Specific Targeting of Brain Tumors with an Optical/Magnetic Resonance Imaging Nanoprobe across the Blood-Brain Barrier

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Abstract

Nanoparticle-based platforms have drawn considerable attention for their potential effect on oncology and other biomedical fields. However, their in vivo application is challenged by insufficient accumulation and retention within tumors due to limited specificity to the target, and an inability to traverse biological barriers. Here, we present a nanoprobe that shows an ability to cross the blood-brain barrier and specifically target brain tumors in a genetically engineered mouse model, as established through in vivo magnetic resonance and biophotonic imaging, and histologic and biodistribution analyses. The nanoprobe is comprised of an iron oxide nanoparticle coated with biocompatible polyethylene glycol–grafted chitosan copolymer, to which a tumor-targeting agent, chlorotoxin, and a near-IR fluorophore are conjugated. The nanoprobe shows an innocuous toxicity profile and sustained retention in tumors. With the versatile affinity of the targeting ligand and the flexible conjugation chemistry for alternative diagnostic and therapeutic agents, this nanoprobe is potentially useful for the diagnosis and treatment of a variety of tumor types.

Introduction

Nanoparticle-based diagnostic and therapeutic platforms have been investigated extensively due to their potential effect on clinical oncology for the early detection, treatment, and staging of tumors (1, 2). Particularly, the use of magnetofluorescent nanoprobes, combined with both high–spatial resolution magnetic resonance imaging (MRI) and quantitative biophotonic fluorescence imaging, could allow for more versatile use of these nanoparticles. However, the implementation of this approach is complicated by the inability of current-generation contrast agents to circumvent biological barriers, such as the blood-brain barrier (BBB) and vascular endothelium, as well as the nonspecific uptake by surrounding tissues and macrophages upon i.v. injection (3, 4).

Serving as the major interface between the blood and the brain, the BBB is responsible for transporting essential nutrients and metabolites to the brain while protecting and regulating its internal environment. As such, the BBB represents one of the most exclusive biological barriers encountered in the treatment of neurologic diseases, limiting the delivery of a vast majority of potential diagnostic agents and therapeutics (5, 6). Although the specific mechanisms for BBB passage have yet to be elucidated, strategies such as the integration of lipophilic substances have been shown to facilitate passive diffusion of macromolecules into the brain parenchyma (7, 8). Alternatively, attachment of ligands such as apolipoproteins (9, 10), transferrin antibodies (11, 12), TAT peptide (13), or α-methyl tryptophan (14), and of positively charged moieties has been reported to promote receptor-mediated and adsorptive-mediated transcytosis for macromolecules to cross the BBB (7, 15, 16). Recent studies in polymeric and liposome nanoparticles indicated that BBB passage by nanoparticulates was dictated by hydrodynamic size, charge, and surface chemistry of the particles (10, 17–23). For example, a recent study evaluated the influence of nanoparticle size on BBB permeability and showed that gadolinium chelated dendrimer nanoparticles with core sizes of <12 nm were able to permeate the tumor BBB, whereas those with large sizes were hindered (20). Cationic albumin nanoparticles have been investigated in many studies and were shown to be effective in permeating the BBB due to their cationic charge (21, 22). Another recent study showed that the hydrophilicity of polymeric nanoparticles could influence BBB permeability (23). Although these advances are encouraging, they were limited to the passive delivery of nanoparticles (i.e., the inability to specifically target tumors), and provided limited information about the influence of nanoparticles on the integrity of BBB and on the safe profiling of these constructs. None, to our knowledge, have been shown to deliver imaging contrast agents specifically to brain tumors. Given the shortcomings of conventional Gd-DTPA–based MR imaging (MRI), including nonspecific tissue contrast and quick clearance (24), the development of a nanoparticle-based imaging agent to overcome these challenges would fulfill a significant clinical need.

Here, we report the development of a targeting nanoprobe that is capable of selectively accumulating in brain tumors across the BBB. We integrated a number of design elements in the development scheme of this nanoprobe that would facilitate the BBB crossing and tumor-specific targeting, including synthesizing core nanoparticles with minimal size, coating nanoparticles with a thin but dense polymer layer, integrating ample chemical functionality, using a small, versatile tumor-specific ligand, and the use of biocompatible materials. The nanoprobe (NPCP-CTX-Cy5.5) is comprised of an iron oxide nanoparticle coated with a PEGylated chitosan–branched copolymer, to which a targeting ligand, chlorotoxin (CTX), and a near-IR fluorophore (NIRF), Cy5.5, were conjugated (Fig. 1). In this design, chitosan is used as a linker and stabilizer. The amino and hydroxyl groups of chitosan’s glucosamine

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backbone serve to anchor the polymer to the iron oxide surface through electrostatic interaction and physical adsorption, alleviating the need for cross-linking agents, while also providing sites for subsequent conjugation of ligands without the need for further chemical modification. The bound chitosan also acts as a sterically stabilizing corona, preventing particle aggregation under physiologic conditions. In vivo, aggregated or opsonized nanoparticles are readily recognized and rapidly cleared from the bloodstream by the reticuloendothelial system before reaching target tissues (25). Polyethylene glycol (PEG) is integrated into the polymer coating to reduce protein adsorption, limit immune recognition, and thereby increase the nanoprobe serum half-life in vivo. In addition to its magnetic property for MRI detection, the nanoparticle coated with the PEGylated chitosan–branched copolymer (NPCP) received selective targeting and optical functionality via covalently linked CTX and Cy5.5, respectively. CTX, a 36–amino acid peptide, was selected as a tumor-targeting ligand due to its strong affinity for tumors of neuroectodermal origin (26, 27). We previously showed that CTX specifically binds to glioma, medulloblastoma, prostate cancer, sarcoma, and intestinal cancer (28). Integration of Cy5.5 into the nanoparticle coating, combined with the presence of the intrinsic superparamagnetic core, makes the nanoprobe a dual contrast agent, detectable by both MR and biophotonic imaging, which expands the scope of its applicability. The NPCP provides flexible functional groups for conjugation of ligands bearing either amine or sulfhydryl reactive groups, including CTX and Cy5.5, as shown in this study.

In addition to the favorable physiochemical properties, these constituents are also selected to facilitate the BBB crossing by the nanoprobe. Amphiphilic PEG with high lipid solubility may increase the endothelial permeability of the nanoprobe and thus facilitate its BBB passage. PEG has been shown to facilitate the BBB permeability of several conjugates (15, 29–31). Positively charged cationic chitosan may interact with the negatively charged brain endothelium via electrostatic interactions to trigger adsorptive-mediated transport across the BBB. Also, the small hydrodynamic size of the nanoprobe is essential for the BBB passage (32, 33). CTX has also been observed to permeate an intact BBB in both animal models and humans with brain tumors (28, 34). We evaluated the capability of this nanoprobe to transverse the BBB, its tumor-specific targeting efficiency, and MRI and optical detectability in a transgenic mouse model, ND2:SmoA1, that closely resembles human medulloblastoma, the most common malignant childhood brain tumor (35). This tumor model arises spontaneously in the cerebellum (36) and maintain an intact BBB (28).

Figure 1. Synthesis and characterization of NPCP-Cy5.5-CTX nanoprobe. A, nanoprobe structure and chemical reaction schematic for the syntheses of (i) PEG-grafted chitosan, (ii) sulfhydryl functionalization of CTX, and (iii) CTX and Cy5.5 conjugation to NPCP. B, X-ray diffraction pattern of NPCP confirming the magnetite (Fe₃O₄) crystalline structure of the nanoprobe. C, Fourier-transformed IR spectra of bare iron oxide nanoparticle, PEGylated chitosan, and NPCP, confirming the successful immobilization of PEGylated chitosan on the surface of the nanoparticles.
Materials and Methods

**Nanoprobe synthesis.** PEG was grafted onto chitosan by alkalination of depolymerized chitosan followed by Schiff base formation (37). Depolymerized chitosan was obtained by oxidative degradation of high-molecular weight chitosan (Mw, 190 kDa Sigma) with sodium nitrite (NaNO₂). The chitosan depolymerization was carried out by reacting 100 mmol/L of aqueous NaNO₂ solution with a 2 wt% chitosan solution (pH 4.5; dilute acetic acid) for 24 h at room temperature. Methoxy-PEG (Mn, 2000 grams/mol/L; Sigma) was first oxidized into PEG-aldehyde and then reacted with primary amines of depolymerized chitosan in the presence of sodium cyanoborohydride. The chemical structure and the purity of the polymer were confirmed by high-performance liquid chromatography and 1H-nuclear magnetic resonance.

The nanoparticles were synthesized in the presence of chitosan-grafted PEG via coprecipitation of ferrous and ferric chlorides with ammonium hydroxide. Specifically, NPCP were synthesized by first dissolving 3.0 grams of PEGylated chitosan in 50 mL of deionized H₂O followed by the addition of an iron chloride solution (4.6 grams FeCl₂·H₂O, and 9.1 grams FeCl₃·H₂O) in 50 mL of deoxygenated deionized H₂O. This mixture was then heated to 80 °C under mechanical stirring and nitrogen bubbling. One hundred milliliters of 7% NH₄OH were then added to the polymer and iron chloride mixture at a rate of 100 mL/h. The resulting black precipitate was dialyzed for 2 to 3 d in H₂O to remove unreacted reagents.

CTX (Alamone Labs) and Cy5.5 (GE Healthcare) were conjugated to the NPCP through the chemical scheme outlined in Fig. 1A. Specifically, 1.75 mg of monoreactive Cy5.5 NHS ester was dissolved in 100 L of anhydrous dimethylformamide (Sigma). The solution was then added to 2 mL of NPCP (2.5 mg/mL, suspended in 0.1 M of sodium bicarbonate; pH 8.5). The suspension was allowed to react for 2 h before the addition of 100 L of succinimidyl iodoacetate (Molecular Biosciences; 50 mg/mL, dissolved in anhydrous DMSO). The resulting solution was allowed to react for an additional 2 h. Excess Cy5.5 and succinimidy iodoacetate were removed from the suspension through gel chromatography using Sephacryl S-200 column (GE Healthcare) equilibrated with 20 mmol/L of sodium citrate and 0.15 mol/L of NaCl buffer at pH 8.0. CTX was functionalized with sulfhydryl groups through reaction with N-succinimidy S-acetylthioacetate (SATA; Molecular Bioscience). To perform this reaction, 40 µL of SATA (1 mg/mL, dissolved in anhydrous DMSO) was added to a 1 mL solution of CTX (1 mg/mL, dissolved in 50 mL bicarbonate buffer; pH 8.5). After reaction for 1 h at room temperature, excess SATA was removed by dialysis against PBS buffer (pH 7.4). Upon purification, SATA was depleted by reacting 100 µL of a 25 mmol/L hydroxylamine with 10 mmol/L of EDTA solution for 1 h at room temperature. The resulting sulfhydryl-modified peptide was then added to the Cy5.5 and succinimidyl iodoacetate–modified NPCP solution, and the mixture was allowed to react for 1 h at room temperature. Unreacted CTX was removed from the suspension through gel filtration chromatography using Sephacryl S-200 column equilibrated with 20 mmol/L of sodium citrate and 0.15 mol/L of NaCl buffer at pH 8.0.

**Animal model.** All mouse studies were conducted with procedures approved by the Institutional Animal Care and Use Committee at the University of Washington and Fred Hutchinson Cancer Research Center. Transgenic ND2:SmoA1 mice were generated on a C57BL/6 background (Charles River Laboratories, Inc.) as described previously (36). Two–genetically altered C57BL/6 mice were used as wild-type controls. Symptomatic and wild-type mice were injected with nanoparticles at 10 mg/kg (n = 3) for in vivo MR and optical studies.

**In vivo MRI.** Multiecho multislice imaging was performed on a 4.7 T magnet. Spin–spin relaxation time T₂ maps were generated by multi-echo images with echo times (Tₑ) ranging from 14 to 68 ms. Details on image processing are provided in the Supplementary Methods and Discussion. Spin–lattice relaxation time T₁-weighted MRI was performed for N2SmoA1 mice before and after the injection of Gd-DTPA to confirm that the BBB was intact in the tumor-bearing mouse model. T₁-weighted images were consecutively acquired five times before Gd-DTPA injection to obtain baseline signal intensities. Serial acquisitions of T₁-weighted images were conducted for 60 min after i.v. injection of Gd-DTPA (0.1 mmol/kg gadopentetate dimeglumine; 5× diluted Magnevist; injection volume, 100 µL; Berlex Laboratories). The imaging variables for T₁-weighted images are as follows: Tₑ/T₁, 500/14.7 ms; number of averaging, 2; matrix, 256 × 128; slice thickness, 1.5 mm; and acquisition time, 2 min. A mouse head coil and a head holder were used to image the N2SmoA1 mice. A slice covering a developed tumor region was selected from eight consecutive slices for each acquisition. Signal intensities were measured on two different regions of interest: one in the cerebellar region and the other outside the brain. Identical experiments were performed on the same mice 48 h after injection of NPCP-CTX nanoprobe.

**In vivo optical imaging.** Biophotonic fluorescence images were acquired on a Xenogen IVIS-100 system (Caliper Life Sciences). Mice were anesthetized with 2.5% isoflurane (VEDCO, Inc.) before they were placed into the imaging chamber and imaged at various time points after injection of the nanoprobe. Relevant organs, tissues, and tumors were dissected from some of the animals and imaged immediately to determine biodistribution. Fluorescence emission was normalized to photons per second per centimeter squared per steradian (p/s/cm²/sr).

**Histology and microscopy.** The whole brains of mice were dissected immediately after the animals were sacrificed and fixed in freshly prepared 10% buffered formalin. Samples were then embedded in paraffin blocks. Half of each brain was sliced along the sagittal plane into 10-µm sections and the remaining half was sliced along the coronal plane, followed by staining with H&E and Prussian blue/Nuclear Fast red using standard clinical laboratory protocols. Microscopic images of tissue were acquired using an Ez600 upright microscope (Nikon) equipped with a CCD color camera.

**Statistical analysis.** The data were expressed as mean ± SD of the mean. A paired t test was used to determine the significance of nanoprobe accumulation as measured by MRI. Statistical significance in biodistribution and toxicity effects were determined using one-way ANOVA followed by a Student’s t test for multiple comparison tests. We considered a P value of <0.05 as statistically significant.

Results and Discussion

**Synthesis and characterization of nanoparticles.** PEGylated chitosan–branched copolymers were prepared by a process of chitosan alkalination followed by Schiff base formation, as reported in our previous work (ref. 37; Fig. 1A, reaction i). CTX was first functionalized with sulfhydryl groups through reaction with SATA (Fig. 1A, reaction ii). Iron oxide nanoparticles were then synthesized through a coprecipitation process and simultaneously coated in situ with the copolymer (Fig. 1A, reaction iii). The functionalized CTX and Cy5.5 were then conjugated to the NPCP via iodoacetate and amide linkages (Fig. 1A, reaction iii), respectively, yielding an average of 16.2 CTX peptides and 1.5 fluorophores per NPCP (Supplementary Table S1), as determined by BCA protein quantification and fluorescence quantification, respectively (38). The mean hydrodynamic size and potential of the resulting nanoparticle coated with copolymer (NPCP) was found to be 33 nm and 4.2 mV, respectively, by dynamic light scattering (Supplementary Table S1 and Supplementary Fig. S1). Transmission electron microscopy of the NPCP (Supplementary Fig. S1, inset) showed the iron oxide cores with a mean diameter of 7 nm. It should be noted that this diameter differs from the hydrodynamic size in that the latter includes the contribution of the polymer coating and hydration of the PEG chains in aqueous solution. Powder X-ray diffraction of the NPCP (Fig. 1B) is consistent with that of crystalline magnetite (Fe₃O₄; JCPDS card no. 19-0629), whereas immobilization of the copolymer on the iron oxide surface was confirmed by Fourier-transformed IR spectroscopy (Fig. 1C; Supplementary Methods and Discussion). In addition, the number of reactive amino functional groups on

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Each NPCP was quantified by N-succinimidyl 3-(2-pyridyldithio)propionate assay and determined to be 30.5 primary amine per nanoparticle. Nanoparticles coated with copolymer were found to be stable (i.e., no agglomeration or loss of functionality) in solutions of physiologic pH for months compared with those coated with chitosan alone, which showed a short shelf-life of a few hours under identical conditions.

To verify the targeting specificity of CTX-conjugated NPCP (NPCP-CTX) for brain tumor cells, in vitro cell uptake experiments were performed. When incubated with 9L rat gliosarcoma cells, NPCP-CTX showed a 6.1 ± 1.1-fold increase in uptake (mean ± SD; P < 0.0001) compared with the nontargeting control nanoprobe (NPCP), and an 11 ± 0.8-fold increase (mean ± SD; P < 0.0001) compared with dextran-coated nanoparticles (Supplementary Fig. S2). Furthermore, the effectiveness of the PEG conjugated on nanoprobe in suppressing macrophage uptake was also evaluated in vivo. Uptake of NPCP by RAW 264.7 macrophages was found to be 12.4 ± 0.7-fold lower (mean ± SD; P < 0.0001) than that of nanoparticles coated with chitosan alone (Supplementary Fig. S3).

Figure 2. In vivo MRI of brain tumors and signal quantification. In vivo MR images of ND2:SmoA1 (A) and wild-type mice (B) acquired before and 48 h after administration of either NPCP-CTX or NPCP nanoprobes. Coronal cross-sections of the frontal lobe (no tumor present) of the cerebral hemisphere and cerebellum (containing tumor tissues) were analyzed. Colorized R2 maps of the brain region were superimposed onto proton density–weighted images. Varying R2 values (s⁻¹) from low (blue) to high (red) were visually represented in colors generated from the gradient (right). Change in R2 was quantified by dividing the change in R2 before and after nanoprobe injection, and by the preinjection R2 response for ND2:SmoA1 (C) and wild-type mice (D). Both targeting (NPCP-CTX) and nontargeting (NPCP) nanoprobe systems were evaluated in ND2:SmoA1 and wild-type mice.

Evaluation of MRI contrast enhancement and in vivo MRI. The magnetic properties of the nanoprobe were evaluated by MRI using the clinically approved, dextran-coated iron oxide nanoparticle, Feridex I.V., as a reference. MR images of agarose samples containing NPCP of varying concentrations (Supplementary Fig. S4A) were acquired over a range of T E to generate a R2 (1/T2) map (Supplementary Fig. S4B). A linear correlation of R2 with particle concentration was observed for both contrast agents (Supplementary Fig. S4C). The R2 relaxivities (slope of R2 versus particle concentration) for NPCP and Feridex I.V. were 472.3 and 243.3 s⁻¹mmol/L⁻¹, respectively. The higher relaxivity exhibited by NPCP compared with the commercial agent qualifies NPCP as a valid MRI contrast agent.

MRI was used to show the specific targeting capability of NPCP-CTX nanoprobe to tumors in vivo and validate the nanoprobe as an MRI contrast agent. The MRI was performed on symptomatic ND2:SmoA1 (Fig. 2A) and wild-type mice (as an animal control; Fig. 2B) receiving either targeting NPCP-CTX or nontargeting NPCP (as a particle control), each administered via tail vein injection. Coronal MR images of the frontal lobe of the
cerebral hemisphere (healthy tissue) and cerebellum (tumor-containing tissue) were acquired over a range of $T_1$ (14–68 ms) before nanoprobe injection and at 48 hours postinjection. Using this series of images, $R_2$ maps were generated and overlaid on proton density images to analyze nanoprobe accumulation. Forty-eight hours after injection of targeting NPCP-CTX, the significant increase in $R_2$ (red) at the periphery of the cerebellum in ND2:SmoA1 mice, compared with the image taken before injection, indicated specific nanoprobe accumulation (Fig. 2A, first column, left), whereas images of the frontal lobe region of these mice did not show discernible $R_2$ shifts between preinjection and postinjection of NPCP-CTX (Fig. 2A, second column, left), indicating minimal or no accumulation of the nanoparticles in healthy brain tissues. These results confirm that the targeting nanoprobe preferentially accumulate in neoplastic tissues. The areas highlighted by NPCP-CTX in the cerebellum coincide well with the tumor regions identified in histologic sections of the same tissue slices stained with H&E (Supplementary Fig. S5).

Quantitative evaluation of NPCP-CTX accumulation further indicated a contrast enhancement of $37.6 \pm 2.4\%$ (mean $\pm$ SD), in terms of $R_2$ increase, in the cerebellum, whereas minimal contrast variations in healthy tissue of the frontal lobe were found before and after nanoprobe injection ($4.3 \pm 1.6\%; P < 0.0001$; Fig. 2C). No apparent contrast enhancement was observed in the cerebellum or frontal lobe of ND2:SmoA1 mice treated with nontargeting NPCP (Fig. 2A, right). Quantitative evaluation also found minimal or no contrast change ($P > 0.05$) in both the cerebellum ($0.7 \pm 0.9\%$) and the frontal lobe ($1.2 \pm 2.5\%$) of ND2:SmoA1 mice receiving NPCP (Fig. 2C). These results indicate that the NPCP has no specific targeting capability due to lack of targeting ligand CTX.

Specific targeting of the NPCP-CTX nanoprobe to tumors was further counter-illustrated with wild-type mice (bearing no tumors) injected with these nanoparticles, which showed no apparent NPCP-CTX accumulation in the brain (Fig. 2B, left). Nontargeting NPCP also showed no apparent accumulation in wild-type mice, as expected (Fig. 2B, right). Quantitative evaluation of both the targeting and control nanoparticles in wild-type mice further confirmed no significant change in $R_2$ in either frontal lobe ($P > 0.05$) or cerebellum ($P > 0.05$) regions after nanoprobe injection (Fig. 2D).

**In vivo optical imaging and histology.** The ability of the NPCP-CTX nanoprobe to specifically target tumors and serve as an optical contrast agent was assessed by quantitative *in vivo* imaging experiments using NIRF imaging with Cy5.5 attached to NPCP-CTX (NPCP-Cy5.5-CTX). First, we determined the correlation between nanoprobe concentration and optical intensity of fluorescence emission, which revealed a linear relation (Supplementary Fig. S6). Then, specific tumor targeting and illumination in symptomatic ND2:SmoA1 mice by the targeting nanoprobe was assessed at 2 and 120 hours (Fig. 3A and B, respectively) postinjection of either targeting NPCP-Cy5.5-CTX or its nontargeting variant, NPCP-Cy5.5 (as control). Preferential accumulation of the NPCP-Cy5.5-CTX in tumors was evident by the significant NIRF signal observed only in the brain tumor regions of the mice receiving NPCP-Cy5.5-CTX at both 2 and 120 hours (the first mouse in Fig. 3A and B, respectively) postinjection. Quantitative analysis of NIRF signal intensity revealed that the accumulation of NPCP-Cy5.5-CTX nanoprobe in the brain tumor was complete by 50 hours postinjection and that the signal intensity remained at a similar level throughout the remaining 70 hours of the 120-hour analysis (Supplementary Fig. S7), indicating prolonged retention of NPCP-CTX-Cy5.5 in tumors. Conversely, significantly lower levels of NIRF signal were detected in the tumors of mice receiving nontargeting NPCP-Cy5.5 2 hours postinjection (the second mouse in Fig. 3A) followed by no detectable signal after 50 hours postinjection (Supplementary Fig. S7 and the second mouse in Fig. 3B). The mice receiving no injection were also presented as references (the third mouse in Fig. 3A and B; Supplementary Fig. S7). *Ex vivo* images of the brains of mice receiving NPCP-Cy5.5-CTX were acquired immediately after whole-body optical imaging of mice, which showed a NIRF signal outlining the medulloblastoma tumor regions (Fig. 3B, inset, the first mouse), demonstrating the ability of this probe to

**Figure 3.** *In vivo* NIRF imaging of autochthonous medulloblastoma tumors in genetically engineered ND2:SmoA1 mice. A and B, fluorescence imaging of medulloblastoma tumors in ND2:SmoA1 mice injected with either NPCP-Cy5.5-CTX or NPCP-Cy5.5, or receiving no injection (left to right). Images were acquired at 2 h (A) and 120 h (B) postinjection. Ex vivo fluorescence images of mice brains from the same mice following necropsy (inset, B). The spectrum gradient bar (right) corresponds to the fluorescence intensity (ps/cm²/sr) of the images.

Cancer Research 2009; 69: (15). August 1, 2009 6204 www.aacrjournals.org
potentially serve as an aid in intraoperative tumor resection. Conversely, no significant levels of fluorescence were detected in the brains of mice receiving NPCP-Cy5.5 (Fig. 3B, inset, the second mouse). These results correlate well with those obtained from MRI (Fig. 2), confirming the selective accumulation of targeting nanoprobes (but not nontargeting nanoprobes) in tumors.

To determine the accuracy of the regions highlighted as tumor tissue by the NPCP-Cy5.5-CTX nanoprobe, histologic analysis (Fig. 4) was performed on the excised brains of the mice after NIRF imaging. The dark purple region in the H&E-stained cerebellum of the ND2:SmoA1 mice clearly outlines the tumor (Fig. 4A), which is absent in the comparable brain sections from the wild-type mouse (Fig. 4B). Higher magnification images of the H&E-stained ND2:SmoA1 mouse brain sections show the difference in cell density and morphology between tumor and healthy tissues (Fig. 4C, first row). The selective accumulation of the NPCP-CTX-Cy5.5 nanoprobe within tumor tissue was evident by the positive iron staining of Prussian blue, which is absent in healthy tissue (Fig. 4C, second row). This result further indicates that the regions illuminated by the nanoprobe in the in vivo optical imaging were neoplastic in nature and nanoparticles accumulated in neoplastic tissue but not in healthy tissue. Brains excised from ND2:SmoA1 mice receiving NPCP-Cy5.5 showed no apparent nanoprobe accumulation in either tumor or normal brain tissue regions (Fig. 4D) 5 days postinjection, consistent with the observation made with in vivo optical imaging.

**Verification of nanoprobes crossing the BBB without compromising it.** In our previous study, we confirmed that the ND2:SmoA1 mouse model possesses an intact BBB (28). The accumulation of our nanoprobes in brain tumors, as shown by both MR and biophotonic imaging, indicated that the nanoprobes overcame the BBB. However, the question remains as to whether the passage of the nanoprobes had compromised the integrity of the BBB. Using the Evans blue exclusion assay, we verified the integrity of the BBB after the nanoprobes had crossed it. Two groups of ND2:SmoA1 mice were subjected to the assay: one group received NPCP-CTX and the other group received no nanoprobe treatment. Both groups were given Evans blue dye, and their brains and lungs/heads were imaged. No permeation of Evans blue into the brains of either group was observed (Supplementary Fig. S8), whereas for comparison, the lungs/heads of the same mice, which lack strict protective barriers such as the BBB, exhibited significant blue staining due to dye permeation. These results (1) verified our previous studies using immunostaining assessment that ND2:SmoA1 mice possess an intact BBB (2, 28), confirming that the passage of the nanoprobe left the BBB intact.

We further verified that the BBB in symptomatic ND2:SmoA1 mice were intact following the passage of the nanoprobe using a Gd-DTPA exclusion method. In this method, the permeation of i.v.-injected gadolinium contrast agent into the brain tissue would indicate a compromised BBB (39). First, T1-weighted brain images were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB. Next, the same mice were injected with NPCP-CTX; to confirm the entry of the nanoprobe into the brains, R2 maps of the cerebella of these mice were acquired for ND2:SmoA1 mice before and after receiving Gd-DTPA injection (Fig. 5A). Compared with the MR images of the mice before receiving Gd-DTPA (Fig. 5A, left), MR images of the cerebellum of these mice after Gd-DTPA administration showed that signal enhancement by Gd-DTPA was confined only to the neural vasculature and did not extend into the brain (Fig. 5A, right), confirming that ND2:SmoA1 mice possess an intact BBB.
mice were acquired before and 48 hours after NPCP-CTX administration (Fig. 5B). The significant $R_2$ increase (red) in the regions of the cerebellum in which tumor tissue was expected after mice receiving NPCP-CTX (Fig. 5B, right), compared with the same regions before nanoprobe injection (Fig. 5B, left), indicates the entry of NPCP-CTX into the brains across the BBB and its accumulation in brain tumors. Then, the same mice were again injected with Gd-DPTA and $T_1$-weighted imaging was repeated before and after Gd-DPTA injection (Fig. 5C). Here again, the signal enhancement by Gd-DPTA was confined only to the neural vasculature, indicating that the BBB retained its integrity after the entry of the nanoprobe into the brain.

**Nanoprobe biodistribution and toxicity profile.** The biodistribution of the nanoparticles in mice receiving either NPCP-Cy5.5-CTX or NPCP-Cy5.5 was determined by ex vivo NIRF signal quantification of excised tissues (tumor, healthy brain tissue, heart, liver, spleen, kidney, and muscle) 120 hours postinjection of nanoparticles (Fig. 6). Here, tissues from mice receiving no nanoprobe injection were also presented to provide NIRF baseline signal intensities (intrinsic signals) for each of these tissues. No marked nanoprobe accumulation was observed in healthy brain, heart, and muscle tissues ($P > 0.05$). NPCP-Cy5.5-CTX and NPCP-Cy5.5 accumulated at a similar level ($P > 0.05$) in each of these organs, indicating that the NPCP-Cy5.5-CTX has no specificity to these nonneoplastic tissues. Conversely, significant accumulation of the NPCP-Cy5.5-CTX nanoprobe ($P = 0.015$), but not the NPCP-Cy5.5 ($P > 0.05$; note the baseline NIRF signals as exhibited by the mice receiving no injection), within tumor tissue further showed high-level specificity and prolonged retention of NPCP-Cy5.5-CTX for tumor tissue. Additionally, the ability of the NPCP-Cy5.5-CTX nanoprobe to discriminate tumor from healthy tissue in the brain, as shown by MR and optical imaging, was further validated by its preferential accumulation within the brain tumor compared with normal brain tissue ($P = 0.0113$). It should be noted that significant nanoprobe accumulation in clearance organs including liver ($P < 0.0001$), spleen ($P < 0.001$), and kidney ($P < 0.0001$) was expected, and was comparable in distribution profile to those reported for other iron oxide nanoparticle systems (40–42).

Because the accumulation of nanoparticles was expected to be the highest in liver, potential toxicity of the accumulated nanoparticles to liver was assessed by a hepatotoxicity assay. The serum aspartate aminotransferase and alanine aminotransferase levels of mice injected with NPCP-Cy5.5-CTX or NPCP-Cy5.5 nanoparticles were measured (Supplementary Fig. S9). No marked elevation of aspartate aminotransferase ($P > 0.05$) and alanine aminotransferase ($P > 0.05$) levels were found in mice receiving either nanoprobe compared with noninjected control mice, suggesting that neither nanoprobe induces liver toxicity at the given dosage.
In summary, we have developed a novel magnetofluorescent nanoprobe that is able to traverse the BBB, specifically target brain tumors, and leave the BBB uncompromised. We revealed the significant difference between targeting and nontargeting nanoprobes in the discrimination of tumor from healthy tissue. The nanoprobe has shown persistent contrast enhancement for as long as 5 days. The MRI detectability combined with NIRF illumination exhibited by the same nanoprobe will potentially allow for more versatile uses of these contrast agents, such as preoperative diagnostics, tumor resection, as well as postoperative assessment with either MR or optical imaging. The developed nanoprobe platform retains the flexibility to conjugate alternative targeting and therapeutic agents, which may be used in the further development of alternative nanoparticle systems for specific applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References


Figure 6. Biodistribution of nanoparticles. Accumulation of nanoparticles in various tissues assessed by NIRF signal measurements of tissues/organs excised from ND2:Sm A1 mice receiving no injection and 120 h after receiving injection of either targeting NPCP-Cys5.5-CTX or nontargeting NPCP-Cys5.
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