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Multifunctional nanoparticle platforms for in vivo MRI enhancement and photodynamic therapy of a rat brain cancer

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Abstract

A paradigm for brain cancer detection, treatment, and monitoring is established. Multifunctional biomedical nanoparticles (30–60 nm) containing photosensitizer externally deliver reactive oxygen species (ROS) to cancer cells while simultaneously enhancing magnetic resonance imaging (MRI) contrast providing real-time tumor kill measurement. Plasma residence time control and specific cell targeting are achieved. A 5 min treatment in rats halted and even reversed in vivo tumor growth after 3–4 days post-treatment.

Keywords: MRI contrast agent; Molecular imaging; Nanoparticles; Cancer; Polyacrylamide (PAA); Polyethylene glycol (PEG); Photodynamic therapy (PDT)

1. Introduction

Brain tumors, or gliomas, are among the deadliest forms of cancer, with a median life expectancy of only 6–10 months [1]. Conventional treatment methods of cancer (i.e. surgical procedure, chemotherapy, and radiotherapy) all have limited success providing long-term cures without severe side-effects. A new cancer therapy paradigm, resulting in an effective and versatile brain tumor treatment, utilizes multifunctional nanoplatforms that bombard malignant cells from the outside, through the external release of reactive oxygen species (ROS). These nanoplatforms also enhance

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simultaneous magnetic resonance imaging (MRI) of the treatment's progress. The multifunctional nanoparticles include, as components, photodynamic agents, MRI contrast enhancers, polyethylene glycol (PEG) for the control of plasma residence time and RGD peptide for specific cell targeting. Here we show in vivo tumor growth was halted and even reversed after photodynamic therapy (PDT) with our nanoplatforms for rats bearing intracerebral 9L tumors. The extracellular positioning of the nanoplatforms prevents multidrug resistance (MDR) by the tumor cells.

PDT is a light-initiated chemotherapy where a drug is activated by light, causing oxidative damage to cells, and eventually resulting in cell death. It has been claimed to have the promise of better selectivity and fewer side effects than radiotherapy and chemotherapy [2,3]. However, like chemotherapy, conventional PDT still suffers from the obstacle of MDR; cancer cells pump the introduced drug molecules back out into the extracellular environment [4]. Conventional PDT uses locally present oxygen to produce singlet (''killer'') oxygen and its oxidizing products, free radicals called ROS, all of which destroy cancer cells [2]. PDT relies on intracellularly delivered photosensitizers, dyes that produce singlet oxygen upon irradiation in the visible range. Consequently, PDT requires a light-accessible tumor. Brain tissue is essentially transparent to light and hence a good environment for PDT developmental studies.

In this work, we describe a new class of photodynamic nanoparticles for extracellular cancer cell kill. They can combine MRI contrast enhancement with targeted PDT, enabling protocols of simultaneous cancer detection, therapy and monitoring. The nanoparticles contain PHOTOFRIN® (porfimer sodium, Axcan Pharma Inc.) as the photosensitizer. They have a polyacrylamide (PAA) core containing both photosensitizers and MRI contrast agents, with a surface-coating of both PEG and molecular targeting groups, for controllable particle residence time and the recognition of the tumor neovasculature, respectively (Fig. 1A). Other recent work describes nanoparticles containing PDT agents but without inclusion of MRI enhancing agents or surface modification and with intracellular delivery [5–7].

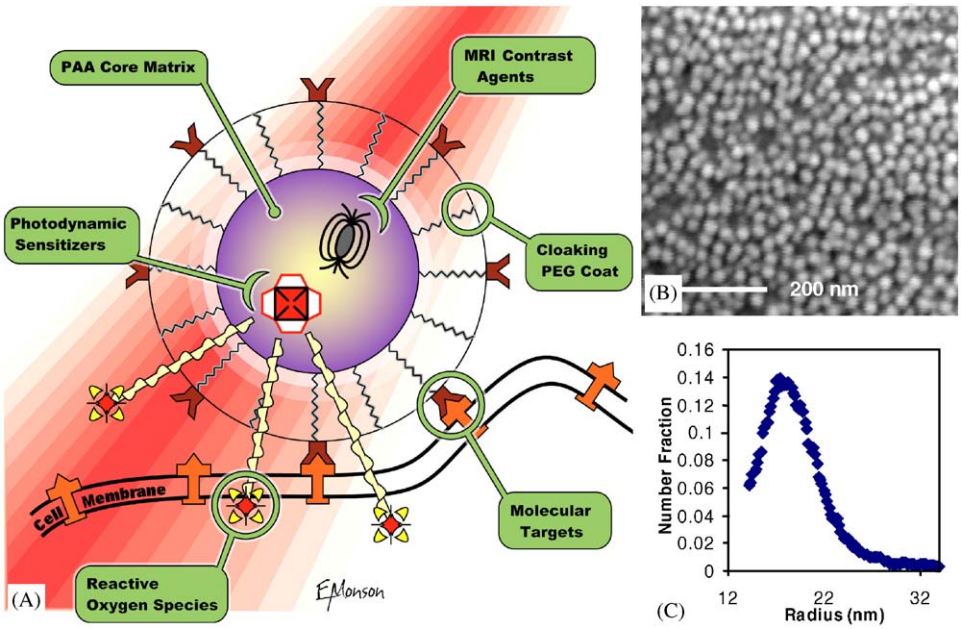

Fig. 1. Overview of nanoparticle platform. (A) Schematic nanoplatform with photodynamic dye, MRI contrast enhancement agent, polyethylene glycol (PEG) cloaking and molecular targeting. (B) A typical SEM image of PAA particles. (C) A typical size distribution result from multi-angle light scattering.

2. Design and synthesis

The design of the nanoparticle is universal, flexible and allows for facile interchange of its active components, including its targeting ligands. The production of PAA nanoparticles is based on previously published nanoemulsion techniques [8]. The morphology of the nanoparticles was determined by SEM (Fig. 1B) and multi-angle light scattering. The average size of PAA nanoparticles in this study ranged from 30 to 60 nm (Fig. 1C).

3. Results and discussion

The efficacy of the nanoparticles as PDT agents depends on the production of singlet oxygen. Therefore, it is important to confirm the production of singlet oxygen from our nanoplatforms and that the amount generated was sufficient to cause cell death. This was demonstrated by both in vitro cell kill and chemical tests. Anthracene-9,10-dipropionic acid, disodium salt (ADPA) was used as a singlet oxygen detection probe for the chemical test [9]. The fluorescence intensity of ADPA decays due to chemical reaction with singlet oxygen produced from the light-activated PHOTOFRIN® that is incorporated in the nanoparticles (Fig. 2A).

In vitro evaluation of the nanoparticles for PDT was done using 9L rat gliosarcoma cells, treated with various concentrations of PHOTOFRIN®-containing nanoparticles (or no particles) and irradiated with a laser source at various intensities (or no light). These treated (or not treated) samples contained propidium iodide and calcein acetoxymethylester dyes to monitor dead and live cells, respectively, with a fluorescent microscope. The cell images under the four different conditions are shown in Fig. 2B. No toxicity was found in control cells treated with laser light only. Increasing the concentration of PDT nanoparticles resulted in an increasing degree of cell death; a minimum particle concentration of 1050 μg/mL was required to produce a recognizable effect under these conditions. It should be noted that a control test with nanoparticles without PHOTOFRIN® was performed for each set of experiments (both spectroscopic detection and cell kill test). The results confirmed us that nanoparticles themselves do not have any therapeutic effect.

The in vivo therapeutic activity of the nanoparticles containing PHOTOFRIN® was also evaluated using rats bearing intracerebral 9L tumors. Diffusion MRI was performed at various time points to monitor changes in tumor diffusion, tumor growth, and tumor load. The diffusion MRI can provide a more quantitative assessment of the tumor than ordinary MRI, by providing the apparent diffusion coefficients for each voxel contained within the tissue. The intensity of each voxel (volume equivalent to pixel) on each image is

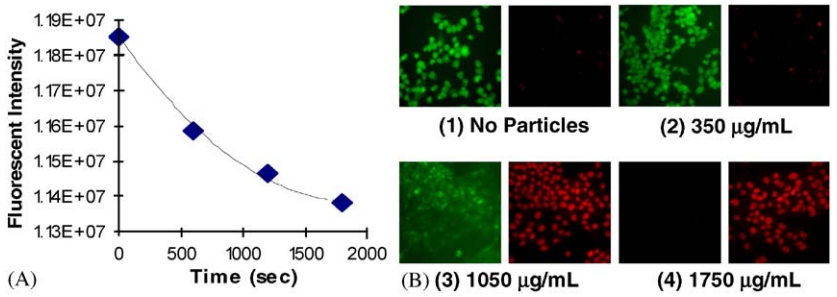

Fig. 2. (A) Detection of singlet oxygen produced by PHOTOFRIN®-containing nanoparticles. The decay of fluorescence intensity of ADPA is a measure of singlet oxygen production and delivery by the nanoparticles. Excitation wavelengths: PHOTOFRIN® (630 nm) and of ADPA (376 nm). (B) In vitro cell kill tests: dose response of PHOTOFRIN® nanoparticles on cultured 9L cells: (1) no nanoparticles present; (2) 350 μg/mL nanoparticles present; (3) 1050 μg/mL nanoparticles present; (4) 1750 μg/mL nanoparticles present. The cells were exposed to 1500 mW of 630 nm laser light for 5 min. Calcein acetoxymethylester and propidium iodide were used to stain live and dead cells, respectively. For each pair of images, left-hand-side images show the live cells (green dots) while right-hand-side images show the dead cells (red dots).

proportional to the mobility of the water within that tissue region. In general, the increase in tumor diffusion values corresponds to a loss of tumor cellularity within the region under study.

The time series of diffusion-weighted MR images show that the untreated (Fig. 3A), as well as laser only treated 9L gliomas (Fig. 3B), continued to grow over the lifespan of the animals. We note that the slight dark region with a central bright spot in post-treatment images of laser-treated tumor cells corresponds to a small area of damage due to insertion of the laser probe. Gliomas treated by the administration of PHOTOFRIN®-containing nanoparticles (Fig. 3C), followed by laser irradiation, produced massive regional necrosis, demonstrated by huge ''bright'' regions in the images, resulting in shrinkage of the tumor mass. Re-growth occurred at 12 days post-treatment.

The diffusion histograms were constructed based on the images and the changes in the mean apparent diffusion coefficient (ADC) and tumor volumes were analyzed. For untreated (Fig. 4A), and laser only treated 9L tumors (Fig. 4B), the overall distribution of diffusion values did not change significantly, nor did the ADC, while the tumors continued to rapidly and exponentially grow over time, indicating that exposure of the tumor to the fiber optic light source did not affect the tumor growth rates or cause sufficient damage to change the tumor water diffusion values. The diffusion histograms for 9L tumors that received PDT treatment using PHOTOFRIN®-containing nanoparticles (Fig. 4C) reveal that this treatment significantly increased the tumor diffusion values, as evidenced by the dramatic right-shift of the histograms. Moreover, ADC was also markedly increased by approximately 50% after treatment,

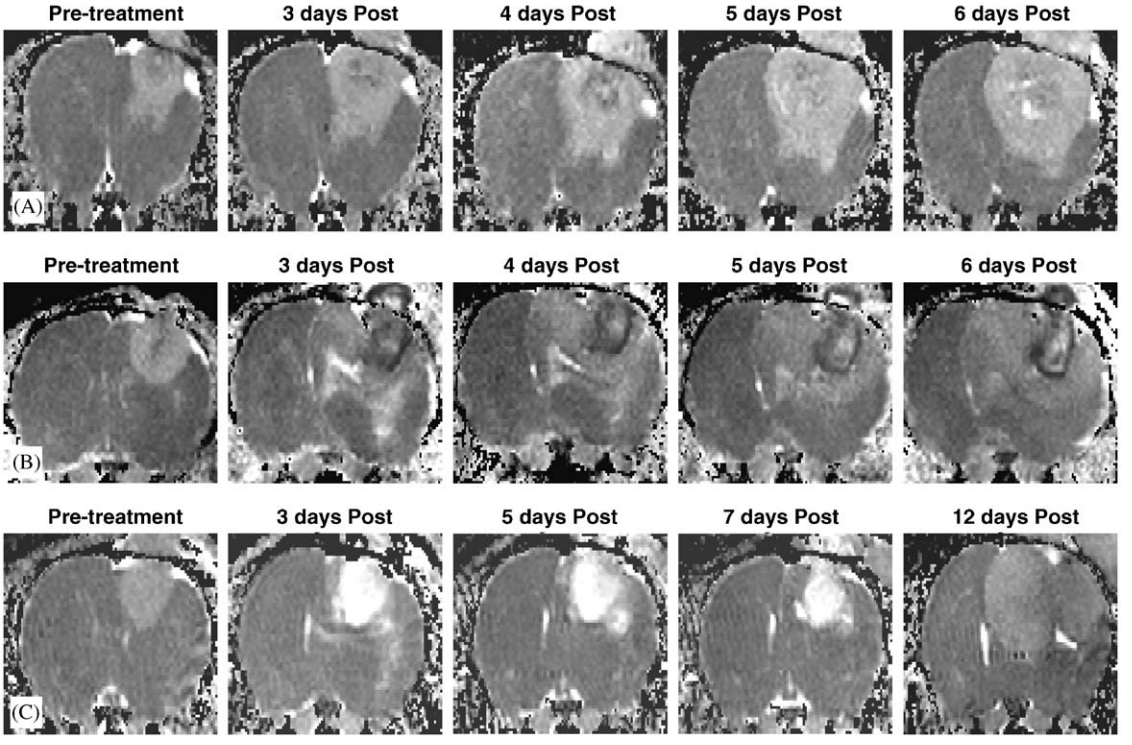

Fig. 3. Time series of the diffusion-weighted MR images of tumor after in vivo PDT: (A) untreated; (B) treated with laser light alone; (C) treated with laser light and PHOTOFRIN®-containing PAA nanoparticles. The images shown here are not diffusion-weighted images but rather computer-generated quantitative diffusion maps wherein the intensity of each pixel (voxel) is proportional to the diffusion values for that voxel. Each series of images is obtained from a representative animal from a group of three.

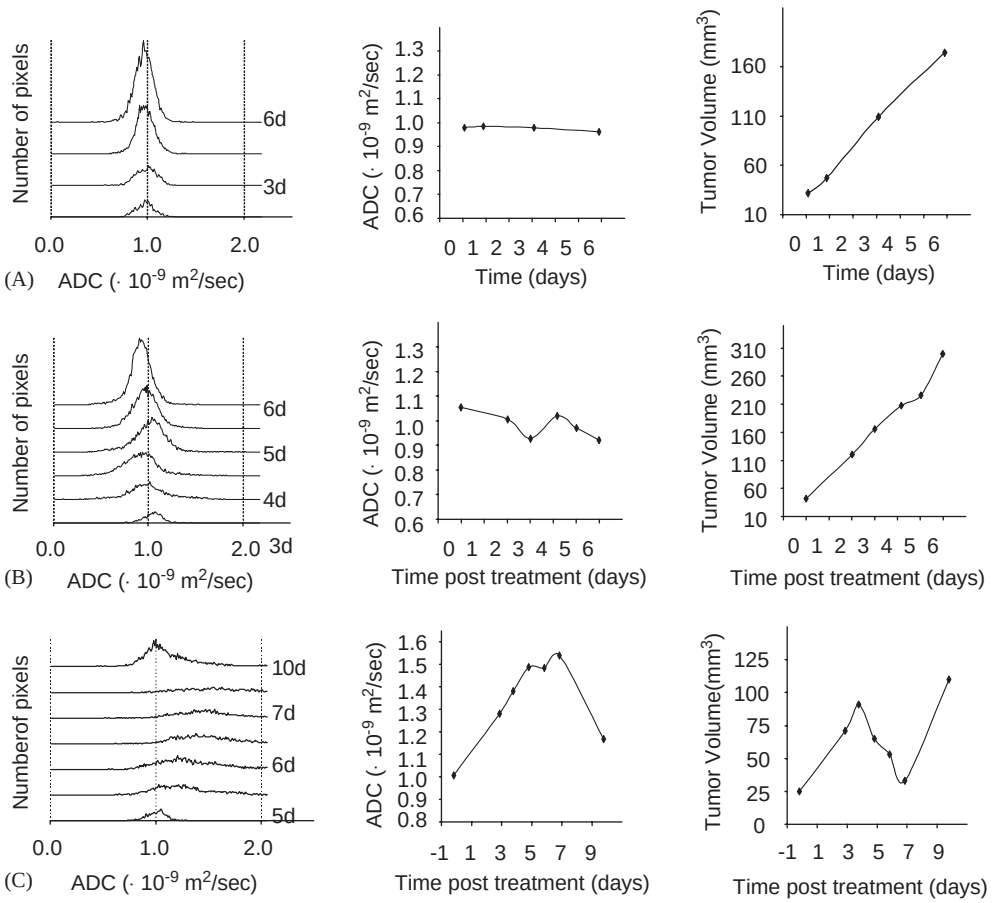

Fig. 4. ADC quantitative analysis of tumor after in vivo PDT. The diffusion histogram means, ADC and tumor volumes over time are shown for (A) untreated tumor; (B) laser treatment only; (C) PHOTOFRIN® PAA nanoparticles and laser light treatment. Each set reveals the data for a representative animal from a group of five.

which indicates a massive cell kill due to treatment. The increase in diffusion is correlated with significant tumor growth retardation and, in many cases, with tumor mass shrinkage. Such increase in average ADC values and the tumor growth retardation were observed for all the tested animals. It can be concluded that administration of these PHOTOFRIN®-containing nanoparticles, followed by light activation, is a viable therapeutic approach for the treatment of brain tumors, which can, furthermore, be successfully monitored with a noninvasive imaging technique.

In vivo toxicology studies with PAA nanoparticles showed no alterations in histopathology or clinical chemistry values for the dose up to 500 mg/kg over 4 weeks. We note that the dose used for the PDT work was 375 mg/kg, which should not produce any toxicity.

The MRI and PDT results indicate a compromised blood-brain barrier, which allows the successful localization of the untargeted nanoparticles in the tumor. However, targeted delivery of a therapeutic agent would be of great value as it would improve efficacy by increasing the amount of therapeutic agent delivered to the site and minimize toxicity by reducing systemic exposure.

Since expression of $\alpha_V\beta_3$ integrins is a common feature of tumor vasculatures [10], we have developed nanoparticles surface-coated with an RGD peptide (cyclic CDC**RGD**CFC), an $\alpha_V\beta_3$

ligand. The RGD peptide was synthesized [11] and attached on the surface of an amine functionalized PAA nanoparticle, using a sandwich of biotin and sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate. Alexafluor 594 dye was included in the nanoparticle matrix to enable fluorescent detection.

We introduced both targeted and non-targeted nanoparticles to MDA-435 cells ($\alpha_V\beta_3$ expressing) and MCF-7 cells ($\alpha_V\beta_3$ non-expressing). The RGD-coated particles specifically bound to MDA-435 cells but not to MCF-7 cells (Fig. 5). These results indicate that these particles retains the ability to specifically bind to $\alpha_V\beta_3$ expressing cells, thus enabling the targeted delivery of the particles for imaging and therapy. We note that there should be no intracellular uptake of the nanoparticles as there is no significant binding of nanoparticles to the cells without targeting moiety.

The loading of the magnetic contrast enhancer into our nanoplatforms further increases the sensitivity of the MRI, thus enabling early cancer therapy monitoring. Our recent work showed that in vitro relaxivity of the iron oxide containing PAA nanoparticles was increased up to approximately 5-fold, compared to other superparamagnetic iron oxide [12]. Also, the length of PEG attached on the surface of the nanoparticles controlled well the in vivo plasma residence time. Non-PEGylated PAA nanoparticles were found to have the fastest plasma half-life of 25–30 min, while PEGylated ones with PEG of molecular weight 10,000 yielded a plasma half-life of about 150 min [12]. The incorporation of all ingredients (molecular targeting, photosensitizer, magnetic contrast agent, PEG) into one nanoplatform is underway due to the flexibility of the synthetic method.

4. Conclusions

In conclusion, extracellular PDT has been demonstrated to be effective on a brain tumor, utilizing a multifunctional nanoparticle platform specially designed for combining in vivo therapy with MRI monitoring of the cancer therapy. The photosensitizers were encapsulated and conserved within the nanoparticle core shell, still showing effective in vitro and in vivo tumor killing. The integrity of nanoparticles was found to be maintained over several months by in vitro degradation studies in PBS at 37 °C.

Because the nanoparticles do not release drugs, in contrast to conventional PDT, the stumbling block of MDR is avoided. The overall effect is a high probability of tumor cell kill. The in vivo tests (rat 9L gliosarcoma model) show the dissolution of the brain tumor upon only 5 min of light therapy. The tumor kill is quickly established by the increased mobility (diffusivity) of the water molecules, monitored simultaneously by spatially resolved magnetic resonance. PEG and RGD surface modifications of the nanoparticles do control the residence time and tumor-specific delivery, respectively.

The multifunctional nanoplatform presents a novel and versatile approach to cancer treatment, as it attacks the tumor cells externally, forgoing the need for drug delivery. A generalization of this approach to other methods of therapy appears to be feasible.

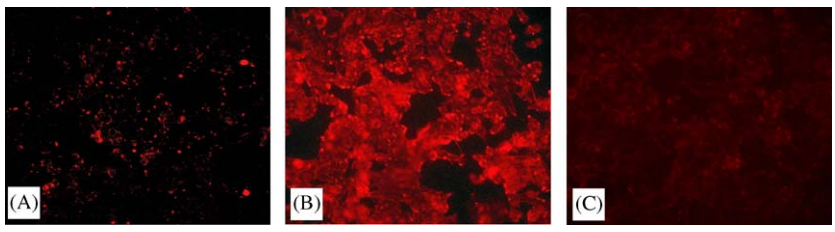

Fig. 5. RGD targeting of fluorescent nanoparticles to cell surface receptors on viable tumor cells. MDA-435 cells were used as a positive control and MCF-7 cells as a negative control. (A) Non-specific binding of non-targeted PAA-nanoparticles to MDA 435 cells. (B) RGD-modified nanoparticles selectively bind to MDA-435 cells. (C) RGD-modified nanoparticles do not bind MCF-7 cells.

5. Methods

For toxicity test, tissues and blood were collected 1, 3, 7, 14, and 28 days after injection of nanoparticles to rats via the caudal vein using a flexible cannula coupled to an automated injection system (Harvard Apparatus). Sera were separated at the time of collection analyzed using a COBAS FARA II fast centrifugal automated enzyme analyzer (Roche Diagnostics). Tissues were fixed in situ by perfusion through the heart with buffered formalin and processed in a Citadel 2000-Tissue Processor (Shandon) embedded using a histocentre-2, (Shandon) sectioned (5 μm), stained (Haematoxylin and Eosin) and analyzed by light microscopy.

For in vivo PDT work, rats bearing intracerebral 9L tumors were anesthetized and 1.0 mL of a 75 mg/mL (in 0.9% NaCl solution) of PHOTOFRIN®-contaning nanoparticles was delivered to the rats by intravenous injection. After ~60 min, laser treatment was applied for 5 min at 700 mW using a fiber optic probe and a Diomed 630 PDT Class IV diode laser (630 ± 3 nm). Untreated tumor-bearing rats and those with laser treatment only (7.5 min, 700 mW) were also monitored by diffusion MRI as controls [12]. For in vitro cell kill test, the same laser was used but at 1500 mW for 5 min.

For targeting study, fixed MDA-435 cells and MCF-7 cells were incubated with targeted and non-targeted nanoparticles for 2 h and then washed three times with PBS to rinse off any unbound nanoparticles before fluorescence imaging.

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