Published in final edited form as:

Small. 2011 May 9; 7(9): 1227–1235. doi:10.1002/sml.201001934.

Covalently Linking Poly(Lactic-co-Glycolic Acid) Nanoparticles to Microbubbles Before Intravenous Injection Improves Their Ultrasound-Targeted Delivery to Skeletal Muscle

Caitlin W. Burke¹, Yu-Han J. Hsiang¹, Eben Alexander IV¹, Alexander L. Kilbanov^{1,4}, and Richard J. Price^{1,2,3}

¹Department of Biomedical Engineering, University of Virginia, Charlottesville, VA

^{2,3}Departments of Radiology & Radiation Oncology, University of Virginia, Charlottesville, VA

⁴The Cardiovascular Division, University of Virginia, Charlottesville, Virginia

Abstract

Intravenously-injected nanoparticles can be delivered to skeletal muscle through capillary pores created by the activation of microbubbles with ultrasound; however, strategies that utilize co-injections of free microbubbles and nanoparticles are limited by nanoparticle dilution in the bloodstream. Here, we tested whether fluorescently-labeled (VT680; far-red fluorophore) nanoparticle [~150nm; poly(lactic-co-glycolic acid)] delivery to skeletal muscle can be improved by covalently linking them to albumin-shelled microbubbles in a composite agent formulation. Studies were performed using an experimental model of peripheral arterial disease, wherein the right and left femoral arteries of BalbC mice were surgically ligated. Four days after arterial ligation, composite agents, co-injected microbubbles and nanoparticles, or nanoparticles alone were administered intravenously and 1 MHz pulsed ultrasound was applied to the left hindlimb. Nanoparticle delivery was assessed at 0, 1, 4, and 24 hrs post-treatment by fluorescence-mediated tomography. Within the co-injection group, as expected, both microbubbles and ultrasound were required for nanoparticle delivery to skeletal muscle. Within the composite agent group, nanoparticle delivery was enhanced 8- to 18-fold over “no ultrasound” controls, depending on the time of measurement. A maximum of 7.2% of initial nanoparticle dose per gram tissue (ID/g) was delivered at 1 hr in the composite agent group, which was significantly greater than in the co-injection group (3.6% ID/g). We conclude that covalently linking 150 nm diameter poly(lactic-co-glycolic acid) nanoparticles to microbubbles before intravenous injection can improve their delivery to skeletal muscle.

Keywords

microbubble; nanoparticles; targeted drug delivery; skeletal muscle

Correspondence: Richard J. Price, PhD, Department of Biomedical Engineering, University of Virginia, Box 800759, Health System, Charlottesville, VA 22908 (rprice@virginia.edu). Fax: (434) 982-3870, Phone: (434) 924-0020..

*Dr. R. J. Price University of Virginia Department of Biomedical Engineering, Radiology, and Radiation Oncology Box 800759, Health System Charlottesville VA, 22908 (USA) Fax: (+1) 434-982-3870 rprice@virginia.edu

C. W. Burke and Y-H J. Hsiang University of Virginia Department of Biomedical Engineering Box 800759, Health System Charlottesville VA, 22908 (USA) Fax: (+1) 434-982-3870

Dr. A. L. Kilbanov University of Virginia Cardiovascular Medicine Box 800500, Health System Charlottesville VA, 22908 (USA) Fax: (+1) 434-982-3183 alk6n@virginia.edu

1. Introduction

Over the past twenty years, substantial research efforts have been invested into the development of gene or drug delivery strategies to stimulate collateral artery growth (therapeutic arteriogenesis) for patients suffering from peripheral arterial disease (PAD).^[1] While growth factor delivery strategies for stimulating therapeutic arteriogenesis have shown success in animal studies and small scale clinical trials; double-blinded controlled trials have shown limited effectiveness to date.^[2,3,4] This lack of efficacy could, at least in part, be explained by insufficient recombinant protein concentration at the site of action and/or poor protein stability. For example, intravenous delivery of proangiogenic growth factors in small animal studies resulted in less than 0.04% of the initial dose delivered per gram of muscle.^[5,6] While intramuscular (IM) delivery techniques increase payload concentration at the site of action, resulting in increased therapeutic efficacy;^[7] IM delivery is invasive, heterogeneously delivers drug in pockets at site of injection, and lacks tissue specificity. Clearly, there is a need for a drug delivery system that can safely concentrate the delivery therapeutics.

One promising method to target and enhance drug and gene delivery is the use of contrast ultrasound (US).^[8–10] It is generally thought that contrast agent microbubble (MB) expansion and collapse in an acoustic field facilitates the delivery of intravascularly administered drugs to tissue by permeabilizing cellular membranes^[11] and/or the microvasculature,^[8,12] and such approaches have been shown to enhance delivery, as compared to systemic administration, to the heart, tumors, skeletal muscle and brain.^[13–16] However, US-MB based approaches in which MBs are administered with a “free drug” are still subject to concerns about drug toxicity, poor aqueous solubility, and/or rapid clearance.^[17,18] To address these concerns and increase payload delivery while reducing off target effects, drugs and/or genes may be incorporated into or onto MB shells.^[19–25] Alternatively, drugs and/or genes can be incorporated into nanoparticles (NPs) and either co-injected with MBs^[26–28] or linked to MB shells.^[29–32]

By linking drugs or genes to MB shells, either chemically or electrostatically, higher payload delivery may be achieved.^[21–25] However, with such formulations, the total payload is limited by shell surface area, the bioactivity of the drug may not be preserved, and the release of the drug upon ultrasound application may not be well-controlled. In many applications, such as the delivery of chemotherapeutics, this could necessitate multiple treatments. By combining MBs with NP drug/gene carriers, drug loading capacity and circulation time may be increased, and the controlled release of the drug/gene from the NP may be achieved. Such approaches have resulted in extravascular drug delivery to heart and skeletal muscle^[26–28] and amplified arteriogenesis after the targeted delivery of NPs bearing fibroblast growth factor-2 to ischemic hindlimb.^[27]

Nonetheless, we and others hypothesize that further improvements in secondary NP delivery may be made by conjugating them to MBs.^[29–32] Indeed, in vitro studies have shown that the application of US results in the targeted deposition of NPs^[29] and liposomes^[30], as well as enhanced transfection with plasmid loaded PEGylated lipoplexes,^[31,32] when such secondary nanocarriers are coupled to the shells of MBs using avidin-biotin bridges. In addition, coating MBs with “stealth” secondary carriers, such as PEGylated lipoplexes, has the potential to increase agent circulation time; thereby increasing the probability for US mediated agent destruction. In this study, covalent coupling between poly(lactic-co-glycolic acid) (PLGA) nanoparticles and microbubbles was done via carbodiimide chemistry to create so-called microbubble-nanoparticle composite agents (MNCAs).

Our objective was to determine whether US-targeted PLGA NP delivery to skeletal muscle may be improved through the use of MNCAs. In a clinical manifestation, NP delivery to skeletal muscle would most likely be performed in the presence of morbidity; therefore, we used a standard experimental model of hindlimb ischemia, which simulates PAD. This model is well-suited for studying collateral artery growth, and versions of this model have been used in pre-clinical studies on ultrasonic microbubble destruction for this purpose.^[21,27,33–36] Overall, our in vivo results from this study show that US application significantly enhances PLGA NP delivery after intravenous MNCA injection and that MNCAs can provide enhanced PLGA NP delivery to skeletal muscle when compared to a MB+NP co-injection strategy.

2. Results and Discussion

2.1. Microbubble-Nanoparticle Composite Delivery Agent Rationale and Characterization

We and others^[2627,28,37] have previously shown that the application of ultrasound to a targeted organ or tissue after the intravascular co-injection of NPs and MBs can result in the delivery of NPs to tissue. Here, we adapted a water-in-oil-in-water method to fabricate biodegradable PLGA NPs loaded with bovine serum albumin to target to ischemic muscle using US-MB approaches. Analysis with a submicron-particle analyzer (Multisizer IIe, Beckman Coulter) revealed a mean NP diameter of $154.9 \pm 79.65\text{nm}$ (Figure 1A). Analysis with a Zetasizer (Zetasizer 3000, Malvern Instruments,) revealed a zeta potential of $-43.3 \pm 3.93\text{ mV}$. Scanning electron microscopy (SEM) images confirmed that the PLGA NPs were spherical (Figure 1B). PLGA NPs exhibited an average of 2400 VivoTMTag680 fluorochromes per NP as determined spectrophotometrically.

While US-MB approaches can generate substantial NP delivery when an arterial injection site just upstream of the target tissue is utilized; this technique is invasive. However, when the co-injection is done intravenously, NP dilution in the bloodstream can reduce effectiveness. Therefore, we reasoned that covalently linking NPs to MBs in a composite agent formulation (MNCA) before intravenous injection would generate greater delivery because it would ensure that a relatively high concentration of NPs would be present at sites where MBs had increased microvessel permeability. To this end, MNCAs were fabricated by coupling PLGA NPs to MBs using carboiimide chemistry linkers. Coupling resulted in an average of 518 ± 236 NPs per MB, as determined by UV-visible spectroscopy (Pharmacia Biotech, Ultrospec 3000). The average mean diameter of MNCAs was determined to be $1.72\text{ }\mu\text{m} \pm 1.28\text{ }\mu\text{m}$, as measured by electrozone sensing (Multisizer-III, Beckman Coulter). An image of a single MNCA in solution is shown in Figure 1C. Flow Cytometry imaging performed on an ImageStream (Amnis Corporation, Seattle, WA), revealed the median MB mean pixel intensity was 171.75 for the MNCA group (Figure 2, *top row*) and 0 for MBs incubated with NPs (Figure 2, *middle row*) as well as for MBs alone (Figure 2, *bottom row*). Results suggest minimal non-specific association of NPs with MBs in the co-injection group, which is not surprising due to the negative charge of both NPs and MBs, and a high degree of NP linkage in the MNCA group.

2.2. Fluorescence Mediated Tomography Scans Illustrate Nanoparticle Delivery to Skeletal Muscle

We then determined whether the delivery of NPs to tissue could be enhanced by the destruction of MBs and/or MNCAs. In this study, VivoTag680TM labeled NPs were administered intravenously (IV) with MBs or coupled to MBs into mice whose hindlimbs had been made ischemic by placing a ligature around the femoral artery. The left ischemic hindlimbs were exposed to pulsed 1MHz ultrasound and FMT was used to analyze NP delivery to skeletal muscle 0, 1, 4, and 24 hours after treatment (Figure 3A). For both the

MNCA (Figure 3A; *top row*) and MB+NP co-injection (Figure 3A; *middle row*) groups, fluorescence intensity was clearly greater on the ultrasound-treated side when compared to the contralateral “no ultrasound” side. While the contralateral “no ultrasound” side exhibited some trace amounts of fluorescence signal, based on results from previous studies,^[26,27,37] it is highly unlikely that circulating ~150nm NPs were crossing the endothelium. Instead, any background fluorescence observed in the contralateral “no ultrasound” hindlimb can be attributed to a population of NPs that stick to the endothelium and remain in vessel lumens, (Figure 3A). While enhanced fluorescence was evident with ultrasound application at all timepoints, it appeared to be especially strong at 0, 1, and 4 hours. Applying ultrasound to IV injected NPs without MBs present yielded little to no increase in fluorescence intensity (Figure 3A; *bottom row*).

Because we made longitudinal FMT measurements over a 24 hr period after US application, our experiments had the potential to capture dynamic changes in NP delivery. Nonetheless, we did not observe any statistically significant differences in NP delivery through time with either approach, suggesting that virtually all NP delivery occurred rapidly after US application. In the context of the MB+NP+US group, one explanation is that the permeabilization effects were transient, which would limit the ability of circulating NPs to collect in the muscle over time. Alternatively, it could imply that MNCA and/or free NP circulation times are relatively short. This would not be surprising because, in this proof-of-principle study, neither the MNCA nor the PLGA NPs were designed to be “stealthy” (i.e. avoid phagocytosis and thus circulate longer), which could be achieved by adding PEG brushes to the NPs. Clearly, this is one area in which improvements in NP delivery could be achieved.

2.3. Nanoparticle Delivery to Skeletal Muscle Is Dependent on Both Ultrasound and Microbubbles When Using an Intravenous Co-Injection Strategy

To determine the percentage of the initial dose of nanoparticles delivered per gram of tissue (%ID/g), fluorochrome concentration within the skeletal muscle was quantitatively analyzed using FMT scans. Results from the co-injection and NP groups are shown in Figure 4. The effect of US application on NP delivery within the co-injection group is illustrated by comparing the MB+NP+US group to the MB+NP group. Here, as expected based on results from previous studies,^[26,27,37] US application caused a significant increase in NP delivery overall. However, no significant differences were observed at individual time points. Meanwhile, the effect of MBs on NP delivery is shown by comparing the MB+NP+US group to the NP+US group. Here, MBs were shown to have a significant effect on NP delivery overall, with pairwise comparisons revealing 150% and 209% increases in NP delivery at 1 and 4 hrs, respectively.

2.4. Ultrasound Facilitates Nanoparticle Delivery to Skeletal Muscle After Intravenous MNCA Injection

We then determined whether acoustic activation of MNCA could be used to increase nanoparticle delivery to skeletal muscle. Quantitative results from FMT scans within the MNCA group are shown in Figure 5. Ultrasound application elicited statistically significant 18-, 8.4-, and 16.3-fold increases in NP delivery at 0, 1, and 4 hrs after treatment, respectively, when compared to contralateral “no ultrasound” controls. Absolute NP delivery was 4.7-, 7.2- and 5.4 % ID/g which is equivalent to 0.19, 0.28, and 0.22 µg of polymer NPs per gram of muscle; depending on the weight of the mouse. Although there appeared to be a trend toward decreasing fluorescence intensity at 4 and 24 hrs in the MNCA+US group, no statistically significant differences in NP delivery were observed between time points. One hour after treatment with MNCA, the liver, kidney, spleen, lungs

and heart were excised and FMT was used to analyze non-specific NP accumulation. Quantitative results from FMT organ scans are shown in Figure 3B.

The delivery of circulating NPs to skeletal muscle is challenging when compared to many other organs (tumor, liver, spleen) due in part to a relatively low blood volume.^[38] In one study, following the IV administration of untargeted particles (20–60nm), only 0.45–0.75 %ID/g accumulated in muscle. This value was ~30-fold less than in liver.^[39] In another study, NPs that were targeted to the $\alpha_v\beta_3$ integrin accumulated ~3-fold more than untargeted NPs in ischemic muscle; however, only 0.25% ID/g was delivered.^[40] In contrast, we achieved 150 nm diameter NP delivery to skeletal muscle in the range of 4.7 to 7.2 %ID/g over a 4 hour period, which is several fold higher than in previous reports. Differences in NP size and composition must be considered when making such comparisons; however, we contend that US-MB techniques may have a significant advantage in targeting NPs to skeletal muscle. Additionally, in contrast to studies by other investigators,^[39,40] we examined NP delivery to adductor muscle in the context of the ischemic hindlimb model, wherein blood flow and/or tissue oxygenation are altered. While it is possible that these conditions may have served to enhance NP delivery, it is important to note that we have shown US-MB-targeted NP delivery to non-ischemic skeletal muscle as well in previous studies^[12,26,27]. Thus, the ability of US and MBs to target NP delivery is not limited to ischemic muscle.

2.5. Coupling Nanoparticles to Microbubbles Before Intravenous Injection Enhances Their Ultrasound-Targeted Delivery to Skeletal Muscle

To determine whether coupling MBs to NPs in the MNCA formulation results in improved NP delivery, comparisons were made between the MNCA+US and MB+NP+US groups (Figure 6). These treatment groups received identical MB and NP concentrations and were exposed to the same US pulsing protocol. Here, the ANOVA revealed a significant overall increase in NP delivery with the MNCA formulation. At 1 hr after US application, NP delivery was 2-fold higher for the MNCA+US group. Once again, no statistically significant differences in NP delivery were observed between different time points.

In general, these results are consistent with in vitro studies performed by other investigators that have demonstrated successful delivery using “composite” formulations in which therapeutic nanocarriers are linked to MBs.^[29–32] Although no statistically significant time-dependent differences in NP delivery were observed, we do note that there was an apparent trend toward decreased NP delivery with time in the MNCA+US group (Figure 6). One possible explanation is that fluorochrome release from the degrading PLGA polymer is enhanced with MNCA agents, perhaps due to differences in NP location in the tissue when compared to the MB+NP+US group. If this is the case, free fluorochrome could be undergoing removal from the muscle tissue via lymphatic pumping. Alternatively, it is possible that some of the MNCA shells with bound NPs are becoming “dislodged” from capillary endothelium due to shear forces created by blood flow. In essence, this would result in some of the fluorochrome signal being washed out of the tissue.

2.6. Distribution of Nanoparticles in Skeletal Muscle After Delivery with Ultrasound and Microbubbles

To determine whether covalently linking the NPs to the MBs affects NP distribution in muscle tissue after delivery, we examined US-treated muscles 1hr after intravenous injection of MNCA (Figure 7; *top row*), MBs+NPs (Figure 7; *middle row*), or NPs (Figure 7; *bottom row*). Nanoparticles labeled with a far-red fluorophore (VT680) have been false colored to red, while capillaries appear green. In general agreement with the FMT data in Figure 6, overall NP delivery appeared significantly greater for the MNCA+US group when compared

to the MB+NP+US group. For the MNCA+US and MB+NP+US groups, many NPs were co-localized with BS-I lectin, indicating delivery to the capillary endothelium. Many NPs were also present in the extravascular space between muscle fibers, indicating that significant delivery to the interstitium had also occurred. In contrast, ultrasound application to NPs in the absence of MBs revealed (Figure 7; *bottom row*) no apparent NP delivery.

Consistent with results from a previous study by our group,^[27] the MB+NP+US co-injection group exhibited NP delivery to both the endothelium and the interstitial space. We contend that NP distribution after co-injection into both of these compartments is not surprising because 2 primary modes of NP delivery with US and MBs are known and are likely to have occurred.^[41–45] In the first mode, MB oscillation reversibly opens pores in cell membranes through microstreaming. Presumably, such a response permitted the transfer of NPs to the endothelium. In the second mode, MB expansion creates high circumferential wall stresses that open pores through the capillary basement membrane,^[41] subsequently allowing NP delivery to the interstitium by convective forces.^[26,37] In comparison to direct intramuscular delivery, we contend that US+MB approaches may confer a significant advantage because they more homogeneously distribute the payload throughout the region of insonation,

With regard to the MNCA+US group, we observed both similarities and differences in NP delivery distribution when compared to the MB+NP+US group (Figure 7). Like the MB+NP+US group, NPs were both co-localized with the endothelial lectin stain and present in the extravascular space. Importantly, this would appear to indicate that US-activated MNCA also elicit both endothelial sonoporation and capillary wall permeabilization. The primary difference between these 2 groups was that many of the endothelial-associated NPs in the MNCA group appeared to be significantly more concentrated. While this could be because the MNCA permit better endothelial delivery through enhanced sonoporation and/or higher local NP concentration, it is also possible that NPs were not always dissociating from MB shell fragments upon insonation, which would lead to NP clustering. Indeed, we have previously shown that MB shell fragments may remain on capillary walls in skeletal muscle after insonation,^[12] and it has been reported that nanobeads that are initially adhered to MB shells remain on MB shell fragments after MB destruction.^[29] On the other hand, in studies utilizing other nanocarrier-microbubble linker strategies, nanocarrier dissociation from insonated MBs has been widely reported.^[30–32] Furthermore, diffuse NP delivery to the extravascular space was also clearly observed in the MNCA group in our study (Figure 7). Based on this evidence, we contend it is likely that NPs dissociated from MBs upon insonation in the MNCA+US group, albeit incompletely and/or inconsistently.

Indeed, going forward, it will be important to better understand and reduce response heterogeneity. Both US settings (i.e. duty cycle,^[46] pulse repetition frequency,^[47] and power^[37,48]) and microbubble parameters (i.e. concentration,^[48, 49] size,^[50] and shell composition) will influence MB oscillation and expansion, permeability enhancement, and subsequent NP transport. For example, fabrication of the MNCA in our study caused their diameters to decrease by ~40 nm when compared to our typical albumin-shelled MBs. Although our results indicate that endothelial and extravascular NP delivery still occurred with the MNCA, both their acoustic response and potential for generating microvascular permeabilization may have been affected.^[51–53] In addition, it will be important to determine the relative effectiveness of MNCA-assisted delivery in different organs, so that appropriate strategies are designed to improve local drug and gene delivery efficiency and in some applications to minimize potential damage to capillaries.^[54]

(3) Conclusions

The focus of this study was an ultrasound-activated delivery agent (MNCA) formulation comprised of ~150 nm diameter PLGA NPs covalently linked to albumin-shelled microbubbles. We tested these microbubble-nanoparticle composite delivery agents in vivo and report two major findings. First, we determined that, after intravenous MNCA injection, the application of 1 MHz ultrasound to ischemic skeletal muscle results in a greater than 8-fold increase in NP delivery to tissue (Figure 5). In absolute terms, at 1 hour after treatment, 7.2% of the initial nanoparticle dose was present in US-targeted muscle. When compared to the few studies that have quantified NP delivery to muscle in terms of %ID/g,^[38–40] this represents more than an order of magnitude increase. Confocal observations (Figure 7; *top row*) revealed that many NPs were delivered to the capillary endothelium; however, many were also present in the extravascular space between muscle fibers. Second, we determined that, in comparison to a strategy in which MBs and NPs were co-injected but not complexed, the MNCA formulation yields a ~2-fold increase in NP delivery within 1 hr after treatment (Figure 6). Based on these results, we conclude that covalently linking nanoparticles to microbubbles in a composite agent formulation may be a useful strategy for concentrating intravenously injected drugs and/or gene-bearing nanocarriers in ultrasound-targeted skeletal muscle.

(4) Experimental Section

Microbubble Fabrication

To prepare albumin MBs, a 1% solution of serum albumin in normal saline was placed in a flask with a blanket of gas (octafluoropropane) above the aqueous phase. The solution was briefly sonicated (30 sec) with an ultrasound disintegrator equipped with an extended ½" titanium probe. This formulation is similar to Optison (GE Healthcare), which is provided in a concentration range of $0.50\text{--}1.2 \times 10^9$ MBs/mL, as determined by a Coulter Counter. Large microbubbles were removed from the preparation by flotation in a vertically positioned syringe. After purification, microbubbles were placed in glass vials, stoppered and sealed under perfluorocarbon atmosphere. Mean MB diameter was $2.19\mu\text{m} \pm 1.36\mu\text{m}$. Analysis with a Zetasizer (Zetasizer 3000, Malvern Instruments,) revealed a zeta potential of -43.3 ± 3.93 mV.

Nanoparticle Fabrication

These methods were adapted from the water-in-oil-in-water emulsion solvent evaporation technique described by Davda^[55] and Chappell.^[27] Prior to NP fabrication, a 2% solution of polyvinyl alcohol (PVA) (Acros Organics, Morris Plains, NJ) was made by dissolving PVA (20 g) in H₂O (1,000 mL) with low heat. On the day of NP fabrication, dichloromethane (50 mL, CH₂Cl₂, D37-500, Fisher Scientific) was added to the PVA solution (2%, 24 mL). The PVA-methylene chloride (MC) solution was centrifuged at 1,000 rpm for 10 min and filtered with a 0.22 μm sterile filter to remove any residual undissolved PVA. A polymer solution was made by dissolving high-molecular weight 85:15 uncapped poly(lactic-co-glycolic acid) (PLGA) (180 mg, Lakeshore Biomaterials, Birmingham, AL) in MC in a glass scintillation vial. An aqueous solution of BSA (15 mg, Jackson Laboratories in PBS (1.5mL) was added and vortexed. The PLGA-MC-BSA solution was placed in an ice bath for 5 min and then sonicated at 45W for 150 s with using a high-power sonicator (Misonix S-3000 Sonicator, Farmingdale, NY). The PLGA-MC-BSA solution was added in two portions with intermittent vortexing to the PVA solution (24 mL). The PVA-PLGA-MC-BSA solution was placed on an ice bath for 5 min and then sonicated again with 60W of energy for 150 s. This solution was stirred overnight. The suspension was then transferred into a thick-walled polycarbonate centrifuge tube and centrifuged at 20,000 rpm for 60 min at 4 °C. After

discarding the supernatant, the pellet was resuspended in distilled water and sonicated for 30 s. The resulting solution was centrifuged again at 20,000 rpm for 60 min at 4 °C, pouring off the supernatant after centrifugation. The NP pellet was resuspended again in distilled water (8 mL) and sonicated for 30 s. To remove large aggregates, the NP solution was centrifuged at 1,000 rpm for 10 min at 4 °C. A sample of the NP suspension was analyzed by a submicron-particle sizer (Nicom 370, Particle Sizing Systems Inc., Santa Barbara, CA), Zetasizer 3000 (Malvern Instruments, Southborough, MA) and by scanning electron microscopy (SEM). The supernatant was collected, frozen at −80 °C for 45 min, and lyophilized for 2 days. The dry NP powder was stored in a desiccator at −80 °C until needed.

Conjugation of VivoTMTag 680 to Nanoparticles

BSA-PLGA NPs were conjugated to fluorochrome–hydroxysuccinimide (VivoTagTM680) using the protocol adapted from VisEn Chemistry Notes. Briefly, a NP solution was prepared at 5.0 mg/mL in 100mM sodium phosphate buffer, pH 7.5. Next, the NP solution was added to VivoTagTM680 such that the concentration of the fluorochrome was approximately 2 mg/mL. The solution was mixed gently, covered from light and allowed to react for 2 hours. Fluorochrome-labeled NPs (VT680-NPs) were purified by centrifugation at 20,000 rpm for 60 min at 4 °C, pouring off the supernatant afterwards. To determine the number of fluorochromes per NP VT680-NPs (0.1 mg) were dissolved in MC (100uL) and combined with PBS (1mL), and stirred under a vacuum hood to allow the solvent to evaporate. Two hours later, the sample was assessed spectrophotometrically. The combination of the concentration of NPs (determined by the mass, density, and mean diameter) and absorbance enabled the determination of the number of fluorochromes per NP. This absorbance value was then subtracted from the absorbance of “blank” BSA-PLGA NPs to obtain the number of fluorochromes per VT680-NPs.

Microbubble-Nanoparticle Composite Agent Fabrication

To fabricate MB-NP composite agents, VT680-NPs were combined with 1 mg of 1-Ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC) and 2.2 mg of N-hydroxy-sulfosuccinimide (Sulfo-NHS) in 0.1 M MES buffer, pH 6. The solution was allowed to react for 1 hr at room temperature. Next, the activated NPs were purified from excess EDC by centrifuging at 20,000 rpm for 60min at 4 °C, pouring off the supernatant after centrifugation. While particles were spinning, albumin MBs were washed three times by centrifugal flotation in degassed PBS to remove excess BSA. Purified VT680-NPs were incubated with washed MBs for 2 hr at room temperature. Subsequent to incubation, MBs conjugated to VT680-NPs (MNCA) were purified from unbound VT680-NPs by washing the suspension by centrifugal flotation at 200g, three times with degassed PBS. Subsequent to washing, the concentration and mean diameter of MNCAs was determined using electrozone sensing (Multisizer-III, Beckman Coulter, Fullerton, Calif) and the number of bound NPs per MB was determined spectrophotometrically. Briefly, a 100uL of concentrated MNCAs were sonicated in a sonicate water bath (Sonicore Instrument Corporation, Farmingdale, NY), and then combined with MC to dissolve VT680 NPs. Absorbance was then determined by UV-visible spectroscopy. Based on the predetermined MB concentration and the number of fluorochromes per NP we were able to determine the mean number of NPs per MB.

Cytometry Imaging

For cytometry imaging, MBs were incubated with or conjugated to VT680-NPs (MNCA), or imaged alone. Imaging was performed on an ImageStream (Amnis Corporation, Seattle, WA) utilizing 658 laser and an Extended Depth of Field (EDF) element. Data were collected using INSPIRE 3.0, and analysis in IDEAS 3.0 software (Amnis Corporation). Focused

single MBs were gated and colocalization of fluorescence from VT680-NPs and was assessed.

Nanoparticle Delivery to Gracilis Muscle by Ultrasonic Microbubble Destruction

All animal studies were approved by the Institutional Animal Care and Use Committee. Four days before treatment, both the left and right femoral arteries of BalbC mice (n=12 total) were ligated distal to the superficial epigastric artery to create ischemia in the lower hindlimbs. On the day of treatment, animals were anesthetized with an intraperitoneal (i.p.) combination injection of ketamine hydrochloride (1.56 mL/kg body weight), xylazine (0.52 mL/kg body weight) and sterilized water (3.12 mL/kg body weight). The tail vein of each animal was cannulated for intravenous (i.v.) administration of either a co-injection of MBs and NPs (n=4 mice; 1×10^5 MBs/g and 0.2 μ g NPs/g body weight in 0.16 mL of 0.9% saline), MNCAs (n=4 mice; 1×10^5 MNCAs/g body weight in 0.16 mL of 0.9% saline) or NPs (n=4 mice; 0.2 μ g NPs/g body weight in 0.16 mL of 0.9% saline) solution. A water based US gel (Aquasonic 100; Parker Laboratories, Inc., Fairfield, NJ) was applied to the skin above the hindlimb, and a 0.75" diameter 1 MHz unfocused transducer (A314S; Panametrics, Waltham, MA) was ultrasonically coupled to the skin. Injection duration (15 mins) and rate (13.33 μ L/min) were controlled by an infusion pump (Harvard Apparatus PHD 2000; Harvard Apparatus, Holliston, MA). The dead space of the tail vein catheter was cleared prior to initiating US pulsing. Each pulse consisted of 5 sets of 300 consecutive 1 MHz sinusoids of 1V peak-to-peak amplitude, separated by 100ms and applied every 5 sec for 12 mins. Sinusoids were each of 1V peak to peak amplitude from a waveform generator (AFG-310; Tektronix, Inc., Beaverton, OR). The waveform signal was amplified by a 55 dB RF power amplifier (ENI 3100LA; Electronic Navigation Industries, Richardson, TX). Maximum peak negative pressure at the focus of the transducer, as measured with a needle hydrophone (Specialty Engineering Associates, Model PVDF-Z44-0400) was 0.75MPa.

Nanoparticle Biodistribution

Twelve days before fluorescent tomographic (FMT) imaging, mice were placed on a low alfa sprout diet (Harlan, Indianapolis IN) to reduce gut autofluorescence. Mice were anesthetized by i.p. combination injection of ketamine hydrochloride (1.56 mL/kg body weight), xylazine (0.52 mL/kg body weight) and sterilized water (3.12 mL/kg body weight) and the imaging site was shaved. Mice were then imaged on the FMT system (VisEn Medical) before and 0, 1, 4, and 24 hrs after US application. Reflectance images were taken in white light and fluorescent modes. Non-invasive FMT was carried out in the 680nm channel. Using the FMT software, 3D reconstructions of the imaging data were preformed utilizing a normalized Born equation. Following reconstruction, volumes of interest (VOI's) were selected by drawing regions of interest (ROI's) in all 3 imaging planes (X, Y, Z). The total fluorochrome concentration due to VT680-NPs was determined per VOI. Uptake of VT680-NPs in hindlimb was calculated as the percentage of the injected dose per gram of tissue (%ID/g). Tissue density was assumed to be 1g/mL. In biodistribution studies, at 1 hr following treatment mice (n=3) were euthanized and spleen, liver, lungs, kidney, and heart were harvested. For each organ harvested the %ID/g was calculated.

Tissue Processing

One hour after treatment, animals were euthanized and the left ventricle was cannulated. Blood was exsanguinated with 10mL of 2% Heparinized Tris Buffered Saline (TBS) with CaCl_2 (0.68mM), followed by 10mL of TBS with CaCl_2 (0.68mM), each for 10 minutes at 100 mm Hg. Following blood removal, tissues were perfusion-fixed with 4% paraformaldehyde in PBS for 30 min. Specimens were excised, embedded in paraffin and cut into 5-micron thick sections. Capillaries were labeled with Bandeiraea simplicifolia lectin (BSI-lectin) conjugated to Alexa 488 (1:200; Sigma Biosciences). Digitized images of

cross-sectioned specimens were acquired using a Nikon Eclipse TE2000-E microscope equipped with confocal accessories (Nikon D-Eclipse C1) using a $\times 60$ Nikon oil immersion objective.

Statistical analysis

Data in Figures 4, 5, and 6 were analyzed by Two-Way analysis of variance (ANOVA). In cases where the ANOVA revealed that significant differences existed among the different levels of a factor, pairwise comparisons were subsequently made use the Holm-Sidak method. Significance was assessed at $P < 0.05$.

Acknowledgments

This study was supported by research grants from the National Institutes of Health (R01 HL74082), The Hartwell Foundation, and The Focused Ultrasound Surgery Foundation.

References

- [1]. Vincent KA, Jiang C, Boltje I, Kelly RA. *Gene Ther.* 2007;14.
- [2]. Grines CL, Watkins MW, Helmer G, Penny W, Brinker J, Marmur JD, West A, Rade JJ, Marrott P, Hammond HK, Engler RL. *Circulation.* 2002; 105:1291. [PubMed: 11901038]
- [3]. Baklanov D, Simons M. *Endothelium.* 2003; 10:217. [PubMed: 14660081]
- [4]. Simons M, Ware AJ. *Nat Rev Drug Discov.* 2003; 2:863. [PubMed: 14668807]
- [5]. Lu E, Wagner WR, Schellenberger U, Abraham JA, Klivanov AL, Woulfe SR, Csikari MM, Fischer D, Schreiner GF, Brandenburger GH, Villanueva FS. *Circulation.* 2003; 108:97. [PubMed: 12821549]
- [6]. Jung KH, Kim DH, Paik JY, Ko BH, Bae JS, Choe YS, Lee KH, Kim BT. *Ann Nucl Med.* 2006; 20:8.
- [7]. Kornowski R, Fuchs S, Leon MB, Epstein SE. *Circulation.* 2000; 101:454. [PubMed: 10653839]
- [8]. Ferrara KW, Pollard R, Borden M. *Annu. Rev. Biomed.Eng.* 2007; 9:415. [PubMed: 17651012]
- [9]. Hynynen K. *Expert Opin. Drug Deliv.* 2007; 4:27. [PubMed: 17184160]
- [10]. Hernot S, Klivanov A. *Adv. Drug Deliv. Rev.* 2008; 60:1153. [PubMed: 18486268]
- [11]. Ward M, Wu J, Chiu J. *J. Acoust. Soc. Am.* 1999; 105:2951. [PubMed: 10335644]
- [12]. Skyba DM, Price RJ, Linka AZ, Skalak TC, Kaul S. *Circulation.* 1998; 98:290. [PubMed: 9711932]
- [13]. Zhang Q, Wang Z, Ran H, Fu X, Li X, Zheng Y, Peng M, Chen M, Schutt CE. *Acad. Radiol.* 2006; 13:363. [PubMed: 16488849]
- [14]. Rapoport NY, Kennedy AM, Sheac JE, Scaifec CL, Nama KH. *J. Control. Release.* 2009; 15:268. [PubMed: 19477208]
- [15]. Hynynen K, McDannold N, Sheikov NA, Jolesz FA, Vykhodtseva N. *Neuroimage.* 2005; 24:12. [PubMed: 15588592]
- [16]. Treat LH, McDannold N, Vykhodtseva N, Zhang Y, Tam K, Hynynen K. *Int. J. Cancer.* 2007; 121:901. [PubMed: 17437269]
- [17]. Chen Y, Jungsuwadee P, Vore M, Butterfield D, St Clair D. *Mol. Interv.* 2007; 7:147. [PubMed: 17609521]
- [18]. Carvalho C, Santos RX, Cardoso S, Correia S, Oliveira PJ, Santos MS, Moreira PL. *Curr. Med. Chem.* 2009; 16:3267. [PubMed: 19548866]
- [19]. Rapoport N, Gao Z, Kennedy A. A. Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy. *J. Natl. Cancer. Inst.* 2007; 99:1095. [PubMed: 17623798]
- [20]. Hitchcock K, Caudellb D, Suttona J, Klegermanc M, Velad D, Pyne-Geithman GJ, Abruzzo T, Cyr Peppar EP, Geng Y-J, McPherson DD, Holland CK. *J. Control Release.* 2010; 144:288. [PubMed: 20202474]

- [21]. Phillips LC, Klibanov AL, Bowles DK, Ragosta M, Hossack JA, Wamhoff BR. *J. Vasc. Res.* 2009; 47:270. [PubMed: 19923850]
- [22]. Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner LR. *Ultrasound Med. Biol.* 2003; 29:1759. [PubMed: 14698343]
- [23]. Bekerredjian R, Chen S, Frenkel PA, Grayburn PA, Shohet RV. *Circulation.* 2003; 108:1022. [PubMed: 12912823]
- [24]. Chen S, Shohet RV, Bekerredjian R, Frenkel P, Grayburn PA. *J. Am. Coll. Cardiol.* 2003; 42:301. [PubMed: 12875768]
- [25]. Borden MA, Caskey CF, Little E, Gillies RJ, Ferrara KW. *Langmuir.* 2007; 23:9401. [PubMed: 17665937]
- [26]. Song J, Chappell JC, Qi M, VanGieson EJ, Kaul S, Price RJ. *J. Am. Coll. Cardiol.* 2002; 39:726. [PubMed: 11849875]
- [27]. Chappell J, Song J, Burke CW, Klibanov AL, Price RJ. *Small.* 2008; 10:1769. [PubMed: 18720443]
- [28]. Vancraeynest D, Havaux X, Pouleur AC, Pasquet A, Gerber B, Beauloye C, Rafter P, Bertrand L, Vanoverschelde J-LJ. *Eur. Heart J.* 2006; 27:237. [PubMed: 16166101]
- [29]. Lum AF, Borden MA, Dayton PA, Kruse DE, Simon SI, Ferrara KW. *J. Control Release.* 2006; 111:128. [PubMed: 16380187]
- [30]. Kheirrolomoom A, Dayton PA, Lum AF, Little E, Paolia EE, Zheng H, Ferrara KW. *J. Control Release.* 2007; 118:275–284. [PubMed: 17300849]
- [31]. Vandenbroucke RE, Lentacker I, Demeester J, De Smedt SC, Sanders NN. *J. Control Release.* 2006; 126:265. [PubMed: 18237813]
- [32]. Lentacker I, Wan N, Vandenbroucke RE, Demeester J, De Smedt SC, Sanders NN. *Molecular Pharmaceutics.* 2008; 6:457. [PubMed: 19718799]
- [33]. Song J, Cottler PS, Klibanov AL, Kaul S, Price RJ. *Am. J. Physiol. Heart Circ. Physiol.* 2004; 287:H2754. [PubMed: 15319212]
- [34]. Chappell J, Song J, Klibanov AL, Price RJ. *Arterioscler. Thromb. Vasc. Biol.* 2008; 28:1117. [PubMed: 18403725]
- [35]. Imada T, Tatsumi T, Mori Y, Nishiue T, Yoshida M, Masaki H. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25:2128. [PubMed: 16051876]
- [36]. Leong-Poi H, Kuliszewski MA, Lekas M, Sibbald M, Teichert-Kuliszewska K, Klibanov AL. *Circ. Res.* 2007; 101:295. [PubMed: 17585071]
- [37]. Price RJ, Skyba DM, Kaul S, Skalak T. *Circulation.* 1998; 98:1264. [PubMed: 9751673]
- [38]. Cieslar J, Huang MT, Dobson GP. *Am. J. Physiol.* 1988; 275:R1530. [PubMed: 9791070]
- [39]. Yang Z, Leon J, Martin M, Harder JW, Zhang R, Liang D, Lu W, Tian M, Gelovani JG, Qiao A, Li C. *Nanotechnology.* 2009; 20:165.
- [40]. Almutairia A, Rossinb R, Shokeenb M, Hagooleyb A, Ananthc A, Capocciac B, Guillaudeua S, Abendscheinc D, Andersonb CJ, Welchb MJ, Frecheta JM. *Nanotechnology.* 2009; 106:685.
- [41]. Meijering BD, Juffermans LJ, Wamel A, Henning RH, Zuhorn IS, Emmer M, Versteilen AM, Paulus WJ, van Gilst WH, Kooiman K, Jong N, Musters RJ, Deelman LE, Kamp O. *Circ. Res.* 2009; 104:679. [PubMed: 19168443]
- [42]. Qin S, Ferrara KW. *Phys. Med. Biol.* 2006; 51:5065. [PubMed: 17019026]
- [43]. van Wamel A, Bouakaz A, Versluis M, de JN. *Ultrasound Med. Biol.* 2004; 30:1255. [PubMed: 15550330]
- [44]. van Wamel A, Kooiman K, Emmer M, Ten Cate FJ, Versluis M, de JN. *J. Control Release.* 2006; 116:e100. [PubMed: 17718938]
- [45]. Nyborg WL. *Adv. Drug. Deliv. Rev.* 2008; 60:1103. [PubMed: 18468716]
- [46]. Qin S, Caskey C, Ferrara KW. *Phys. Med. Biol.* 2009; 54:R27. [PubMed: 19229096]
- [47]. Tran BC, Seo J, Hall TL, Fowlkes JB, Cain CA. *IEEE. Trans. Ultrason. Ferroelectr. Freq. Control.* 2003; 50:1296. [PubMed: 14609069]
- [48]. Miller DL, Li P, Dou C, Gordon D, Edwards CA, Armstrong WF. *Radiol.* 2005; 237:137.
- [49]. Song J, Klibanov AL, Hossack JA, Price RJ. *Invest. Radiol.* 2008; 43:322. [PubMed: 18424953]

- [50]. Choi JJ, Feshitan JA, Baseri B, Wang S, Tung Y-S, Borden MA, Konofagou EE. IEEE Trans. Biomed. Eng. 2010; 57:145. [PubMed: 19846365]
- [51]. El-Sherif DM, Wheatley MA. J Biomed. Mat. Res. 2003; 66:347.
- [52]. Caskey CF, Stieger SM, Qin S, Dayton PA, Ferrara KW. J. Acoust. Soc. Am. 2007; 122:1191. [PubMed: 17672665]
- [53]. Allen J, Maya D, Ferrara KW. Dynamics Ultra. Med. Biol. 2002; 28:805.
- [54]. Stieger SM, Charles CF, Adamson RH, Qin SP, Curry F-RE, Wisner ER, Ferrara KW. Radiol. 2007; 243:112.
- [55]. Davda J, Labhasetwar V. Int. J. Pharm. 2002; 233:51. [PubMed: 11897410]

Figure 1. Characterization of MNCA. *A*: Number weighted NiComp NP size distribution. *B*: Scanning electron microscopy image of PLAGA NPs. *C*: Epifluorescence-transmitted light merge image of a single composite delivery agent (MNCA) comprised of 100nm PLAGA polymer NPs adhered to a contrast agent microbubble (MB) using carbodiimide chemistry linkers. MB is approximately 2 μ m in diameter.

Figure 2.

Specific and Nonspecific Interactions of NPs with MBs. *Left column:* Image cytometry scatter plots of microbubble (MB) area vs. fluorescence intensity. *Middle Column:* Brightfield images of MBs. *Right Column:* Cy5.5 images of MBs and/or NPs. *Top row:* Composite agent (MNCA). *Middle row:* Microbubbles (MBs) incubated with nanoparticles (NPs). *Bottom row:* Microbubbles (MBs). Arrows denote MB presented in the middle and right columns. Stars denote individual MBs visualized in middle and right columns. The median MB mean pixel intensity was 171.75 for the MNCA group and 0 for MBs incubated with NPs and MBs alone.

Figure 3.

Fluorescence-mediated tomography scans showing NP delivery to hindlimb skeletal muscle at various time points after ultrasound application and NP biodistribution 1hr following MNCA infusion. A: *Top row*: Composite agent (MNCA) injection. *Middle row*: Co-injection of microbubbles (MBs) and nanoparticles (NPs). *Bottom row*: NP injection without microbubbles. Ultrasound treated regions are denoted with white asterisks. Enhanced fluorescence intensity is evident with US application for the MNCA and MB+NP groups. B:

Bar graph of fluorochrome concentration [% initial dose (ID) per gram of tissue] in “off-target” tissues and organs, as determined by FMT at 1 hr after treatment with MNCA.

Figure 4. Microbubbles (MBs) and ultrasound (US) facilitate nanoparticle (NP) delivery to skeletal muscle after intravenous injection. Bar graph of fluorochrome concentration [% initial dose (ID) per gram of tissue] as a function of time. *pairwise comparisons with Holm-Sidak *t*-tests indicate significantly different than NP+US group at same time point ($P < 0.05$). **Analysis of variance indicates significantly different than MB+NP ($P < 0.05$), but no pairwise differences exist.

Figure 5.

Ultrasound application enhances nanoparticle (NP) delivery to skeletal muscle following the intravenous injection of MNCAs. Bar graph of fluorochrome concentration [% initial dose (ID) per gram of tissue] as a function of time. *pairwise comparisons with Holm-Sidak *t*-tests indicate significantly different than MNCA group at same time point ($P < 0.05$).

Figure 6.

Composite agent (MNCA) formulation yields greater ultrasound-mediated nanoparticle delivery to hindlimb skeletal muscle than co-injections of microbubbles (MBs) and nanoparticles (NPs). Bar graph of fluorochrome concentration [% initial dose (ID) per gram of tissue] as a function of time. *pairwise comparisons with Holm-Sidak *t*-tests indicate significantly different than MB+NP+US group at same time point ($P < 0.05$).

Figure 7.

Confocal images of cross-sectioned skeletal muscle at 1 hr after ultrasound application. *Top row:* Composite agent (MNCA) injection. *Middle row:* Co-injection of microbubbles (MBs) and nanoparticles (NPs). *Bottom row:* NP injection without microbubbles. *Left column:* Capillaries labeled with BS-I lectin. *Middle column:* VT680-conjugated nanoparticles (NPs) that have been delivered to tissue. *Right column:* Merge images showing delivered NPs (red) with respect to capillaries (green). Filled arrows denote co-localization of NPs and capillary endothelium. Open arrows denote regions where NPs have been delivered beyond the endothelium to the interstitial space between muscle fibers.