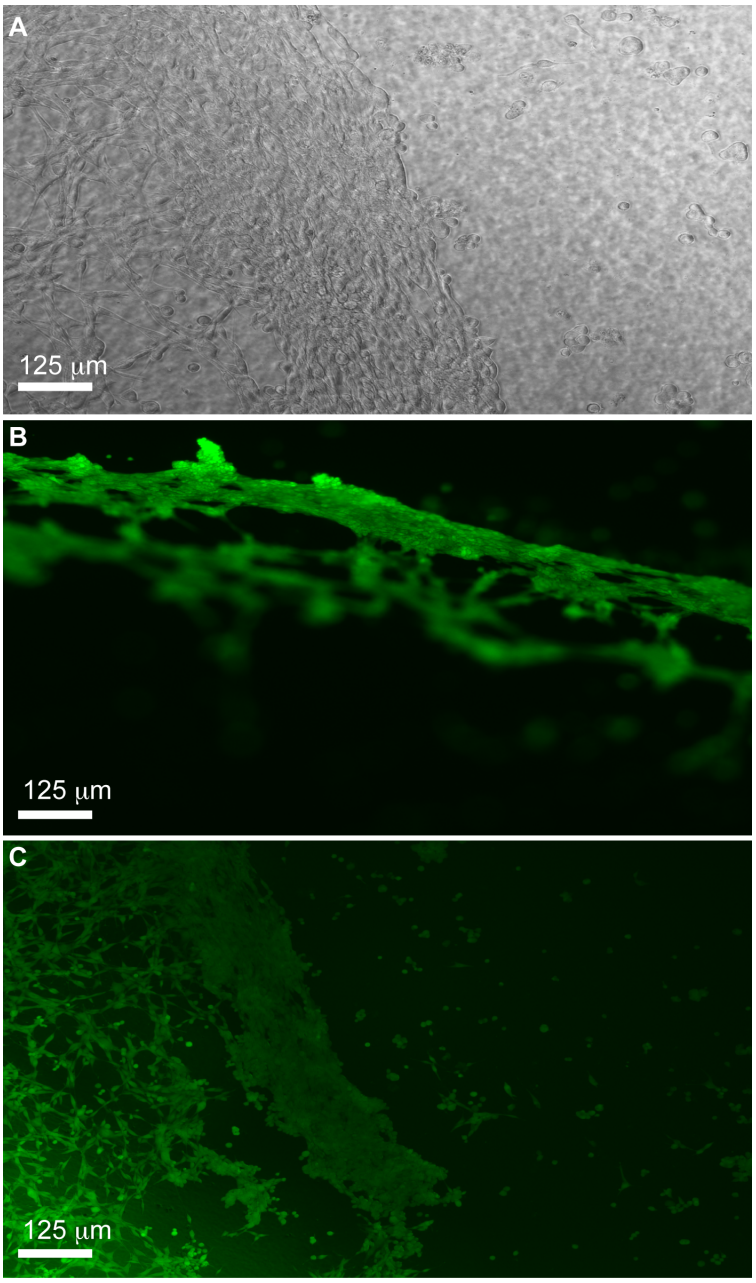


Figure S9. Fluorescent microscopy images of NIH 3T3 cells 2 days after cell seeding on Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1) coassembled hydrogels. A. Brightfield image (4× magnification). B. LIVE/DEAD stained cells (live cells = green, dead cells = red) (4× magnification); image is viewed from the edge of the gel indicating that the cells remain primarily at the hydrogel surface and fail to penetrate into the gel matrix. C. Image of LIVE/DEAD stained cells (live cells = green, dead cells = red) (10× magnification).

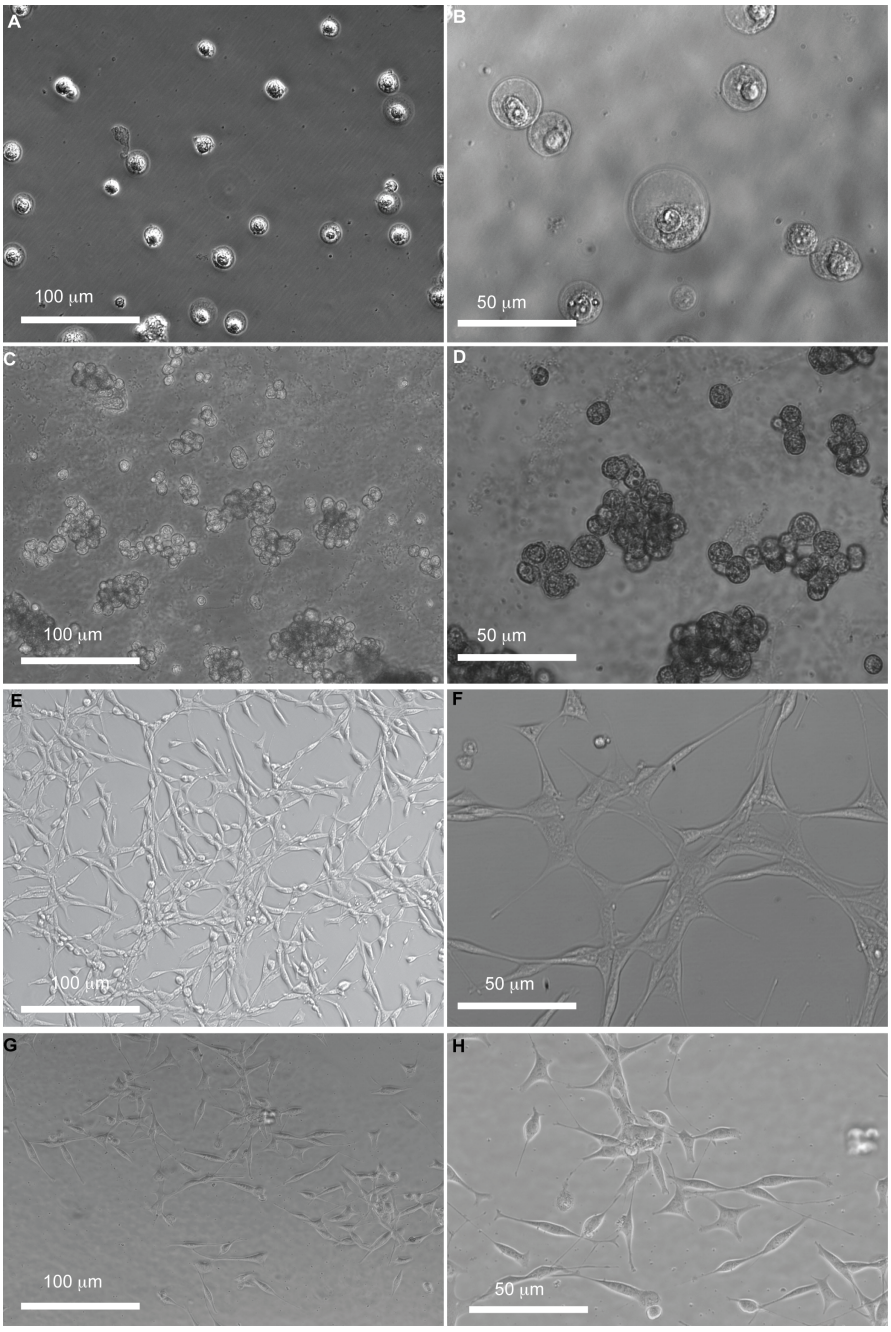


Figure S10. Brightfield microscopy images of NIH 3T3 cells seeded on: A. Fmoc-3F-Phe-Asp-OH hydrogel (10× magnification); B. Fmoc-3F-Phe-Asp-OH hydrogel (20× magnification); C. Fmoc-3F-Phe-Arg-NH_2 (10× magnification); D. Fmoc-3F-Phe-Asp-OH (20× magnification); E. on tissue culture treated plates (10× magnification); F. on tissue culture treated plates (20× magnification); G. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1) hydrogel (10× magnification) H. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1) hydrogel (20× magnification).

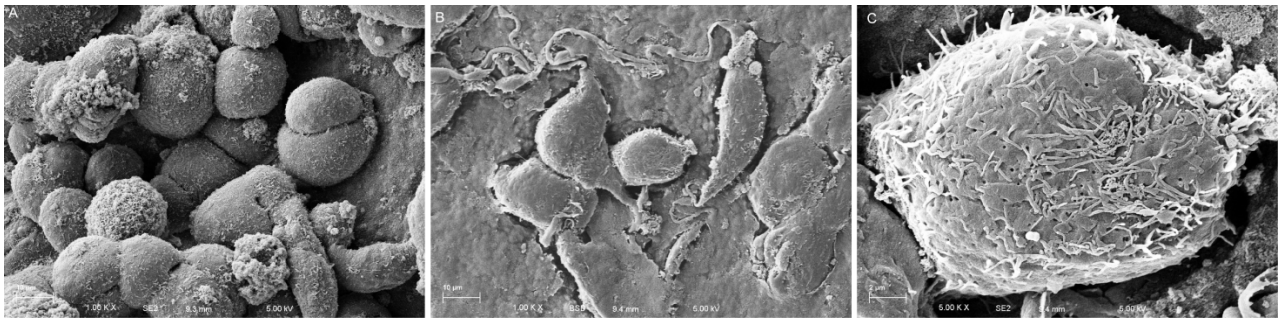


Figure S11. SEM micrographs of interaction between NIH 3T3 fibroblast cells and Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1) coassembled hydrogel after 2 days. A. 1000× magnification; B. 1000× magnification; C. 5000× magnification.

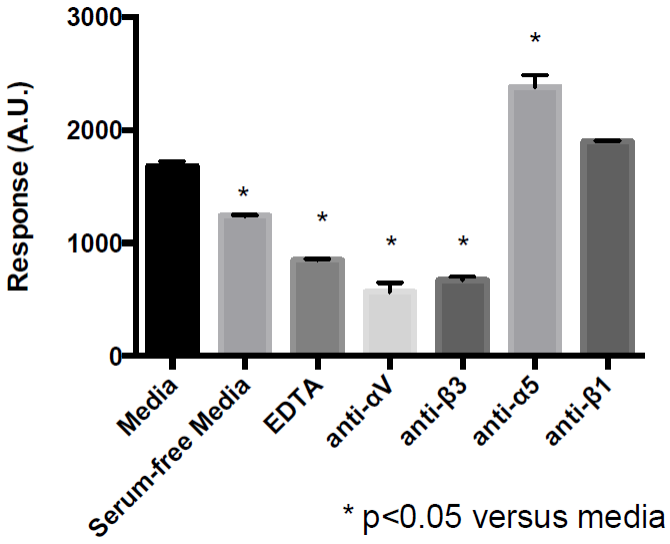
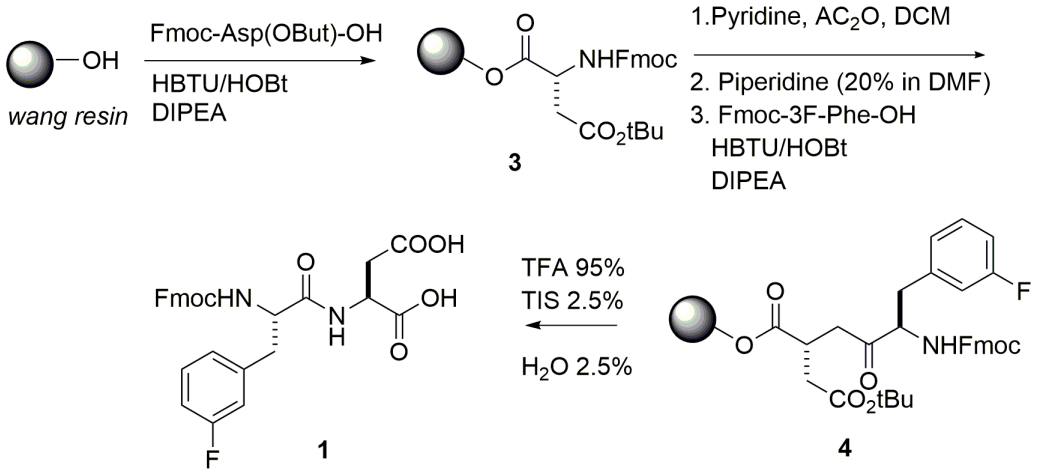


Figure S12. Results from integrin-blocking cell adhesion assay. Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1) coassembled hydrogels were incubated with integrin blocking antibodies (anti-αV, anti-β3, anti-α5, and anti- β1). Cells were then seeded on these hydrogels and incubated for 2 h at 37 ºC. Cells in treated tissue-culture plates were used as a positive control (Media) and EDTA-treated cells were used as negative controls (EDTA). Untreated cells on 1:1 hydrogels were also assessed. After 2 h of incubation, cells were stained with 2 mM calcein to detect live cells. It was found that cells incubated on anti-αV and anti-β3 treated hydrogels exhibited reduced viability, consistent with an RGD-integrin binding mechanism for cell adhesion.

Experimental Section

General. Unless stated otherwise, reactions were performed in flame-dried glassware under a positive pressure of argon or nitrogen. Commercial grade reagents and solvents were used without further purification except where noted. Water for gelation experiments was purified using ananopure filtration system (Barnstead, NANOpure Diamond 0.2 μm filter, 18 Ω). NMR spectra were recorded on a 500 MHz spectrometer. ^{1}H and ^{13}C NMR chemical shifts are reported as δ using residual solvent as an internal standard. ^{19}F NMR spectra were collected on a Brüker Avance-400 MHz NMR instrument. ^{19}F NMR chemical shifts are reported as δ using CF_3COOH (-76.55 ppm) as an internal standard.

Scheme S1. Solid-phase synthesis of Fmoc-3F-Phe-Asp-OH (**1**).



The Fmoc-3F-Phe-Asp-OH (**1**) dipeptide was synthesized using manual solid-phase peptide synthesis using standard Fmoc protection techniques. Wang resin (Novabiochem) was utilized and loaded using standard methods with O-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-

hexafluoro-phosphate (HBTU)/N-hydroxybenzotriazole (HOBt) activation. After the completion of amide bond couplings, the dipeptide was cleaved from the resin by treatment with trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5) for 2 h at room temperature. HPLC purification of the cleaved dipeptide was performed with a LC-AD HPLC system equipped with a variable wavelength absorbance detector (Shimadzu Scientific Instruments, Columbia, MD) using a reverse phase C18 column (Gemini 10 m 110A AXIA packed). A binary gradient of water (0.1% TFA) and acetonitrile (0.1% TFA) at 10 mL min^{-1} was used and the column eluents were monitored by UV absorbance at 215 and 254 nm. The purity of collected fractions was confirmed by analytical HPLC using an RP-C18 column (BEH300 10 μM, 4.6×250 mm; Waters). The purified peptide fractions were pooled, frozen, and lyophilized. Peptide identity was verified using matrix-assisted laser desorption ionization mass spectroscopy and then high resolution mass spectroscopy.^{1}H NMR (500 MHz, DMSO) δ 12.64 (s, 2H), 8.44 (d, J = 9 Hz, 1H), 7.91 (d, J = 8 Hz, 2H), 7.70-7.65 (m, 3H), 7.45-7.43 (m, 2H), 7.36-7.31 (m, 3H), 7.24-7.14 (m, 2H), 7.10-7.03 (m, 1H), 4.63 (d, J = 5 Hz,1H), 4.38-4.34 (m, 1H), 4.21-4.18 (m, 3H), 3.10 (d, J =17 Hz 1H), 2.84-2.65 (m, 3H) ppm; ^{13}C NMR (125 MHz, DMSO) δ 173.7, 171.7, 163.3, 161.5, 159.3, 159.0, 158.8, 158.6, 157.2, 156.2, 144.2, 144.1, 141., 141.5, 141.1, 130.3, 130.2, 128.1, 127.5, 125.8, 125.8, 125.6, 120.6, 118.6, 116.5, 116.3, 116.2, 113.7, 113.5, 66.2, 56.5, 52.5, 47.0, 37.6, 30.0, 25.7 ppm; ^{19}F NMR (376 MHz, DMSO) δ -115.81 ppm; HRMS (ESI-TOF) (*m/z*) [MNa^{+}] calcd for $C_{28}H_{25}FN_2O_7Na$ 543.1543 found 543.1543.

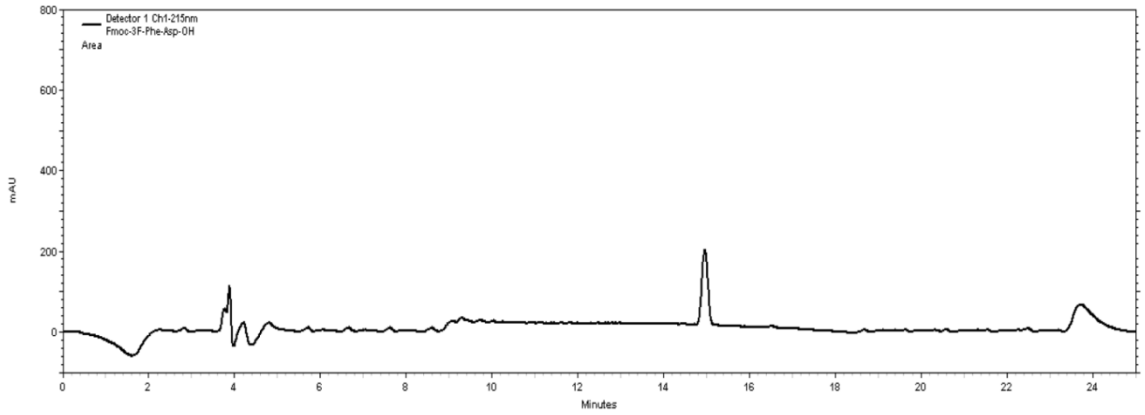


Figure S13. Analytical HPLC trace of Fmoc-3F-Phe-Asp-OH.

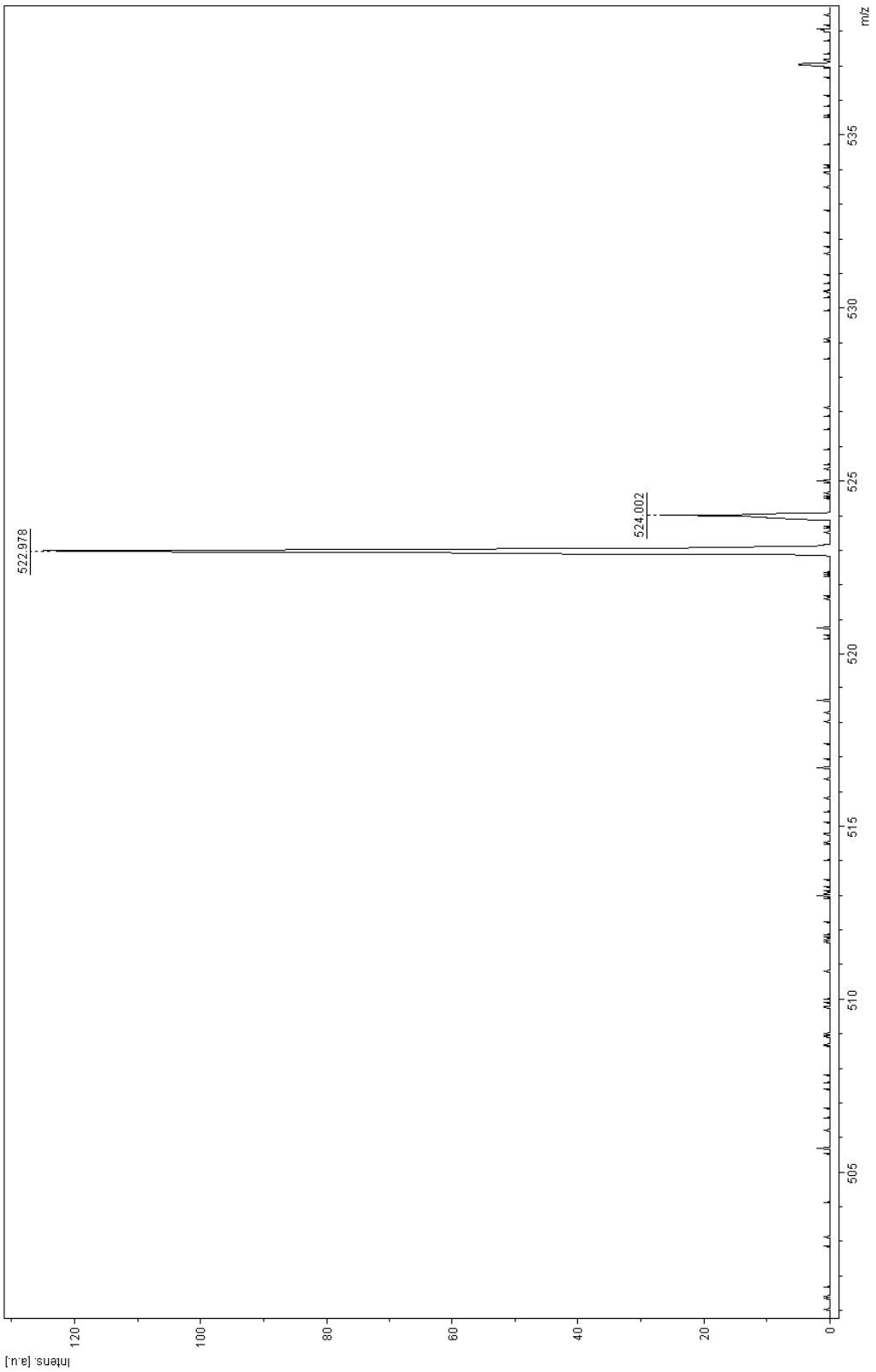
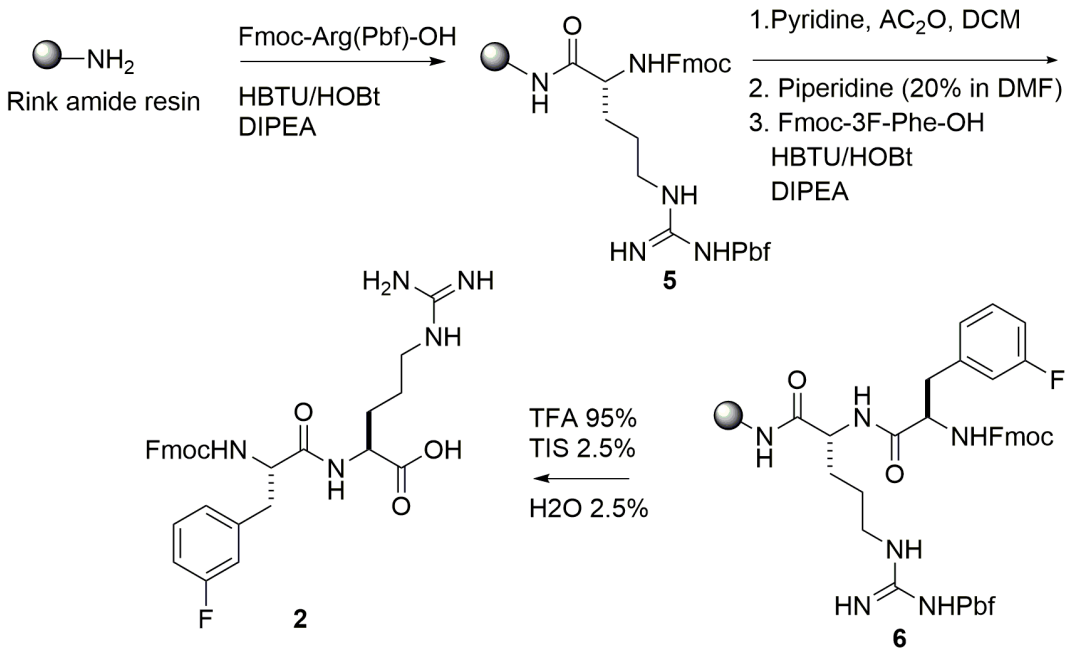


Figure S14. MALDI-TOF spectrum of Fmoc-3F-Phe-Asp-OH.

Scheme S2. Solid-phase synthesis of Fmoc-3F-Phe-Arg-NH_2 (**2**).



The Fmoc-3F-Phe-Arg-NH_2 di-peptide (**2**) was synthesized using manual solid-phase peptide synthesis with standard Fmoc methods. Rink amide resin (Advanced Chem Tech) was utilized and loaded using standard HBTU/HOBt activation. After the completion of couplings, the dipeptide was cleaved from the resin by treatment with trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5) for 2 h at room temperature. HPLC purification of the cleaved dipeptide was performed with a LC-AD HPLC system equipped with a variable wavelength absorbance detector (Shimadzu Scientific Instruments, Columbia, MD) using a reverse phase C18 column (Gemini 10 m 110A AXIA packed). A binary gradient of water (0.1% TFA) and acetonitrile (0.1% TFA) at 10 mL min^{-1} and the column eluents were monitored by UV absorbance at 215 and 254 nm. The purity of collected fractions was confirmed by analytical HPLC using an RP-C18 column (BEH300 10 mM, 4.6 250 mm; Waters). The purified peptide

fractions were frozen and lyophilized.^{1}H NMR (500 MHz, DMSO) δ 8.16 (d, J = 9 Hz, 1H), 7.91 (d, J = 7.5 Hz, 2H), 7.72-7.63 (m, 5H), 7.46-7.30 (m, 4H), 7.23-7.15 (m, 2H), 7.07-7.03 (m, 2H), 4.35-4.23 (m, 5H),2.71-2.80 (m, 1H),3.14-3.10 (m, 3H),1.74 (s, 1H), 1.59-1.52(m, 3H) ppm; ^{13}C NMR (125 MHz, DMSO) δ 172.7, 172.1, 171.8, 163.4, 161.5, 158.7, 158. 5, 156.2, 156.2, 155.3, 144.2, 144.1, 141.6, 141, 141.1, 130.3, 130.2, 128.3, 128.1, 127.5, 125.9, 125.8, 125.7, 120.5, 118.5, 117.5, 116.5, 116.4, 1 15.2, 113.6, 113.5, 111.5, 66.8, 66.2, 57.0, 56.1, 49.1, 47.0, 40.9, 37., 36.5 ppm; ^{19}F NMR (376 MHz, DMSO) δ -116.66 ppm; HRMS (ESI-TOF) (*m/z*) [MH^+] calcd for $C_{30}H_{34}FN_6O_4$ 561.2626 found 561.2624.

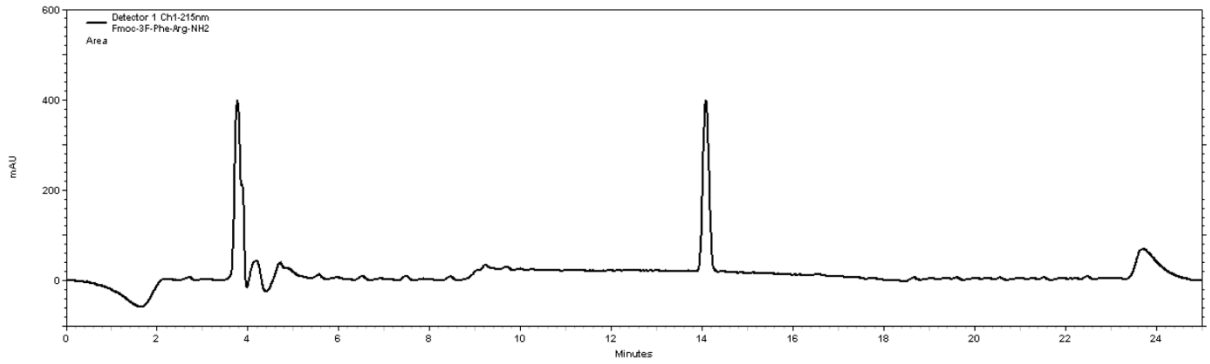


Figure S15. Analytical HPLC trace of Fmoc-3F-Phe-Arg-NH_2.

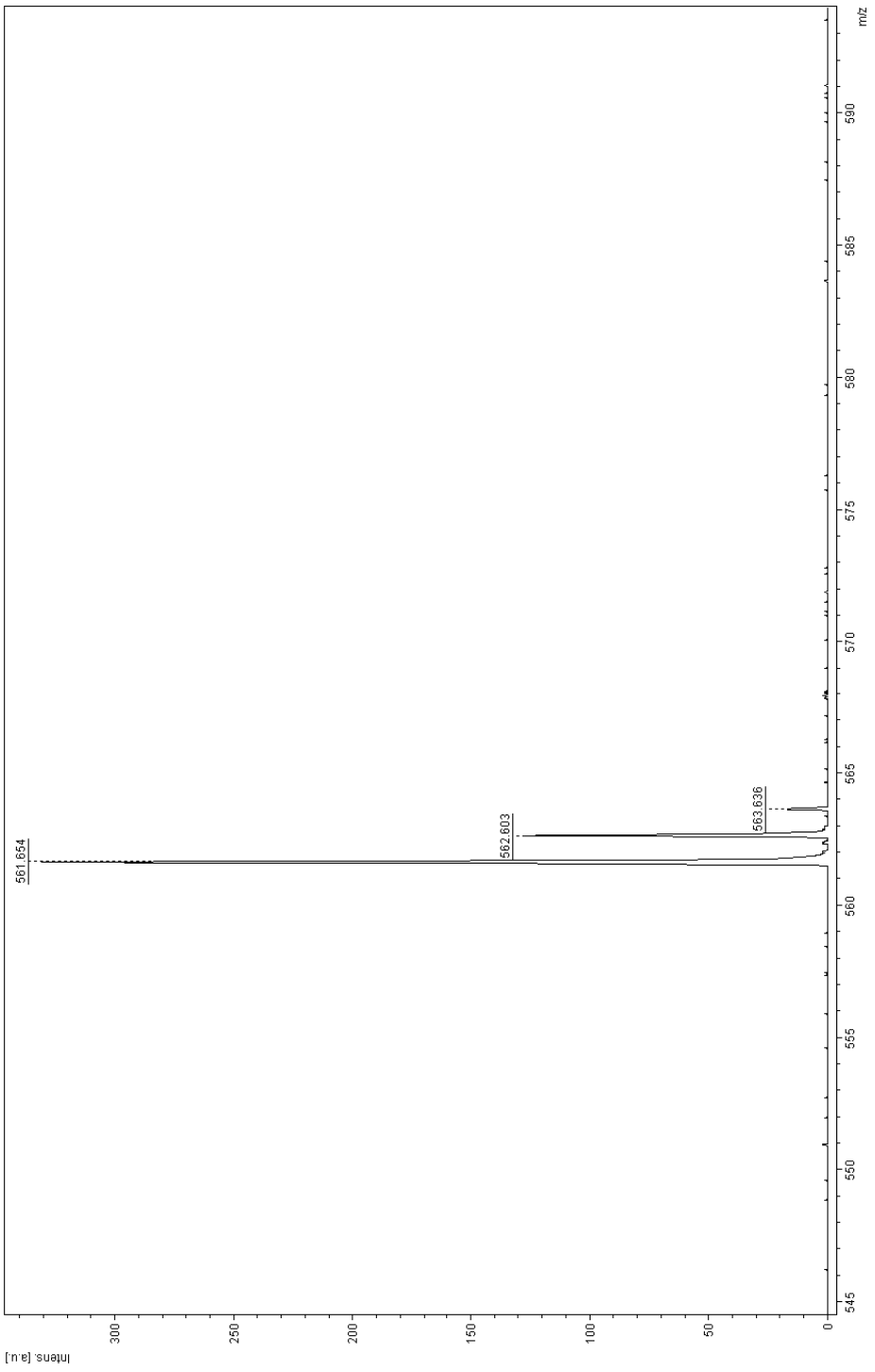


Figure S16. MALDI-TOF spectrum of Fmoc-3F-Phe-Arg-NH_2.

Materials and Methods

Coassembly and Hydrogelation Conditions

Each Fmoc amino acid derivative was dissolved in dimethyl sulfoxide (DMSO; Aldrich) at 247 mM. The DMSO stock solutions were then diluted into water and gently mixed using a pipette to a final amino acid concentration of 9.8 mM in 2% DMSO/H_2O (*v/v*). Coassembly experiments were conducted by mixing 247 mM DMSO stock solutions of each monomer in different ratios. The coassembly mixtures were diluted into water to a final concentration of 9.8 mM in 2% DMSO/H_2O (*v/v*). Following dilution, each sample was allowed to stand at room temperature. Each sample formed an opaque suspension that became an optically transparent hydrogel over time (see Table S1).

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on an AVIV 202 circular Dichroism spectrometer. Coassembly mixtures were prepared by mixing (247 mM) DMSO solutions in 1:1 ratios prior to dilution with unbuffered water to final concentrations of 9.8mM Fmoc amino acid in 2% DMSO/H_2O (*v/v*). The solutions were immediately transferred into a 0.1 mm path length quartz cuvette (Hellma), and gelation was allowed to proceed in the cuvette. Spectra were obtained from 350 to 190 nm with a 1.0 nm step, 1.0 nm bandwidth, and 6 s collection time per step at 25 ºC. The AVIV software was used for background subtraction and data smoothing with a least-squares fit.

Transmission Electron Microscopy (TEM)

Gel samples (10 μL) were applied directly onto 200 mesh carbon-coated copper grids and allowed to stand for 1 min. Excess gel was carefully removed by capillary action (using filter paper), and then the grids were stained with a saturated solution of freshly filtered uranyl acetate

(10 μL for 2 min). Excess stain was removed by capillary action, and the grids were allowed to air-dry for 10-15 min. Fibrils were imaged using a Hitachi 7650 transmission electron microscope with an accelerating voltage of 80 kV. Digital images were captured using an attached 11 megapixel Gatan Frlangshen digital camera system. Fibril dimensions were determined using ImageJ64 (http://rsbweb.nih.gov/ij/) software (fibril dimensions were reported as the average of at least 100 measurements of unique fibrils with the standard deviation about the mean).

Sedimentation Analysis to Determine Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH Ratios in the Assembled Hydrogel Fibril Networks

Hydrogels of Fmoc-3F-Phe-Arg-NH_2:Fmoc-3F-Phe-Asp-OH (1:1, 3:2, 7:3, 4:1, 9:1 ratios were assessed) were mechanically agitated by vortex in order to induce precipitation of the assembled fibril network. The precipitated fibrils were separated from soluble monomer by centrifugation. The resulting precipitate was dissolved in DMSO to disassemble the fibrils and the resulting solution was assessed by HPLC analysis. The concentrations of Fmoc-3F-Phe-Arg-NH_2 and Fmoc-3F-Phe-Asp-OH in the precipitated fibrils was determined by integration of the peak area and correlation to a standard concentration curve for each dipeptide.

Rheology

Rheological measurements were conducted on a Discovery HR-2 hybrid rheometer operating in oscillatory mode, using a 20 mm parallel plate geometry equipped with a solvent trap filled with silicon oil to prevent evaporation. DMSO stock solutions of Fmoc amino acid/Fmoc amino acid coassembly mixtures were diluted into water (9.8 mM, 2% DMSO/H_2O (*v/v*)) and immediately applied on to the Peltier plate; a dynamic time sweep was immediately performed at 25 ºC over

30–60 min with an angular frequency of 6.283 rad s^{-1} and 0.2% strain. A dynamic frequency sweep was performed from 0.1–100 rad s^{-1} using 0.2% strain at 25 ºC. All the measurements were performed in the linear viscoelastic region of each gel. The reported values for storage modulus (G′) and loss modulus (G″) are an average of at least three runs.

Cell culture

Mouse embryonic fibroblast cells (NIH 3T3) were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM;Gibco by Life Technologies) supplemented with 10% (*v/v)* fetal bovine serum (FBS; Atlanta Biologicals) and 1% *v/v* Penicillin/Streptomycin/fungizone solution (PSF; Thermo Scientific). Cells were cultured in tissue culture treated T-75 flasks in a 5% CO_2-humidified incubator at 37 ºC. Cells were seeded at 1×10^6 cells/flask and when confluent, incubated with 1% Trypsin-EDTA (Gibco by Life Technologies) to detach from the flask surface. Then cells were suspended in culture media to neutralize the trypsin and centrifuged at 1000 rpm for 5 min at 25 ºC to pellet the cells. The media was aspirated and the cell pellet was re-suspended in fresh cell culture media to a final volume of 5 mL. 10 μL of this solution was mixed with 90 μL of Trypan blue (Sigma) to distinguish live cells from dead cells for cell counting using a hemocytometer.

Cell culture on gels (2D)

The Fmoc-3F-Phe-Arg-NH_2 and Fmoc-3F-Phe-Asp-OH materials were sterilized by UV exposure and hydrogelation was initiated by dilution of sterile filtered DMSO stock solutions into water (4% DMSO in water, *v/v*). The resulting opaque solutions (100 μL) were transferred into a 24-well plate and allowed to form hydrogels. Coassembled Fmoc-3F-Phe-Arg-NH_2/ Fmoc-3F-Phe-Asp ionic complementary hydrogels were perfused with cell culture media

(DMEM from Gibco) at 37 ºC. After 24 h of perfusion, cell culture media was refreshed and NIH 3T3 cells were seeded on the hydrogels (50,000 cells/cm^2). The media was refreshed daily. Cell adhesion and morphology was observed using optical microscopy and fluorescence microscopy (Nikon).

Cell attachment Assays

Cell density was quantitatively investigated using LIVE/DEAD staining (Life Technologies). Cells were incubated in LIVE/DEAD reagents (2 mM calcein AM and 4 mM ethidium homodimer-1, Invitrogen) in cell media for 20–40 min. After 20–40 min incubation at 37 ºC images were taken using an inverted fluorescence microscope (Nikon Eclipse Ti 2000) with excitation filters of 450-490 nm for calcein AM (green = live cells), and 510-560 nm for ethidium homodimer (red = dead cells).

Cell fixation

Cells cultured on hydrogels formed on a round coverglasswere fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, post-fixed using the same buffer in 1.0% osmium tetroxide, then transitioned through a graded series of ethanol exchanges (3×). The slides were then transitioned into a graded series of 100% ethanol mixed with hexamethyldisilazane (HMDS) and finally with three exchanges of 100% HMDS. The last change was allowed to evaporate off of the cover glasses overnight in a fume hood.

Scanning Electron Microscopy (SEM)

The dried round cover glass, mounted onto an aluminum stub and sputter coated (2 min) with gold at 1Å/s using a low vacuum sputter coating (DENTON VACUUM Desk II) system under 100 mTorr pressure in 15 mA current to achieve conformal conductive coating on the samples.

Images were taken using a Zeiss Aurgia Supra40-VP field emission scanning microscope set up in backscatter electron detector mode with an accelerating voltage of 5 kV or with the accelerating voltage as on micrographs presented.

Integrin blocking adhesion assay

A 96-well plate was coated with Arg/Asp (1:1) coassembled hydrogels. NIH 3T3 cells were pre-incubated in suspension (after trypsinization) with integrin-blocking antibodies (anti-α5, -αv, -β3, -β1, (25 μg/mL), BD Biosciences) or ethylenediamine tetraacetic acid (EDTA, Thermo Scientific™) (20 mM) for 45 min. Cells (~10×10^3 cells/cm^2) were seeded on hydrogels and incubated for 2 h at 37 ºC; adherent cells were stained with 2 mM calcein AM (Invitrogen) for 20-40 min. Fluorescence intensity of the seeded cells was quantified using a TECAN infinite plate reader with excitation at 490 nm and emission at 520 nm. Untreated cells adhered to Arg/Asp (1:1) gel and untreated cells adhered to tissue culture treated 96-well plate were used as positive controls while EDTA-treated cells were negative controls. Untreated cells seeded on hydrogels in the presence of serum-free DMEM and untreated cells in tissue culture treated 96-well plate under serum-free DMEM were also assessed for comparison.

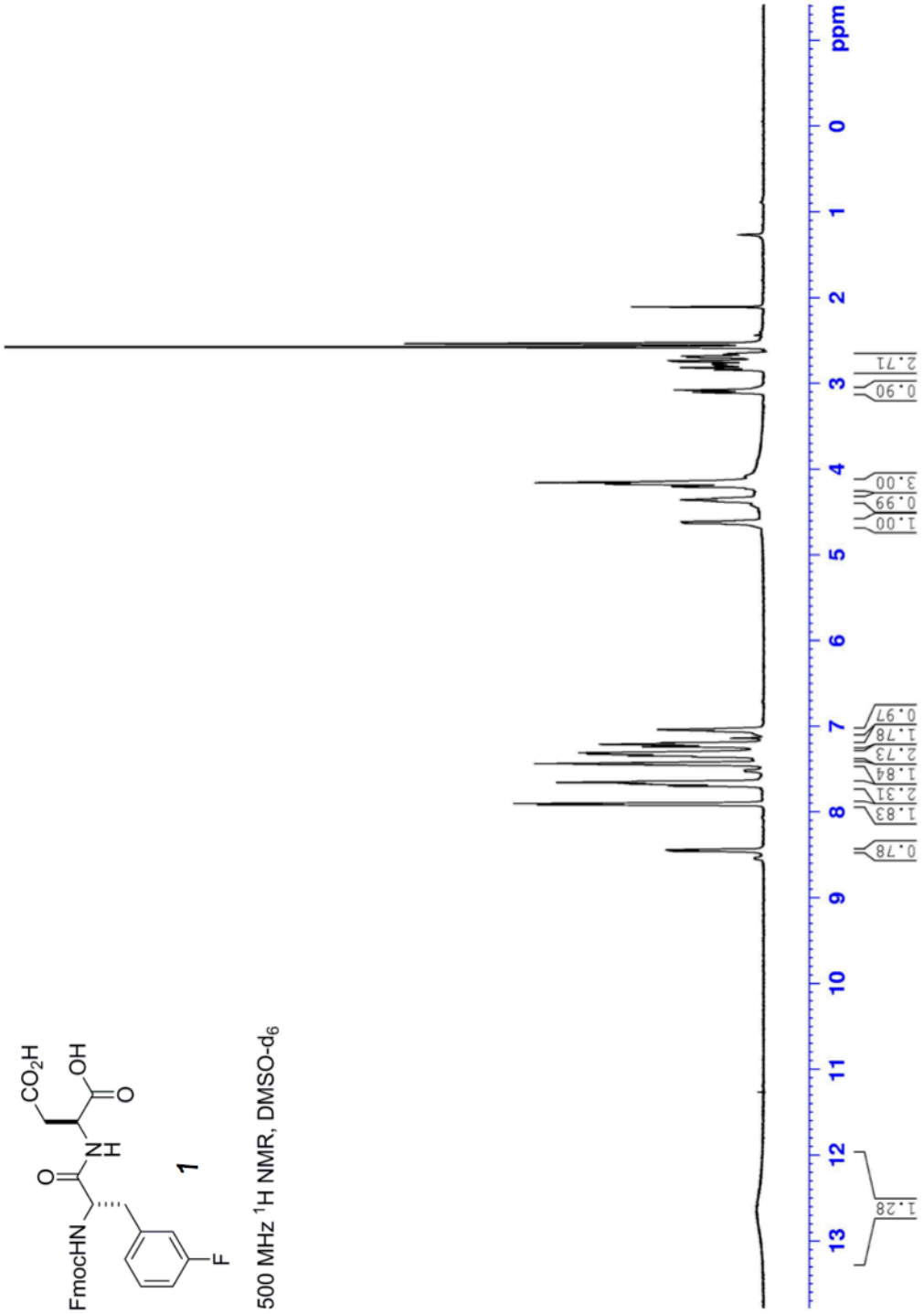


Figure S17. ^{1}H NMR of Fmoc-3F-Phe-Asp-OH.

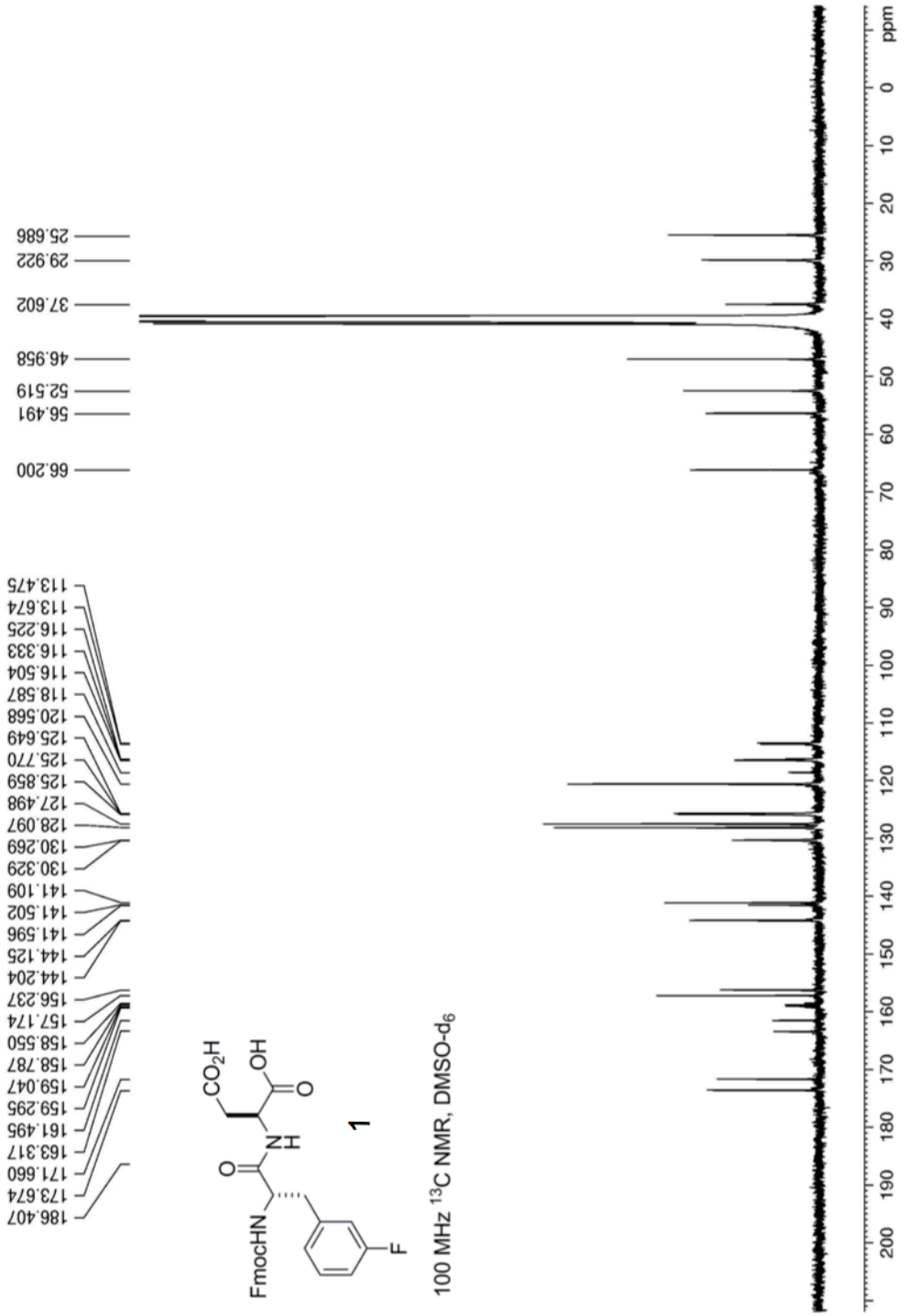


Figure S18. ^{13}C NMR of Fmoc-3F-Phe-Asp-OH.

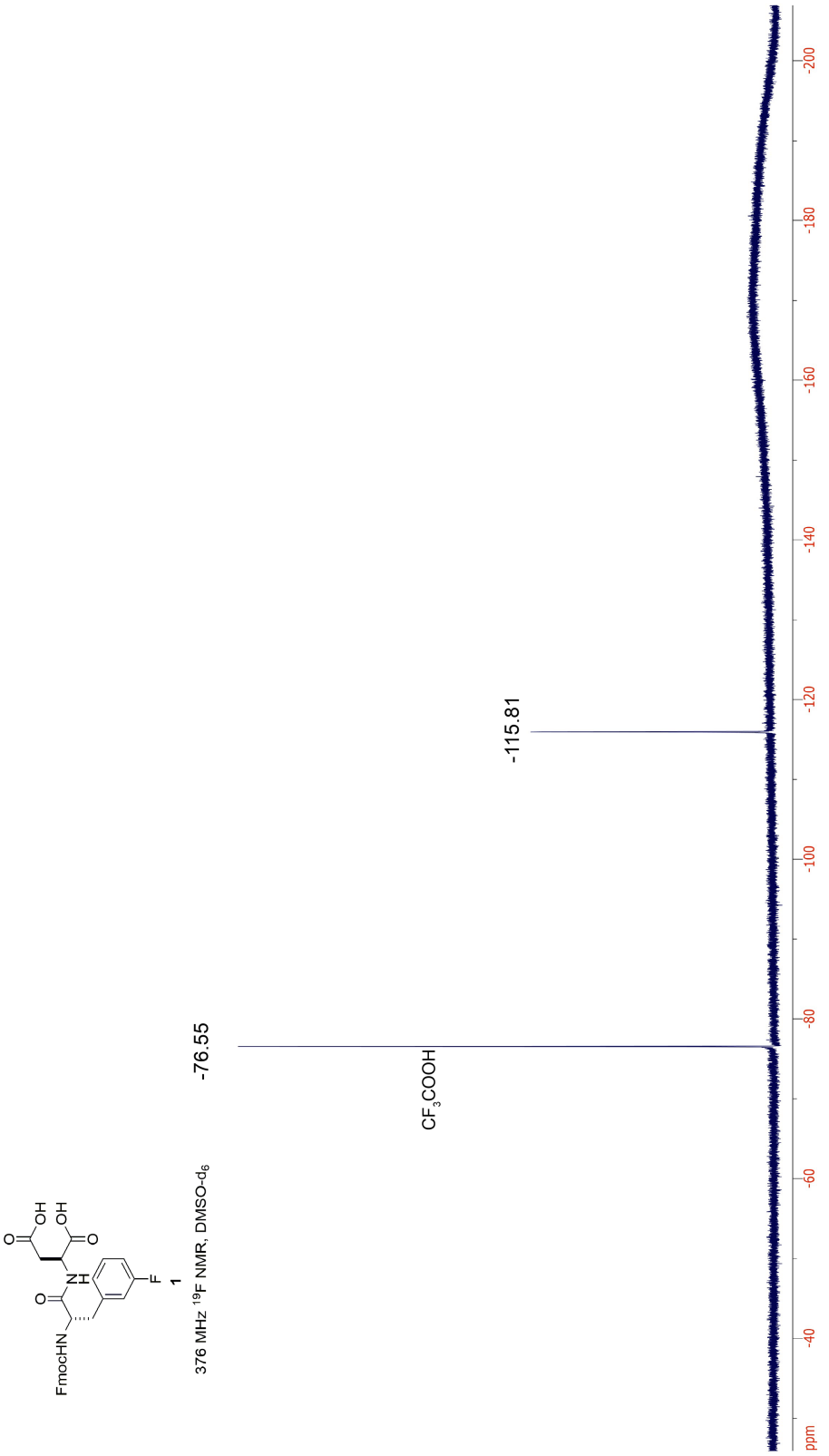


Figure S19. ^{19}F NMR of Fmoc-3F-Phe-Asp-OH.

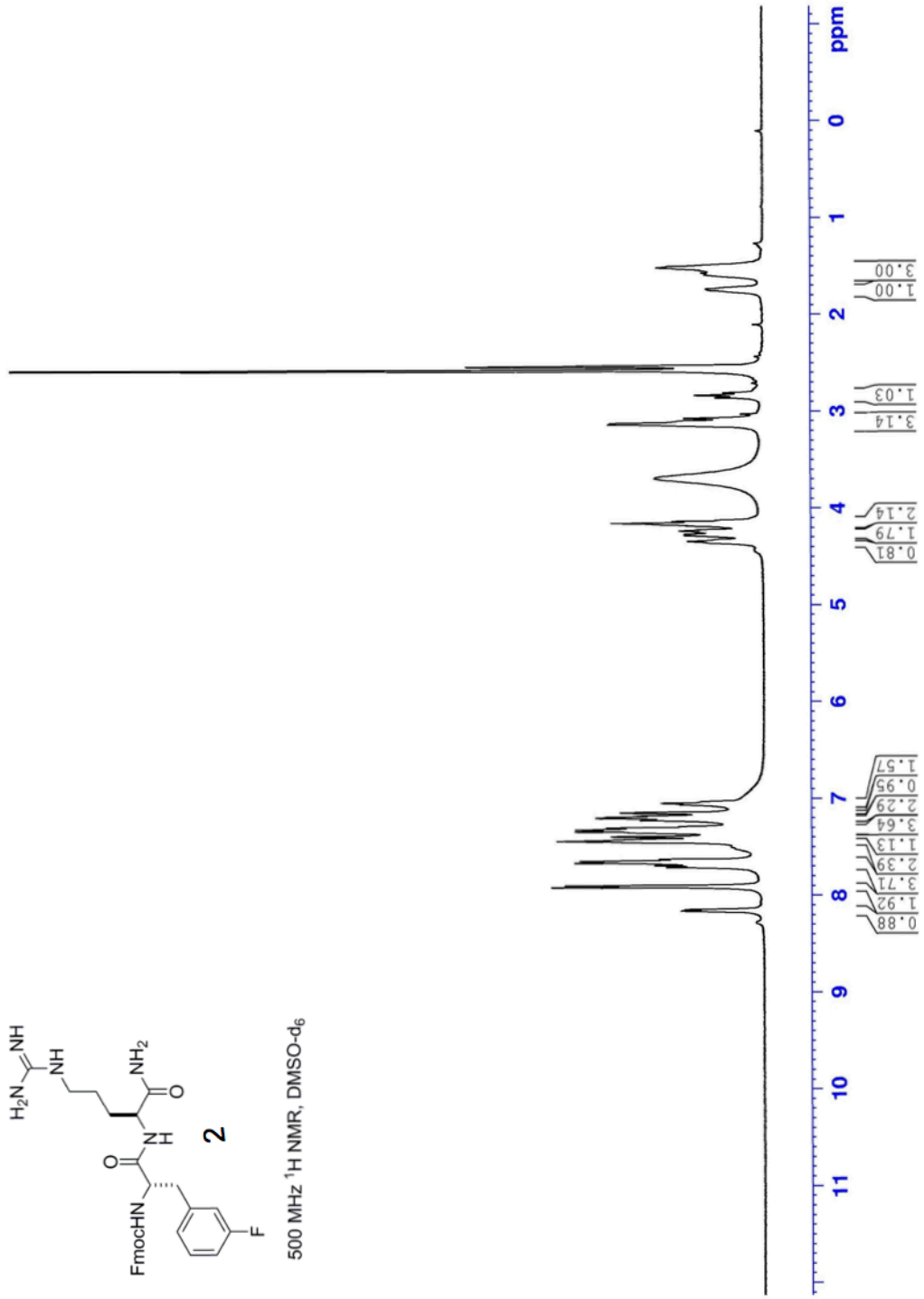


Figure S20. ^{1}H NMR of Fmoc-3F-Phe-Arg-NH_2.

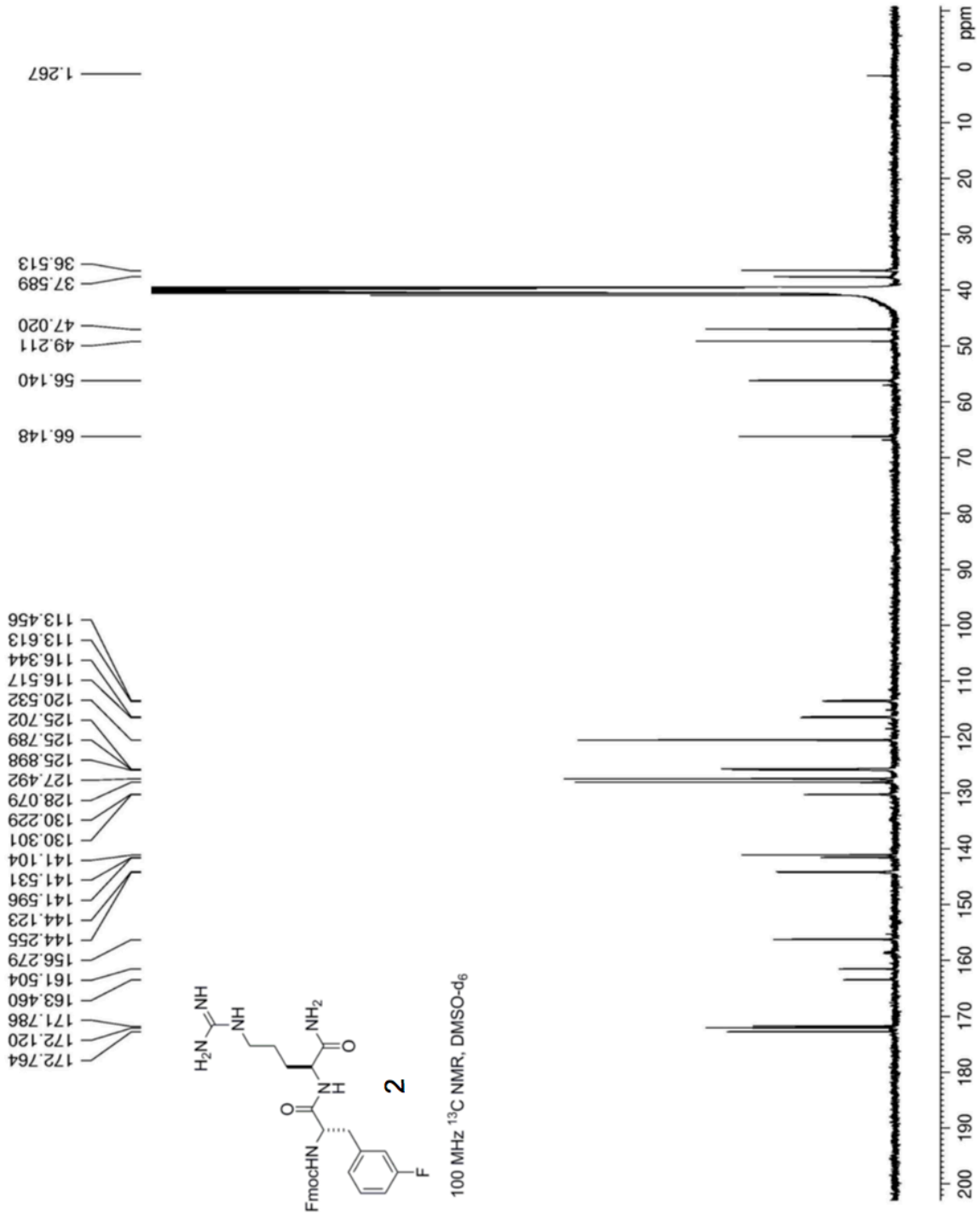


Figure S21. ^{13}C NMR of Fmoc-3F-Phe-Arg-NH_2.

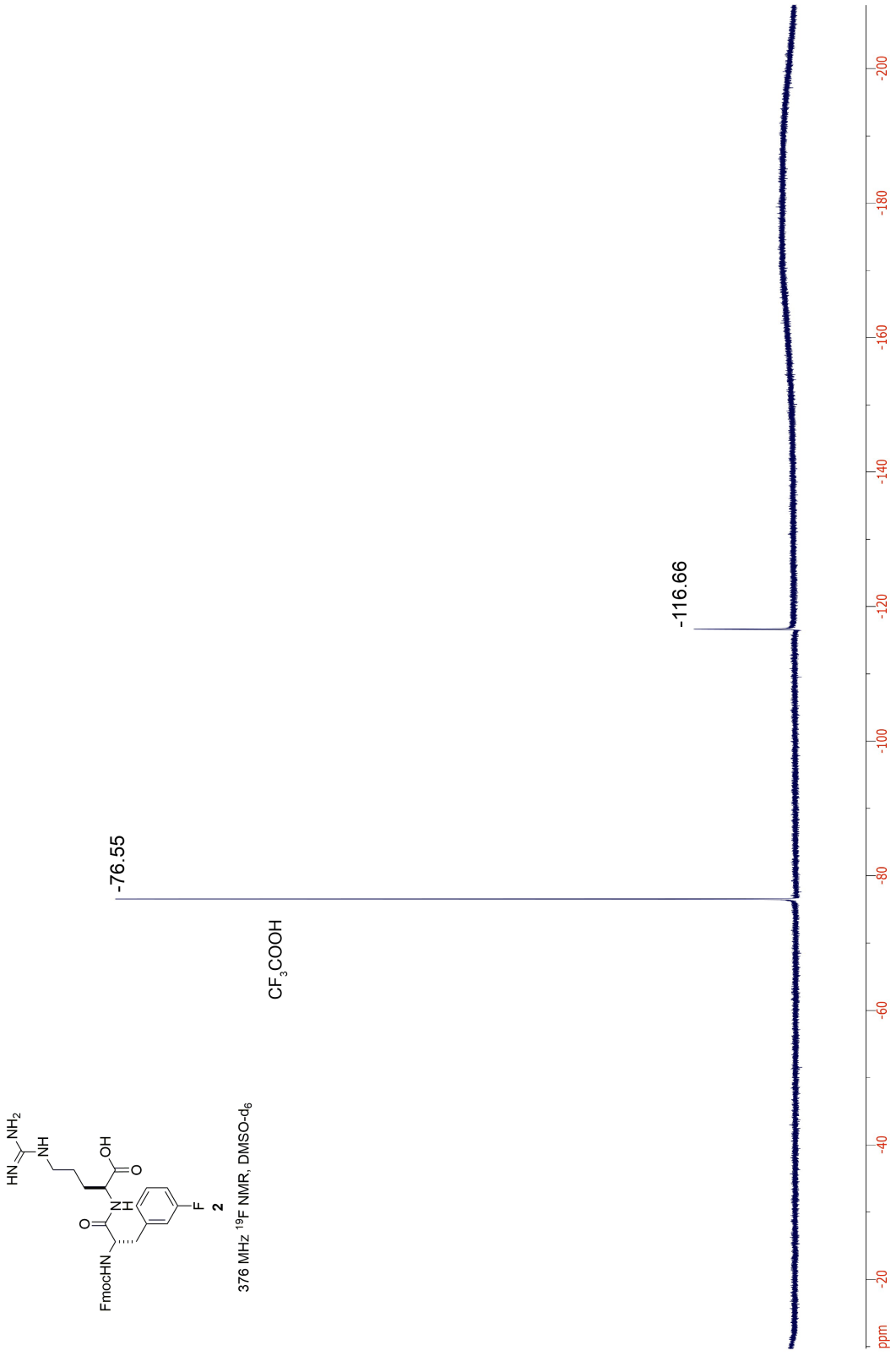


Figure S22. ^{19}F NMR of Fmoc-3F-Phe-Arg-NH_2.