Electronic Supplementary Material (ESI) for Polymer Chemistry.

Enzyme-Targeted Nanoparticles for Delivery to Ischemic Skeletal Muscle

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Electronic Supplementary Information

1. **Supplemental Methods**
2. **Supplemental Figures S1-S11**

Supplemental Methods

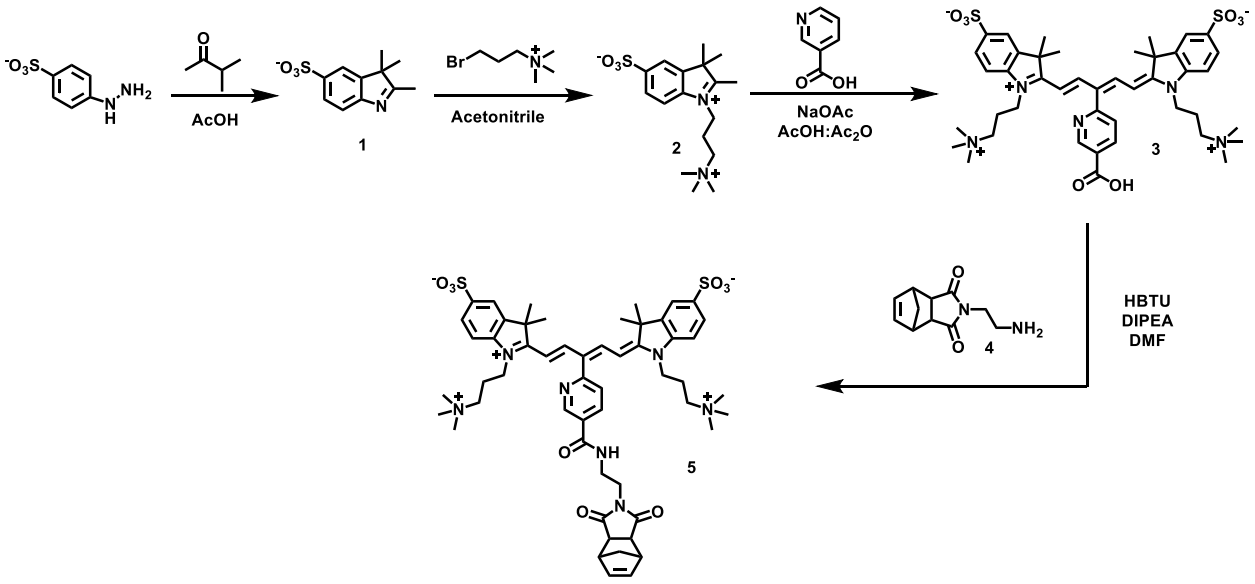
General Methods

All reagents were purchased from commercial sources and used without further purification. Peptides were synthesized using an AAPPTEC Focus XC automated synthesizer. Amino acids were purchased from AAPPTEC and NovaBiochem. *N*-(Glycine)-*cis*-5-nrbornene-*exo*-dicarboximide,[1] and (IMesH2)(C5H5N)2(Cl)2Ru=CHPh[2] were prepared according to published procedures. All polymerizations that were monitored by NMR ran in J. Young NMR tubes (5 mm diameter) in a glove box under an inert atmosphere (N_2) using DMF-d_7 from a sealed vial (Cambridge Isoptopes). All other polymerizations were ran in flame dried vials using anhydrous DMF under an inert atmosphere (N_2). Analytical HPLC was performed on a Jupiter Proteo90A phenomenex column (150 x 4.60 mm) using a Hitachi-Elite LaChrom L-2130 pump equipped with a UV-Vis detector (Hitachi-Elite LaChrome L-2420). PREP HPLC was performed on Jupiter Proteo90A Phenomenex column (2050 x 25.0 mm) on a Waters DeltaPrep 300 system. HPLC was ran using water with 0.1% TFA as buffer A, and acetonitrile with 0.1% TFA as buffer B. Purified peptide-monomers were confirmed by analytical HPLC and ESI-MS in the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. Polymer dispersities and molecular weights were determined by size-exclusion chromatography (phenomenex Phenogel 5u 10, 1k-75k, 300 x7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (0.05 M LiBr in DMF)) using a Shimadzu pump equipped with a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 MW polystyrene standard. ^{1}H (400 MHz) NMR spectra were recorded on a Varian Mercury Plus spectrometer. Chemical shifts were reported in ppm relative to the solvent residual proton peaks**.**

Peptide Synthesis

Peptides were synthesized using standard solid phase peptide synthesis (SPPS) using FMOC-chemistries on an AAPPTec Focus XC automated synthesizer. Peptides were synthesized using Rink Amide MBHA resin to prepare protecting group free peptides. In general, the resin was swelled in DMF for 1-2 hours. The resin was then deprotected 2x10 minutes with 20% 4-methylpiperidine. Following deprotection, the peptide was washed 3X2 minutes with DMF. The first amino acid was activated with HBTU and DIPEA in a ratio of 3:2.9:6 for amino acid:HBTU:DIPEA for 10 minutes, followed by addition to the resin. The coupling was mixed for 45 minutes. After coupling, the resin was washed 3X2 minutes with DMF, and the process was repeated for each remaining amino acid. To prepare ROMP monomers, the final amino acid coupling was to *N*-(Glycine)-*cis*-5-norbornene-*exo*-dicarboximide. The peptide was cleaved from resin using a mixture of TFA/H_2O/TIPS (95:2.5:2.5) for 45 minutes. The peptide was precipitated in cold diethyl ether followed by dissolving in buffer A (H_2O with 0.1% TFA) with minimal amounts of buffer B (ACN with 0.1%TFA). Once dissolved, the peptide was purified by reverse phase HPLC and analyzed by analytical HPLC and ESI-MS to confirm purity. ESI-MS: Calc. M+H: 1648.76, found: 1648.31.

Zwitterionic Cy 5 Synthesis

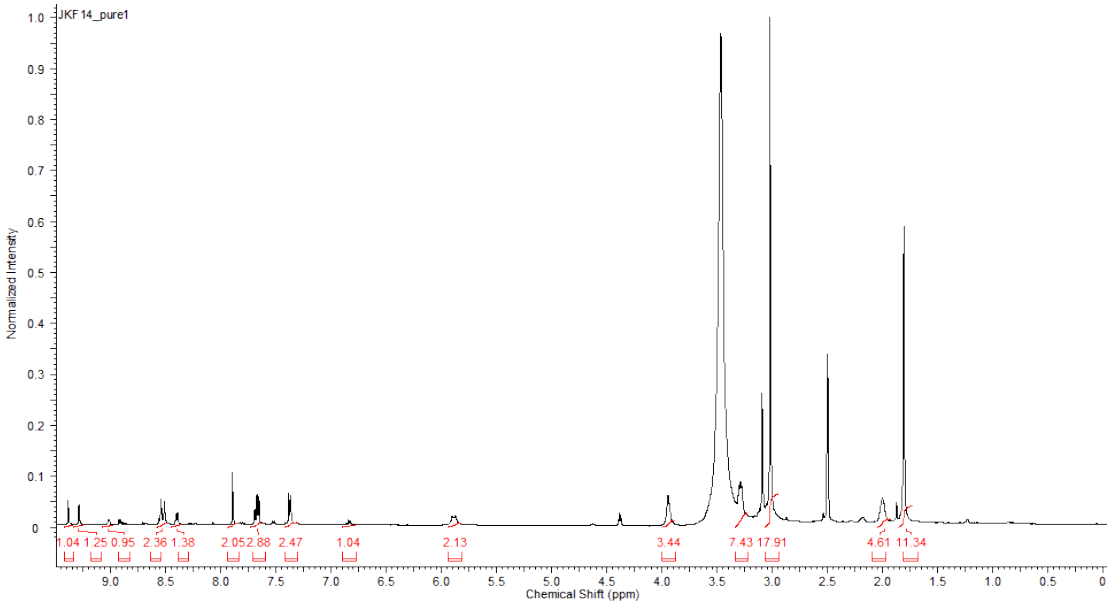


Synthetic scheme for zwitterionic cy 5 monomer.

1 and **2** have been synthesized previously.[4] **4** has been synthesized previously.[5]

2-((1E,3Z)-3-(5-carboxypyridin-2-yl)-5-((E)-3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate (3)

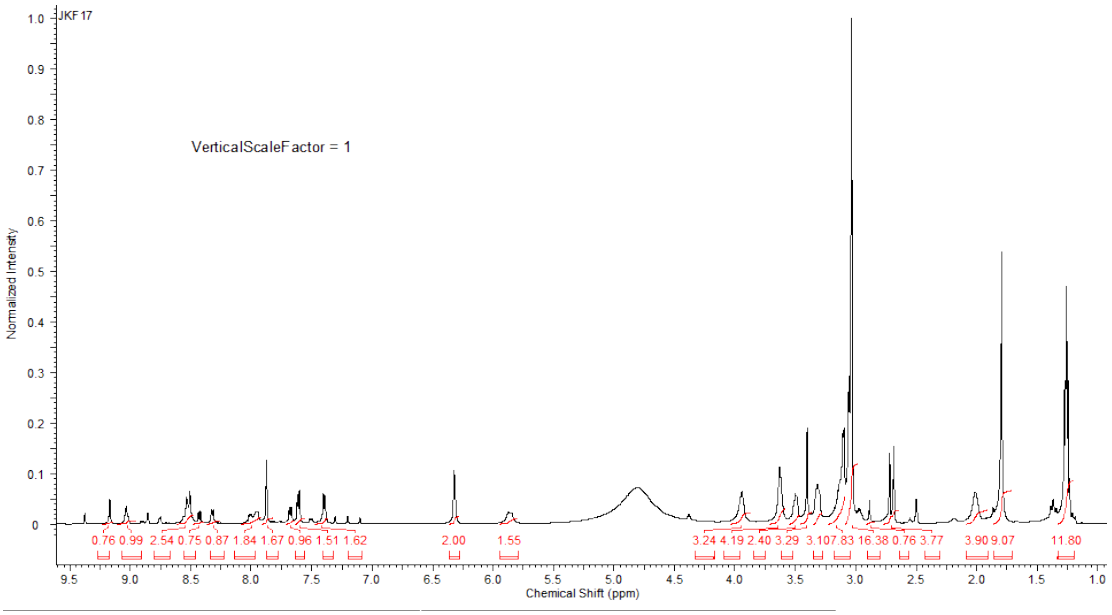
2 (1.97 mmol, 2.2 equiv), 6-(1,3- dioxopropan-2-yl)nicotinic acid (0.895 mmol, 1 equiv), and sodium acetate (6.265 mmol, 7 equiv) were dissolved in 4 mL of a 1:1 mixture of acetic anhydride and acetic acid followed by heating to reflux for 18 hours. Solvent was removed by rotary evaporation which resulted in a blue solid. The solidwas dissolved in water with 0.1% TFA and purified by reverse phase HPLC using a gradient from of 10-60% buffer B. ESI-MS: Calcd for $C_{47}H_{56}N_5O_8S_2^+$ m/z 834.3565, found m/z . 832.43.



^{1}H NMR of 3.

2-((1E,3Z)-5-((E)-3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)-3-(5-((2-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisoindol-2-yl)ethyl)carbamoyl)pyridin-2-yl)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate (5)

3 (.0179 mmol, 1equiv) was activated with HBTU (0.0179 mmol, 1 equiv.) and DIPEA (.1076 mmol, 6 equiv.) in DMF for 10 minutes. **4** (.0538 mmol, 3 equiv.)was added to the reaction followed by stirring at room temperature for 2 hours. The reaction was precipitated in cold diethyl ether, dissolved in water with 0.1% TFA and purified by reverse phase HPLC using a gradient 10%-60% acetonitrile in 0.1% TFA in water with 0.1% TFA. The pure fractions were lyophilized to obtain a blue powder. MS: Calcd for $C_{54}H_{68}N_7O_9S_2^+$ m/z 1022.4541, found 1022.43 m/z



^{1}H NMR of **5**.

Polymerization Kinetics

To determine the kinetics of polymerization for Nor-Peptide monomer and the Nor-Glycine monomer, the polymerization of each was monitored by ^{1}H NMR. A standard protocol for the experiment involved dissolving the monomer (0.0275 mmol, 20 equiv.) in DMF_{d7} and transferring to an NMR tube under N_2 atmosphere. An initial NMR spectrum was acquired, followed by addition of the (0.0014 mmol, 1 equiv), and acquiring spectrum. The polymerization was monitored until complete monomer consumption. The kinetics of the polymerization were determined by integrating the monomer olefinic chemical shift and the polymer olefinic chemical shift at the various time points.[6] A corrected integral for each was calculated by subtracting the background integration. The background signal of the monomer was determined using the final NMR spectrum where no monomer is present. The background of the polymer integral was determined by the initial NMR and subtracted from the polymer integral at all timepoints. The percent conversion was then determined using the polymer and monomer integrals (percent conversion = (polymer/(monomer + polymer))*100. The number of monomers polymerized was determined by dividing polymer concentration by initial monomer concentration and multiplying by the DP targeted.

Polymerization of Block and Blend Copolymers

All polymerizations were carried out under inert atmosphere in a glove box. A typical polymerization protocol used to generate the block copolymer of phenyl$_{25}$-*b*-peptide$_3$ involved mixing the phenyl monomer (0.04 mmol, 20 equiv.) with the catalyst initiator (0.002 mmol, 1 equiv.) in DMF$_{d7}$ for 20 minutes. A small (~20 uL) aliquot was removed and quenched with ethyl vinyl ether to analyze block length by SEC-MALS. Next, the peptide monomer (0.006 mmol, 3 equiv.) was added to the reaction. After 30 minutes, if the polymer was to be labeled with NIR dye, the dye (0.0008 mmol, 0.4 equiv.) was added to the polymerization for 1 hour. Polymers were quenched with ethyl vinyl ether (10 equiv.). Polymers were analyzed by SEC-MALS to determine molecular weight and poly dispersities.

For the preparation of the blend copolymer phenyl$_{25}$-*b*-peptide$_3$-*co*-glycine$_4$, the phenyl monomer (0.04 mmol, 20 equiv.) was mixed with the catalyst initiator (0.002 mmol, 1 equiv) in dry DMF$_{d7}$. After 20 minutes, the peptide monomer (0.006 mmol, 3 equiv) was added to the polymerization. To make the blend copolymer, 1 equiv of glycine (0.008 mmol, 4 equiv) was added to the polymerization every 2 minutes, which is the time necessary to polymerize one peptide monomer). After final addition of glycine, NIR dye monomer (0.0008 mmol, 0.4 equiv.) was added for the polymers that required a NIR label for 1 hour, then termination ethyl vinyl ether. Before each addition of subsequent blocks, a small aliquot (~20 uL) was removed from the polymerization reaction, quenched with ethyl vinyl ether and analyzed by SEC MALS to determine molecular weight and dispersity.

Polymers were precipitated in cold diethyl ether 3 times, collected by centrifugation and dissolved in a mixture of 1:1 H_2O:ACN and lyophilized to afford dry polymer.

Particle Formation

PPAs were dissolved in dry DMF at a concentration of 2 mg/mL. 1x DPBS was added to the particle mixture at a rate of 1 mL/hr with rapid stirring until a final concentration of 1 mg/mL. The particle mixture was then transferred to a 10000 MWCO dialysis tubing and dialyzed into DPBS for 3 days with 3 buffer changes. For *in vivo* studies, particles were then staril filtered using 0.22 mm filters followed by concentration with sterilized Millipore centrifugal units with 10000MWCO filters. Particles that were unlabeled were characterized by DLS and TEM. Particles that were labeled were characterized by electron microscopy only due to the absorbance of the DLS laser by the NIR dyes.

For cryoEM characterization, Quantifoil R2/2 400 mesh holey carbon TEM grids were cleaned and made hydrophilic using air plasma from a glow discharge system (2 minutes, 20 mA). 3 μL of a 10 mg/mL sample was pipetted onto each grid and blotted for approximately 3 seconds to make a thin film of liquid on the surface of the grid. Immediately afterwards, the grid was directly plunged into liquid ethane and transferred into liquid nitrogen. Cryo-TEM imaging was performed on a FEI Tecnai G2 Sphera operated at 200 keV. Low electron dose imaging procedures were used in order to prevent beam damage of the samples.

In Vitro Enzyme Assay

Particles (100 mM with respect to peptide) were incubated with 1 mM thermolysin in 1x DPBS buffer. The cleavage was monitored by RP-HPLC for 14 hours. An initial RP-HPLC trace was taken, followed by the addition of thermolysin, the subsequent HPLC analysis occurred every 1.5 hours.

Hindlimb Ischemia Surgery and Perfusion Imaging

All animal procedures were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of California, San Diego and the American Association for Accreditation of Laboratory Animal Care. Studies were done with 32 female

Sprague Dawley rats ranging in weights from 200-250g. For the hindlimb ischemia surgery, animals were anesthetized with 2.5% isoflurane gas using a Kent Scientific PhysioSuite ventilator. Unilateral ischemia damage was applied to the animals' right limb by ligation and removal of segments from the femoral artery and vein starting proximal to the epigastric artery as described previously.[7] First, the surgical site was shaved and cleaned with betadine and isopropanol along with delivery of 1% lidocaine subcutaneously along the incision site. The contralateral skin was also shaved for equivalent IVIS imaging since fur can interfere with IVIS. The targeted vessels were visualized by creating an incision overlying the proximal, medial portion of the right hindlimb. The femoral artery and vein were dissected away from the femoral nerve and ligated to target the proximal removal of a 2 cm segment. Any major branches along the 2 cm segment, including the epigastric artery, were cauterized or ligated prior to excision to minimize bleeding. The entry incision through the skin was then closed using a mattress suture (5-O silk). Postoperatively, all animals were regularly montored and analgesia (Buprenorphine at 0.05 mg/kg) was administered immediately upon regaining consciousness.

Immediately after surgery, perfusion measurements were made using a laser speckle contrast analysis system (PeriMed, Stockholm, Sweden) to ensure adequate ischemia compared to the healthy limb. All readings were done with the following settings: working distance of 20 cm. high point density, 0.42 images/s, frame rate of 25 images/s, recorded with averaging of 50 images, effective frame rate of 0.5 images/s, and intensity filter from 0.20-10.0. The animals were transferred to the PeriMed immediately after hindlimb ischemia surgery and were maintained at 2.5% isoflurane and on a heated insulated deck. Readings were continuously taken for a minimum of 20 minutes or until the perfusion measurements reached a plateau. Regions of interest were utilized to target each hairless plantar sole region individually for assessment at the equilibrium time point. Percent perfusion was calculated as a ratio of the ischemic limb (right) to the healthy limb (left). Measurements were taken before and immediately after surgery.

NP Injection and IVIS Tracking

For all animal studies, animals were randomized after the hindlimb ischemia surgery. For the injection time point pilot study, animals were injected with Cy5.5-labeled NPs at 1 (n=2), 3 (n=2), 5 (n=2), or 7 (n=2) days post-ischemia. For the block versus blend study, animals were injected with Cy5.5-labeled block (n=3) or blend (n=3) NPs at 4 days post-ischemia. For the zwitterionic long term retention study, $NP_{5.5}$ (7 day retention: n=4; 28 day retention: n=4) or NP_{zw5} (7 day retention: n=4; 28 day retention: n=8) were injected 4 days post-ischemia. For all injections, NP concentration was 400nmol with respect to polymer in a 1mL injection volume.

For the tail vein injections, animals were anesthetized using 2.5% isoflurane. Their tails were heated for 1 minute with a heating pad to induce vasodilation. Tails were sterilized by scrubbing with isopropanol. NPs were injected intravenously with a 27G needle through either the right or left tail vein after verifying the vein was accessed via flashback. Venous puncture attempts were not totaling more than 3 per side. Animals with failed injections were excluded from the study.

NP targeting was monitored using an *In Vivo* Imaging System (IVIS) Spectrum 200 (Perkin Elmer). For *in vivo* monitoring, the transillumination mode was used to capture signal deeper in the tissue. Animals were anesthetized with 2.5% isoflurane and placed in the IVIS chamber in a supine position. Their legs were angled outward to expose the ischemic region. Excitation/emission and exposure was 675nm/720nm and 10 seconds for $NP_{5.5}$ and 640nm/680nm and 5 seconds for NP_{zw5}. Each leg (ischemic and healthy) was imaged individually using a 3x3 pinhole square (9 images per limb). Overview images were created using the Perkin Elmer Living Image software. Ischemic to healthy muscle fluorescence

ratios were calculated by drawing circular regions of interest around the thigh (gracilis) muscle, measuring the total normalized transmission fluorescence (NTF) efficiency for each limb, and normalizing to the healthy limb internally for each animal. After the final *in vivo* time point, animals were euthanized with lethal injection of sodium pentobarbital. For *ex vivo* fluorescence, organs were harvested after euthanasia, placed on a black laminated sheet, and read for fluorescence on IVIS using epi-fluorescence mode with the same excitation/emission filters as for *in vivo* imaging, but with an exposure time of 0.1 sec. Radiant efficiency for each organ ([p/s/cm^2/sr]/[μW/cm^2]) was internally normalized to the healthy muscle of each animal to be able to compare between different fluorescent dye species.

Immunohistochemistry and Confocal Imaging

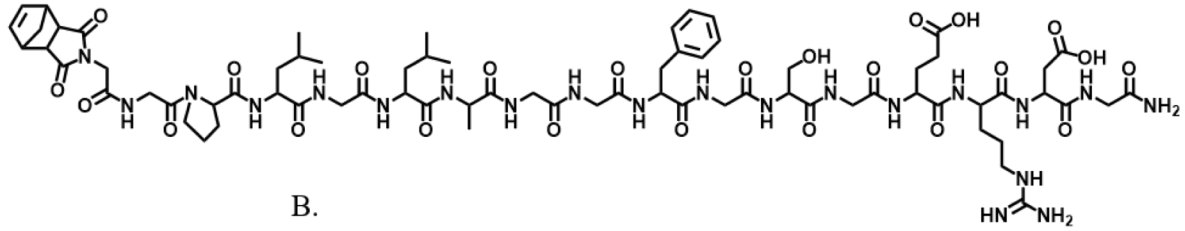
After *ex vivo* IVIS imaging, both ischemic and healthy gracilis muscle from each animal was flash frozen in liquid nitrogen-chilled isopentane and blocked in OCT for cryosectioning in the transverse orientation. Sections of 10 μm thickness were taken at 4 locations spanning the majority of the muscle. For immunohistochemistry, sections were thawed, fixed with ice-cold acetone, blocked with 5% bovine serum albumin (Gemini Bio-Products), and permeabilized with 0.3% Triton X-100 (Sigma) before incubation with primary antibody (rabbit polyclonal anti-laminin 1:100, abcam), secondary antibody (AlexaFluor-488 goat anti-rabbit 1:500, Invitrogen), and Hoechst 33342 (ThermoFisher Scientific. Sections were mounted using FluorMount and were imaged on a Nikon A1R Confocal microscope using a 20x air objective. Confocal settings were as such: 1 frame/sec, simultaneous scan, 1.2 Airy unit pinhole, scan area of 1024x1024 pixels, 0.34 μm Nyquist pixel size. For each laser the hv, offset, and power were respectively: DAPI 85, -10, 0.85; FITC 66, -10, 0.4; Cy5 97, -9, 14.44 (Cy5.5 NPs); Cy5 84, -9, 14.44 (zwCy5 NPs).

Statistical Analysis

For the injection time point pilot, data was analyzed with a two-way ANOVA with post-ischemia injection time point and post-injection imaging time point as factors with a post-hoc Tukey multiple comparisons test. Block versus blend data and zwitterionic versus charged data were analyzed with a Student's T-test at each time point post-injection. *Ex vivo* biodistribution data was analyzed with a Student's T-test for each organ. Data are means±SEM unless otherwise noted. Significance was accepted at $p < 0.05$.

Supplemental Figures S1-S11

A.



B.

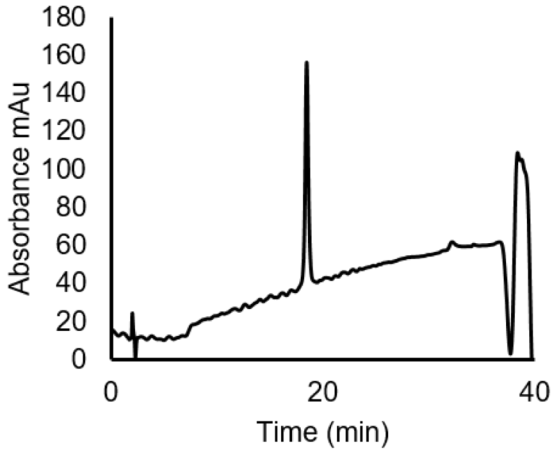


Figure S1. (A) peptide structure of sequence NorbornylG-GPLGLAGGFGSGERDG. (B) analytical HPLC trace of purified peptide.

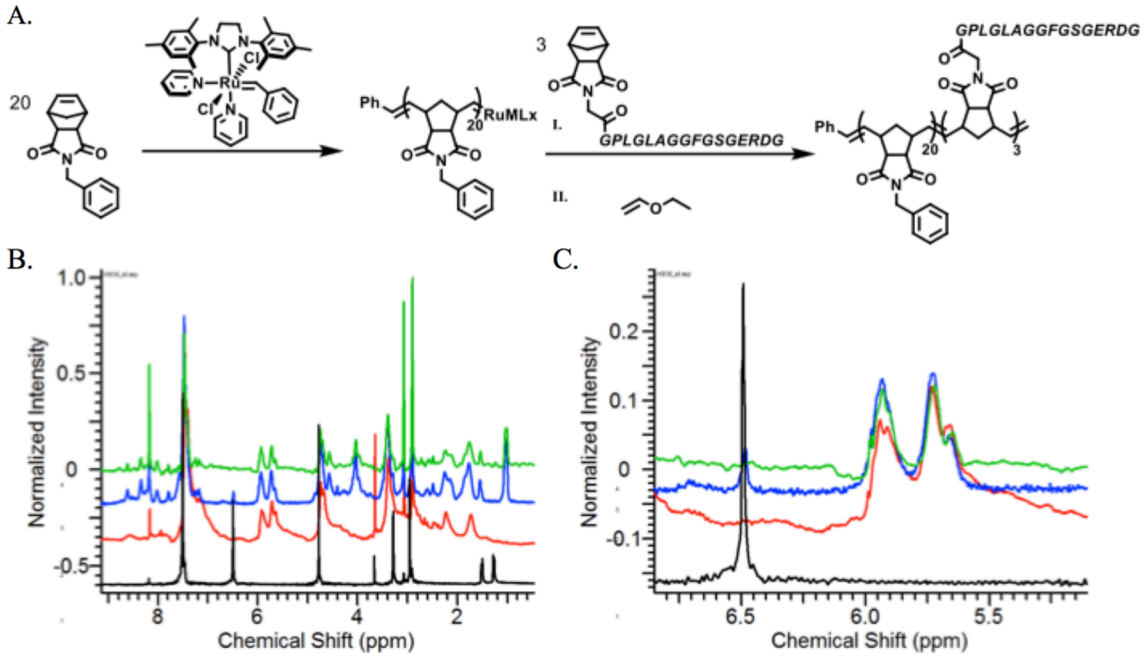


Figure S2. Preparation of block PPAs. (A) Scheme for the preparation of PPAs. (B) Full ^{1}H NMR spectrum for the PPA polymerization. Black is the initial NMR of the phenyl monomer. Red is the NMR after polymerization of the phenyl monomer where the singlet olefin chemical shift at 6.5 ppm has been consumed, and a new doublet chemical shift between 5.5 and 6 ppm has appeared corresponding to the cis/trans olefin protons on the polymer backbone. Blue is the NMR after peptide addition. Green is the final NMR showing complete consumption of the peptide. (C) The ^{1}H NMR spectrum showing only the region corresponding to the olefinic protons.

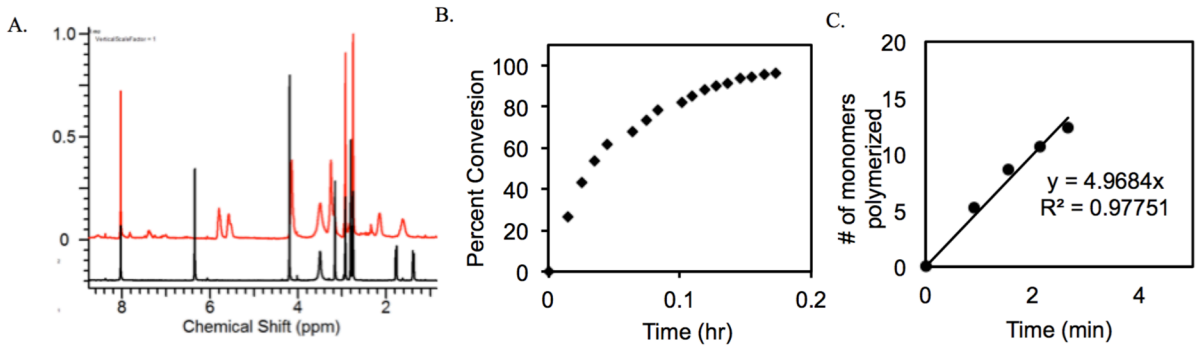


Figure S3. Polymerization kinetics of the norbornene glycine monomer. (A) ^{1}H NMR before (Black) and after (Red) complete monomer consumption. (B) The percent conversion of the monomer over time. (C) The number of monomers polymerized vs time.

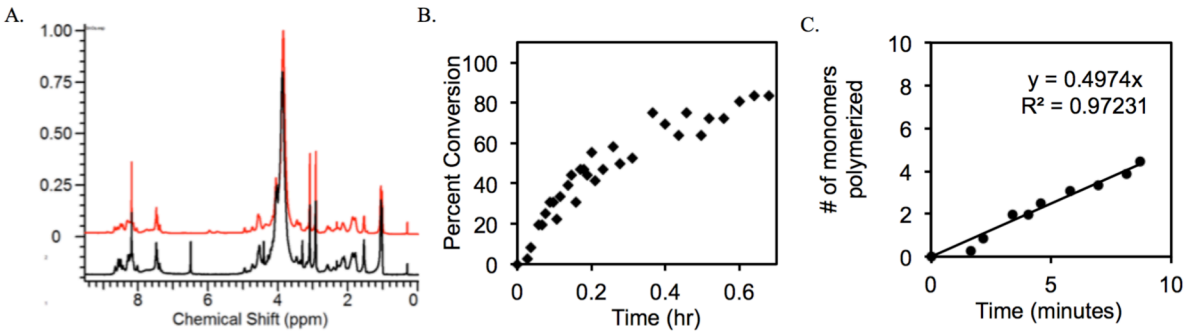
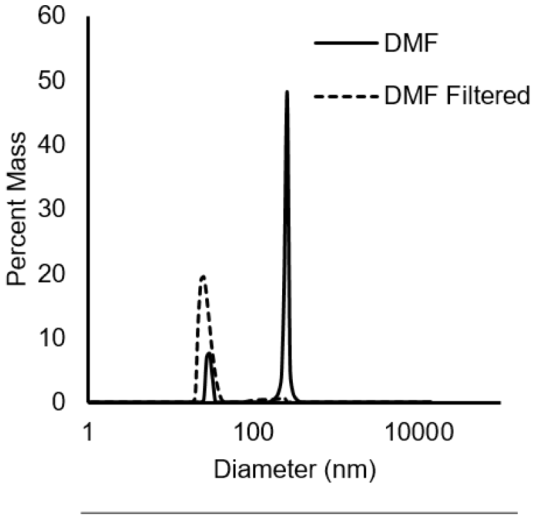


Figure S4. Polymerization kinetics of the norbornene peptide monomer. (A) ^{1}H NMR before (Black) and after (Red) complete monomer consumption. (B) The percent conversion of the monomer over time. (C) The number of monomers polymerized vs time.



	Diameter (nm)	Percent Mass
Unfiltered	29.1	18.8
	243.1	81.2
Filtered	26.5	94.5
	164.7	5.5

Figure S5. DLS of particles prepared from PPA in DMF. Note that particles that were filtered showed more monodispersed DLS data, likely due to the removal of dust and other large aggregates in solution.

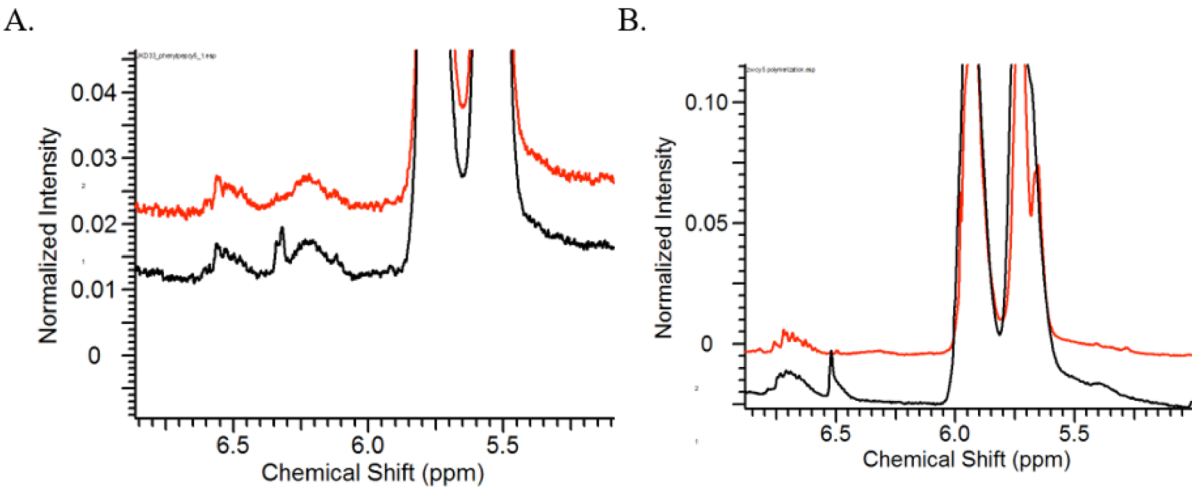


Figure S6. (A) ^{1}H NMR showing the spectrum immediately after the addition of Nor-$Cy_{5.5}$ (black) and 1 hour after the addition of Nor-$Cy_{5.5}$ (red). The small chemical shift around 6.3 ppm corresponds to the olefin protons on the Nor-$Cy_{5.5}$ monomer. The chemical shift is no longer present after 1 hour indicating the consumption of Nor-$Cy_{5.5}$. (B) ^{1}H NMR showing the spectrum immediately after the addition of Nor-Cy_{zw5} (black) and 1 hour after the addition of Nor-Cy_{zw5} (red). The small chemical shift around 6.3 ppm corresponds to the olefin protons on the Nor-Cy_{zw5} monomer. After 1 hour, the chemical shift is no longer present indicating the consumption of Nor-Cy_{zw5}.

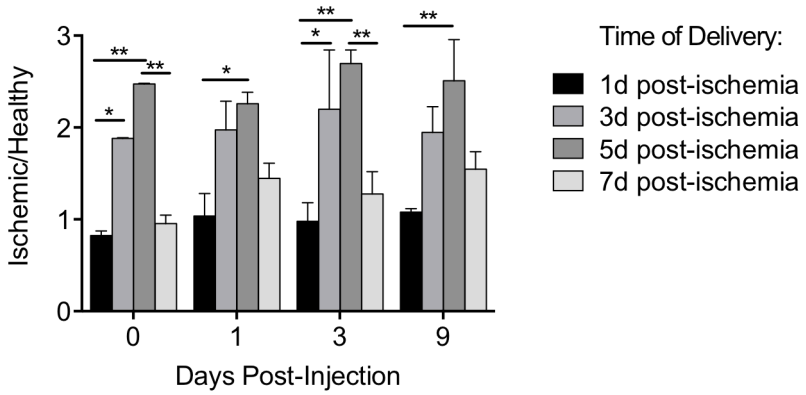


Figure S7. Delivery of PPA NPs after hindlimb ischemia. Cy5.5-labeled PPA NPs were injected at multiple time points (n=2 per group) after hindlimb ischemia and were monitored for 9 days post-injection to determine optimal delivery time point. Data is presented as mean±SEM. Analyzed with two-way ANOVA and post-hoc Tukey's multiple comparisons test. *p<0.05, **p<0.01.

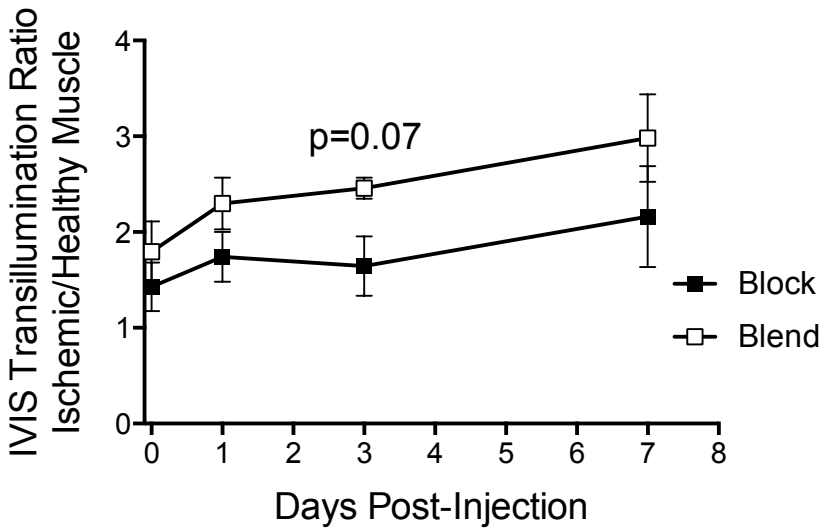


Figure S8. Targeting of block versus blend NPs after hindlimb ischemia. Cy5.5-labeled PPA NPs with the hydrophilic block consisting of just peptide ("block") or peptide mixed with spacer ("blend") were injected 4 days post-hindlimb ischemia (n=3). Animals were monitored for 7 days post-injection. Blend NPs targeted better than block NPs as indicated by a higher ischemic-to-healthy fluorescence ratio. Data is presented as mean±SEM. Data was analyzed with Student's T-test at each time point.

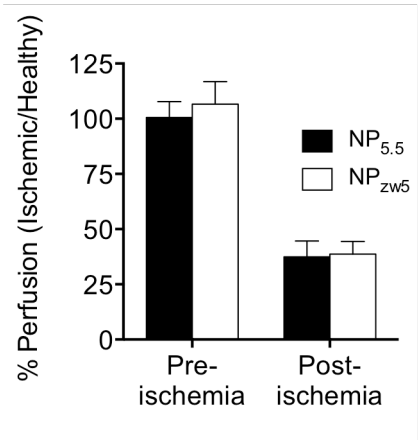


Figure S9. Blood perfusion pre- and post- hindlimb ischemia surgery as determined by laser speckle contrast analysis. Perfusion to the hindlimb was measured using laser speckle contrast analysis immediately before (pre-ischemia) and after (post-ischemia) the hindlimb ischemia surgery in order to ensure an adequate and similar degree of ischemia between experimental groups. N=8 for $NP_{5.5}$, n=12 for NP_{zw5}.

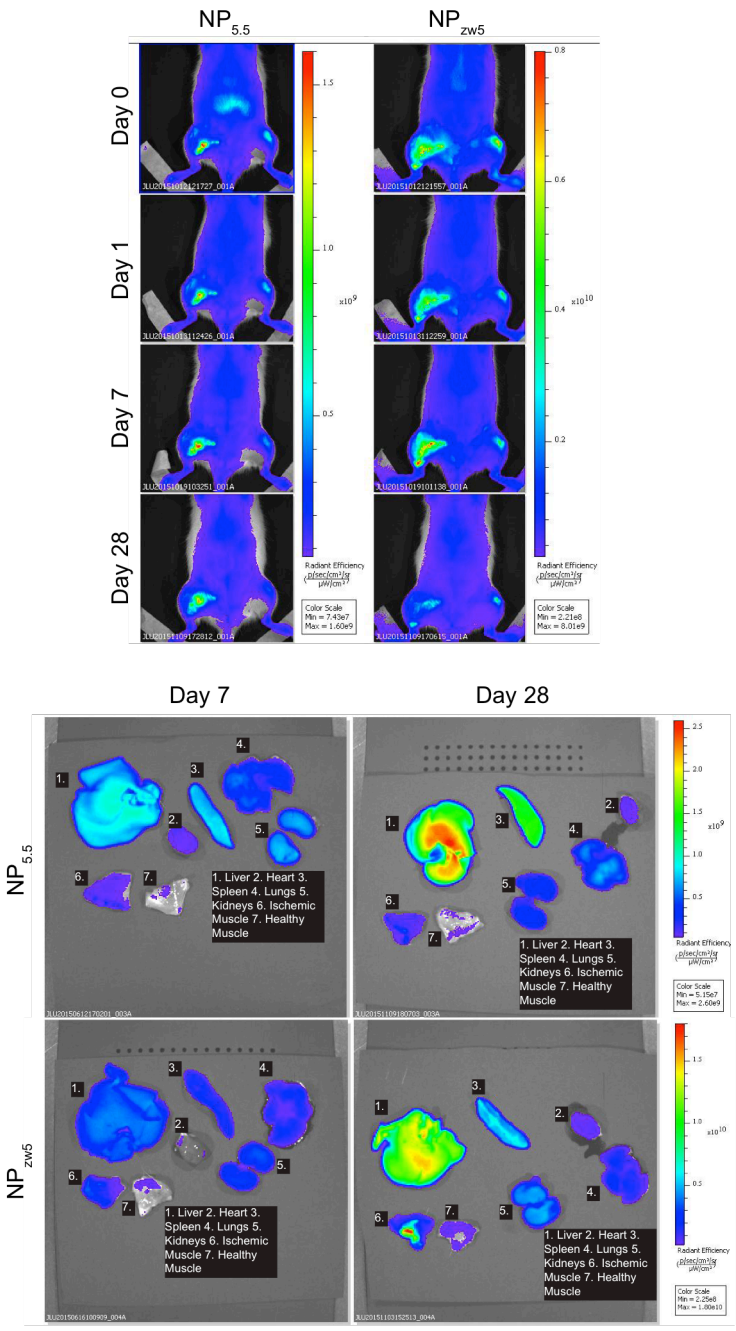


Figure S10. Top: representative IVIS timecourse images for $NP_{5.5}$ (left) and NP_{zw5} (right) groups. Bottom: representative IVIS *ex vivo* images at day 7 (left) and day 28 (right) post-injection. Each image represents a single animal throughout the time course. Cy5.5 and Cy5 are shown on different intensity scales to account for the differences in fluorescent intensity between the different dyes at their unique excitation/emission filters.

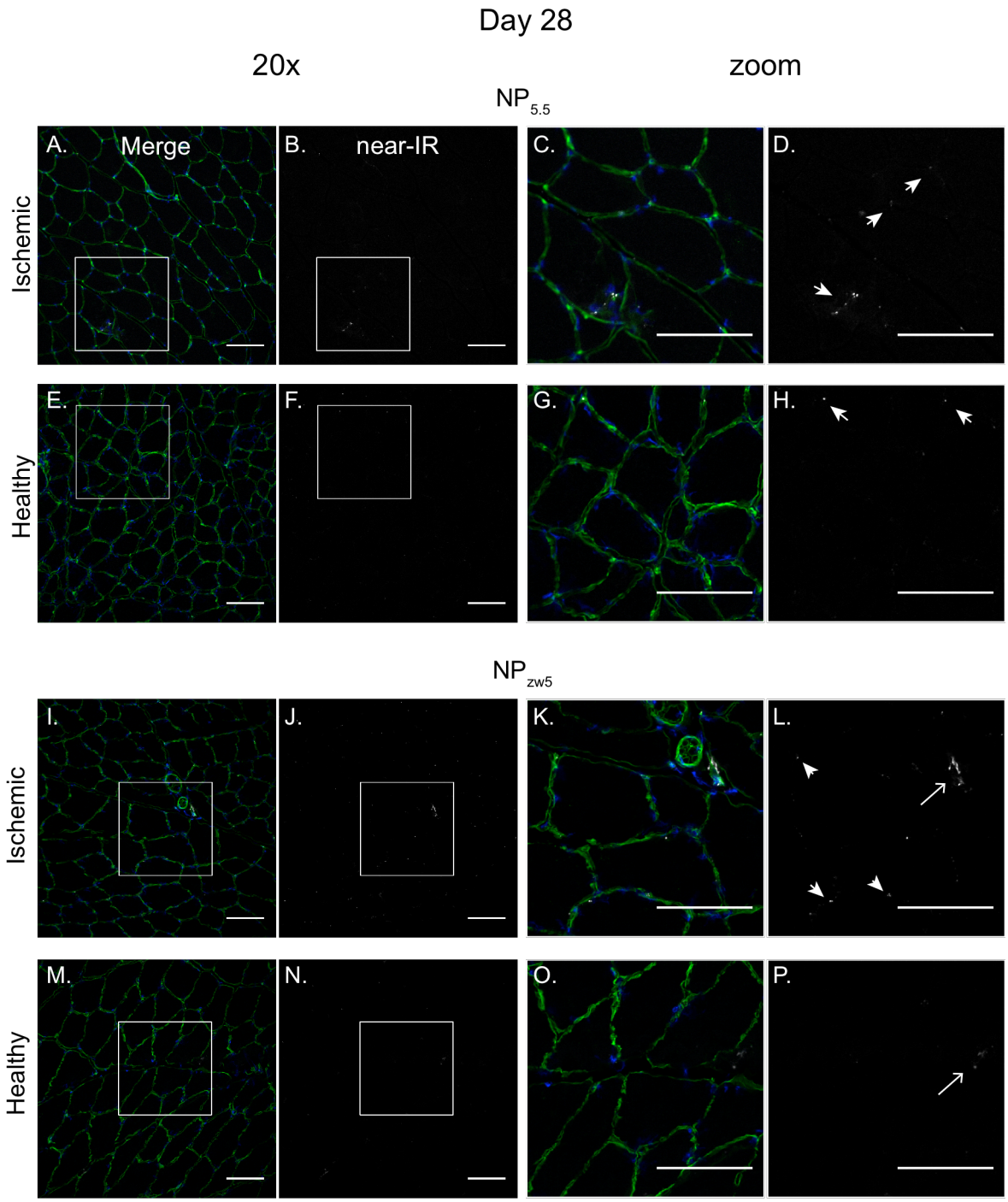


Figure S11. *Ex vivo* histology of ischemic and healthy muscle at 28 days post-injection. Immunohistochemistry of fresh frozen muscle cross sections stained for laminin (green) and nuclei (blue) shows nanoparticles (white) dispersed primarily through ischemic, and not healthy, muscle in both $NP_{5.5}$ (A-H) and NP_{zw5} (I-P) treated groups. Images on the left (A, B, E, F, I, J, M, N) show 20x views and images on the right (C, D, G, H, K, L, O, P) are zoomed in from the corresponding boxes on the left. (A, C, E, G, I, K, M, O) Three channel images. (B, D, F, H, J, L, N, P) Near-IR channel only. Thin arrows indicate perimysial localization while thick arrowheads indicate endomysial localization. Scale bars are 100 μm.

Supplemental References

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