Vascular Targeted Nanoparticles for Imaging and Treatment of Brain Tumors

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Abstract

Purpose: Development of new therapeutic drug delivery systems is an area of significant research interest. The ability to directly target a therapeutic agent to a tumor site would minimize systemic drug exposure, thus providing the potential for increasing the therapeutic index.

Experimental Design: Photodynamic therapy (PDT) involves the uptake of a sensitizer by the cancer cells followed by photoirradiation to activate the sensitizer. PDT using Photofrin has certain disadvantages that include prolonged cutaneous photosensitization. Delivery of nanoparticles encapsulated with photodynamic agent specifically to a tumor site could potentially overcome the drawbacks of systemic therapy. In this study, we have developed a multifunctional polymeric nanoparticle consisting of a surface-localized tumor vasculature targeting F3 peptide and encapsulated PDT and imaging agents.

Results: The nanoparticles specifically bound to the surface of MDA-435 cells in vitro and were internalized conferring photosensitivity to the cells. Significant magnetic resonance imaging contrast enhancement was achieved in i.c. rat 9L gliomas following i.v. nanoparticle administration. Serial magnetic resonance imaging was used for determination of pharmacokinetics and distribution of nanoparticles within the tumor. Treatment of glioma-bearing rats with targeted nanoparticles followed by PDT showed a significant improvement in survival rate when compared with animals who received PDT after administration of nontargeted nanoparticles or systemic Photofrin.

Conclusions: This study reveals the versatility and efficacy of the multifunctional nanoparticle for the targeted detection and treatment of cancer.

Photodynamic therapy (PDT) relies on the selective uptake of a photosensitizing molecule in a tumor relative to the surrounding normal parenchyma followed by exposure to the appropriate wavelength of light to activate the photosensitizer (1). When activated by light irradiation, the photosensitizer interacts with molecular oxygen to produce a cytotoxic, short-lived species known as singlet oxygen. PDT elicits both apoptotic and necrotic responses within treated tumors and produces microvascular injury leading to inflammation and hypoxia. Photofrin, a complex mixture of porphyrin oligomers, is one of the most efficient photosensitizers approved for PDT of cancer (2). However, Photofrin can cause prolonged skin photosensitization, where patients are required to avoid direct exposure to sunlight for a period of 4 to 6 weeks. Current strategies under development include attempts to direct the photosensitizing agent to the tumor by active targeting approaches, such as peptide conjugates and antibodies (3–7), incorporation within liposomes (8, 9), and encapsulation within polymeric nanoparticles (10–14) in an attempt to deliver higher local concentrations at the therapeutic site.

A recent report of a sub-100 nm dynamic nanoparticle platform composed of polyacrylamide, which could be loaded with a photoactivatable agent (methylene blue) for the specific purpose of singlet oxygen delivery, has revealed that this is a promising approach for killing tumor cells (11). Moreover, this nanoparticle platform was also recently used for encapsulation of iron oxide (15), a superparamagnetic magnetic resonance imaging (MRI) contrast agent (16, 17). Intravenous administration into rats with intracranial 9L gliomas was well tolerated and provided excellent tumor contrast enhancement (15), suggesting that this technology could be adapted for numerous applications, including a combined imaging and therapeutic agent. In addition, chemical modification of the nanoparticle

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Note: G.R. Reddy and M.S. Bhojani contributed equally to this work.

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surface also provides the possibility for attachment of tumor-targeting molecules. The ultimate goal of these modifications is the development of a multifunctional nanoparticle capable of delivery of diagnostic and therapeutic agents to tumor while potentially reducing the interaction of the therapeutic payload from the systemic biological milieu, thus decreasing the unwanted side effects.

Proper principle of using a targeting molecule to direct a therapeutic agent to the tumor site was reported using a homing peptide-directed drug delivery approach by coupling doxorubicin to an Arg-Gly-Asp (RGD) peptide (18). RGD motif is present in ligands of several integrin family members and is capable of targeting to certain tumors and tumor endothelial cells (19). The doxorubicin-RGD peptide conjugate was found to be very effective in tumor growth inhibition and was less toxic than nonconjugated doxorubicin (18). For this current study, we have selected a vascular homing peptide, F3, which has been reported to have cell-penetrating properties (20–24). F3 is a 31-amino acid sequence of the NH2-terminal fragment of human high-mobility group protein 2, which was discovered using phage-displayed cDNA libraries (20). It was shown that FITC-labeled F3 peptide accumulated on the surface and then translocated to the nucleus of MDA-MB-435 both in vitro and in xenograft studies (20, 22). For this tumor-specific uptake and nuclear localization of F3 peptide, expression of nucleolin, a shuttle protein that traffics between membrane and nucleus located on the cell surface, is a prerequisite (22). Cell surface nucleolin is a specific marker for angiogenic endothelial cells within tumor vasculature (22). Further evidence for the possibility of using F3 to target nanoparticles was provided in a recent study wherein quantum dots (<10 nm) were targeted to tumor blood vessels in human xenograft tumors (21).

Tumor response to therapy is traditionally assessed by radiographic evaluation of tumor dimensions required weeks to months after treatment. Diffusion MRI has emerged as a viable imaging biomarker for early detection of treatment response in experimental (25) and clinical tumors (26, 27). This approach relies on the ability of MRI to quantify the Brownian motion (diffusion) of water within tissues (28). Diffusion MRI has recently been reported for detection of PDT in animal models (29, 30), and we have therefore examined changes in tumor diffusion values in this current study involving the use of nanoparticles for PDT of gliomas. PDT as an adjuvant therapy has been evaluated both experimentally and clinically in the treatment of several cancer types. PDT is an interesting approach for the treatment for malignant gliomas as >80% recur locally despite conventional treatments. PDT thus offers a localized treatment approach in which improvement in local control of malignant cerebral gliomas may result in significantly improved survival. Several studies have been reported on the application of PDT for the treatment of brain tumors (31–37). In fact, a recent report has suggested that there was an association between the amount of photosensitizer uptake within the tumor with survival in glioblastoma patients (37). More recently, it was reported that PDT of primary and recurrent gliomas resulted in an increase in patient median survival (38). These encouraging results provide the rationale for improving PDT of malignant gliomas through enhancement of local delivery of the photosensitizing agent to achieve a higher level of singlet oxygen generation within the tumor mass.

In this current study, we prepared an F3-targeted polymeric nanoparticle formulation consisting of encapsulated imaging agent (iron oxide or fluorescent) and photosensitizer (Photofrin). This formulation was evaluated in a series of in vitro experiments for its ability to produce singlet oxygen, target the nucleolin cell surface receptor, and confer photosensitivity. In vitro studies revealed that F3-targeted nanoparticles were bound to, internalized, transported, and concentrated within tumor cell nuclei. Photoactivation of the nanoparticles resulted in the loss of cell viability. In vivo studies revealed that iron oxide/Photofrin-encapsulated F3-targeted nanoparticles could be detected in i.c. 9L gliomas using MRI. Administration of laser light through a fiber optic applicator into the tumor site resulted in significant therapeutic benefit, with F3-targeted nanoparticles providing a significantly increased survival time over that of nontargeted Photofrin-encapsulated nanoparticles or Photofrin alone. Overall, the polymeric multifunctional nanoparticle formulation reported here was found to be a versatile drug delivery vehicle for the delivery of imaging contrast agents and therapeutics in a targeted manner.

Materials and Methods

Synthesis of Photofrin- and iron oxide–encapsulated nanoparticles. Photofrin-encapsulated or Photofrin- and iron oxide–encapsulated amine-functionalized nanoparticles were prepared by adapting the procedure reported previously for iron oxide nanoparticles (15). Briefly, Photofrin-encapsulated nanoparticle was synthesized by mixing an aqueous solution containing acrylamide (27%, w/v), N,N-methylene (bis)methacrylamide (9%, w/v), 3-aminopropylmethacrylamide (5%), and Photofrin (20 mg) with hexane solution containing Brij 30 (polyoxyethylene-4-lauryl ether, 7.1%) and bis(2-ethylhexyl)sulfosuccinate (3.5%). For nanoparticles harboring both Photofrin and iron oxide, 180 mg iron oxide was added to above mixture. The nanoparticle synthesis was initiated by ammonium persulfate (10%) and TEMED. The reaction mixture was concentrated to a thick residue, which was washed with ethanol to produce a fine brown powder of nanoparticles.

PEGylation of nanoparticles. Amine-functionalized, Photofrin-encapsulated and/or iron oxide–encapsulated polyacrylamide nanoparticles (500 mg, 0.2 mmol amine groups) were suspended in 25 mL of 0.15 mol/L sodium bicarbonate solution (pH >7.8) and sonicated for 20 minutes. The suspended clear brown solution was filtered through 0.2-μm filters, and the filtrate was transferred into a round-bottomed flask. The nanoparticle solution was treated with succinimidyl succinate ester of PEG2000 (1 g), and the reaction solution was gently stirred for 2 hours at room temperature. The brown solution was transferred into an Amicon (Millipore, Billerica, MA) stirred cell equipped with a 500,000 MWCO polyethersulfone filter membrane and thoroughly dialyzed with PBS (5 × 180 mL) to remove any excess PEG2000 and concentrated to approximately each mL that contain 100 mg of particles. The material was used for in vivo experiments.

Conjugation of F3 peptide to nanoparticles. A clean glass vial (20 mL size) was charged with amine-functionalized, Photofrin-encapsulated and/or iron oxide–encapsulated nanoparticles (400 mg contain 2.8 mg Photofrin, 0.16 mmol amine groups) and 20 mL PBS. The mixture was sonicated for 20 minutes, and the solution was thoroughly washed with argon-purged PBS (pH 7.4). The concentrated nanoparticle solution (23.8 mg/mL) was transferred into a clean glass vial, the solution was treated with sulfo-SMCC (16 mg), and the reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was further treated with succinimidyl succinate ester of PEG2000 (750 mg), and the reaction solution was gently stirred for 2 hours at room temperature under argon atmosphere. The reaction mixture was dialyzed with a
500,000 MWCO filter membrane against argon-purged PBS (5 × 180 mL) to remove any unreacted succinimidyl ester, and the concentrated solution (20 mg/mL) was directly used in the next step. In the mean time, a clean 20 mL glass vial was charged with F3 peptide (24 mg, KDEPQRSDLARPSAPPKPEPKPKPAK) in PBS (5 mL) and treated with 2-iminothiolane (1 mg). After a 30-minute stirring, the above nanoparticle solution was added to the peptide solution and stirred for 15 hours. The solution was further treated with L-cysteine (5 mg, 0.04 mmol) for 30 minutes to block any unreacted SMCC groups and then dialyzed against PBS (8 × 150 mL) in a stirred cell equipped with a 500,000 MWCO filter membrane to remove any unreacted peptide molecules. The solution was concentrated to a final concentration of 66 mg/mL.

**Labeling of nanoparticles with Alexa Fluor 594.** A 20 mL glass vial was charged with amine-functionalized nanoparticles (200 mg, 0.08 mmol amine groups) in 10 mL PBS (pH 7.4). The solution was sonicated for 10 minutes and filtered through 0.2-μm syringe filter. The solution was treated with 1 mg of carboxy succinimidyl ester of Alexa Fluor 594. The mixture was gently stirred overnight at room temperature. The reaction mixture was extensively dialyzed against PBS (8 × 80 mL) using a 500,000 MWCO polyethersulfone filter membrane and concentrated. The filtrate was tested by UV spectrophotometer for the presence of any unbound fluorophore, and the dialfiltration was continued until no fluorescence was found in the filtrate.

**Labeling of F3-targeted nanoparticles with Alexa Fluor 594.** The fluorescent-labeled F3-targeted nanoparticles were prepared as mentioned in the Photofrin-encapsulated nanoparticles. The F3-targeted nanoparticle solution (20 mg/mL) was passed through 0.2-μm filters and used for in vitro experiments.

**Particle sizing by multianalyte light scattering and scanning electron microscopy.** The particle size measurements were done by multianalyte light scattering methods. The average calculated molecular weight of the nanoparticles was 2,000 kDa. A dilute solution (1 mg/mL concentration) was used to measure particle sizing. The data were collected as an average of two 5-minute cycles. The samples were further analyzed by scanning electron microscopy and elementary analyses.

**Chemical detection of singlet oxygen produced from Photofrin nanoparticles.** The \( \Delta \Omega \) production of the Photofrin nanoparticles was determined chemically by using 1,3-diphenylisobenzofuran (DPBF) as a singlet oxygen detection probe (39, 40). The quantitative analysis was made based on the kinetic model analogous to the one for anthracene-9,10-dipropionic acid disodium salt (41). In brief, the fluorescence intensity of DPBF decreases in the presence of singlet oxygen due to chemical reaction to form a nonfluorescent endoperoxide. The fluorescent decay follows the first-order kinetics, and the decay constant \( k \) is proportional to the quantum yield of \( \Omega \) under a preset experimental condition.

Briefly, 0.2307 mg/mL DPBF-embedded nanoparticles in ethanol-H2O (50:50) solution were prepared. DPBF nanoparticle solution (200 μL) was added to 2 mL of pure ethanol, ethanol-H2O (75:25) mixture, ethanol-H2O (50:50) mixture, and ethanol-H2O (25:75) mixture and water. Each sample had been stood for 5 minutes in the dark, and fluorescence emission of DPBF was measured with 412 nm excitation wavelength.

**Cell culture and treatments.** MDA-MB-435 human breast carcinoma cell lines were routinely grown in DMEM supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 292 μg/mL l-glutamine (all from Invitrogen, Carlsbad, CA). For measuring cytotoxicity of F3-targeted Photofrin-embedded nanoparticles, \( 1 \times 10^6 \) MDA-MB-435 cells, grown on 25-mm coverslips in 100-mm dish for 2 to 3 days, were incubated with 2, 5, or 10 μmol/L nanoparticles for 4 hours at 37°C with slow intermittent rocking. Four-hour time point was selected after initial screening of the dose of nanoparticles and time of incubation for maximal cytotoxicity after laser irradiation. For laser irradiation, coverslips with cells were placed in a 60-mm dish and gently washed thrice with serum-free DMEM and then fresh serum and phenol red indicator-free medium was added. A Diomed 630 PDT Laser (Diomed Inc., Andover, MA) was used for all in vitro and in vivo illumination studies. A custom optical diffuser fiber consisting of a 100-μm core silica optical fiber, a proximal SMA-type connector, and a distal light diffusing tip 1 cm in length and 0.1 mm in diameter was used. The fiber was designed to distribute light energy uniformly over the length of the diffuser tip. Cultured cells were irradiated with a 1,500 mW light exposure for 5 minutes. The wattage and exposure time were determined after initial screening wherein cells were irradiated with laser light ranging from 500 to 2,000 mW for 1 to 30 minutes. The cell viability was then monitored using calcine-AM and propidium staining (both obtained from Molecular Probes, Inc., Eugene, OR). Red nuclei and green cells in a random field were counted. Data presented represent the percentage live cells ± SD from three separate experiments.

For monitoring cellular uptake and subcellular localization of nanoparticles, MDA-MB-435 cells were incubated with 10 μmol/L fluorescently labeled nanoparticles for 4 hours and, following three washes, monitored under a Nikon fluorescent microscope (Nikon, Melville, NY) attached to CoolSNAP CCD camera (Roper Scientific, Tucson, AZ). The cells were counterstained with membrane-permeable SYTO 11 or Hoechst 33342 (both from Molecular Probes). Gray scale images were acquired and colorized (red for nuclei and green for nanoparticles) using MetaMorph software (Molecular Devices Corp., Sunnyvale, CA).

**Animal model.** Rat 9L glioma cells (Brain Tumor Research Center, University of California, San Francisco, CA) were grown as monolayers in minimal essential medium supplemented with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 95%/5% air/CO2 atmosphere. Cells were harvested and resuspended for tumor implantation at a concentration of 10³/5 μL. I.c. 9L tumors were induced in male Fisher 344 rats weighing between 125 and 150 g. Briefly, 9L cells (10⁴) were implanted in the right forebrain at a depth of 3 mm through a 1-mm Burr hole. The surgical field was cleaned with 70% ethanol, and the burr hole was filled with bone wax to prevent extracerebral extension of the tumor. Animals were imaged using MRI beginning at 12 days after cell implantation to select tumors between 30 and 60 μL in volume for in vivo studies.

**In vivo pharmacokinetic brain tumor studies.** Nontargeted (n = 9) and F3-targeted (n = 4) nanoparticle preparations were given to i.c. 9L glioma (60-100 μL) rats as a suspension (~40 mg/mL) in normal saline by tail vein injection at a dose of 200 μg nanoparticles/kg body weight (~7 mg Fe/kg and 7 μg Photofrin/kg). Briefly, an Angiocath was placed in the tail vein of the animal flushed with 10 units/mL of heparin and a preprime infusion line of nanoparticles was connected to the Angiocath. Particles were injected over 30 seconds during single-slice dynamic magnetic resonance scanning done using a horizontal bore, 7 Tesla Varian animal imaging system (Varian, Palo Alto, CA). All images were acquired using a 38-mm quadrature rat head coil and a field of view of 35 × 35 mm over a 128 × 128 matrix and using a slice thickness of 2 mm. The number of averages was varied depending on the repetition time used. T1- and T2-weighted images were acquired using a spin-echo sequence. For T1-weighted images, the echo time (TE) was set to 9 milliseconds and the repetition time (TR) was varied between 100 milliseconds and 10 seconds. In T2-weighted images, TR was fixed to 2 seconds and TE was set to 60 milliseconds. Gradient-echo images were acquired with TR fixed at 1 second, and TE of 5 milliseconds. To determine the distribution and pharmacokinetic behavior of the nontargeted and targeted nanoparticle preparations, magnetic resonance images were obtained using dynamic T2*-weighted gradient-echo MRI. After animal preparation as described above, axial slices of the rat brain were acquired using a 30 × 30 mm field of view over a 128 × 128 matrix, a 1-mm-thick slice, TR = 10 milliseconds, and TE = 5 milliseconds. Precans were acquired before i.v. injection of particles and immediately following the injection, and the dynamic course of signal decrease was followed by continuous acquisition of...
follows changes in tumor diffusion changes for up to 2 weeks after PDT. Every 2 to 3 days thereafter using T2-weighted and diffusion MRI to filled with bone wax and the skin was closed. Animals were imaged of the probewas lowered slowly to extend to the base of the tumor. Laser exposure was given at a setting of 750 mW for 7.5 minutes. Treatment were used to determine the depth of placement for the fiber optic probe, which was attached to a rodent stereotaxic frame. The tip of the probe was lowered slowly to extend to the base of the tumor mass. Laser exposure was given at a setting of 750 mW for 7.5 minutes. Following light activation, the probe was removed and the hole was filled with bone wax and the skin was closed. Animals were imaged every 2 to 3 days thereafter using T2-weighted and diffusion MRI to follow changes in tumor diffusion changes for up to 2 weeks after PDT.

Animal survival and statistics. Survival of the five groups (control, laser only, Photofrin, nontargeted PDT, and targeted PDT) was compared using a log-rank test for the difference in median survival time from initiation of treatment at a nominal significance level of 0.05. Secondarily, pair-wise log-rank tests on select groups were done to determine which groups have significantly different median survival times from one another.

Results

In vitro studies. The tumor-homing peptide, F3, selectively targets to tumor cells and angiogenic vasculature. To assess the ability of the F3 peptide to target nanoparticle to tumors, we produced multifunctional nanoparticles (Fig. 1). These polymeric nanoparticles encapsulated Photofrin and iron oxide crystals within the core matrix. Polyethylene glycol was attached to the surface of the nanoparticle along with the targeting peptide. Chemical and spectrophotometric analysis of the particles revealed the presence of ~4% iron and 4.9 μg Photofrin/mg nanoparticles. This value corresponded to an average of six molecules of Photofrin/molecule of nanoparticle. The optimum number of fluorochrome per molecule of nanoparticles that yielded the highest signal intensity when measured by fluorimeter was 10. Increasing the number of fluorochrome per nanoparticle did not increase fluorescence intensity possibly due to fluorescence quenching (results not shown). The average number of F3 peptide, when analyzed by quantitative amino acid analysis, revealed an average of 30 molecules of F3 peptide per molecule of nanoparticles. Scanning electron microscopy studies of particles revealed a relatively uniform size distribution (Fig. 1B), which was quantified using laser sizing showing an average particle diameter of ~40 nm (Fig. 1C).

The ability of the Photofrin-encapsulated nanoparticles to produce reactive oxygen species (O$_2$) on laser light exposure was evaluated. Results from 0.5 and 1.0 mg nanoparticles/mL suspensions are shown in Fig. 1D. These data reveal that a concentration-dependent increase in singlet oxygen production was obtained. The fluorescent decay of the DPHBE in light-activated Photofrin nanoparticle solutions showed that the singlet oxygen produced from this polymeric nanoparticle formulation is able to exit the particles into the surrounding medium. This feature is critical for achieving tumor cell kill in subsequent experiments.

To investigate if these F3-tagged nanoparticles could be used as a vehicle to deliver cytotoxic agents to tumor cells, Photofrin-embedded nanoparticles were synthesized (Fig. 1). When these nanoparticles, with or without F3 peptide tag, were incubated with MDA-435 cells for a very brief period (5 seconds), rinsed with PBS, and irradiated with laser, no Photofrin-mediated cytotoxicity was observed. In fact, most cells were live (calcein-AM positive) with no detectable loss of cell membrane integrity (Fig. 2A and D). These results revealed that the 5-minute laser irradiation of MDA-435 cells produced no cytotoxicity. Furthermore, when the nanoparticles were incubated for 4 hours without irradiation with laser light, no detectable cytotoxicity was observed, suggesting that nanoparticles alone were not cytotoxic to MDA-435 cells (Fig. 2B and D). The combination of 4 hours of incubation of F3-tagged nanoparticles and laser light resulted in cytotoxicity wherein 90% of MDA-435 cells were killed as monitored by the presence of propidium iodide–stained red nuclei (Fig. 2C and D). However, this combination of 4 hours of incubation and irradiation with laser did not induce cell death when similar nanoparticles lacking the F3 peptide were incubated with MDA-435 cells. The cell death induced by nanoparticles embedded with Photofrin was dose dependent when monitored in the range of 2 to 10 μmol/L of nanoparticle concentration (Fig. 3A and B).

To monitor cellular uptake and subcellular distribution, the F3-targeted nanoparticles were labeled with a fluorescent dye, Alexa Fluor 594, and incubated with MDA-435 cells for 4 hours. Fluorescence microscopic analyses revealed that these nanoparticles bound the cell surface and were also internalized into the nucleus (Fig. 3C, top). The specificity of the targeting of the F3 peptide was shown by incubating non-F3-targeted fluorescent nanoparticles with MDA-435 cells for 4 hours. No specific signal from these nanoparticles was observed (Fig. 3C, bottom). Taken together, these data revealed that F3 peptide could carry nanoparticles as cargo to cancer cells and the cellular uptake and nuclear distribution seem to be similar to those previously reported with fluorescently coupled F3 peptide (20, 22).

Pharmacokinetic imaging of nanoparticles in vivo. Administration of untargeted and F3-targeted iron oxide–embedded nanoparticles was undertaken in rats harboring i.c. 9L gliomas. Shown in Fig. 4A and B are representative images from two independent animals. The images in Fig. 4A were collected from a rat following i.v. administration of non-targeted nanoparticles. Images shown were acquired at 0, 10, 60, and 120 minutes following injection, revealing that significant tumor contrast enhancement was achieved at 10
minutes. Results following administration of targeted nanoparticles are shown in Fig. 4B, which reveal that the contrast enhancement was increased in both magnitude and duration for this preparation. Quantification of tumor contrast half-life and contrast-to-noise ratios for these experiments is summarized in Table 1. The F3-targeted nanoparticles had ~3-fold prolonged tumor transit time ($P < 0.001$). Moreover, the presence of the F3-targeting moiety also resulted in a significantly improved contrast-to-noise ratio of ~2-fold at 1 hour ($P < 0.01$) and 2 hours ($P < 0.008$) after contrast administration. These data reveal that the nanoparticles can be delivered intravascularly to the tumor site and that the presence of the F3-targeting moiety confers a significantly greater amount of nanoparticle accumulation and longer duration within the tumor.

**Therapeutic applications of nanoparticles in vivo.** The ability of the various nanoparticle formulations to be used for tumor treatment was evaluated using diffusion MRI as well as survival as quantitative end points. Shown in Fig. 4C-H are single anatomic T2-weighted magnetic resonance images with color overlays representing the apparent diffusion coefficient values for each tumor at 8 days after treatment intervention. Figure 4C

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**Fig. 1.** Schematic representation and characterization of a multifunctional nanoparticles. A, the nanoparticles developed during this study harbored multifunction units. The core of the nanoparticle was synthesized from polycrylamide, which was embedded with photodynamic dyes (Photofrin) and/or imaging agents (magnetite/fluorochrome). Polyethylene glycol linker and a molecular address tag (F3 peptide) were attached to target these nanoparticles to cancer cells. B, size distribution of the polycrylamide nanoparticle was determined using a scanning electron microscopy. C, the overall size distribution was measured using a laser scanning light scattering size system. D, production of singlet oxygen from the Photofrin-containing nanoparticle formulation was measured by the first-order decay of fluorescence of DPIBF in 0.5 and 1 mg/mL Photofrin nanoparticle solutions in 1:1 ethanol-water mixture after 5 minutes of irradiation at 630 nm (2 mW). The decay constant $k$ obtained for 0.5 and 1 mg/mL nanoparticle solutions was 0.0014 and 0.0023 s$^{-1}$, respectively.
reveals that no significant increase in apparent diffusion coefficient values was observed following insertion of the laser fiber optic tip into the center of the tumor and serves as the experimental control group. An additional group of animals was used to investigate if administration of laser light alone had an effect on tumor diffusion values or survival. The mean change in tumor diffusion values increased slightly (<10%) in animals that had the laser tip inserted into the tumor site followed by exposure to laser light as shown (Fig. 4D and I). Administration of Photofrin either itself (Fig. 4E and I) or within a nontargeted nanoparticle matrix (Fig. 4F) followed by laser activation resulted in similar and significant increased tumor diffusion values (Fig. 4I). However, administration of F3-targeted Photofrin-encapsulated nanoparticles resulted in the most significant increase in mean tumor apparent diffusion coefficient values (Fig. 4G and I) as shown in the images acquired at day 8. The image in Fig. 4H was from the same animal shown in Fig. 4G but reimaged at 40 days after treatment, which revealed a high diffusion value indicative of a cystic cavity that was confirmed at the end of the experimental period.

Evaluation of the effects of each of the treatment groups on animal survival was accomplished and is shown in Fig. 5. Overall, there was a statistically significant difference in

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**Fig. 2.** Cytotoxicity induced by F3-tagged Photofrin-embedded nanoparticles (NP) and laser irradiation. MDA-435 cells were incubated with nanoparticles with or without F3 tag and irradiated with 1,500 mW of laser for 5 minutes. The Photofrin-mediated cytotoxicity was then monitored by labeling cells with calcein-AM (green, live cells) and propidium iodide (dead, red cells). A, incubated for 5 seconds. B, incubated for 4 hours without laser irradiation. C, incubated for 4 hours with laser irradiation. Bar, 20 μm. D, columns, cells in randomly selected fields were counted and plotted as percentage live cells±SD. Bar, 20 μm.
median survival time between the nontargeted \((n = 9)\) versus F3-targeted \((n = 5; P = 0.02)\) and the Photofrin \((n = 6)\) versus the F3-targeted nanoparticle group \((P = 0.01)\). There was no significance in animal survival between the control \((n = 5)\) versus laser-only \((n = 9)\) groups nor the Photofrin versus the nontargeted nanoparticle groups. A median survival time from treatment of 8.5 days for the laser-only group and 7.0 days for the untreated control group was observed. There was no statistically significant difference between the control group and the laser-only group \((P = 0.348)\). The animals from the Photofrin group had a median survival time of 13.0 days, whereas animals treated with the F3-targeted Photofrin nanoparticles had a median survival of 33 days with three animals surviving past 60 days. Two of these three animals were found to be disease-free 6 months following treatment, whereas one recurred ectopically from tumor spread s.c. on the surface of the head at the injection site but had no i.c. involvement.

**Discussion**

The goals of this study were to synthesize and evaluate a multifunctional polymeric nanoparticle formulation for use as a vascular-targeted imaging and drug delivery vehicle. We found that synthetic microemulsion preparations of these nanoparticles could be readily achieved, yielding particles with a mean diameter of \(~40\) nm (range, 10-200 nm). In vitro characterization studies revealed that exposure of Photofrin-encapsulated nanoparticles to 630 nm laser light produced significant amounts of singlet oxygen species in a concentration-dependent manner. These results provided direct evidence that Photofrin encapsulated within the nanoparticle matrix

![Fig. 3.](image-url)
could be photoactivated to yield cytotoxic singlet oxygen. It was hypothesized that targeting Photofrin nanoparticles using the F3 vascular homing peptide could provide for more efficient killing of tumors through similar internalization of the nanoparticle as was previously observed for quantum dots (21). 

In vitro studies clearly revealed that binding and internalization of multifunctional nanoparticles was achieved with nanoparticles prepared with the F3 homing peptide attached on the surface of the nanoparticle (see Fig. 1). These results reveal a remarkable aspect of the F3 peptide—that it was able to facilitate internalization and nuclear localization of our polymeric nanoparticle, which was 6 to 20 times larger than previous F3-linked quantum dots (21). Thus, the F3 homing peptide seems to be very useful for the delivery of vascular agents using nanoparticle vehicles.

The ability of the F3 homing peptide to improve tumor localization of the nanoparticles in vivo was also evaluated by comparing the pharmacokinetics of nontargeted and F3-targeted nanoparticles by quantification of tumor contrast changes using MRI. Dynamic scanning MRI revealed that F3-targeting resulted in an increase in tumor half-life from 39 to 123 minutes. Not only were targeted nanoparticles found to be retained at the tumor site longer, but the amount of contrast enhancement was also significantly increased by ~2-fold, indicating that increased uptake of nanoparticles occurred at the tumor site (see Table 1). Taken together, these data reveal that the F3-targeting moiety significantly enhanced tumor nanoparticle localization, which would be predictive of increased drug delivery potentially translating into improved treatment outcome. In fact, improved treatment efficacy was observed in animals treated with F3-targeted nanoparticles, which exhibited a significantly enhanced overall survival compared with animals treated with nontargeted Photofrin-encapsulated particles or Photofrin alone. Moreover, 40% of animals treated with F3-targeted Photofrin nanoparticles were found to be tumor-free at the end of the study (60 days after treatment). An interesting finding was that animals receiving nontargeted Photofrin-encapsulated nanoparticles had similar magnetic resonance diffusion changes and survival enhancement as animals treated with Photofrin. However, Photofrin-treated animals required a 24-hour delay following i.v. administration before light exposure versus only 1 hour after Photofrin-encapsulated nanoparticles were given.

Diffusion MRI was used to evaluate changes in tumor diffusion properties for each of the treatment groups of animals. The peak change in diffusion observed at 8 days following treatment revealed that animals treated with F3-targeted nanoparticles had the largest increase in tumor diffusion values of ~40%. A correlation of the magnitude of diffusion shift with animal survival has been recently reported (42). This is consistent with the finding here in which animals treated with F3-targeted nanoparticles were found to have the largest increase in diffusion values and were also found to have the longest survival time over the other treatment groups. Moreover, there was no statistical difference between the survival of animals treated with Photofrin and those treated with nontargeted Photofrin-encapsulated nanoparticles in terms of diffusion changes and overall survival. This finding again confirms the consistent reliability of the diffusion measurement as an early predictor of treatment outcome. These nanoparticles were produced to contain both a magnetic resonance contrast agent (iron oxide) along with a therapeutic agent (Photofrin). The ability of vascular targeting along with imaging capability while carrying a payload of a drug by these nanoparticles proves for a multifunctional nanoparticle technology that can be adapted for other diagnostic or therapeutic purposes in future studies.

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**Fig. 4.** Imaging and monitoring of therapeutic efficacy using multifunctional nanoparticles in 9L brain tumors. A and B, top, T2-weighted coronal images through the tumor of two different rats that reveal the anatomic extent of the tumor mass. Fast spin-echo images of the tumor following administration of (A) nontargeted nanoparticles and (B) F3-targeted nanoparticles obtained at the time points indicated.
systemic PDT has been reported to be only effective when it leads to complete ischemia of solid tumors through the prerequisite localization in the intravascular space. However, encapsulation and targeting of Photofrin-encapsulated nanoparticles directly to the tumor vasculature produced a significantly improved treatment outcome in this present study. Therefore, previous and ongoing development of photoactivatable compounds have focused on evaluation of large compounds (80-200 kDa) to be retained within tumor intravascular space. Therefore, the prerequisite of developing novel photosensitizers that have combined properties of both vascular targeting as well as high singlet oxygen yield dramatically increases the difficulty of identifying suitable molecules. However, the ability to use our polymeric nanoparticle vehicle to target a photosensitizer to tumor intravascular space now provides an opportunity to focus on development of photoactivatable molecules that have high singlet oxygen yield ($\Phi_A \geq 0.3$; ref. 46) and high wavelengths of absorption ($\lambda_{abs} > 700$ nm). The lack of the need for having the molecule of high molecular weight and tumor homing should provide new opportunities for developing novel agents for improving PDT. Furthermore, compounds that were found to be excellent photosensitizers but were limited by inherent systemic toxicity

**Table 1.** Pharmacokinetic imaging using MRI of nontargeted and F3-targeted iron oxide–encapsulated nanoparticles in i.c. 9L tumors after i.v. administration

<table>
<thead>
<tr>
<th></th>
<th>Nontargeted (n = 9)</th>
<th>F3 targeted (n = 4)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life (min)</td>
<td>39 ± 3</td>
<td>123 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CNR 1 hour after</td>
<td>3.2 ± 0.6</td>
<td>5.8 ± 0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>CNR 2 hours after</td>
<td>2.4 ± 0.6</td>
<td>5.2 ± 0.5</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Abbreviation: CNR, contrast-to-noise ratio (relative to baseline).
could be reevaluated in the context of polymeric nanoparticle encapsulated delivery as the nanoparticle matrix removes the therapeutic molecule from direct interaction with the physiologic milieu.

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