Published in final edited form as:

Nano Res. 2011 November ; 4(11): 1163–1173. doi:10.1007/s12274-011-0166-1.

Targeted Blue Nanoparticles as Photoacoustic Contrast Agent for Brain Tumor Delineation

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Abstract

Distinguishing a tumor from non-neoplastic tissue is a challenging task during cancer surgery. Several attempts have been made to use visible or fluorescent agents to aid in the visualization of a tumor during surgery. We describe a novel method to delineate brain tumors, using a highly sensitive photoacoustic imaging technique that is enhanced by tumor-targeting blue nanoparticles serving as a contrast agent. Experiments on phantoms and on rat brains, *ex vivo*, demonstrate the high sensitivity of photoacoustic imaging in delineating tumors containing contrast agent at a concentration much lower than needed for visualization by the naked eye. The limit of detection of the system for the nanoparticles is about 0.77 $\mu\text{g/mL}$ in water (equivalent to 0.84 $\mu\text{mol/L}$ Coomassie Blue dye). The present exploratory study suggests that photoacoustic imaging, when used with strongly optical absorbing contrast agents, could facilitate cancer surgery intraoperatively by revealing the distribution and extent of the tumor.

Keywords

Brain tumor; surgery; targeted nanoparticles; F3 peptide; Coomassie Blue dye; photoacoustic imaging

1. Introduction

For most patients affected by brain tumors, surgical excision is one of the core components of treatment. Of the several types of brain tumors, such as craniopharyngiomas, ependymomas, medulloblastomas, and meningiomas, gliomas are the most common type of primary brain tumor especially in adults. The quality of life and survival of patients depends, in part, on the extent of surgical resection of the brain tumors [1]. During surgical resection, identifying the tumor boundary by eye can be misleading due to the heterogeneous nature

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and indistinguishable appearance of the glioma. Pre-operative images of the tumor, obtained by MRI (magnetic resonance imaging) or CT (computed tomography) scans after administering intravenous contrast agents, are commonly used to aid the surgeons. However, due to the dynamic geometry of tumor tissue as well as normal brain during surgery, none of these pre-imaging technologies enables reliable intraoperative guidance for brain tumor resection surgery. Another approach to aid the visualization of the tumors during the surgical procedure employs dyes to demarcate the tumor boundaries. Several visual and fluorescent dyes such as Coomassie Blue, Bromophenol Blue, Indocyanine Green, fluorescent porphyrins, and fluorescein have been used for this purpose [2–9]. However, there are negative side effects generated by the systemic injection of the dye, especially when the concentration of the dye needs to be high enough for visualization by eye. Furthermore, in order to visualize fluorescent dyes, special illumination, and a dark background are essential, but these are likely to interfere with the surgical procedure. Therefore, keeping the dye concentration as low as possible, while still providing sufficient contrast for differentiating tumors from background tissue, is still a major challenge. Moreover, based on the direct visualization of the dyes, during the surgical procedure the surgeon can only evaluate the surface of the brain but has no access to three-dimensional (3D) morphological tissue information including the depth and extension of the tumor in subsurface brain.

Photoacoustic imaging (PAI), as an emerging hybrid imaging modality that combines the merits of both light and ultrasound, may provide an alternative approach for mapping the optical contrast agents in the brain and for guiding the surgical resection of tumors. In PAI, a short-pulsed laser source is used to illuminate the tissue sample, and the photoacoustic waves excited by thermoelastic expansion due to light absorption are then measured to reconstruct the optical absorbers distribution in the sample. Therefore, while a PAI image presents the optical contrast in the biological tissues, its resolution is primarily limited by the bandwidth of the detected photoacoustic waves [10]. Because ultrasonic waves are much less scattered in biological tissues than are optical waves, PAI can depict subsurface tissue structures much more accurately than optical imaging. Like conventional optical imaging technologies, PAI presents high sensitivity in imaging versatile contrast agents. However, unlike fluorescent imaging, PAI does not require a dark background, which is especially favorable for intraoperative tumor delineation during a surgical operation that has to be carried out under abundant lighting conditions. Employing laser-based PAI, imaging of the dynamic distributions of Indocyanine Green (ICG) in the circulatory system in small animal brains has been achieved *in vivo* [11]. More recently, noninvasive mapping of sentinel lymph nodes and lymphatic vessels in rats *in vivo* has been realized using PAI enhanced by ICG contrast agent [12]. These studies have demonstrated the high sensitivity, good spatial resolution and satisfactory imaging depth of PAI in localizing and quantifying optical contrast agents in living tissues [13–15].

Reported in this article is an exploratory study on tumor delineation by PAI that is aided by tumor-targeting blue nanoparticles (NPs) as an optical contrast agent. The hydrogel NPs are made of an inert polyacrylamide (PAA) matrix with covalently linked Coomassie Blue (CB) dye molecules, and their surfaces are conjugated with F3 peptides for active tumor targeting. The PAA nanoparticles are biocompatible, chemically inert, with a long plasma circulation lifetime and nontoxic, which makes them suitable as nanodevices for efficient imaging of

tumors [16, 17]. Compared to the free Coomassie Blue dye, the nanoparticles can be selectively targeted to specific tumors or tumor vasculature. They have the ability to act as a platform for delivering contrast agents and drugs into tumors *in vivo* [18, 19]. Previously, our lab has used CB-containing nanoparticles to delineate brain tumors visually [20]. We use blue nanoparticles, as blue offers the best contrast with the red (blood) background, and is most suitable for visual delineation. In this work, we investigated the possibility of imaging and delineating tumors based on PAI, while using the same CB nanoparticles as the PAI contrast enhancement agent. We focused especially on the use of PAI for tumors treated with a very low dose of nanoparticles and, hence, not differentiable by eye. The method presented here can be used as a complement to visual delineation, especially in areas or at levels indistinguishable by eye.

Highly sensitive imaging of the nanoparticles in the tumor was possible using a home-made PAI system. We used reconstruction-based PAI for our imaging purpose. Compared to the reflection model, reconstruction-based PAI using an array of transducers for reconstruction-based PAI enables real time imaging. Also, for a reconstruction-based PAI system, the image quality, including resolution, and pixel number is not limited by the number of transducers but instead by the bandwidth and receiving angle of the transducer, making it more economical and affordable. We first explored the sensitivity of this system by imaging nanoparticles in phantoms. The lowest concentration that was detectable by the PAI was about 0.01 mg/mL. At this concentration, the nanoparticle-containing inclusions in the phantoms cannot be differentiated from the background by the naked eye. Following the experiments on phantoms, the PAI setup was used to image *ex vivo* rat brains containing tumors that had been treated with various doses of nanoparticles. The high sensitivity of the PAI enabled a successful delineation of tumors that did not show any color contrast, compared to the background brain tissues, when observed by the eye. This exploratory study suggests that PAI, enhanced by tumor-targeting nanoparticle contrast agents for delineating tumors, has the potential for reliable preoperative or intraoperative guidance of surgical resection of glioma.

2. Materials and methods

2.1 Chemicals and materials

Acrylamide, methylene-bis-acrylamide (MBA), dioctylsulfosuccinate (AOT), Brij 30, hexane, ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), bovine serum albumin (BSA) (30% w/v), *L*-cysteine, CB derivative, *N,N*-dimethylformamide (DMF), and glycerol dimethacrylate (GDMA) were all acquired from Sigma–Aldrich (St. Louis, MO). 3-(aminopropyl)methacrylamide hydrochloride salt (APMA) was obtained from Polysciences Inc. (Warrington, PA). Ethanol (95%) was acquired from Decon labs, Inc (King of Prussia, PA). *N*-hydroxysulfosuccinimide (sulfo-NHS) and succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) were acquired from Pierce Biotechnology (Rockford, IL). F3-Cys peptide (PKAARALPSQRSRPPEKAKKPPDKPAPEKKKC) was purchased from SynBioSci

(Livermore, CA). All solutions were prepared in 18-M Ω water purified in a Barnstead 1 Thermolyne NANOpure II system. All chemicals and materials were used as received.

2.2 Photoacoustic setup

A schematic illustration of the experimental setup is shown in Fig. 1. A tunable dye laser (ND6000, Continuum) pumped by the second harmonic of a pulsed Nd:YAG laser (Powerlite, Continuum) was employed to provide pulsed light with a pulse width of 5 ns, a tuning range of 578–610 nm using R610 dye, and a pulse energy of 90 mJ at a wavelength of 590 nm (the optical absorption peak of the nanoparticles). After being expanded and homogenized, the laser beam covered an area of about 30 cm² on the surface of the sample with an incident energy density of 3 mJ/cm², well below the American National Standard Institute (ANSI) safety limit of 20 mJ/cm².

The ultrasound signal created due to the laser induced thermo-elastic expansion was detected by a high-sensitivity, wide-bandwidth (132.63% at –6 dB with a center frequency of 9.01 MHz) ultrasonic transducer (V312, Panametrics) that was cylindrically focused with a focal length of 0.75 in. Using the transducer with a cylindrical focusing, resolution along the Z-axis was 0.46 mm. The signal from the transducer was amplified and then digitized by an oscilloscope. Driven by a computer controlled stepper motor, the transducer scanned circularly around the sample with a step size of 3 degrees and a number of steps of 120 (i.e., 360 degrees of total view angle). The images were collected very close to the surface of the tumors. With all the data collected, a two-dimensional (2D) cross sectional image of the sample was built using a modified back-projection algorithm [21, 22]. For this prototype study we used a single transducer. However an ultimate clinical device would probably involve a transducer array, possibly ring shaped.

2.3 Preparation of nanoparticles

The nanoparticle contrast agents for PAI were prepared by a reverse nanoemulsion polymerization method as shown in Fig. 2 [16]. The monomer solution was prepared using 8.6 mmol of acrylamide and 0.25 mmol of APMA dissolved in 1.0 mL of water. The dye solution was prepared by dissolving 0.12 mmol of CB-derivatized acrylamide in 400 μ L of DMF and then 360 μ L of GDMA were added. This monomer solution and the dye solution were combined and sonicated before adding them to 120 mL of a hexane solution containing 28.5 mmol Brij-30 and 8.77 mmol dioctylsulfosuccinate. The two solutions were emulsified by stirring them for 20 min under an inert atmosphere. The reaction was initiated by using 150 μ L of TEMED and 240 μ L of APS. The APS was freshly prepared by dissolving it in water 50% (w/v). The solution was further stirred for 2 h under an inert atmosphere, to complete the polymerization. The hexane was removed by rotary evaporation, using a Rotavapor-R (Brinkmann Instruments) and then ethanol was added to the residue. The surfactants and excess dye were removed by washing the particles several times with ethanol and then with water in an Amicon ultra-filtration cell (Millipore Corp., Bedford, MA) using a 300 kDa filter and then freeze-dried with a 5 L ModulyoD freeze dryer (Thermo Fisher Scientific). Thus, prepared CB-containing PAA nanoparticles were surface-conjugated with F3 peptide so as to enhance tumor selective staining. The F3 peptide is a 31-amino acid fragment of HMNG-2 (human high-mobility group-2) protein and is known for its tumor

homing capability towards neucleolin-over-expressing tumor and tumoric endothelial cells [23–25]. Affinity towards tumor cell lines has been previously investigated in our lab [26, 27]. The freeze-dried PAA nanoparticles (50 mg) were dissolved in pH 7.4 phosphate buffered saline (PBS) (2.5 mL), treated with polyethylene glycol(PEG)–SMCC (2 μ mol) and stirred at room temperature for 30 min. The reaction mixture was then subjected to thorough washing to remove any unreacted ligands and concentrated to ~20 mg/mL. F3-Cys peptides (0.06 μ mol) was added to the concentrated nanoparticle solution which was gently stirred overnight (> 8 h) at room temperature and treated with *L*-cysteine (1 μ mol) for another 2 h. The resultant solution was thoroughly washed with water and PBS in an Amicon cell and then freeze dried.

3. Results and discussion

3.1 Nanoparticle characterization

The size of the nanoparticles in dried powder form was measured by scanning electron microscopy (SEM) while the size distribution of the nanoparticles in aqueous solution was obtained by dynamic light scattering (DLS) using a Beckman Coulter DelsaNano C zeta potential/submicrometer size analyzer. The particle size of the dried form was approximately between 15–40 nm and the average particle size in aqueous solution was 80 nm (Figs. 3(a) and 3(b)). The CB dye loading per nanoparticle was determined by comparing the optical absorbance of the nanoparticle suspension of a known concentration with those of free CB dye at different known concentrations. The measured CB contents per nanoparticles thus obtained were about 7.0% by mass. The prepared nanoparticles are highly suspendable in water and PBS (> 50 mg/mL). The nanoparticles had a maximum absorption of around 590 nm as shown in Fig. 3(c).

Moreover, these nanoparticles are quite stable and the nanoparticle suspension in PBS showed no change in spectral characteristics and no precipitate formation after storing in the refrigerator for 6 months. No dye leaching out of the nanoparticles was observed after incubation for 72 h in 9% albumin containing PBS at 37 °C.

We compared the staining efficiency of the F3-targeted nanoparticles with the non-targeted nanoparticles *in vitro*. 9L cells (glioma) and MCF7 cells (breast cancer) were incubated with both F3-targeted and non-targeted CB nanoparticles in separate wells in a six-well plate. After incubation for 30 min, the cells were washed thoroughly and their absorbance at 620 nm was observed using a plate reader. We saw a significant amount of staining of the 9L cells that were incubated with the F3-targeted nanoparticles, as compared to the non-targeted ones (Fig. 3(d)). The 9L cells incubated with non-targeted nanoparticles did not show significant staining. The MCF7 cells showed little staining with either the F3-targeted or non-targeted nanoparticles (Fig. 3(d)). While cells incubated with free CB dye also stained well, there was no specific/selective staining towards any cell line such as 9L vs. MCF7 (Fig. 3(d)). The reason for the high staining is attributed to the high affinity of this dye towards any protein. Thus, this *in vitro* experiment clearly demonstrates the advantage of using specifically targeted nanoparticles, compared to free dye, for selective and efficient staining of gliomas.

3.2 Phantom imaging

In order to examine the detection limit of our nanoparticle contrast agent by the PAI system, gel phantoms with inclusions containing different concentrations of the nanoparticle contrast agent were imaged. Two phantoms (#1 and #2) were made by boiling 2.5 g of agarose in 100 mL of water, with each having four cylindrical inclusions, as shown by their photographs in Figs. 4(a) and 4(b), respectively. In phantom #1, the concentrations of the nanoparticles in the four inclusions were 0.5 mg/mL, 0.25 mg/mL, 0.2 mg/mL, and 0.125 mg/mL. At these concentrations, the four inclusions can be easily recognized in the sample photograph. In phantom #2, the concentrations of the nanoparticles in the four inclusions were 0.1 mg/mL, 0.04 mg/mL, 0.02 mg/mL, and 0.01 mg/mL. By observing the phantom by naked eye, the inclusions with concentrations of 0.1 mg/mL and 0.04 mg/mL are just barely distinguishable; however, the inclusions with concentrations of 0.02 mg/mL and 0.01 mg/mL are not distinguishable from the background. The imaging of the phantoms with higher concentration of nanoparticles (Fig. 4(a)) was performed at a slightly lower amplifier gain (35 db), compared to the one with low concentration of nanoparticles (Fig. 4(b)), due to signal saturation of the signal over 35 db. The rest of the measurements were taken at 45 db. The PAI images of the two phantoms are shown in Figs. 4(c) and 4(d), respectively. All the inclusions in the two phantoms can be recognized clearly, including the two most diluted inclusions that cannot be differentiated from the background by the naked eye. We have imaged additional phantoms with inclusions containing even further diluted concentrations of nanoparticle contrast agent. When the concentration was 0.005 mg/mL or lower, the contrast-to-noise ratio in imaging the inclusions was less than 1, i.e., the inclusions could not be recognized in the image any more. Using our current PAI system, the maximum sensitivity in visualizing the nanoparticle contrast agent is on the order of 0.01 mg/mL, which is equivalent to 0.7 $\mu\text{g/mL}$ or 0.84 $\mu\text{mol/L}$ CB dye at an incident energy density of 3 mJ/cm^2 . We obtained signal-to-noise (S/N) ratios of 11, 11, 9, and 5, respectively, for each inclusion in the phantom with high concentration of nanoparticles (Fig. 4(c)). For the phantom with low concentration nanoparticle inclusions, we obtained fairly high S/N ratios of 11, 5, 3, 2, respectively, for each of the inclusions (Fig. 4(d)).

3.3 *Ex vivo* imaging

The *in vivo* feasibility tests were performed on a rat brain tumor window (BTW) model—a rat with a cranial glass window and a 9L glioma implanted tumor, for visual observation of the rat brain. The BTWs were installed in Sprague Dawley rats weighing 250 to 350 g. The tumor model used was an orthotopic model, i.e., gliomas were grown in the rat brain by implanting the 9L cells about 2 mm deep into the inner table of the skull and then subsequently closing the skin, as reported before [2]. Implantation of the tumor in the rats was part of a separate study that aims to establish visual tumor delineation. The nanoparticle contrast agents conjugated with the tumor-targeting F3 peptide were administered intravenously into the right femoral vein of live Sprague Dawley rats with developed brain tumors, as described previously [2, 20]. The animals were sacrificed by CO_2 asphyxiation, 6–10 days after nanoparticle administration. At the conclusion of each delineation experiment, the brain and intact tumor were removed from the skull of the rat. Gross brains and tumors were embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek, Zoeterwoude, Netherlands) before being frozen at -80°C for preservation.

Previously, tissue distribution studies (liver, kidney, lungs, heart, spleen, brain, and the bone marrow) have been performed by our group after a single intravenous bolus injection of ^{14}C labeled non-targeted and PEGylated nanoparticles into the tail veins of healthy male rats [28]. It was found that the liver was the major site of deposition among the organs, while the lungs and kidneys were secondary sites of deposition, for both formulations. In term of concentrations, the majority of the organs contained levels of radiolabel that were roughly comparable, with the exception of the brain in which an approximate 10-fold reduction was observed [28]. Our other previous studies showed that, in the case of tumor-bearing rats, the nanoparticles can be efficiently delivered intravascularly to the brain tumor site [18]. Moreover, the same studies revealed that the presence of the F3-targeting moiety results in a significantly greater amount of nanoparticle accumulation and longer duration within the tumor, leading to significant enhancement in image contrast and therapeutic efficacy of the nanoparticles [18]. Our previously published MRI images of rat brain tumors [18] show that non-targeted PAA nanoparticles are not retained inside the tumors beyond 2 h after IV injection, whereas the F3-targeted PAA nanoparticles show significant retention several days later. In the recent delineation protocols [20] and those reported here, we waited several days post-injection before sacrificing the animals. Given this significantly long time, the retention of non-targeted nanoparticles inside the tumors would be negligible. Therefore, we did not study a control group with non-targeted nanoparticles for the *in vivo* visual delineation protocol [20], nor in this current *ex vivo* photoacoustic delineation protocol, but limited ourselves to studying controls with no nanoparticles. In the first study, three tumors, were embedded at different locations in an excised rat brain specimen and then imaged simultaneously, as shown in Fig. 5(a). The three tumors were harvested from three different rats: two of them were injected with different dose of nanoparticles (250 mg/kg of rat mass and 125 mg/kg of rat mass) and the third rat was not injected with the nanoparticle and thus was used as control. The tumor, extracted from the rat injected with a dose of 250 mg/kg of rat mass, containing a higher dose of nanoparticles, was slightly bluish. It could be differentiated from the background upon close visual inspection; however, the other tumor, containing the lower dose of nanoparticles, did not show a distinct color and could not be differentiated from the background brain tissue by the naked eye. A PAI image of the three tumors embedded on the brain is shown in Fig. 5(b). We can clearly distinguish the two tumors that were treated with the nanoparticle contrast agent, including the one that is not distinguishable by eye. In comparison with the two nanoparticle-containing tumors, the control tumor that was extracted from a rat not injected with any nanoparticle, did not show any enhanced signal.

In another experiment, we imaged quite a few individual tumors, each in their original brain specimen. Figure 6(a) shows a sample photograph of a rat brain containing a tumor containing a low dose of nanoparticles (125 mg/kg of rat mass). At this dose, the color contrast between the tumor and background brain tissue is not sufficient to differentiate the tumor by the eye. However, as shown in Fig. 6(b), the tumor can be clearly recognized in the PAI image, facilitated by the superior sensitivity of PAI in imaging the nanoparticle contrast agent. We have also observed strong signals from some specific areas of the tumor, which is likely due to the higher local density of the nanoparticles in those areas, as a result of inhomogeneous uptake of the contrast agent by the tumor tissue. For comparison with Fig.

6(b), Fig. 6(c) shows the image of a tumor in another specimen that does not contain any nanoparticles. The intrinsic contrast of the tumor is quite similar to that of the rest of the brain. Thus, the outline of the brain tumor is barely visible from the background, which is in stark contrast to the photoacoustic images of the tumors targeted with the blue nanoparticles. We found an average S/N ratio of around 2.0 for the photoacoustic images of the tumor. The current method can also be used to quantify the size of the tumors. We performed 2D imaging and so, in our measurements, we were able to estimate the cross section of the rat brain tumors with an accuracy of ± 1 mm. However we expect that any 3D PAI will enable us to estimate the complete tumor size with similar accuracy (± 1 mm).

This study was performed to show a proof of principle for our blue nanoparticle-aided PAI for the purpose of tumor delineation and thus only eight tumors (two containing a high dose of nanoparticles, three containing low dose of nanoparticles, and three control tumors with no nanoparticles) were imaged, and all the images were taken *ex vivo*. We did not see any enhanced PA signal from the three control tumors, whereas all the five tumors that had been targeted with the nanoparticles at both high and low concentrations showed a significantly increased PA signal. We recognize that in order to fully quantify the feasibility of PAI aided by nanoparticles for actual clinical pre- and intra-operative imaging of brain tumors, *in vivo* experiments involving a much large number of animals are preferable. However, we believe that this preliminary study on *ex vivo* animal models has demonstrated the potential of PAI in delineating brain tumors targeted with our blue nanoparticles.

Also with a maximum absorption at 590 nm, the blue nanoparticles show a strong optical contrast with respect to the background tissue, and hence, can be clearly visualized by eye. For this reason, using the blue nanoparticles to delineate the tumor boundary will be highly advantageous to surgeons. The aim of this study is to develop a complementary technique to current visual tumor delineation, presenting tumor locations with better accuracy whenever the visual contrast is not sufficient. Therefore, using the same contrast agent for both PAI and visual delineation is the best strategy and is what we examined in this study. However, contrast agents working in the visible region of the optical spectrum are not the most suitable for *in vivo* PAI due to the strong intrinsic absorption from the blood. In comparison, near-infrared region contrast agents with absorption from 700 nm to 950 nm should be a better choice considering the weak absorption of blood and, as a result, the low background signals from the brain. In the future, nanoparticle contrast agents with broader absorption spectra or dual absorption peaks, one around 590 nm while the other in the near-infrared spectral region, could provide better solutions.

4. Conclusion

We have demonstrated the feasibility of imaging brain tumors by using PAI aided by tumor-targeting blue PAA nanoparticles as a contrast agent. These nanoparticles provide excellent contrast enhancement when imaged by our PAI system. Facilitated by the intrinsically high sensitivity of PAI, brain tumors containing contrast agent with concentrations that are too low to be visualized by the naked eye could still be recognized clearly in the PAI images. Benefiting from both high sensitivity and excellent spatial resolution, PAI could contribute to future surgical procedures on gliomas by aiding the surgeons in delineating the tumors in

the brain, without the need for the high dose of contrast agent that is necessary for visual delineation. We note that reducing the dose of administered nanoparticles by a factor of four or more would have high clinical significance. Although the performance of PAI in imaging the brain tumor was only validated through 2D imaging experiments in this study, PAI allows high resolution 3D imaging and good penetration depth up to several centimeters in strongly optically scattering tissues [10]. We thus expect PAI to also be useful for delineating the 3D distribution and morphological shape of tumors in the brain. Moreover, since the nanoparticle contrast agents are biocompatible, chemically inert and have a long circulation lifetime, they could also be designed to target other specific cell membrane receptors found in various types of cancer cells, such as prostate and bladder cancers, and thus could act as a demarcation agent for other cancerous tumors.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grant No. R33CA125297 (RK) and National Natural Science Foundation of China (NSFC) grant No. 11028408 (XW). We thank Dr. Z. Xie and Dr. J. Rajian for their help during photoacoustic imaging. We also like to extend our sincere thanks to Dr. M. Nie for his help during nanoparticle synthesis. We would also like to thank Mr. Dah-Luen Huang for developing the BTW in the rats.

References

1. Sanai N, Berger MS. Glioma extent of resection and its impact on patient outcome. *Neurosurgery*. 2008; 62(4):753–766. [PubMed: 18496181]
2. Orringer DA, Chen T, Huang DL, Armstead WM, Hoff BA, Koo YE, Keep RF, Philbert MA, Kopelman R, Sagher O. The brain tumor window model: A combined cranial window and implanted glioma model for evaluating intraoperative contrast agents. *Neurosurgery*. 2010; 66:736–743. [PubMed: 20305495]
3. Ozawa T, Britz GW, Kinder DH, Spence AM, VandenBerg S, Lamborn KR, Deen DF, Berger MS. Bromophenol blue staining of tumors in a rat glioma model. *Neurosurgery*. 2005; 57(5):1041–1047. [PubMed: 16284574]
4. Britz GW, Ghatan S, Spence AM, Berger MS. Intracarotid RMP-7 enhanced indocyanine green staining of tumors in a rat glioma model. *J Neurooncol*. 2002; 56(3):227–232. [PubMed: 12061728]
5. Hansen DA, Spence AM, Carski T, Berger MS. Indocyanine green (ICG) staining and demarcation of tumor margins in a rat glioma model. *Surg Neurol*. 1993; 40(6):451–456. [PubMed: 7694381]
6. Shinoda J, Yano H, Yoshimura S, Okumura A, Kaku Y, Iwama T, Sakai N. Fluorescence-guided resection of glioblastoma multiforme by using high-dose fluorescein sodium. *J Neurosurg*. 2003; 99(3):597–603. [PubMed: 12959452]
7. Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: A randomised controlled multicentre phase III trial. *Lancet Oncol*. 2006; 7(5):392–401. [PubMed: 16648043]
8. Moore GE. Fluorescein as an agent in the differentiation of normal and malignant tissues. *Science*. 1947; 106(2745):130–131.
9. Veisoh M, Gabikian P, Bahrami SB, Veisoh O, Zhang M, Hackman RC, Ravanpay AC, Stroud MR, Kusuma Y, Hansen SJ, Kwok D, Munoz NM, Sze RW, Grady WM, Greenberg NM, Ellenbogen RG, Olson JM. Tumor paint: A chlorotoxin: Cy5.5 bioconjugate for intraoperative visualization of cancer foci. *Cancer Res*. 2007; 67(14):6882–6888. [PubMed: 17638899]
10. Xu M, Wang LV. Time-domain reconstruction for thermoacoustic tomography in a spherical geometry. *IEEE T Med Imaging*. 2002; 21(7):814–822.
11. Wang X, Ku G, Wegiel MA, Bornhop DJ, Stoica G, Wang LV. Noninvasive photoacoustic angiography of animal brains *in-vivo* with near-infrared light and an optical contrast agent. *Opt Lett*. 2004; 29(7):730–732. [PubMed: 15072373]

12. Kim C, Song KH, Gao F, Wang LV. Sentinel lymph nodes and lymphatic vessels: Noninvasive dual-modality *in vivo* mapping by using indocyanine green in rats—volumetric spectroscopic photoacoustic imaging and planar fluorescence imaging. *Radiology*. 2010; 255(2):442–450. [PubMed: 20413757]
13. Kim C, Cho EC, Chen J, Song KH, Au L, Favazza C, Zhang Q, Cobley CM, Gao F, Xia Y, Wang LV. *In vivo* molecular photoacoustic tomography of melanomas targeted by bioconjugated gold nanocages. *ACS Nano*. 2010; 4(8):4559–4564. [PubMed: 20731439]
14. De la Zerda A, Zavaleta C, Keren S, Vaithilingam S, Bodapati S, Liu Z, Levi J, Smith BR, Ma T, Oralkan O, Cheng Z, Chen X, Dai H, Khuri-Yakub BT, Gambhir SS. Carbon nanotubes as photoacoustic molecular imaging agents in living mice. *Nat Nanotechnol*. 2008; 3(9):557–562. [PubMed: 18772918]
15. Li P, Shieh D, Wang C, Shieh D, Wei C, Liao C, Ding A, Wu Y, Poe C, Jhan S. *In vivo* photoacoustic molecular imaging with simultaneous multiple selective targeting using antibody-conjugated gold nanorods. *Opt Express*. 2008; 16(23):18605–18615. [PubMed: 19581946]
16. Lee YEK, Kopelman R, Smith R. Nanoparticle PEBBLE sensors in live cells and *in vivo*. *Annu Rev Anal Chem*. 2009; 2:57–76.
17. Orringer DA, Koo YEL, Chen T, Kopelman R, Sagher O. Small solutions for big problems: The application of nanoparticles to brain tumor diagnosis and therapy. *Clin Pharmacol Ther*. 2009; 85(5):531–534. [PubMed: 19242401]
18. Reddy GR, Bhojani MS, McConville P, Moody J, Moffat BA, Hall DE, Kim G, Koo YE, Woolliscroft MJ, Sugai JV, Johnson TD, Philbert MA, Kopelman R, Rehemtulla A, Ross BD. Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res*. 2006; 12(22):6677–6686. [PubMed: 17121886]
19. Winer I, Wang S, Lee YEK, Fan W, Gong Y, Burgos-Ojeda D, Spahlinger G, Kopelman R, Buckanovich RJ. F3-Targeted Cisplatin-Hydrogel nanoparhticles as an effective therapeutic that targets both murine and human ovarian tumor endothelial cells. *in vivo Cancer Res*. 2010; 70(21): 8674–8683. [PubMed: 20959470]
20. Orringer, DA., Sagher, O., Kopelman, R., Koo, YEL. Dye loaded nanoparticle. US Patent. 20,100,098,637. Apr 22. 2010
21. Xu M, Wang LV. Universal back-projection algorithm for photoacoustic-computed tomography. *Phys Rev E*. 2005; 71(1):016706.
22. Wang X, Pang Y, Ku G, Xie X, Stoica G, Wang LV. Non-invasive laser-induced photoacoustic tomography for structural and functional imaging of the brain. *in vivo Nat Biotechnol*. 2003; 21(7):803–806. [PubMed: 12808463]
23. Porkka K, Laakkonen P, Hoffman JA, Bernasconi M, Ruoslahti E. A fragment of the HMGN₂ protein homes to the nuclei of tumor cells and tumor endothelial cells *in vivo*. *Proc Natl Acad Sci*. 2002; 99(11):7444–7449. [PubMed: 12032302]
24. Akerman ME, Chan WC, Laakkonen P, Bhatia SN, Ruoslahti E. Nanocrystal targeting *in vivo*. *Proc Natl Acad Sci*. 2002; 99(20):12617–12621. [PubMed: 12235356]
25. Ruoslahti E, Duza T, Zhang L. Vascular homing peptides with cell-penetrating properties. *Curr Pharm Des*. 2005; 11(28):3655–3660. [PubMed: 16305501]
26. Orringer DA, Koo YEL, Chen T, Kim G, Hah HJ, Xu H, Wang S, Keep R, Philbert MA, Kopelman R, Sagher O. *In-vitro* characterization of a targeted, dye-loaded nanodevice for intraoperative tumor delineation. *Neurosurgery*. 2009; 64(5):965–972. [PubMed: 19404156]
27. Koo YEL, Reddy GR, Bhojani M, Schneider R, Philbert MA, Rehemtulla A, Ross BD, Kopelman R. Brain cancer diagnosis and therapy with nano-platforms. *Adv Drug Deliv Rev*. 2006; 58(14): 1556–1577. [PubMed: 17107738]
28. Wenger Y, Schneider RJ II, Reddy GR, Kopelman R, Jolliet O, Philbert MA. Tissue distribution and pharmacokinetics of stable polyacrylamide nanoparticles following intravenous injection in the rat. *Toxicol Appl Pharmacol*. 2011; 251(3):181–190. [PubMed: 21134391]

Figure 1.
Schematic illustration of the PAI setup

Figure 2. Synthesis of targeted Coomassie Blue-loaded nanoparticles, through a reverse nano-emulsion polymerization process followed by the attachment of the tumor-homing F3 peptide

Figure 3. Characterization of the Coomassie Blue-attached nanoparticles. (a) The size distribution of the nanoparticles in aqueous medium, as obtained from the DLS reading. (b) SEM image of the nanoparticles. (c) Normalized extinction (absorption + scattering) spectrum of the nanoparticles and the free dye. The nanoparticle spectral curve does not go to the baseline due to significantly higher scattering, compared to the free dye. (d) The 9L glioma cell and MCF7 cell targeting efficiency of the F3-attached nanoparticles. The dye stains the cells, irrespective of the cell line, due to its high affinity towards the proteins. However the F3-targeted nanoparticles stain the 9L cells significantly more than the MCF7 cells. Note that the non-targeted nanoparticles stain the targeted cells (9L) significantly less, while for the non-targeted cells (MCF7) both targeted and non-targeted particle staining is equally poor, as expected

Figure 4.

(a) and (b): Photographs of phantoms #1 (concentration of nanoparticle (clockwise): 0.5 mg/mL, 0.25 mg/mL, 0.2 mg/mL, and 0.125 mg/mL) and #2 (concentration of nanoparticles (anti-clockwise): 0.1 mg/mL, 0.04 mg/mL, 0.02 mg/mL, and 0.01 mg/mL), respectively. (c) and (d): PAI images of the phantoms #1 and #2, respectively (taken with an amplifier gain of 35 db and 45 db, respectively) (scale $3\text{ cm} \times 3\text{ cm}$)

Figure 5.
(a) Photograph of a rat brain with three embedded tumors. (b) PAI image of the brain (scale $2\text{ cm} \times 2\text{ cm}$)

Figure 6.

(a) Photograph of a rat brain containing a tumor treated with nanoparticles. (b) PAI image of the sample (scale $2\text{ cm} \times 2\text{ cm}$). (c) Photograph of the control tumor in the brain containing no contrast agent. (d). PAI image of the sample (scale $2\text{ cm} \times 2\text{ cm}$). We do not see any signal from the control tumor