Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Information

Materials

All fluorenylmethyloxycarbonyl chloride (Fmoc)-protected amino acids were purchased from AAPPTec (Louisville, KY, USA) except for Fmoc-Lys(N_3)-OH, which was purchased from Chem-Impex (Wood Dale, IL, USA). Piperidine, O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluoro-phosphate (HBTU), Fmoc-Rink-amide 4-methylbenzhydryalmine (MBHA) resin, and diisopropylethylamine (DIEA) were also purchased from AAPPTec. Diethyl ether (DEE), trifluoroacetic acid (TFA), acetonitrile (ACN), N,N-dimethyl formamide (DMF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), anhydrous N-methyl pyrrolidine (NMP), deuterated dimethyl sulfoxide (DMSO-d_6), diisopropylcarboimide (DIC), sodium hydroxide (NaOH), and isopropyl alcohol (IPA) were purchased from VWR (Radnor, PA, USA). Triisopropylsilane (TIS), biotin, poly(caprolactone) (PCL) (M_W 14,000 Da), deuterated dichloromethane (CD_2Cl_2), bovine serum albumin (BSA), polyoxyethylenesorbitan monolaurate (TWEEN 20), streptavidin-conjugated fluorescein isothiocyanate (FITC), dibenzocyclooctyne-cyanine3 (DBCO-Cy3), Triton-X, N-acetyl cysteine, papain, ethylenediaminetetraacetic acid (EDTA), Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and Oxyma were purchased from MilliporeSigma (Burlington, MA, USA). Dithiothreitol (DTT) was purchased from AGTC bioproducts (Alachua, FL, USA). Ninhydrin test kit was purchased from Anaspec (Fremont, CA, USA), while p-maleimidophenylisocyanate (PMPI) was purchased from Chem-Impex. PCL (M_W 80,000 Da) was generously provided by Polysciences, Inc. (Warrington, PA, USA). 1,1,1,3,3,3,-Hexafluoro-2-propanol (HFIP) was purchased from Oakwood Chemical (Estill, SC, USA), and phosphate buffer saline (PBS) tablets were purchased from AMRESCO (Solon, OH, USA). All cell culture reagents were purchased from ThermoFisher Scientific (Waltham, MA, USA) and all cell culture plasticware were from VWR, unless otherwise stated. Trypsin ethylenediaminetetraacetic acid (EDTA) was purchased from ATCC (Manassas, VA, USA). Antibiotic/antimycotic was purchased from Caisson Labs (Smithfield, UT, USA), while calf thymus DNA (CTDNA) was purchased from VWR. Quan-iT™ Picogreen® dsDNA Assay Kit and rhodamine-phalloidin were purchased from ThermoFisher Scientific. Paraformaldehyde (PFA) was purchased from Alfa Aesar (Haverhill, MA, USA) and Hoescht 33258 was purchased from MilliporeSigma.

Peptide Synthesis and Purification

CYGGGRGDSK(biotin) (RGDS(biotin)) and CYGGGRGESK(azide) (RGES(azide)) peptides with sequences shown in Fig. S1 were synthesized at a 1 mM scale using standard Fmoc solid phase

peptide synthesis (SPPS) techniques using a CEM Liberty Blue automated microwave peptide synthesizer (CEM Corporation, Matthews, NC, USA). For all amino acids added on the automated microwave peptide synthesizer, coupling reactions were activated using 3 molar equivalents each of DIC and Oxyma. Both peptides were synthesized on Fmoc-Rink-amide 4-methylbenzhydryalmine (MBHA) resin (100-200 mesh, 0.67 mmol/g functionalization). For the RGDS(biotin), an Fmoc-Lys(Mtt)-OH was coupled manually using methods described below (see *Biotinylation of RGDS(biotin)*), and the remaining amino acids were coupled using the automated microwave peptide synthesizer.

Peptides were cleaved from the resin in a solution of 95% (v/v) TFA, 2.5% (v/v) TIS, 2.5% (v/v) ultrapure water, and 2.5% (w/v) DTT for 3 hours. TFA was removed using rotary evaporation, and the product was precipitated in cold DEE. The precipitate was collected using centrifugation then allowed to dry overnight under vacuum.

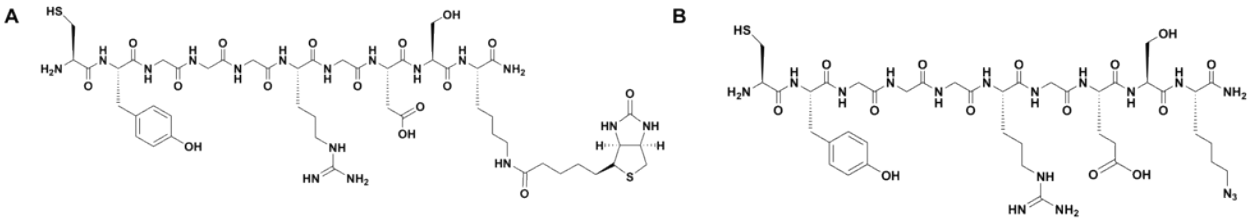


Fig. S1 Chemical structure of (A) RGDS(biotin) and (B) RGES(azide) peptides.

The crude peptides were dissolved in acidic mobile phase (95% (v/v) ultrapure water, 5% (v/v) spectroscopic grade ACN, 0.1% (v/v) TFA), sonicated until fully solubilized, and passed through a 0.45 µm filter before purification. Peptides were purified using reversed-phase preparative high-performance liquid chromatography (HPLC; Agilent 218 Prep HPLC, Agilent Technologies, Santa Clara, CA, USA) with a mobile phase gradient of 5%/95% ACN/water to 100% ACN with 0.1% TFA on an Agilent 5 Prep-C18 column (150 x 21.2 mm, 5 µm pore size, 100 Å particle size). Fractions were collected separately, rotary evaporated to remove excess ACN, and lyophilized with a Labconco Freezone freeze dryer (Labconco, Kansas City, MO, USA). The masses for each purified peptide were verified using electrospray ionization mass spectrometry (ESI-MS; Applied Biosystems 3200 Q Trap, Foster City, CA, USA). The purified peptides were also evaluated by HPLC with an Agilent 5 Prep-C18 analytical column (150 x 4.6 mm, 5 µm pore size, 100 Å particle size). ESI-MS and analytical HPLC spectra for each purified peptide are shown in Fig. S2 and S3.

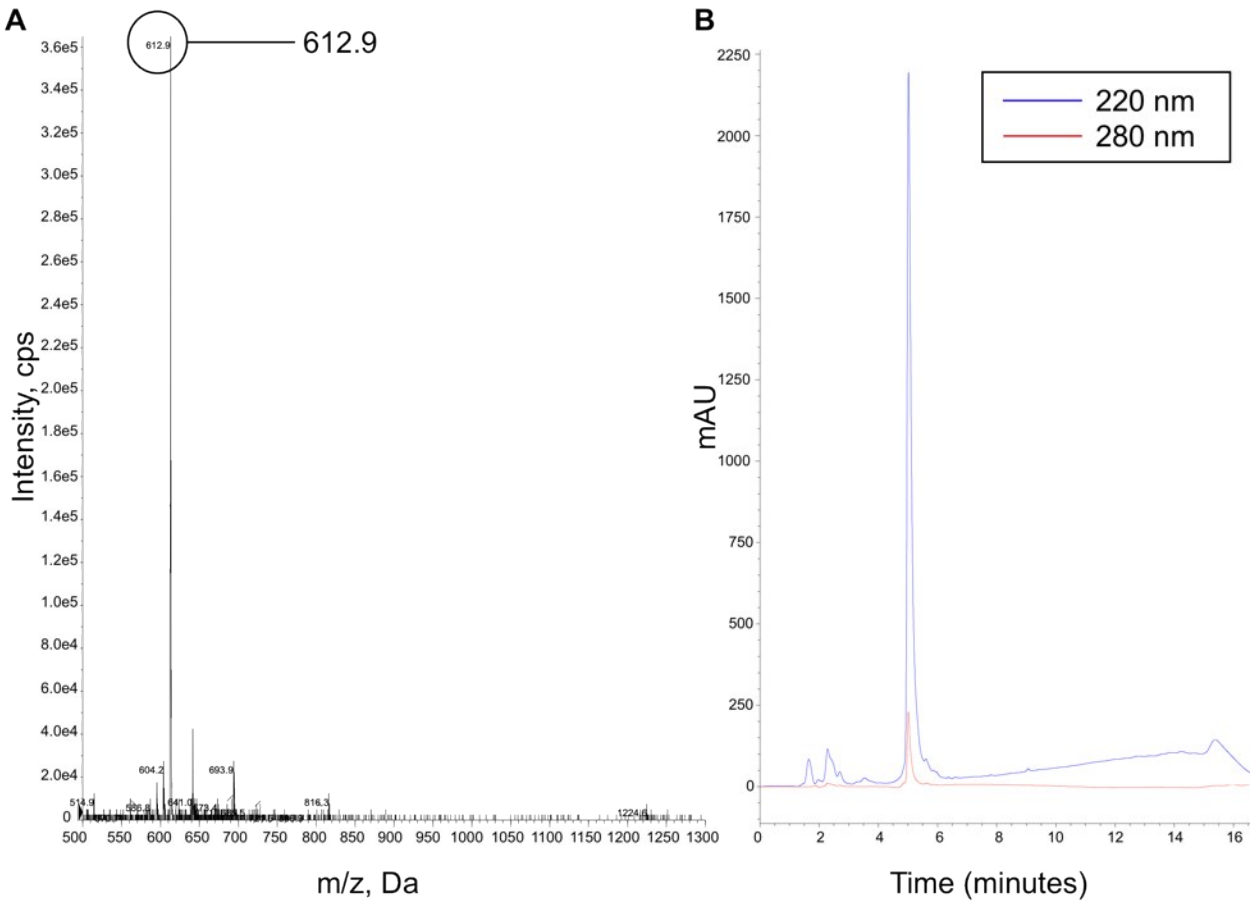


Fig. S2 (A) ESI-MS of purified RGDS(biotin) (MW 1224 g/mol) showing the [M/2 + H] ion and (B) corresponding analytical HPLC of RGDS(biotin).

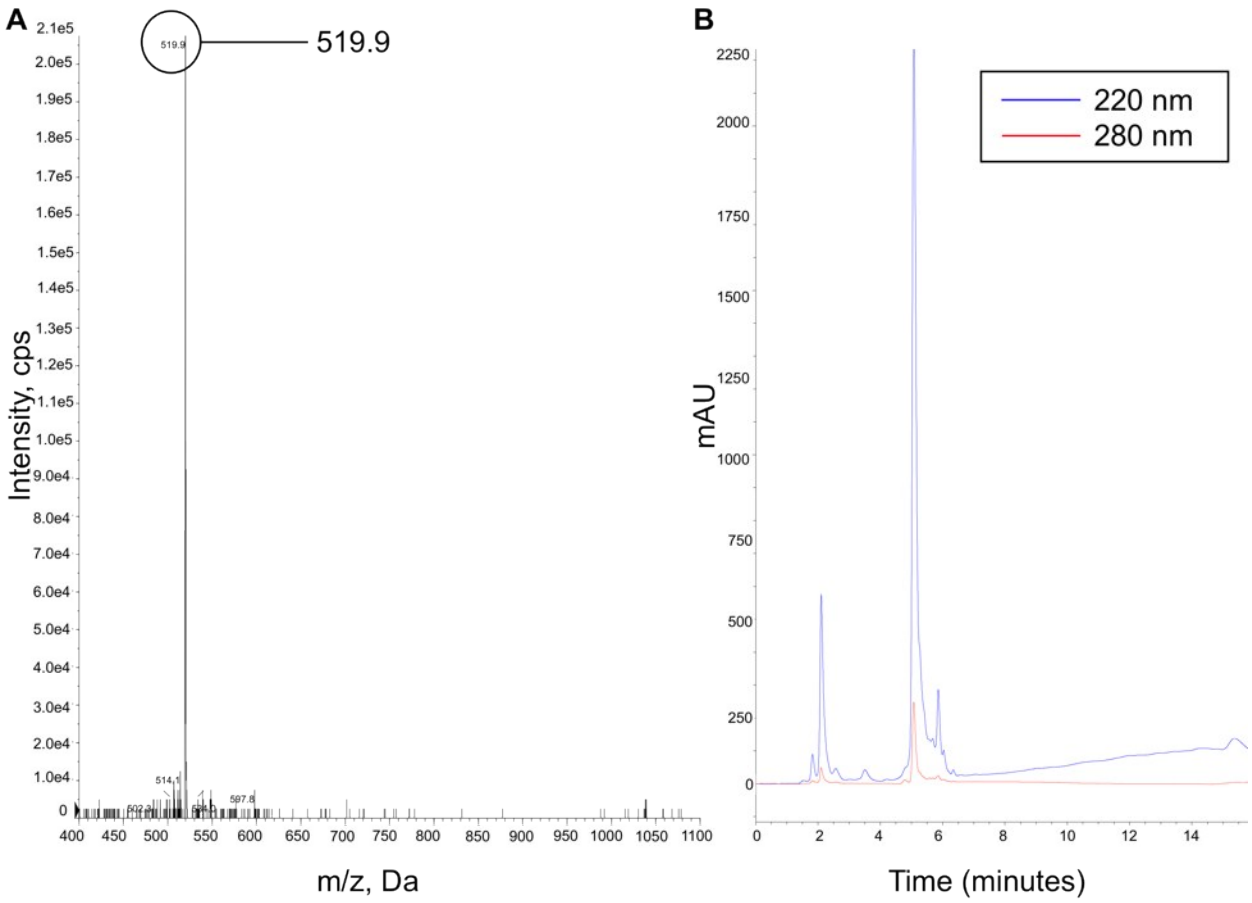


Fig. S3 (A) ESI-MS of purified RGES(azide) (MW 1038 g/mol) showing [M/2 + H] ion and (B) corresponding analytical HPLC.

Biotinylation of RGDS(biotin)

For RGDS(biotin), Fmoc-Lys(Mtt)-OH was manually coupled to Fmoc-Rink-amide MBHA resin in a 100 mL synthesis vessel using standard Fmoc SPPS techniques.[1] Manual SPPS was performed using a 100 mL peptide synthesis vessel (Chemglass Life Sciences, Vineland, NJ, USA) with agitation on a wrist-action shaker (Burrell, Pittsburgh, PA, USA). The Fmoc protecting group on the resin was removed with 20% (v/v) piperidine in DMF followed by thorough washing with DMF and DCM. Fmoc-Lys(Mtt)-OH (4 molar equivalents) was activated with 3.95 molar equivalents of HBTU and 6 molar equivalents of diisopropylethylamine (DIEA) in DMF and added to the resin. After reacting for 2 hours, the resin was washed with DMF and DCM. A ninhydrin test was conducted at room temperature for 30 minutes to monitor the presence of free amines and confirm successful coupling of the Fmoc-Lys(Mtt)-OH. The Mtt protecting group was removed from the lysine side chain with a solution of 90% (v/v) DCM with 5% (v/v) TFA and 5% (v/v) TIS. This Mtt deprotection solution was added to resin in the synthesis vessel, which was placed on the wrist

action shaker for 2 minutes, drained, and washed thoroughly with DCM. The solution turned bright yellow, indicating Mtt removal, and the deprotection and washing steps were repeated until the solution was clear. Biotin was dissolved at 2 molar equivalents with 1.98 molar equivalents of HBTU and 3 molar equivalents of DIEA in 50:50 DMF:DMSO. The biotin coupling solution was added to the resin and allowed to react for 3 hours before washing thoroughly with DCM and DMF. This coupling step was repeated before conducting a ninhydrin test at room temperature to verify successful coupling of biotin. The remaining amino acids were coupled using the automated microwave peptide synthesizer as described above.

Synthesis of peptide-PCL conjugates

RGDS(biotin)-PCL and RGES(azide)-PCL conjugates were synthesized using methods similar to those previously described.[2] Briefly, PCL diol (M_w 14,000 Da) was dissolved at 80 mg/mL in anhydrous NMP under nitrogen. The heterobifunctional linker PMPI was separately dissolved in anhydrous NMP at 15 molar equivalents to the PCL diol and added dropwise to the PCL solution while stirring under nitrogen. The reaction was continued overnight before adding the resulting PCL-maleimide (PCL-mal) to DEE to remove excess PMPI and precipitate the product. To conjugate the peptide, PCL-mal was re-dissolved in anhydrous NMP under nitrogen. The peptide was dissolved in DMSO at 8 molar equivalents to the PCL-mal then added dropwise to PCL-mal solution and reacted overnight to form the peptide-PCL conjugate. The resulting conjugate was precipitated in DEE, washed with ultrapure water or 50 mM NaOH to remove excess peptide, and dried under vacuum overnight prior to analysis. Each synthesis step was confirmed by ^{1}H NMR using a Bruker Biospin Advance III HD 400 MHz NMR spectrometer. PMPI and PCL-mal were dissolved in CD_2Cl_2. Proton assignments of the PMPI and PCL-maleimide were based on published spectra.[2] NMR showed the PMPI was successfully conjugated to the PCL to form PCL-mal (Fig. S4). The peptides and peptide-PCL conjugates were dissolved in DMSO-d_6. ^{1}H-NMR showed peptides were successfully conjugated to the PCL-mal to form each respective peptide-PCL conjugate (Fig. S5 and S6).

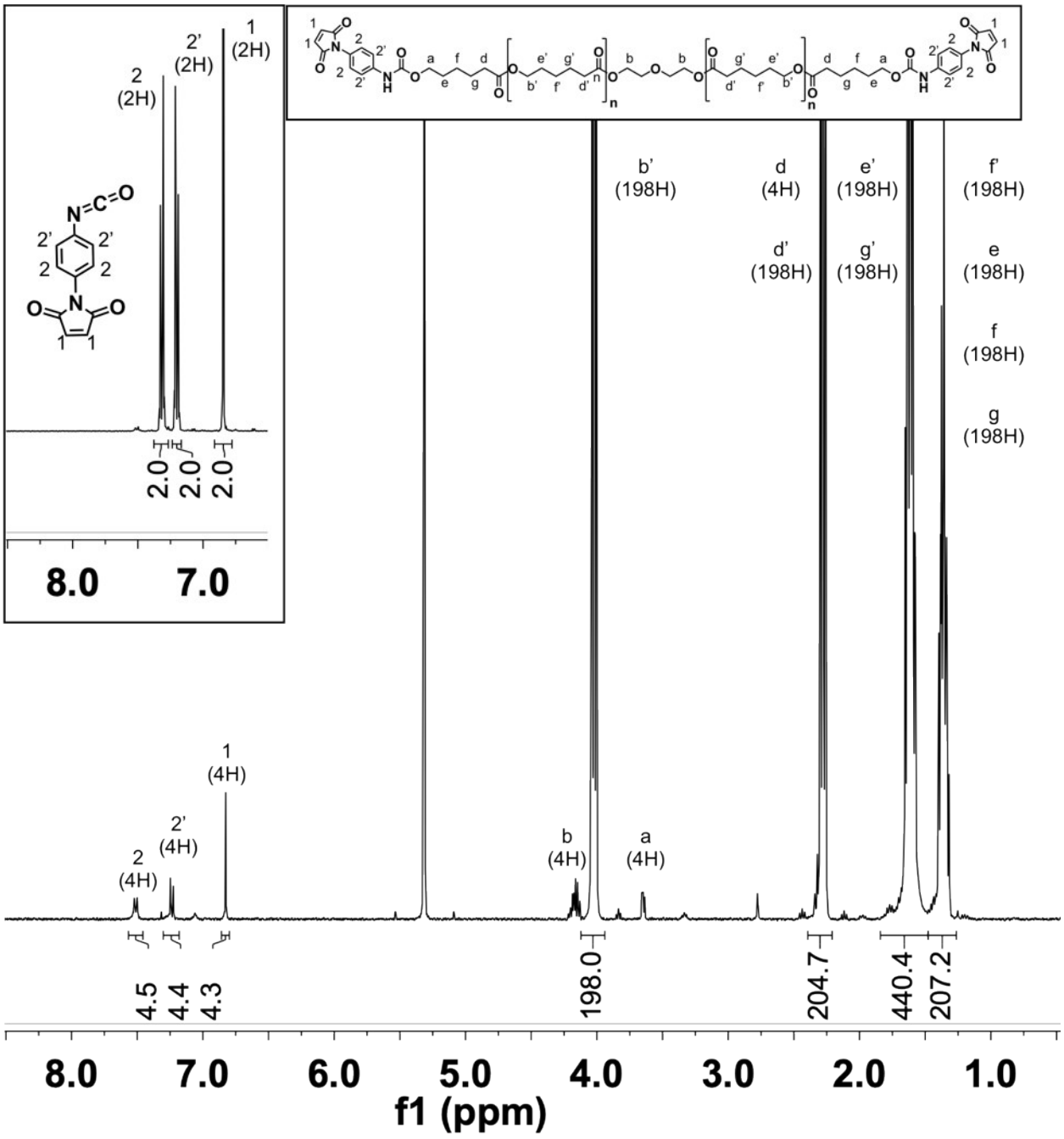


Fig. S4 ^{1}H NMR (400MHz, CD_2Cl_2, TMS) and corresponding chemical structures of p-maleimidophenyl isocyanate (PMPI; inset) with chemical shift assignments *6.84 (2H, s, maleimide vinyl, 1), 7.20 (2H, d, Ar-H ortho to isocyanate, 2'), 7.32 (2H, d, Ar-H ortho to maleimide, 2)* and PCL-maleimide with chemical shift assignments *6.82 (4H, s, maleimide vinyl, 1), 7.24 (4H, d, Ar-H ortho to isocyanate, 2'), 7.51 (4H, d, Ar-H ortho to maleimide, 2), 4.02 (198H, m, -O-CH_2-, b'), 2.27 (205H, m, -CO-CH_2-, d, d'), 1.61 (440H, m, -CH_2-, e, g'), 1.36 (207H, m, -CH_2-, f', e, f, g).*

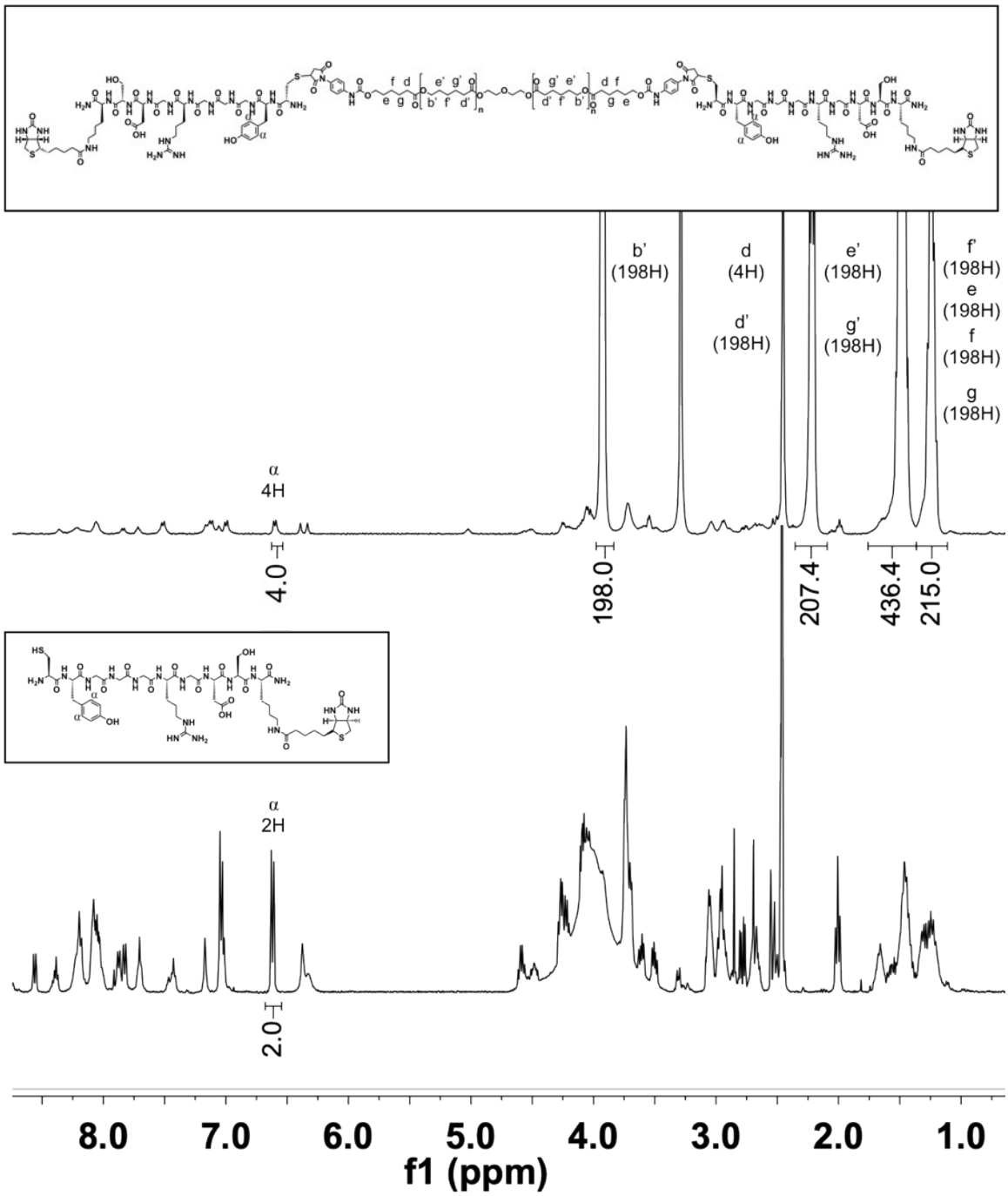


Fig. S5 ^{1}H NMR (400MHz, DMSO-d_6, TMS) and corresponding chemical structures of RGDS(biotin)-PCL (top) with chemical shift assignments *6.61 (4H, d, Ar-H, α), 3.93 (198H, m, -O-CH_2-, b'), 2.22 (207H, m, -CO-CH_2-, d, d'), 1.49 (436H, m, -CH_2-, e, g'), 1.24 (215H, m, -CH_2-, f', e, f, g)* and RGDS(biotin) (bottom) with chemical shift assignment *6.62 (2H, m, Ar-H, α).*

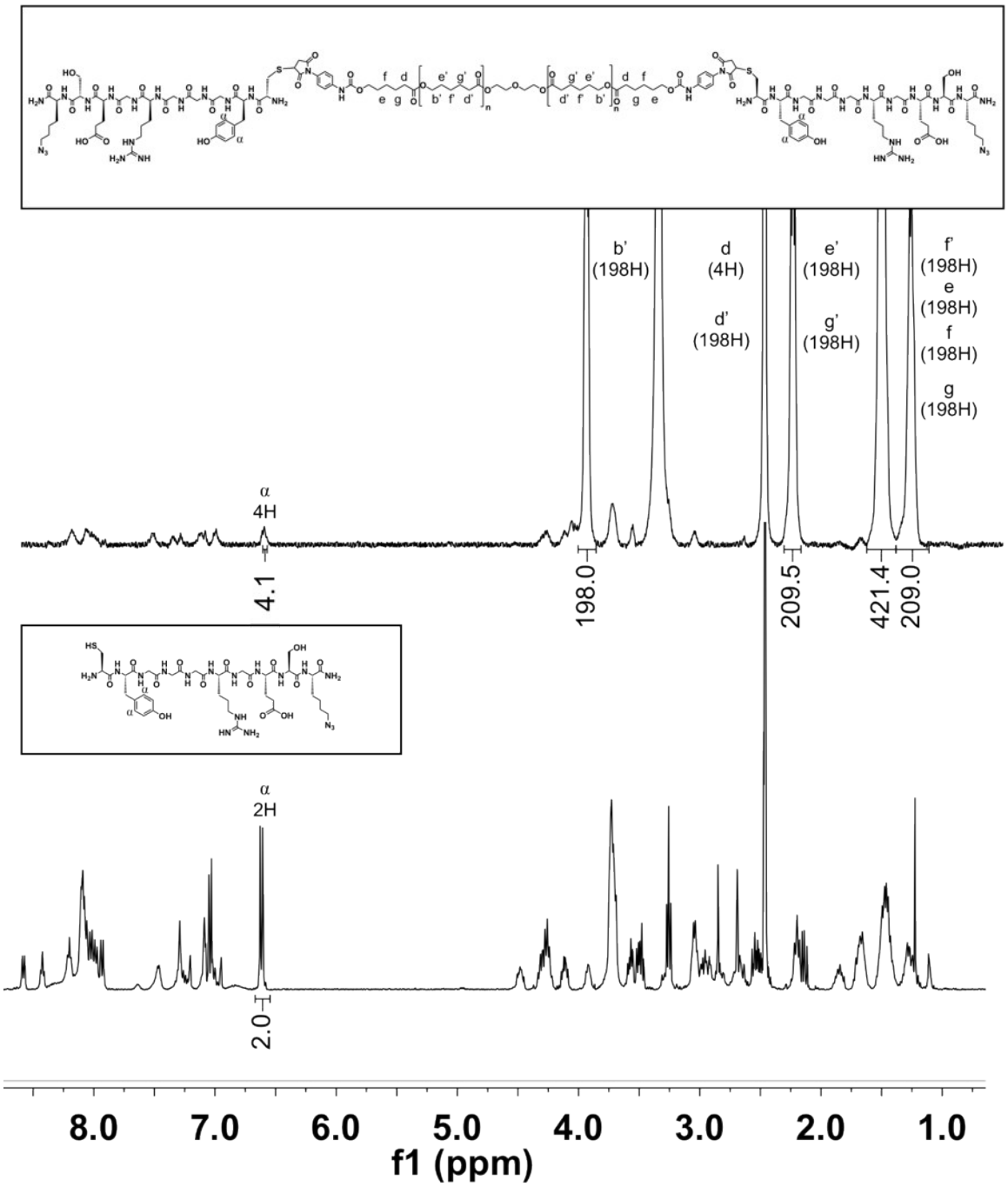


Fig. S6 ^{1}H NMR (400MHz, DMSO-d_6, TMS) and corresponding chemical structures of RGES(azide)-PCL (top) with chemical shift assignments *6.61 (4H, d, Ar-H, α), 3.94 (198H, m, -O-CH_2-, b'), 2.23 (210H, m, -CO-CH_2-, d, d'), 1.50 (421H, m, -CH_2-, e, g'), 1.25 (209H, m, -CH_2-, f', e, f ,g)* and RGES(azide) (bottom) with chemical shift assignment *6.61 (2H, m, Ar-H, α)*.

Peptide conjugation to PCL was also confirmed with Fourier Transform Infrared (FTIR) spectroscopy using a PerkinElmer Spectrum 100 Spectrometer (PerkinElmer Inc., Waltham, MA, USA). FTIR spectra were taken with a scanning wavenumber range from 4000 to 600 cm^{-1} and peaks were analyzed. The IR transmittance peaks at 3200 cm^{-1} and 1630 cm^{-1} indicating amide bonds[3] were present in both RGDS(biotin)-PCL and RGES(azide)-PCL conjugates but not the unmodified PCL (Fig. S7 and S8). The RGES(azide)-PCL conjugate also showed a transmittance peak at 2100 cm^{-1}, representing the azide[4] (Fig. S8).

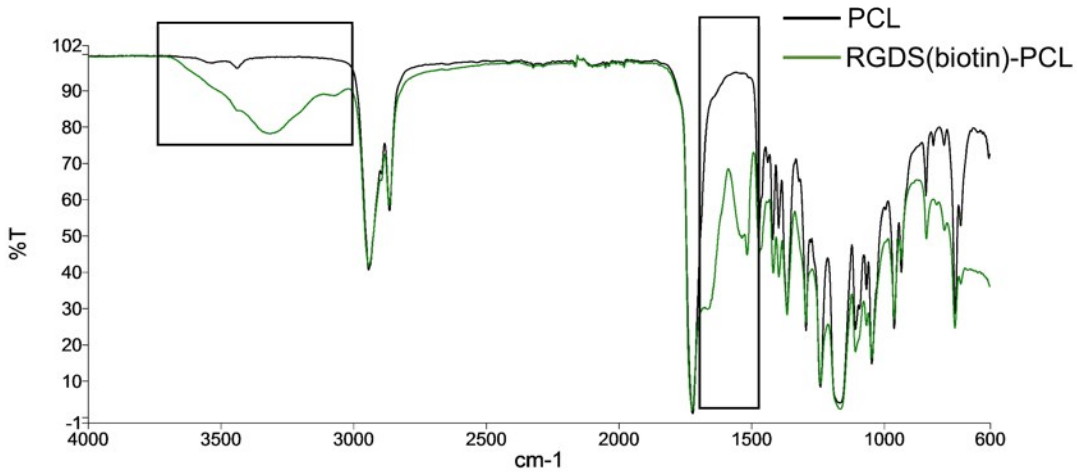


Fig. S7 FTIR of unmodified PCL (black) and RGDS(biotin)-PCL (green) conjugate demonstrating successful conjugation of RGDS(biotin) peptide to PCL. Black boxes highlight the IR transmittance peaks at 3200 cm^{-1} and 1630 cm^{-1} that indicate the amide bonds associated with the peptide.

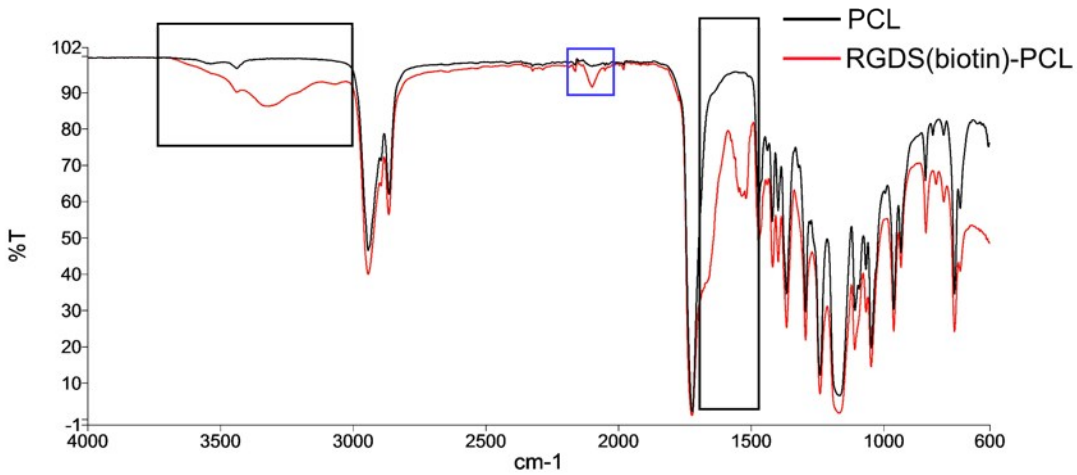


Fig. S8 FTIR of unmodified PCL (black) and RGES(azide)-PCL (red) conjugate demonstrating successful conjugation of RGES(azide) peptide to PCL. Black boxes highlight the IR

transmittance peaks at 3200 cm^{-1} and 1630 cm^{-1} that indicate the amide bonds associated with the peptide and the blue box shows the azide peak at 2100 cm^{-1} from the RGES(azide).

3D printing with peptide-PCL conjugates

PCL-based inks were created by dissolving unmodified PCL (M_w 80,000 Da) in HFIP at 37% (w/v) with RGDS(biotin)-PCL or RGES(azide)-PCL at 0, 1, 5, or 20 mg/mL. All inks were prepared directly in a 3 mL barrel with a tip cap (Nordson EFD, East Providence, RI) sealed with parafilm. For PCL only inks, unmodified PCL and HFIP were added before placing a SmoothFlow piston (Nordson EFD), capping the end of the syringe, and sealing with parafilm. For conjugate-containing inks, peptide-PCL conjugates were first dissolved in HFIP, vortexing the solution for 30 seconds to ensure full dissolution. Unmodified PCL was then added to the conjugate-based solution before adding the piston, capping the end, and sealing the syringe. All inks were vortexed before shaking on a wrist-action shaker for 48 hours. The resulting inks were taken off the shaker for at least 24 hours prior to 3D printing.

A 3-axis EV Series Automated Dispensing System (Nordson EFD) was used for solvent-cast 3D printing. A program or code of movement commands was developed in MATLAB for each scaffold to generate a cross-hatched pattern with an area of 5 mm x 5 mm and 160 μm programmed fiber spacings. The syringe barrels containing the ink were fitted with a 32-gauge (100 μm inner diameter) blunt-tip needle (Nordson EFD) and inserted into a high-pressure HP3cc Dispensing Tool (Nordson EFD). For multi-peptide scaffolds, two printer heads were used to print each peptide-PCL conjugate in alternating layers or alternating fibers. All inks were printed using a Performus I pressure controller (Nordson EFD) set to 10 psi. The HP3cc dispenser multiplies the pressure by 7-fold for a final printing pressure of 70 psi. The printing speed used to fabricate scaffolds was 0.4 mm/s for the first layer and 0.2 mm/s from the second layer on. All 3D-printed samples were left to dry in a fume hood overnight.

FTIR of peptide-functionalized scaffolds

Surface functionalization of scaffolds was confirmed with Fourier transform infrared (FTIR) spectroscopy with a Perkin Elmer Spectrum 100 Spectrometer (PerkinElmer inc.). Scaffolds samples (3 mm x 3 mm) with 90 μm programmed fiber spacings were printed using methods described above. FTIR spectra were taken of RGDS(biotin)-PCL and RGES(azide)-PCL scaffolds at concentrations of 0, 1, 5, or 20 mg/mL with scanning wavenumber range from 4000 cm^{-1} to

600 cm^{-1}. An increase in IR peak intensity at 3200 cm^{-1} and 1630 cm^{-1} indicates amide bonds corresponding to increasing peptide-PCL concentration in both RGDS(biotin)-PCL scaffolds and RGES(azide)-PCL scaffolds (Fig. S9 and S10).

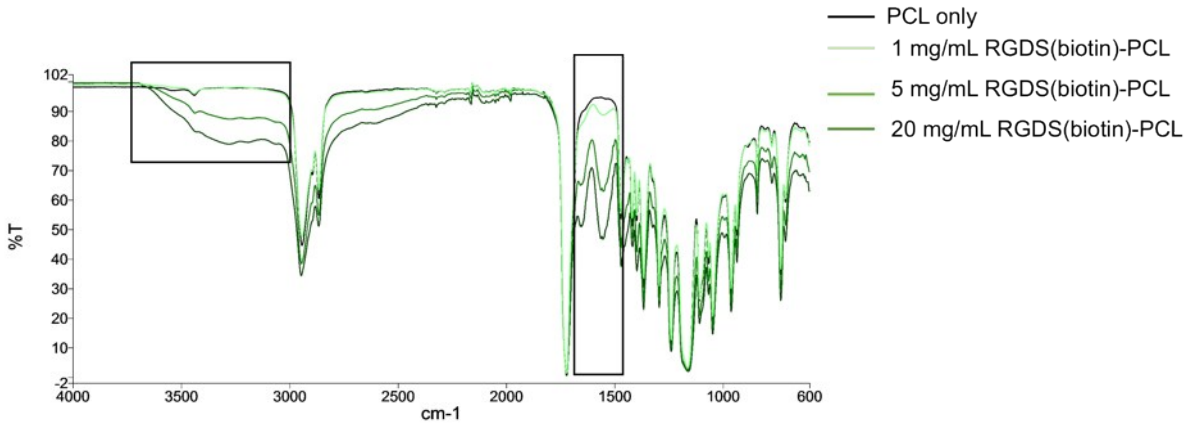


Fig. S9 FTIR of scaffolds 3D-printed with PCL only or PCL with 1, 5, or 20 mg/mL RGDS(biotin)-PCL showed an increase in IR peak intensity at 3200 cm^{-1} and 1630 cm^{-1} indicating an increase in RGDS(biotin) peptide concentration on the scaffold surface that corresponded with increasing conjugate concentration in the ink.

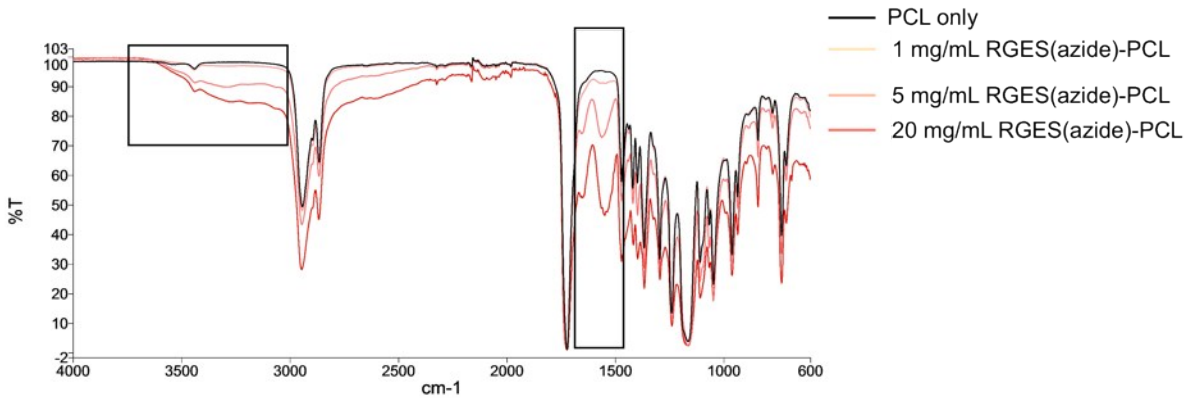


Fig. S10 FTIR of scaffolds 3D-printed with PCL only or PCL with 1, 5, or 20 mg/mL RGES(azide)-PCL showed an increase in IR peak intensity at 3200 cm^{-1} and 1630 cm^{-1} indicating an increase in RGES(azide) peptide concentration on the scaffold surface that corresponded with increasing conjugate concentration in the ink.

SEM of peptide-functionalized scaffolds

Samples were mounted on 12-mm aluminum stubs using carbon tape, then sputter-coated with iridium using a sputter coater (Electron Microscopy Sciences EMS575X, Hatfield, PA, USA). Samples were imaged using a scanning electron microscope (SEM) (LEO 1550 SEM; Zeiss, Peabody, MA, USA). Scaffolds were imaged using a secondary electron detector with an accelerating voltage of 5 kV.

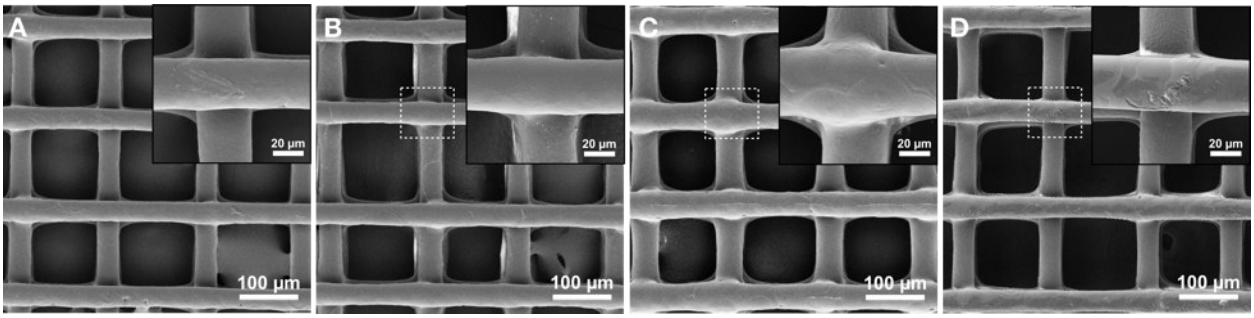


Fig. S11 Representative scanning electron microscopy (SEM) images of scaffolds 3D-printed with inks containing (A) 1 mg/mL RGDS(biotin)-PCL, (B) 1 mg/mL RGES(azide)-PCL, (C) 5 mg/mL RGDS(biotin)-PCL, or (D) 5 mg/mL RGES(azide)-PCL with higher magnification shown in the inset. This demonstrated that scaffold morphology and topography was unaffected by addition of the conjugates.

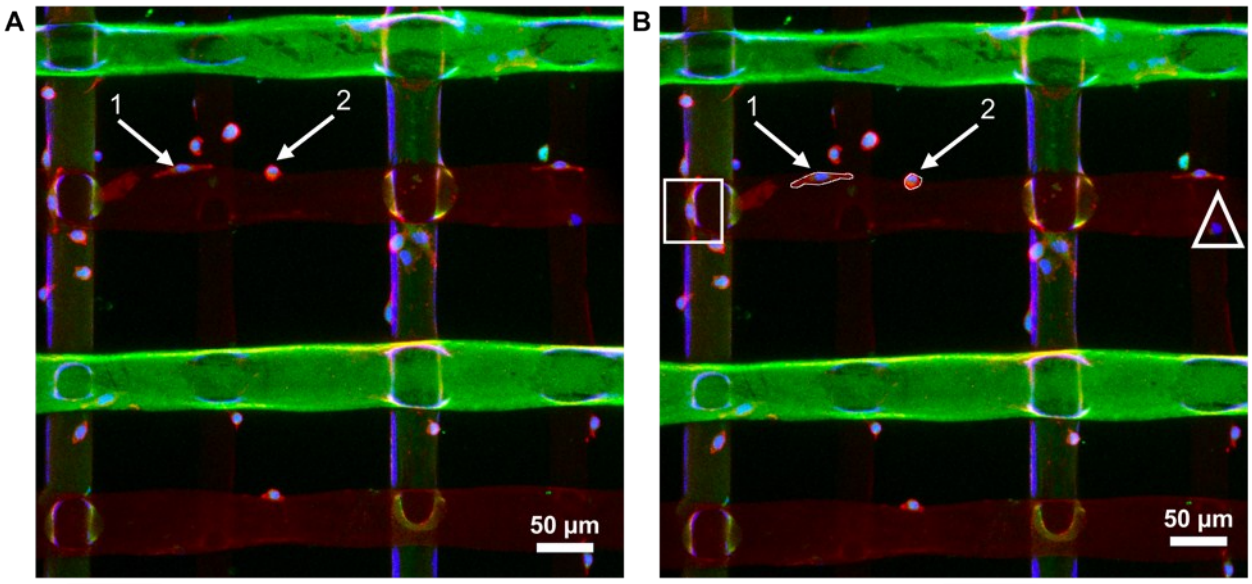
Fluorescence microscopy and image processing

All RGDS(biotin)-PCL and PCL only scaffolds were labeled with streptavidin-conjugated fluorescein isothiocyanate (FITC) in order to visualize the distribution of the biotinylated peptide on the surface of the 3D-printed fibers. All labeling steps were completed with minimal exposure to light at room temperature. Scaffolds were blocked for 30 minutes in PBS with 0.5% BSA (w/v) and 0.05% (v/v) TWEEN 20 and incubated in 0.3 µM streptavidin-FITC in the blocking solution for 1 hour. Scaffolds were washed twice in PBS for 30 minutes then stored in ultrapure water overnight prior to imaging. All RGES(azide)-PCL and PCL only scaffolds were labeled with dibenzocyclooctyne-Cyanine3 (DBCO-Cy3). Samples were incubated in PBS with 0.2% (v/v) TWEEN 20 and 0.2% (v/v) Triton-X for 60 minutes before adding 50 mM DBCO-Cy3 in PBS with 0.5% BSA for 1 hour. After the reaction, each sample was washed for 10 minutes with PBS, ultrapure water, 0.2% TWEEN and 0.2% Triton-X in PBS, 50% IPA, 100% IPA, and 50% IPA. Scaffolds were stored in ultrapure water prior to imaging. Alternating-layer and alternating-fiber scaffolds were first labeled with streptavidin-FITC before labeling with DBCO-Cy3 following steps described above.

Fluorescently-labeled scaffolds were imaged on a Nikon C2+ laser scanning confocal fluorescence microscope (Nikon, Melville, NY, USA). Samples were placed flat on a glass slide, and z-stacks of ~5 μm slices were obtained using a 488 nm (FITC) or 561 nm (Cy3) laser or white light for fluorescence and bright-field images, respectively. Bright-field and fluorescent images were taken concurrently during z-stack acquisition. For alternating-fiber and alternating-layer scaffolds, z-projections at maximum intensity of eight slices per scaffold were used to ensure that the alternating patterns and fluorescence were clearly visible for all fiber layers. To compare fluorescence across peptide-PCL conjugate concentrations, five individual peptide-functionalized fluorescently-labeled scaffolds printed using two different ink batches per scaffold group were imaged on a Nikon Eclipse Ts2R fluorescent microscope with same settings and exposure times maintained for all samples. To quantify fluorescence intensity, fluorescent images were converted to greyscale images and mean grey values were measured across the scaffold cross-sections using Fiji, an image processing distribution of the open source program ImageJ.

Evaluation of cell adhesion and morphology on alternating fiber scaffolds

Alternating-fiber dual-peptide scaffolds were labeled with streptavidin-FITC and DBCO-Cy3 prior to cell culture and minimally exposed to light. After cell culture, samples were incubated in rhodamine-phalloidin and Hoechst 33258 to label actin filaments and cell nuclei, respectively. For quantitative comparison of cell attachment and morphology, five randomly-selected areas on each scaffold were imaged on a Nikon C2+ laser scanning confocal microscope, and the fluorescent images were processed using Fiji. A sub-region with dimensions of 480 μm x 480 μm was selected from each fluorescent image to ensure an equal representation of both RGDS(biotin) and RGES(azide) peptide-functionalized fibers. Cell attachment was quantified by manually counting cell nuclei present on RGDS(biotin)-functionalized fibers (green) and RGES(azide)-functionalized fibers (red) (Fig. S12A). Cells that appeared at the junction between RGDS(biotin) and RGES(azide) peptide-functionalized fibers were not counted, as shown by the square in Fig. S12B. To quantify cell spreading, cell area and aspect ratios of the counted cells were calculated by using the freehand selection tool in Fiji (Figs. S12B and S12C). Cell spreading was defined cell with areas greater than 400 μm^2 and/or aspect ratios greater than 2:1 as indicated by the red and blue boxes in Fig. S12C with cell 1 denoting a spread cell and cell 2 denoting a rounded cell. Cells that were not stained by phalloidin were also excluded because their cell area could not be quantified. An example of this is highlighted by the triangle in Fig. S12B.



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	Area	Mean	Min	Max	Perim.	Major	Minor	Angle	Circ.	AR	Round	Solidity
1	991	46.371	6	152	209.087	90.374	13.962	12.136	0.285	6.473	0.154	0.816
2	258	111.326	42	181	60.721	19.594	16.765	89.057	0.879	1.169	0.856	0.920

Fig. S12 (A, B) Representative confocal microscopy images demonstrating quantification of attachment and spreading of NIH3T3 mouse fibroblasts seeded on alternating-fiber scaffolds presenting both RGDS(biotin) and RGES(azide) peptides. Scaffolds were labeled with streptavidin-FITC (green) and DBCO-Cy3 (red) to differentiate RGDS(biotin)-PCL and RGES(azide)-PCL fibers, respectively. Samples were incubated in rhodamine-phalloidin (red) and Hoechst 33258 (blue) to label actin filaments and cell nuclei, respectively (B) Cell spreading was quantified using the freehand selection tool on Fiji to calculate cell area and aspect ratios of cells that were counted. Cells that appeared at a junction between fibers (square) and cells with no visible phalloidin staining (triangle) were excluded from cell adhesion and cell spreading quantification. (C) Measured cell area (red square) and aspect ratio (blue square) calculated for cell 1 and 2 using the freehand selection tool in Fiji. Cell spreading was defined as cell areas greater than 400 μm^2 and/or aspect ratios greater than 2:1.

Works Cited

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