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MR and fluorescence imaging of doxorubicin loaded nanoparticles using a novel *in vivo* model

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Abstract

We report here the *in vivo* combined-modality imaging of multifunctional drug delivery nanoparticles. These dextran core-based stealth liposomal nanoparticles (nanosomes) contained doxorubicin, iron oxide for MRI contrast, and Bodipy for fluorescence. The particles were long-lived *in vivo* due to surface decoration with polyethylene glycol (PEG) and the incorporation of acetylated lipids which were UV cross-linked for physical stability. We developed a rodent dorsal skinfold window chamber which facilitated both MRI and non-destructive optical imaging of nanoparticle accumulation in the same tumors. Chamber tumors were genetically labeled with DsRed-2 that enabled the MR images, the red fluorescence of the tumor, and the blue fluorescence of the nanoparticles to be co-localized. The nanoparticle design and MR imaging developed with the window chamber were then extended to orthotopic pancreatic tumors expressing DsRed-2. The tumors were MR imaged using iron oxide-dextran liposomes and by fluorescence to demonstrate the deep imaging capability of these nanoparticles.

Keywords

Multifunctional nanoparticle; MRI; dorsal skinfold window chamber

INTRODUCTION

Iron oxide MRI contrast agents are attractive potential candidates for incorporation into nanoparticles^{1, 2}, as on a molar basis they are the most detectable and effective medium for changing the contrast in MRI images as demonstrated both theoretically³ and experimentally⁴⁻⁷. In addition, iron oxide contrast agents are currently approved for human

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use^{8, 9}. Hence, we and others have been investigating the development of liposomal formulations that incorporate iron oxide and therapeutic agents².

The loading of iron oxide inside nanoparticles presents significant challenges due to its hydrophobicity, so that combining iron oxide with a drug payload inside nanoparticles could result in suboptimal loading efficiencies and particle instability. Positioning the iron oxide on the exterior of the particle would inhibit the attachment of stealth and targeting moieties. Attaching the iron oxide to a small chain nanoparticle is feasible, but might result in limited contrast and drug delivery. We sought to develop nanosomal particles having a dextran core, a coating material typically used to render iron oxide hydrophilic, and thereby facilitating the internalization of iron oxide into the particle¹⁰. Dextran-core nanoparticles loading both hydrophilic and hydrophobic drugs have been described previously, so potentially dextran can favorably influence both iron oxide and drug payload loading^{11, 12}. Three critical factors associated with our iron oxide-dextran core design were (1) successful loading of iron oxide into the nanoparticle, (2) the stability of the nanoparticle outer shell, and (3) modulation of the final size of the nanoparticle. Successful control of these factors required adjustments in assembly protocol and the specifics of outer shell composition.

We describe here first the formulation of an iron oxide–dextran core nanoparticle, which exhibits iron oxide loading, successful loading of a doxorubicin drug payload, mechanical durability, and resistance to opsonization. The outer shell composition and assembly protocol were optimized to allow efficient loading of iron oxide dextran spheres and maximize particle survivability. In addition the analysis of these multifunctional nanoparticles required the development of an *in vivo*, multi-modality imaging platform that allows for non-destructive, serial observations of particles and tumors for drug delivery and imaging. In order to demonstrate *in vivo* nanoparticle survivability and the capacity of the nanoparticles to be MRI imaged at tumors, we refined and extended a rodent tumor model based on early reports of a MRI compatible optical tissue window chamber^{12, 13}. Our MRI window chamber allowed MR imaging of the nanoparticles and tumors, and for the first time, incorporated optical imaging for the non-destructive validation of the MRI acquisitions. This facilitated and guided the extension of our nanoparticles and MRI methods to an orthotopic tumor model, in which pancreatic tumors deep within the body were MR imaged before and after nanoparticle injection. The chamber design was in general improved to accommodate 0.5 cm tumors in the mouse dorsal skinfold, and to minimize local host tissue trauma.

METHODS

Nanoparticle Design and Composition

Overview—The liposomal nanoparticle platform was based on a dextran core to accept a multitude of agents, including an iron oxide based MR imaging contrast agent and therapeutics. The outer shell was exposed to UV radiation at 310 nm to mildly crosslink the lipid moieties and stabilize the nanoparticle for longevity. The composition of the shell structure described in Table 1 includes three types of lipids and (i) polyethylglycol moieties to escape immune surveillance (stealth) ^{14–18}, and (ii) UV radiation crosslinkable acetylene groups for stability^{19–21} (Table 1). An advantage of a liposomal nanoparticle is that it rapidly enters tumor cells by endocytosis, and is carried into the lysosome of the cell, where low pH disrupts the liposomal shell to release the payload, in this case doxorubicin. The doxorubicin, a small molecule then diffuses through the cell and enters the nucleus by diffusion, and binds Topoisomerase II which causes cell death ^{22–23}.

Design Rationale—The production of drug or iron oxide loaded dextran hydrogel particles has been reported previously^{11, 21, 24}, and the methods described apply for both

hydrophobic and hydrophilic drugs. In our work we incorporated both iron oxide and dextran within a lipid shell for two reasons; (1) the particles could carry hydrophilic and hydrophobic drugs, and be detected with MRI, and (2) outer shell constituents such as PEG (immune escape)¹⁴⁻¹⁷, targeting moieties, and fluorophores could be added without affecting payload capacity. The lipid shell constituents were mixed from chloroform solutions, and after removal of the chloroform by rotavaporation, the lipid films blended with the core iron oxide-dextran. This was followed by high energy sonication and extrusion of the mixtures through 100 nm pore polycarbonate membranes. All of these procedures are easily adaptable for large scale production. The nanoparticle structure is shown schematically in Figure 1.

Particle Assembly Procedure—The assembly sequence was as follows: (1.) Treatment of iron oxide dextran core nanospheres with doxorubicin-HCL dissolved in PBS for 24 hours, followed by purification of the core structure using G25 size exclusion chromatography. (2.) Extensive sonication at 20-30 watts. (3.) Preparation of the lipid nanoshell mixture [Table 1.]. (4.) Chloroform evaporation in a Bueche rotavap system to form a lipid film. (5.) Addition of the core at 1-5 mg/ml of suspension, to the lipid film followed by high energy sonication. (6.) Extrusion of the lipid coated core sequentially through 400, 200 and 100 nm polycarbonate membranes. (7.) UV radiation for 3-5 min under a mercury arc lamp. (8.) Sterile filtration of the final nanovesicle through 0.2 μ m sterile filters and storage at 4°C.

Confirmation of general structure and loading

Particle Size—A Malvern Zetasizer was used to measure the size of the nanoparticles (Figure 3A). The nanoparticle suspension was diluted in 1/10 in MilliQ water, and 100 μ l of the dilution was sized using light backscattering.

Particle Structure and MR relaxivity—The BODIPY fluorophore labeled nanoparticle suspension was subjected to a magnetic field, the particles concentrated to one location in the vial, and the vial solution was then aspirated and replaced. This washing procedure was repeated five times. A control solution of non-fluorescently labeled nanoparticles had free BODIPY fluorophore added to the solution and then was subjected to the same washing procedure. Subsequently, in order to demonstrate dextran incorporation, we prepared rhodamine labeled, empty liposomes (control) and a second batch of liposomes with FITC-labeled dextran (2 M Mol. Wt.). We then ran each group through a size exclusion column (Sephacrose 4B) and collected fractions. Further confirmation of liposome size and structure was obtained with SEM. The particle samples were diluted 1/10 in milliQ water, drying them onto silicon wafers, and then imaging with an electron microscope. The finished nanoparticles were adhered to an inert surface (silicon wafers) and imaged on a Philips XL-30 electron microscope to 30,000X. Particular emphasis was placed on confirming that the iron oxide was internal to the nanoparticles and that the outer shell was spherical.

Iron oxide-dextran nanoparticles and the iron oxide-dextran core nanosomes were subjected to relaxivity measurements. Initially, the Fe concentration in the iron oxide-dextran nanoparticles and iron oxide-dextran core nanosomes were measured using Perkin-Elmer 3700 Optical Emission Plasma Spectrometer. Relaxivity measurements were performed using a 3T GE MRI system. T_2 relaxation rates of diluted samples of nanoparticles and nanosomes were measured using multi-echo Spin Echo (SE) protocols with TE = 11, 22, 44, 88, 120 ms and TR=2000 ms. R_2 relaxivity values were calculated from these measurements according to,

$$\left(\frac{1}{T_2 \text{ (sample)}} - \frac{1}{T_2 \text{ (tapwater)}} \right) = R_2 \times [CM]$$

Doxorubicin loading into dextran core particles—Doxorubicin hydrochloride was dissolved in PBS and agitated with the iron oxide dextran particles overnight, followed by purification of the mixture using size exclusion chromatography. The resultant purified nanoparticle suspension was methanol extracted to separate the doxorubicin from the dextran core. The drug concentration was measured by fluorescence at 484 nm excitation and 584 nm emission, using a TECAN fluorescence spectrophotometer.

Design and Fabrication of the MRI Compatible Window Chamber

Window chamber materials—Previous generations of chambers were fabricated from Teflon coated aluminum²⁵ and from titanium²⁶. The MR compatible chamber we developed also had to be biocompatible, have a high strength to weight ratio, remain rigid when machined to the necessary small thicknesses, and also offer a low ratio of thermal conductivity to minimize cooling of the chamber tissue. These requirements are satisfied by a commercially available plastic polymer, Delrin®. The physical characteristics of Delrin® are given in Table 2. While the functionality of the skinfold window chamber design was increased, the use of Delrin® reduced the weight, contrasting with previous generation titanium chambers. Moreover, Delrin® is inexpensive, and can be easily molded.

Dimensions and design features—Some of the MR compatible chambers previously reported¹³ were of comparatively small diameter and could not accommodate a 0.5 cm tumor along with a 0.5 cm ring of surrounding host tissue. Our comparatively larger chambers have a window diameter of 1.2 cm with, and are designed to cause less tissue damage. We sought to avoid the use of bolts penetrating the skin, a key feature in terms of minimizing local tissue trauma. Hence, the Delrin® was machined into two complementary plates that when implanted on a subject were held together with two clips and Teflon® screws (Figure 2A). Each plate had two grooves to accommodate the clips. For additional stability of the chamber apparatus, three wings with grooved holes for screws were machined into top border of the plates. The plates were secured together with Teflon screws which were positioned outside of the dorsal skinfold. Aside from suture material used to attach the outer margin of the skinfold to the top edge of the chamber plates, the skinfold was not penetrated in any way. This was important, as it served to simplify the surgery and reduce tissue trauma. Additional openings were provided to secure the chamber to a holder that restrained the subject, and that fixed the chamber to the holder for light microscope imaging. The window plate was machined with grooves for the glass cover slip. The glass coverslip rested on a 0.254 mm thick lip and was retained with a thin 1.2 cm diameter Delrin® ring (Figure 2A).

In vivo Experiments

All animal protocols used in this study were evaluated and approved by the University of California San Diego IACUC committee. All protocols were strictly followed, aseptic surgical technique was used, and the mice were treated humanely according to the NIH guidelines for the care and use of laboratory animals. Every effort was made to ensure that the mice were as comfortable as possible.

Chamber Implantation—The chamber incorporates a glass window on one side and intact skin on the other. The dorsal skin was elevated into a longitudinal fold and a disc of skin, fat, fascia and one skin-retractor muscle layer was removed from one side of the fold

over the A0 vessels^{27, 28} (Figure 2A). The skin surrounding the exposed tissue field was coated with topical antibiotic ointment. The two chamber plates sandwiched the skinfold and maintained it in a vertical configuration. A sterile 12 mm diameter glass coverslip was placed over the exposed tissue, and the outer margins of the skin fold were sutured to openings along the periphery of the chamber plates. The plates were held together using a clip and sutures. After implantation the mice were gently warmed, allowed to recover under direct supervision, received Buprenex® subcutaneously for analgesia, and were provided SMZ antibiotic in their drinking water.

Tumor Implantation—A Lewis Lung Carcinoma (LLC) tumor line stably transfected to express DsRed-2 was cultured, then harvested and resuspended in cold PBS at 200,000 cells per μL . The mouse was secured, lightly anesthetized with ketamine-medetomidine SQ, the chamber coverslip was removed, and 1×10^6 cells (5 μL) was injected into the chamber tissue with a 25 μL Hamilton syringe. A fresh, sterile coverslip was secured over the chamber tissue and the animal was allowed to recover. For the orthotopic pancreatic implantation study, in order to MR image tumors deep inside the body, cultured MiaPaCa-2 cells expressing DsRed-2 were implanted as a 1 μL suspension of 100,000 cells in the tail of the pancreas of a different set of mice, using a 5 μL Hamilton syringe fitted with 28 gauge needle inserted via a surgical incision in the flank. The flank was reapproximated and sutured, and the animal was allowed to recover²⁹⁻³¹.

Magnetic Resonance and Optical Imaging

Mice with tumors growing in their dorsal skinfold window chambers were MR imaged at 7T for the demonstration of nanoparticle accumulation and MR detectability, and subsequently mice with tumors implanted inside the body, specifically in the tail of the pancreas, were MR scanned.

General MR Setup—Each mouse was anesthetized with 1.5% isoflurane and placed within the holding cradle shown in Figure 2B. A respiratory bellows was used to monitor respiration and a nose cone delivered the inhalation anesthetic. Imaging was performed using an in-house 1.5 cm diameter surface coil that was tuned and matched prior to imaging (Figure 2B). The mouse was positioned at the isocenter of the scanner magnet, and the bore was warmed to 36°C. The nanoparticle suspension was injected intravenously via the tail vein after the first series of images was acquired.

Chamber imaging at 7T—The MR scanner was a Bruker 7-T 210 mm bore small animal scanner (Bruker Biospin Corporation, WI). Respiration was monitored remotely. A fast 3-D scan was used to localize over the chamber, and then a T2-weighted RARE sequence was performed. The general imaging parameters were: FOV = 2.5 cm, slice thickness = 1.5 mm, Matrix = 256×256, TE = 56.7 ms, TR = 7151 ms, averages = 6. The T2-weighted images were acquired prior to injection with nanoparticles, and again at 2 and 24 hours after injection.

Deep Body Imaging at 7T—A birdcage type coil was used for imaging orthotopic tumors implanted in the pancreatic tail. A fast 3-D scan was used to localize over the chamber, and then a T2-weighted RARE sequence was performed. The imaging parameters were: FOV = 2.5 cm, slice thickness = 1.5 mm, Matrix = 256×256, TE = 56.7 ms, TR = 7151 ms, averages = 6.

Optical Imaging—In order to validate the MRI findings, chamber tissues were imaged immediately after MRI with a standard brightfield - fluorescence microscope, and a laser scanning two-photon (2-P) microscope. For imaging the mouse was secured within a

perforated plastic restraining tube which was placed within a custom designed holder. The window chamber was bolted to the holder via a stainless steel plate, and the chamber window was both brightfield and fluorescence imaged at 1x, 4x, and then 2-P imaged at 20x. For the orthotopic tumors the mouse was euthanized, the pancreas removed, and imaging performed with a 2-P microscope at 20x.

RESULTS

Physical Characterization of the Liposomal Nanoparticles

Various physical measurements were in agreement and confirmed that the nanoparticles had the predicted layered structure with an iron oxide core, dextran middle core, and intact lipid outer shell, and that they were of the intended diameter.

Particle Size—The results presented in Figure 3A represent the average of 3 Zetasizer measurements. The average diameter was approximately 100 nm, as indicated by the half-width center of the curve depicted in the graph, and the particle distribution index (PDI) was approximately 0.25.

Particle Integrity, Structure and MR relaxivity—Fluorescence washing experiments using a magnetic field to concentrate the nanoparticles confirmed that the control vial contained no fluorescence, while the solution in the test vial was fluorescent after five washes, indicating that it contained intact, fluorescent particles. Moreover, Figure 3B shows that rhodamine labeled empty liposomes and liposomes containing FITC-labeled dextran exited the Sepharose 4B size exclusion column as a single peak, and that there was not a second elution peak. This is important in that it shows that the FITC-dextran was not dissociated into smaller FITC labeled fragments, or free FITC, which would have generated later eluting secondary peaks.

Further confirmation of liposome size and structure was obtained with SEM. The image shown in the leftmost panel of Figure 3C is 30,000x, and is digitally magnified 2x in the middle panel. The dark center of each visible sphere represents the iron oxide core, and the external ring is the lipid outer shell. The dusky material between the iron oxide and the outer shell is the dextran hydrogel. Atomic spectral measurements confirmed the presence of iron oxide, shown in the rightmost panel of Figure 3C.

The Fe concentration of the iron oxide-dextran nanoparticles and the iron oxide-dextran core nanosomes was, respectively, 172 µg/mL and 167 µg/mL, before dilution. Following dilution with water, the Fe concentration was 12.7 µg/mL and 12.9 µg/mL, respectively. Table 3 lists the the relaxivities of the particles and two commercial products³². Note that the relaxivity values of the two particles were almost identical, and were quite close to the two commercial iron contrast agents Feridex® (Endorem) and Resovist®. Figure 3D depicts the MR images of vials containing the iron oxide-dextran nanoparticles and iron oxide-dextran core nanosomes, versus water alone, and illustrates that iron oxide caused significant signal dropout.

Doxorubicin loading into dextran core particles—The uptake of doxorubicin into the dextran core nanoparticles was at least 60 µg per ml of particle suspension.

MR Imaging of particles in window chambers—The 7 Tesla scanner T2-weighted MR images acquired 2 hours after intravenous nanoparticle injection revealed that the tumor blood vessels were darkened and the chamber tissue assumed a mottled appearance due to the iron oxide contained within the nanoparticles. This persisted to 24 hours as shown by the 24 hour MRI (Figures 4A and 4B). The bottom two panels of Figure 4B (i and ii) show the

entire chamber before and after nanoparticle injection, with flanking histograms of pixel grayscale versus pixel count (lower grayscale value is darker). The middle two panels (iii and iv) show tumor vessel ROIs with respective histograms, and the two top panels (v and vi), depict the major feeding vessel image pixels that have been segmented out of the image, together with their histograms. The pixel grayscale ratio of before versus after nanoparticle injection, goes from 1 : 0.8 for the entire chamber images, to approximately 1:1 for the ROI, indicating a darkening restricted to the ROI. The vessels themselves are the darkest with a ratio, before vs. after nanoparticles, of 1.6 : 1. This indicates that the vessel darkening is a local event due to the nanoparticles, and not an artifact of generalized darkening of the entire image. Some extravasation undoubtedly occurred after 24 hours, which might explain the ROI darkening. In any event, these results indicate that the nanoparticles could be detected *in vivo* with MRI, and that they accumulated within major feeding tumor vessels.

MR Imaging of particles in orthotopically implanted tumors—The 7T scanner detected nanoparticles in the pancreas and body wall of MiaPaCa-2 tumors in live subjects. Figure 5A shows MR images before and 24 hours after liposome injection. The two large image panels show the entire mouse abdomen, and reveal a darkening of the tumor and associated vasculature due to the iron oxide containing nanoparticles. The histograms at the bottom of each large image, reveal only a 1.6% difference in mean pixel intensity overall. However, the associated regions of interest (smaller upper image panels) that are centered over the tumor, clearly show darkening of the tumor tissue, and their histograms indicate a 25% decrease in mean pixel intensity after nanoparticle injection. The ratio of mean pixel gray value for the entire body images, before versus after nanoparticle injection, is approximately 1 : 1. However that ratio changes to 1.33 : 1 for the tumor ROIs, indicating that the tumor image darkening is due to local tumor effect, and not simply an artifact-based on darkening of the entire image. The whole-body images and the ROI images also suggest that by 24 hours nanoparticles collected in the spleen, kidney and urinary bladder as these organs appear darker. These organ accumulations may potentially be reduced by attaching a moiety to the nanoparticles that specifically targets one or more tumor cell surface markers²¹.

Optical Imaging—The MRI results were validated optically using the window chamber, as the BODIPY labeled particles were visible in the LLC tumor, using a standard fluorescence microscope and the confocal and 2-P microscopes. In Figure 4C, left panel, the confocal tumor image reveals blue labeling of the tumor by the BODIPY-bearing nanoparticles. The right panel includes red and blue channels, and indicates heavy accumulation of nanoparticles (blue) in blood vessels and around tumors cells (red). The particles could be imaged at 24 hours after injection, indicating that they survived within the circulation and accumulated intact in the tumor. The window chamber results predicted the orthotopic (deep body, pancreas) result, as the BODIPY labeled nanoparticles were imaged within the tumor vessels and could be seen extravasating into surrounding tissue, as depicted by the two-photon image in Figure 5B.

DISCUSSION

In this paper we demonstrate the feasibility of producing iron-oxide-dextran nanoparticles which have a long *in vivo* circulating time, and that can be imaged by MRI and fluorescence microscopy *in vivo*. Acetylated lipids were incorporated into the nanosome surface structure and UV cross-linked to enhance *in vivo* physical stability. The particles also exhibited good drug loading due to the dextran core (doxorubicin 60 µg/ml). Importantly, a MR compatible tissue - tumor window chamber platform was developed. This chamber for the first time provided direct, nondestructive optical validation of the MR imaging data. This system was designed to allow serial imaging over an extended time period. Such an advantage could be

exploited to facilitate optimization of nanoparticle surface features, the degree of tumor targeting, deep MR imaging capabilities, and other characteristics.

Ultimately, the ability to track particles with MRI has potential clinical utility in tumor treatment by facilitating external activation of the nanoparticle payload at an optimal time, rather than by relying on the variable timing of particle tumor penetration and cellular lysosomal disruption of the liposomal outer shell. Nanoparticles might be activated at a specific time by externally applied ultrasound, internal or external light, or via injection with a bolus of functionalized, partner nanoparticles. Accordingly, the mapping and optimization of the MR relaxivity - contrast of our liposomes, together with the introduction of tumor targeting and evaluation of the therapeutic potential of our composite nanoparticle in preclinical tumor models, is in progress and will be the subject of a future publication.

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Figure 1.

Schematic diagram of a multifunctional nanoparticle for MR contrast and drug delivery. The inner core is comprised of iron oxide encapsulated by crosslinked dextran which is the site of drug loading. The outer shell of the nanoparticle is decorated with PEG and can be covalently attached to targeting moieties. The particle undergoes sterile filtration through 0.2 μm sterile filters and has a shelf life of at least 4 weeks.

Figure 2.

A - MRI compatible – optical dorsal skinfold window chamber. The schematic drawings depict the complementary Delrin® plates separately and clamped together. The photographs show the chamber implanted on a mouse with a light colored, amorphous tumor visible in the center. The subcutaneous tissue with microvessels is clearly visible. The securing clips have been removed to more clearly show the skinfold.

B - Mouse coil and holder. The tunable coil is placed against the window chamber as depicted in the photograph. The tubing at the left contains a line for delivery of inhalation anesthetic and a line for evacuation of exhaled gases. The air bladder permits respiration to be monitored via a transducer outside the MR scanner.

Figure 3.

A - Nanoparticle size determination. The actual diameter of 113 nm diameter was measured by a Zetasizer system. The output graph showing intensity versus particle size is shown. (Note that peak intensity does not correspond to mean particle diameter.)

B - Size exclusion column data showing normalized fluorescence versus fraction collected. There is a single early peak for rhodamine controls and FITC-dextran containing liposomes, which together with the absence of a later, second elution peak, shows that unincorporated FITC-dextran was not present.

C - Left panel is a SEM of the iron oxide – dextran nanoparticles, and is digitally magnified in the middle panel. The spherical lipid shell can be seen in many of the particles, especially in the middle panel. Bar at bottom of A is 500 nm, and shows that the nanoparticles are about 100 nm in diameter. The far right panel is the SEM compositional mapping of the scanned particle constituents, showing that iron is a component of the particles.

D - T2-weighted Spin Echo image of the samples in different vials for TE=88 ms (TR=2000 ms). Water in right vial, iron oxide-dextran in left vial and iron-dextran core nanosomes in middle vial. Note the images of the iron containing particles exhibit signal dropout relative to water alone.

Figure 4.

A and B. Both 4A and 4B show images of the MRI compatible window chamber for nanoparticle development purposes. After injection the tumor and tumor vessels are more heavily delineated and there appears to be extravasation of nanoparticles into the surrounding tissue (Figure 4A and 4B). In Figure 4B, panels i and ii show the entire chamber before and after nanoparticle injection, with flanking histograms of pixel grayscale versus pixel count (lower grayscale value is darker). Panels iii and iv show tumor vessel ROIs with respective histograms, and panels (v and vi depict the major feeding vessel image pixels that have been segmented out of the image, together with their histograms. The pixel grayscale ratio of before versus after nanoparticle injection, goes from 1 : 0.8 for the entire chamber images, to approximately 1:1 for the ROI, indicating a darkening restricted to the ROI. The vessels themselves are the darkest with a ratio, before vs. after nanoparticles, of 1.6 : 1. Note the shifting to the left (darker gray scale values) of the segmented vessel histogram after nanoparticle injection.

C. Shows two confocal microscope images with heavy concentrations of the BODIPY labeled nanoparticles (blue). The left panel is a confocal acquisition with only the blue laser and the blue channel active. Note the blue staining due to the BODIPY labeled nanoparticles. The right panel shows a red channel acquisition at 10x blended with the blue channel acquisition. Note the extensive blue staining of tumor blood vessels and stroma.

Figure 5A.

The two large image panels show T2 weighted abdominal MR acquisitions before (left) and 24 hours after (right) nanoparticle injection. The histograms at the bottom of each large image reveal very little change ($< 2\%$) in terms of mean pixel gray level. However the two smaller image panels, each showing an ROI encompassing the tumor, reveal darkening after nanoparticle injection. This is supported by the respective ROI histograms, which indicate a substantial ($\approx 25\%$) change in mean gray level. The ratio of mean pixel intensity before versus after nanoparticle injection is approximately 1:1 for the image of the torso, while it increases to 1.33:1 for the tumor ROIs. This indicates that the tumor darkening is not due to a generalized artifact.

Figure 5B.
2-photon image of the removed tumor shown in 5A, with red labeled nanoparticles in the tumor vessels and extravasating into the surrounding tissue.

Table 1

Nanoparticle outer shell constituents.

Constituent	Percent of Outer Shell
BODIPY Fluor (DOPE)	2
DSPC- PEG 2000	10
UV Crosslinkable acetylated DOPE	5
(-PEG-DOPE)	5
Cholesterol	20
DOPE	30
DSPC	28

DOPE = dioleophosphadylethanolamine, DSPC = distearoylphosphatylcholine BODIPY=6-(4,4-difluoro-5-(2-thienyl-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy-acetyl)aminohexamido-DOPE.

Table 2

Physical properties of Delrin® versus titanium chamber material.

Parameter	Delrin®	Titanium
Material composition	Polyoxymethylene	Titanium, Aluminum, Vanadium
Thermal conductivity (Btu. in/ft ² .hr°F)	2.6	46.5
Tensile Strength (lb/in ³)	10000/0.0515=194.17e3	138000/0.16 = 862.5e3
Can be molded	Yes	Yes
Biocompatible	Yes (not for permanent implant)	Yes

Table 3

Iron concentration and relaxivity of water, nanoparticles, and MR contrast media

Solution	Iron Concentration (µg/mL)	T ₂ (ms) @ 3T	R ₂ (1/mM*s) @ 3T
Tap Water	0	1374	-
Iron oxide-dextran	12.7	39	109.55
Nanosomes	12.9	41	102.44
Feridex/Endorem ¹			93
Resovist ¹			143