Published in final edited form as:

Polym Chem. 2017 September 14; 8(34): 5212–5219. doi:10.1039/C7PY00568G.

Enzyme-Targeted Nanoparticles for Delivery to Ischemic Skeletal Muscle

J. L. Ungerleider^{a,†}, J. K. Kammeyer^{b,†}, R. L. Braden^a, K. L. Christman^{a,‡}, and N. C. Gianneschi^{b,‡}

^aDepartment of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, CA, USA 92037

^bDepartment of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA, USA 92093

Abstract

The targeted delivery of enzyme-responsive nanoparticles to specific tissues can be a valuable, minimally invasive approach for imaging or drug delivery applications. In this study, we show for the first time enzyme-directed assembly of intravenously (IV) delivered nanoparticles in ischemic skeletal muscle, which has applications for drug delivery to damaged muscle of the type prevalent in peripheral artery disease (PAD). Specifically, micellar nanoparticles are cleavable by matrix metalloproteinases (MMPs), causing them to undergo a morphological switch and thus aggregate in tissues where these enzymes are upregulated, like ischemic muscle. Here, we demonstrated noninvasive *in vivo* imaging of these IV-injected nanoparticles through near-infrared dye labeling and *in vivo* imaging (IVIS) particle tracking in a rat hindlimb ischemia model. Polymer peptide amphiphilic nanoparticles were synthesized and optimized for both MMP cleavage efficiency and near-IR fluorescence. Nanoparticles were injected 4 days after unilateral hindlimb ischemia and were monitored over 28 days using IVIS imaging. Nanoparticles targeted to ischemic muscle over healthy muscle, and *ex vivo* biodistribution analysis at 7 and 28 days post-injection confirmed targeting to the ischemic muscle as well as off target accumulation in the liver and spleen. *Ex vivo* histology confirmed particle localization in ischemic but not healthy muscle. Altering the surface charge of the nanoparticles through addition of zwitterionic dye species resulted in improved targeting to the ischemic muscle. To our knowledge, this is the first study to demonstrate the targeted delivery and long term retention of nanoparticles using an enzyme-directed morphology switch. This has implications for noninvasive drug delivery vehicles for treating ischemic muscle, as no minimally invasive, non-surgical options currently exist.

Keywords

Nanoparticles; hindlimb ischemia; matrix metalloproteinases; zwitterionic

[†]Denotes equal contributions.

[‡]Co-corresponding author.

Introduction

Peripheral artery disease (PAD) is a type of cardiovascular disease that afflicts nearly 30 million patients in the western world.¹ PAD is caused by atherosclerotic plaque-occluded vessels in the lower limbs, which can lead to pain, muscle atrophy, and potential amputation.² Current treatments for PAD patients involve revascularization such as balloon angioplasty and arterial bypass. However, both options have high morbidity rates and low 5 year patency rates.^{3, 4} Thus, over 200,000 patients in the U.S. and Europe require lower extremity amputations annually.⁵ There is a strong unmet clinical need for treating the ischemic muscle through improving blood perfusion and reducing muscle atrophy and tissue necrosis. Oftentimes the location of atherosclerotic lesions can prevent access via surgical or vascular intervention.^{6, 7} Moreover, treating a single atherosclerotic lesion may not be desirable because the disease affects the entire tissue. Therefore, a desirable treatment for patients with PAD would involve a minimally invasive approach using angiogenic, arteriogenic, and/or myogenic therapies. Additionally, targeting molecular mechanisms of the disease would be beneficial for early stage treatment, since the patients treated with surgical interventions have already developed severe ischemia, atrophy and necrosis. Nanoparticle (NP) therapies would be desirable because they are minimally invasive, systemically deliverable, and can target to specific hallmarks of disease progression. One option could be to augment revascularization procedures through intra-arterial delivery of nanoparticles at the time of intervention (e.g., balloon angioplasty), or potentially intravenously (IV) via venipuncture for patients who are ineligible for vascular interventions.

NP drug delivery has recently shown promise in preclinical models of cardiovascular disease.⁸ In a hindlimb ischemia model, Mooney and coworkers demonstrated effective delivery of vascular endothelial growth factor (VEGF) on gold NPs to ischemic tissue through the enhanced permeability and retention (EPR) effect known to be characteristic of both cancerous and ischemic tissues.^{9, 10} Another targeting strategy consists of ultrasound-microbubble rupturing to improve NP uptake into the ischemic limb.¹¹ However, neither of these particles was targeted to any particular molecular hallmarks of PAD tissue beyond leaky vasculature.

Recently, NPs comprised of peptide polymer amphiphiles (PPAs) were shown to aggregate in diseased tissue where matrix metalloproteinases (MMPs) are upregulated.^{12, 13} Specifically, these NPs consist of copolymers with hydrophobic and hydrophilic block regions that assemble into micellar nanoparticles.^{12, 14, 15} The hydrophilic block contains a peptide sequence recognized and cleaved by MMP-2/9. Upon cleavage, the particles undergo a morphological switch from NPs to a micron scale aggregate-like scaffold at the tissue of interest because of the change in amphiphilicity of the PPA. MMP-9 is upregulated in the skeletal muscle of humans¹⁶ and animals¹⁷ with critical or hindlimb ischemia, respectively, thus indicating that this targeted nanoparticle system may be a clinically viable option for treatment of ischemic tissues in patients with PAD. We have previously shown that this system initially designed for cancer diagnosis and treatment can also be applied to non-invasive NP delivery to ischemic myocardium.¹³ Furthermore, the system is easily tunable and has been conjugated to a variety of imaging and therapeutic functionalities,^{18–20} providing proof of concept for minimally-invasive drug delivery and subsequent targeted

retention. Our goal was to design enzyme-targeted PPA NPs and demonstrate their delivery and optimal biodistribution for targeting to ischemic muscle in a preclinical model of PAD. We synthesized near-IR labeled enzyme-responsive micellar NPs and demonstrated their enzyme responsiveness and targeting capability *in vivo* in a rat hindlimb ischemia model. This is the first study to show proof-of-concept for NP targeting and retention in ischemic muscle as a potential therapeutic vehicle for PAD patients.

Experimental

PPA synthesis and characterization

Detailed polymer synthesis and characterization methods are included in the supplemental material. Briefly, PPAs were synthesized via ring opening metathesis polymerization (ROMP) using $(\text{IMesH}_2)(\text{C}_5\text{H}_5\text{N})_2\text{Ru}=\text{CHPh}$. PPAs were prepared by first polymerizing N-benzyl-*cis*-5-norbornene-*endo*-2,3-dicarboximide (phenyl monomer) until complete monomer consumption, followed by the addition of N-(Peptide)-*cis*-5-norbornene-*exo*-dicarboximide (peptide monomer; peptide = GPLGLAGGFGSGERDG). First order polymerization kinetics of N-(glycine)-*cis*-5-norbornene-*exo*-dicarboximide (glycine monomer) and peptide monomer were determined by monitoring polymerization by ^1H NMR. For blend copolymers, N-(Glycine)-*cis*-5-norbornene-*exo*-dicarboximide was added every 2 minutes. After complete monomer consumption, polymers were either quenched with ethyl vinyl ether (EVE), or labeled with a near IR cyanine dye monomer and quenched with EVE after complete monomer consumption. PPAs were characterized using size exclusion chromatography coupled with multiangle light scattering to determine molecular weight and dispersity indices.

NP synthesis and characterization

PPAs were dissolved in dry DMF at 2 mg/mL concentration. 1x DPBS was then added to the solution at 1 mL/hr with rapid stirring to reach a final concentration of 1 mg/mL. NPs were then dialyzed into 1x DPBS to remove organic solvent. NPs were analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM) to determine size and morphology. Enzyme cleavage was determined by incubating NPs with thermolysin and characterizing the morphology by TEM.

In vivo studies

Detailed methods related to *in vivo* and *ex vivo* testing of the NPs are included in the supplemental material. All animal procedures were performed in compliance 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. The protocols under which animal experiments were performed were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. Female Sprague Dawley rats received unilateral hindlimb ischemia through ligation and excision of the femoral artery and vein as previously described.²¹ Immediately after surgery, perfusion measurements were made using a laser speckle contrast analysis system (PeriMed) to ensure adequate ischemia compared to the healthy limb. A pilot study was first performed to optimize the post-ischemia delivery time point. At 1, 3, 5,

or 7 days post-ischemia, animals received a tail vein injection of Cy5.5-labeled PPA NPs (400 nmol, n=2 per time point). Animals were regularly monitored at 2 hours, 1, 3, and 9 days post-injection using the transillumination mode on an *In Vivo* Imaging System (IVIS) Spectrum 200 (Perkin Elmer). On day 9 post-injection, animals were euthanized after the last imaging session.

After optimizing the injection time point, the rats received unilateral hindlimb ischemia through ligation and excision of the femoral artery and vein. Four days later, PPA NPs (400 nmol, end-labeled with either Cy5.5 [NP_{5.5}] or zwitterionic Cy5 [NP_{zw5}]) were injected in the tail vein. Animals were imaged using IVIS transillumination at day 0, 1, 3, 7, 14, 21, and 28 post-injection. Excitation/emission and exposure was 675nm/720nm and 10 seconds for NP_{5.5} and 640nm/680nm and 5 seconds for NP_{zw5} chemic to healthy muscle fluorescence ratios were calculated by drawing circular regions of interest in the Perkin Elmer Living Image Software around the thigh (gracilis) muscle, measuring the normalized transmission fluorescence (NTF) efficiency, and normalizing to the healthy limb internally for each animal. Animals were euthanized on day 7 (n=4) or day 28 (n=4 NP_{5.5}, n=8 NP_{zw5}) post-injection, and their organs were harvested 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 ($[p/s/cm^2/sr]/[\mu W/cm^2]$) was internally normalized to the healthy muscle of each animal to be able to compare between different fluorescent dye species.

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. Sections were stained for laminin (primary rabbit polyclonal anti-laminin 1:100, abcam; secondary AlexaFluor-488 goat anti-rabbit 1:500, Invitrogen) and Hoechst 33342 (ThermoFisher Scientific). Sections were imaged on a Nikon A1R Confocal microscope.

Results and discussion

Herein, we sought to investigate the use of MMP responsive NPs for noninvasive targeting and retention in a preclinical model of PAD. Our group has previously developed MMP-targeted PPA NPs and shown these particles target effectively to damaged tissue in xenogeneic tumors^{12, 15} and myocardial infarction models,¹³ both of which are known to have upregulated MMPs. We sought to further investigate this targeting strategy in an animal model of PAD to determine if these particles could target ischemic skeletal muscle after intravenous injection. Because of the nature of the polymeric materials, we were able to tune the design of the NPs by optimizing enzyme cleavage efficiency, and by incorporating differentially charged dyes, which altered the targeting to ischemic tissue. We first demonstrated targeted assembly of our original PPA NP design in a rat hindlimb ischemia model. We then undertook the synthesis and conjugation of a zwitterionic Cy5 to investigate whether a zwitterionic charge on the particles could improve targeting to ischemic muscle.

Synthesis of PPA NPs

PPAs were synthesized via ROMP using a ruthenium based initiator ((H₂IMES)-(pyr)₂(Cl)₂Ru=CHPh, I, Scheme 1). Similar to previous MMP responsive PPAs, we used a N-benzyl-*cis*-5-norbornene-*endo*-2,3-dicarboximide (phenyl monomer) for the hydrophobic block. However, we chose to investigate the composition of the hydrophilic block. Previous MMP responsive nanoparticles were prepared from block copolymers where the hydrophilic block contained only the N-(Peptide)-*cis*-5-norbornene-*exo*-dicarboximide peptide monomer; peptide = GPLGLAGGFGSGERDG, Figure S1). Recently, it was shown that homopolymers of peptides prepared via ROMP were resistant to proteolysis due to the dense packing of the peptide brush.²² Proteolysis could be enhanced by preparing a copolymer of a peptide with a small “spacer”. Inspired by these results, we were interested in comparing the original design (block copolymers) to a new design where the hydrophilic peptide monomer was copolymerized with a small “spacer” monomer, N-(Glycine)-*cis*-5-norbornene-*exo*-dicarboximide (Scheme 1).

Block PPA (where the hydrophilic portion contains only the peptide monomer) and blend PPA (where the hydrophilic portion contains a random copolymer of peptide monomer and glycine spacer monomer) were prepared by ROMP (Figure 1A, Figure S2). To prepare blend PPAs, the first order polymerization kinetics of each monomer were determined by ¹H NMR. Initial rate of glycine spacer monomer was determined to be 17.4 hr⁻¹ or 1 monomer every 0.20 minutes (Figure 1B, Figure S3). Initial rate of peptide monomer was determined to be 3.7 hr⁻¹ or 1 monomer every 2.01 minutes (Figure 1C, Figure S4). Block and blend PPAs were analyzed by size exclusion chromatography coupled with a multi angle light scattering detector (SEC-MALS) to determine molecular weight (M_n) and dispersity index (M_w/M_n) (Figure 1D–E). The resulting PPAs assembled into micellar nanoparticles (NPs) through the slow addition of DPBS buffer to a polymer solution in DMF, followed by dialysis into DPBS buffer, and resulting NPs were characterized by dynamic light scattering (DLS, Figure S5) and TEM. Indeed, when incubated with thermolysin, a model protease, the blend PPAs reached ~35 percent cleavage over 14 hours whereas the block copolymer reached ~25 percent, which supports previous studies showing increased sensitivity to protease cleavage when the peptide block is less dense (Figure 2A).²² For our *in vitro* enzyme assays, we chose thermolysin as a proxy for MMP because it is more reliable than commercially available MMPs and we have shown that it can cleave the same recognition sequence as MMP2/9.²³ Moreover, similar particle systems with the same enzymatic recognition sequences proved to be MMP responsive.^{12, 13, 15}

For *in vivo* tracking of NPs, PPAs needed fluorescent labeling. We chose to utilize NIR fluorescent, polymethine cyanine fluorophores (Cy) as NIR fluorophores allow for deeper tissue imaging with minimal tissue autofluorescence. We synthesized a negatively charged Cy5.5 analog bearing a norbornene moiety (Nor-Cy_{5.5}) to incorporate the fluorophore into ROMP polymers (for synthesis, see supplemental methods). Cy fluorophores can self-quench if positioned in close proximity,^{24–26} such as when fluorophore-labeled polymers assemble into micellar nanoparticles. Labeling each polymer in the particle could potentially lead to self-quenching and a reduced fluorescence signal. To prepare Cy-labeled PPAs, polymers were prepared as stated previously, then after complete monomer consumption of

the hydrophilic block, Nor-Cy_{5.5} monomer was added to the polymerization. To prepare NPs, labeled and unlabeled PPAs assembled at various ratios to determine the proper ratio to minimize self-quenching. Quenching was high with 1:0 ratio of labeled to unlabeled polymer, as seen with minimal fluorescent intensity (Figure 2B). Optimal fluorescence intensity was observed at a 1:1 ratio of labeled to unlabeled polymer, indicating this ratio exhibited the least amount of self-quenching while maintaining a high intensity fluorescent signal.

An additional zwitterionic NIR Cy fluorophore (Nor-Cy_{zw5}) was also synthesized. It has been shown that zwitterionic materials can be “stealthy” *in vivo* due to less uptake in the monocyte phagocytic system (MPS) and overall better circulation half-life.^{27–30} The current polymer system contained an overall negative charge at neutral pH, therefore we were interested in creating a more zwitterionic polymer for NP preparation. For assembled materials, a completely zwitterionic polymer may not be desirable because of difficulty obtaining a stable morphology due to lack of charge repulsion.³¹ Therefore, we envisioned labeling the polymer with a zwitterionic Cy5 monomer to improve the neutral charge character of the PPA and resulting surface of the assembled NPs. Full polymerization of either dye species into the PPAs was verified with ¹H NMR (Figure S6), and successful assembly of PPAs into NPs (NP_{5.5} and NP_{zw5}) was characterized by TEM (Figure 2C–D). Note that due to the laser wavelength of the light scattering instrumentation, no DLS data could be obtained of dye-labeled particles. NPs were confirmed to be responsive to thermolysin enzyme cleavage by TEM (Figure 3). NPs underwent a morphological switch only in the presence of active enzyme but not denatured enzyme, indicating they could form micron-scale aggregates after cleavage of the enzyme-labile peptide substrate. The addition of near-IR dyes and optimization of fluorescence quenching in this enzyme-targeted NP system allowed for non-invasive monitoring of the particle aggregation *in vivo*.

***In vivo* targeting**

NPs were first tested in a hindlimb ischemia model pilot study to optimize the injection timepoint for optimal MMP activity and thus targeting. Ryu *et al.* demonstrated that the highest MMP activity in the tissue would be around 5 days post-ischemia induction using an albumin based probe.³² However, there is variation inherent to the hindlimb ischemia model originating with different surgical technique, species used, and even between animal strains of the same species.^{33, 34} We validated optimal targeting in a pilot study by delivering NP_{5.5} at 1, 3, 5, or 7 days after the induction of hindlimb ischemia. Targeting to the ischemic limb was monitored through an *In Vivo* Imaging System (IVIS) by measuring fluorescence of the near-IR dye over 9 days post-injection. Targeting was determined by comparing the ratio of fluorescence of the ischemic limb compared to healthy limb. It was found that particles delivered at 3 and 5 days post-ischemia had the highest NP targeting (Figure S7). Thus, we chose to inject our particles at 4 days post-ischemia for the remaining studies to ensure we were following the timecourse of maximum MMP activity and thus particle efficacy.

We next sought to examine if improved enzyme cleavage *in vitro* would correlate with increased NP targeting *in vivo*. Cy-5.5-labeled block or blend NPs (same as those characterized in Figure 1 and Figure 2A) were injected IV 4 days post-ischemia and NPs

were monitored for 7 days post-injection using IVIS. Blend particles showed a trend of better targeting than block particles to the ischemic muscle (Figure S8). Thus, this suggests that less dense peptide packing can cause increased enzyme cleavage *in vitro* and improved targeting *in vivo*.

To examine the effect of NP surface charge on targeting and long term retention to the ischemic limb, NP_{5.5} and NP_{zw5} were administered IV four days after hindlimb ischemia injury and monitored by IVIS to either 7 or 28 days post-injection. PeriMed laser speckle contrast imaging confirmed that the degree of ischemia was similar in both experimental groups (Figure S9). Through IVIS monitoring at regular intervals post injection, we showed both NPs target to the ischemic limb as shown by an ischemic/healthy ratio above 1 (Figure 4A, Figure S10). The zwitterionic NPs targeted better to the ischemic limb at each time point, although this trend was not significant. This is the first study to show long term NP retention in ischemic skeletal muscle over at least 28 days, which will be significant for delivering therapeutics for prolonged release. This is consistent with previous studies showing enzyme responsive NP retention in infarcted myocardium for at least 28 days post-injection.¹³

After 7 or 28 days, organs were analyzed *ex vivo* using IVIS and normalized to healthy muscle fluorescence to make comparisons between dyes (Figure 4B, Figure S10). As expected with any NP therapy, the PPA NPs have significant accumulation in the liver and spleen. In agreement with *in vivo* IVIS data, we observed significantly higher fluorescence in the ischemic gracilis muscle of animals treated with NP_{zw5} compared to NP_{5.5} at 7 days post-injection, but this only trended higher at 28 days. This could be because zwitterionic NPs have a longer half-life, thus allowing for more efficient targeting to the ischemic limb through longer circulation time. Furthermore, there were no differences in fluorescent signal in any of the satellite organs between dye species, although we expected to see less NP_{zw5} trafficking to the liver and spleen. However, similar PPA NPs were shown not to cause any inflammatory reaction in off target organs through histopathological analysis,¹³ suggesting there are no significant toxicity concerns, although more through safety evaluation will be needed.

Immunohistochemistry of cross sections from ischemic and healthy muscle confirmed the presence of NPs in the ischemic muscle at a greater frequency than their presence in healthy contralateral muscle (Figure 5, Figure S11). Fluorescence from NP aggregates was apparent in the extracellular space of the endomysium and perimysium, which was visible through co-staining with laminin, a basement membrane protein ubiquitously expressed in skeletal muscle. This spatial localization is expected due to NP aggregation in the presence of extracellular MMP activity. NP fluorescence was present in the ischemic muscle at both 7 (Figure 5) and 28 days (Figure S11) post-injection for both NP_{5.5} and NP_{zw5}. Aggregates colocalized with extracellular laminin seen out to 28 days post-injection shows proof of concept of scaffold formation for possible prolonged, slow release of therapeutics.

Limitations

Although this study is a promising advance in the field of targeted NP delivery, there are several limitations. First, the NP system in its current form shows off target accumulation in

the liver and the spleen. Although this is a common hallmark of NP therapeutics, safety studies with therapeutic conjugated NPs will need to be evaluated in these organs to confirm no off target side effects. Second, the enzyme responsive NPs used for this study are not degradable; future studies will need to evaluate degradable systems. This study only examined a single delivery time point, but multiple injections over the course of months or years could be desirable for certain therapeutic regimens or for patients with chronic lesions. However, the long-term retention of our NP system could reduce the need for multiple injections due to the potential for long term, sustained release from NP aggregates. Lastly, although we did not explore therapeutic molecule delivery here, it is important to note that the PPA system is amenable to the incorporation of a variety of therapeutics. In our hands, similar types of PPA particles have been prepared that contain small molecule drugs in the core,¹⁸ or other peptides or nucleic acids in the shell.²⁰ Thus, this system can be easily modified for future therapeutic delivery.

Conclusions

MMPs are upregulated in animal models of hindlimb ischemia as well as ischemic skeletal muscle of human patients with PAD.^{16, 17} This study demonstrated that these enzymatic biomarkers of damaged ischemic muscle can be harnessed for systemic delivery, intramuscular targeting, and long term retention of an enzyme responsive NP. In this study, we synthesized MMP-responsive, PPA NPs with varied surface charge. We demonstrated that surface charge of the NPs had a significant effect on targeting to ischemic tissues. This study demonstrates proof of concept for the utility of enzyme-targeted vehicles for noninvasive delivery to ischemic skeletal muscle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the use of the UCSD Cryo-Electron Microscopy Facility, which is supported by NIH grants to Dr. Timothy S. Baker and a gift from the Agouron Institute to UCSD. We would like to thank Mollie Touve for TEM assistance and Billy Pistel and Andrea Carlini for their help with enzyme cleavage assays. This work was funded by a NIH Director's Transformative Research Award to KLC and NCG (R01HL117326). JLU would like to acknowledge NIH training grant and fellowship support through T32EB009830 and F31HL136082.

Notes and references

1. Hirsch AT, Haskal ZJ, Hertzner NR, Bakal CW, Creager MA, Halperin JL, Hiratzka LF, Murphy WRC, Olin JW, Puschett JB, Rosenfield KA, Sacks D, Stanley JC, Taylor JLM, White CJ, White J, White RA, Antman EM, Smith JSC, Adams CD, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Hunt SA, Jacobs AK, Nishimura R, Ornato JP, Page RL, Riegel B. Journal of the American College of Cardiology. 2006; 47:e1–e192. [PubMed: 16386656]
2. Regensteiner JG, Wolfel EE, Brass EP, Carry MR, Ringel SP, Hargarten ME, Stamm ER, Hiatt WR. Circulation. 1993; 87:413–421. [PubMed: 8425290]
3. Aihara H, Soga Y, Mii S, Okazaki J, Yamaoka T, Kamoi D, Shintani Y, Ishikawa T, Investigators RR. Circulation journal: official journal of the Japanese Circulation Society. 2014; 78:457–464. [PubMed: 24292129]

4. van de Weijer MAJ, Kruse RR, Schamp K, Zeebregts CJ, Reijnen MMPJ. *Seminars in vascular surgery*. 2015; 28:112–121. [PubMed: 26655055]
5. Lawall H, Bramlage P, Amann B. *Thrombosis and Haemostasis*. 2010; 103:696–709. [PubMed: 20174766]
6. Dormandy J, Heeck L, Vig S. *Seminars in vascular surgery*. 1999; 12:142–147. [PubMed: 10777241]
7. Marston WA, Davies SW, Armstrong B, Farber MA, Mendes RC, Fulton JJ, Keagy BA. *Journal of vascular surgery*. 2006; 44:108–114. [PubMed: 16828434]
8. Suarez S, Almutairi A, Christman KL. *Biomaterials science*. 2015; 3:564–580. [PubMed: 26146548]
9. Kim J, Cao L, Shvartsman D, Silva EA, Mooney DJ. *Nano Let*. 2011; 11:694–700. [PubMed: 21192718]
10. Weis SM. *Current Opinion in Hematology*. 2008; 15:243–249. [PubMed: 18391792]
11. Chappell JC, Song J, Burke CW, Klivanov AL, Price RJ. *Small (Weinheim an der Bergstrasse, Germany)*. 2008; 4:1769–1777.
12. Chien MP, Thompson MP, Barback CV, Ku TH, Hall DJ, Gianneschi NC. *Advanced materials*. 2013; 25:3599–3604. [PubMed: 23712821]
13. Nguyen MM, Carlini AS, Chien MP, Sonnenberg S, Luo C, Braden RL, Osborn KG, Li Y, Gianneschi NC, Christman KL. *Advanced materials*. 2015; 27:5547–5552. [PubMed: 26305446]
14. Chien MP, Thompson MP, Lin EC, Gianneschi NC. *Chemical science (Royal Society of Chemistry)*. 2010. 2012; 3:2690–2694.
15. Chien MP, Carlini AS, Hu D, Barback CV, Rush AM, Hall DJ, Orr G, Gianneschi NC. *Journal of the American Chemical Society*. 2013; 135:18710–18713. [PubMed: 24308273]
16. Baum O, Ganster M, Baumgartner I, Nieselt K, Djonov V. *Journal of vascular research*. 2007; 44:202–213. [PubMed: 17337906]
17. Muhs BE, Plitas G, Delgado Y, Ianus I, Shaw JP, Adelman MA, Lamparello P, Shamamian P, Gagne P. *Journal of Surgical Research*. 2003; 111:8–15. [PubMed: 12842442]
18. Callmann CE, Barback CV, Thompson MP, Hall DJ, Mattrey RF, Gianneschi NC. *Advanced materials*. 2015; 27:4611–4615. [PubMed: 26178920]
19. Rush AM, Thompson MP, Tatro ET, Gianneschi NC. *ACS nano*. 2013; 7:1379–1387. [PubMed: 23379679]
20. Rush AM, Nelles DA, Blum AP, Barnhill SA, Tatro ET, Yeo GW, Gianneschi NC. *Journal of the American Chemical Society*. 2014; 136:7615–7618. [PubMed: 24827740]
21. Ungerleider JL, Johnson TD, Hernandez MJ, Elhag DI, Braden RL, Dzieciatkowska M, Osborn KG, Hansen KC, Mahmud E, Christman KL. *J Am Coll Cardiol: Basic Transl Sci*. 2016; 1:32–44.
22. Blum AP, Kammeyer JK, Yin J, Crystal DT, Rush AM, Gilson MK, Gianneschi NC. *Journal of the American Chemical Society*. 2014; 136:15422–15437. [PubMed: 25314576]
23. Ma CD, Adamiak L, Miller DS, Wang X, Gianneschi NC, Abbott NL. *Small*. 2015; 11:5747–5751. [PubMed: 26418129]
24. Weissleder R, Tung CH, Mahmood U, Bogdanov A. *Nat Biotech*. 1999; 17:375–378.
25. Mahmood U, Weissleder R. *Molecular Cancer Therapeutics*. 2003; 2:489–496. [PubMed: 12748311]
26. Ogawa M, Kosaka N, Choyke PL, Kobayashi H. *Cancer research*. 2009; 69:1268–1272. [PubMed: 19176373]
27. Choi HS, Nasr K, Alyabyev S, Feith D, Lee JH, Kim SH, Ashitate Y, Hyun H, Patonay G, Strekowski L, Henary M, Frangioni JV. *Angewandte Chemie (International ed in English)*. 2011; 50:6258–6263. [PubMed: 21656624]
28. Ladd J, Zhang Z, Chen S, Hower JC, Jiang S. *Biomacromolecules*. 2008; 9:1357–1361. [PubMed: 18376858]
29. Chang Y, Liao SC, Higuchi A, Ruaan RC, Chu CW, Chen WY. *Langmuir*. 2008; 24:5453–5458. [PubMed: 18399670]
30. Xiao W, Lin J, Li M, Ma Y, Chen Y, Zhang C, Li D, Gu H. *Contrast media & molecular imaging*. 2012; 7:320–327. [PubMed: 22539402]

31. Barnhill SA, Bell NC, Patterson JP, Olds DP, Gianneschi NC. *Macromolecules*. 2015; 48:1152–1161.
32. Ryu JH, Shin JY, Kim SA, Kang SW, Kim H, Kang S, Choi K, Kwon IC, Kim BS, Kim K. *Biomaterials*. 2013; 34:6871–6881. [PubMed: 23773822]
33. Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, Ziegelhoeffer T, Brandt U, Pearlman JD, Swartz HM, Schaper W. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2006; 26:520–526.
34. Chalothorn D, Faber JE. *Physiological genomics*. 2010; 42:469–479. [PubMed: 20551146]

Figure 1. Preparation of polymer peptide amphiphiles (PPAs) for *in vivo* studies. (A) Chemical structure of block and blend PPAs. (B–C) First order polymerization kinetics for norbornene glycine monomer (B) and norbornene peptide monomer (C). (D–E) SEC-MALS trace for the block (D) and blend (E) polymers.

Figure 2.

Preparation of near-IR labeled NPs. (A) Block vs. blend cleavage kinetics when incubated with thermolysin, a model protease, as determined by HPLC. (B) Fluorescence intensity of NPs with a ratio of labeled-to-nonlabeled PPAs of 1:1 or 1:0. PPAs were labeled with the negatively charged Nor-Cy_{5.5}, and fluorescence intensity was found to be the greatest when a 1:1 ratio of labeled-to-unlabeled NPs was prepared. (C–D) CryoEM of particles labeled with negatively charged Cy 5.5 (C) and particles labeled with zwitterionic Cy 5 (D). Scale bar is 50 nm.

Figure 3. Characterization of morphology of NPs after treatment with thermolysin, a model protease. TEM images show monodispersed NPs after denatured enzyme treatment, but with active enzyme treatment NPs appear to be large micronscale aggregates. Scale bar is 100 nm.

Figure 4.

Targeting of near-IR NPs over 28 days. (A) PPA NPs were injected 4 days after hindlimb ischemia and monitored *in vivo* at 0, 1, 3, 7, 14, 21, and 28 days post-injection using IVIS. Half of the animals (n=4 for each group) were harvested at day 7; n=4 (NP_{5.5}) and n=8 (NP_{zw5}) animals were followed for the full 28 day study. Data is reported as the ratio of ischemic to healthy muscle fluorescence as measured through the IVIS transillumination setting. (B) *Ex vivo* organs were analyzed using IVIS epifluorescence to determine biodistribution. Average radiant efficiency [(P/sec/cm²/sr)/(μW/cm²)] was measured for each organ and internally normalized to the healthy muscle to account for differences in fluorescent intensity between cyanine dyes. NP_{5.5} and NP_{zw5} were compared using Student's t-test at each time point or for each organ. *p<0.05.

Figure 5.

Ex vivo histology of ischemic and healthy muscle at 7 days post-injection.

Immunohistochemistry of fresh frozen muscle cross sections stained for laminin (green) and nuclei (blue) shows NPs (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 μ m.

Scheme 1.

Synthesis of blend PPAs labeled either with the negatively charged Cy 5.5 or zwitterionic Cy5 using ROMP with the initiator **I**.