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Focused ultrasound delivery of Raman nanoparticles across the blood-brain barrier: Potential for targeting experimental brain tumors

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Abstract

Spectral mapping of nanoparticles with surface enhanced Raman scattering (SERS) capability in the near-infrared range is an emerging molecular imaging technique.

We used magnetic resonance image-guided transcranial focused ultrasound (TcMRgFUS) to reversibly disrupt the blood-brain barrier (BBB) adjacent to brain tumor margins in rats. Glioma cells were found to internalize SERS capable nanoparticles of 50 nm or 120 nm physical diameter. Surface coating with anti-epidermal growth factor receptor antibody or non-specific human immunoglobulin G, resulted in enhanced cell uptake of nanoparticles *in-vitro* compared to nanoparticles with methyl terminated 12-unit polyethylene glycol surface. BBB disruption permitted the delivery of SERS capable spherical 50 or 120 nm gold nanoparticles to the tumor margins. Thus, nanoparticles with SERS imaging capability can be delivered across the BBB non-invasively using TcMRgFUS and have the potential to be used as optical tracking agents at the invasive front of malignant brain tumors.

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Background

Nanoparticles designed for concurrent diagnosis and therapy are potentially useful agents in the medical management of cancer.¹ The application of this nanotechnology in the setting of malignant brain tumors is of interest given that such particles could be used in the detection of tumor margins to facilitate maximal surgical resection and in the delivery of therapeutic agents. Gold nanoparticles (GNPs) can serve as a scaffold for multi-functionality² and can enhance local radiation effects,³ act as agents for thermotherapy,⁴ or be used to deliver therapeutic antibodies,⁵ chemotherapeutic agents,⁶ and small interfering RNAs.⁷

One of the major obstacles to the medical use of nanoparticles in the brain is the absence of a robust parenchymal distribution of nanoparticles administered intravenously.⁸⁻¹¹ The blood-brain barrier (BBB) which is formed by brain capillary endothelial cell tight junctions, luminal glycocalyx, basal lamina, and astrocytic foot processes serves as a barrier to nanoparticle transit from the vascular lumen to the brain parenchyma.¹² Disruption of the BBB as a method of delivery of macromolecules to the brain has been achieved with multiple intravenous or intra-arterial agents;¹³⁻¹⁶ however, targeted BBB disruption was not previously possible with these approaches.

Transcranial focused ultrasound has been shown to disrupt the BBB in a focal and reversible manner and its potential application to brain tumor therapy has been recently demonstrated in rat models.^{17, 18} Advances in intracranial targeting precision have allowed the safe and effective use of transcranial focused ultrasound for the production of lesions in deep structures of the human brain.^{19, 20} Using MRI-guided transcranial FUS (TcMRgFUS) we have previously demonstrated that polyethylene glycol (PEG) coated 50 nm GNPs, which are in the size range for imaging by SERS, can be delivered across the cerebral blood vessel wall into the normal rat brain parenchyma.²¹ Spectral mapping of gold nanoparticles having surface enhanced Raman scattering (SERS) tags with excitation wavelengths in the near-infrared (NIR=700-800 nm) range is a viable molecular imaging technique *in-vitro* and *in-vivo*.²²⁻²⁴ We hypothesized that GNPs with the potential for visualization by NIR Raman scattering could be delivered to the tumor and its borders across the BBB. The rationale for targeting the tumor margin was to demonstrate the specific delivery of multifunctional GNPs to the region of invading cells that have escaped the primary tumor mass – a common histopathological feature of glioblastoma (GBM).

NIR-SERS capable silica shell GNPs have been observed to distribute to human GBM xenografts in mice after tail vein administration.²⁵ We sought to further study the interaction of these particles with human GBM cell lines and primary glioma stem cell cultures as well as with normal fetal human astrocytes. Since the epidermal growth factor receptor (EGFR) has been used as a target for nanoparticle homing to tumors²² and EGFR is expressed at high levels in GBM,²⁶ we hypothesized that functionalizing NIR-SERS capable silica shell GNPs with anti-EGFR antibody would render these particles capable of human GBM cell labeling and tracking.

Methods

Cell culture

The following cell types were cultured as described in Supplementary Methods: rat gliosarcoma cell line 9L; rat C6 glioma; human GBM cells U87, A172, U251, U373; normal fetal human astrocytes; primary oligodendroglioma tumor cells BT2012036; and GBM adherent stem cell line GLINS1.²⁷

Nanoparticle synthesis and characterization

GNPs (50 nm) coated with PEG (MW 2000 Da) were purchased from Nanocs, New York, USA. Silica coated SERS-reporter gold nanoparticles were purchased from Cabot Security Materials, Massachusetts, USA (see Supplementary Methods for physical properties). PEG GNPs (50 nm) were tagged with crystal violet as described by Qian *et al.*²² Functionalization of SERS-reporter gold nanoparticles was performed using thiol-reactive chemistry to covalently link Panitumumab (Amgen Inc., CA, USA), methylpolyethylene glycol (12-carbon), or non-specific human immunoglobulin G to the silica surface as previously described.²⁸ Nanoparticle concentration was determined by absorbance at 544 nm using a NanoDrop 1000 UV-Vis spectrophotometer (NanoDrop products, Wilmington, DE, USA). High-resolution UV-Vis spectra were obtained using a Cary 50 spectrophotometer (Agilent, Santa Clara CA) and Raman spectra were acquired in a quartz cuvette on a Raman spectrometer (Advantage Series, DeltaNu, Laramie WY). Nanoparticle size distribution was assessed by Nanoparticle Tracking Analysis (NanoSight Ltd, Amesbury, UK). Zeta potential measurements were acquired in a disposable folded capillary cell using phase-analysis light scattering (Zetasizer Nano ZS, Malvern, UK).

Assessment of tumor cell uptake of SERS-tagged gold nanoparticles

A172 cells were grown on 18 mm coverslips at a density of 50,000 cells per coverslip and incubated 24 hours with 3.9×10^{10} crystal violet adsorbed 50 nm PEG2000 GNPs. Internalization of anti-EGFR antibody conjugated SERS-tagged nanoparticles in U251, A172, U87, BT2012036, GLINS1, and normal fetal human astrocyte cells was assessed at varying nanoparticle concentrations. The cells were washed with PBS and fixed with 4% paraformaldehyde prior to mounting on glass slides. Images were obtained using the inVia Raman microscope (Renishaw) with either 638 nm or 785 nm excitation laser and Differential Interference Contrast bright light microscopy or confocal fluorescence microscopy. Image processing was performed with Renishaw WiRE 3.3 and Adobe Photoshop. Spectral graphs were generated from Raman data on Prism 6 (GraphPad Software, Inc., CA, USA).

Fluorescence microscopy

To label the actin cytoskeleton, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X in PBS, and blocked with 1% BSA, and incubated with Phalloidin Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:500 in 1% BSA overnight. To assess CD63 co-localization with GNPs, cells were exposed to either mPEG-SERS420 or α EGFR-SERS440 at a ratio of 3000 particles per cell for 24 hours, fixed,

permeabilized, blocked, and then immunolabeled with mouse anti-human CD63 IgG1 monoclonal antibody 1:500 (Developmental Studies Hybridoma Bank, The University of Iowa) and Phalloidin Alexa Fluor 549 (Invitrogen) 1:500 in 1% BSA overnight. This was followed by incubation with anti-mouse IgG Alexa Fluor 488 (Invitrogen) 1:500 in 1% BSA for 1 hour at room temperature. Coverslips were mounted on glass slides using DAPI hard set mounting media (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence confocal microscopy was performed on an Olympus IX81 inverted fluorescence microscope (Supplementary Methods). Costes co-localization coefficient²⁹ was determined on at least 10 cells per experiment at 60X magnification for each cell line and the experiment was repeated with different culture sets twice for each cell line. The means of the co-localization coefficients were compared between groups by student's t-test with $P < 0.05$ chosen as statistically significant.

Western blot

Protein was isolated using whole cell lysis buffer, separated on a 7.5% polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with anti-EGFR mouse monoclonal IgG1 antibody at 1:500 dilution (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in TBS-T containing 5% skim milk and 3% BSA and 1:2000 horseradish peroxidase conjugated anti-mouse IgG was used as the secondary antibody (Cell Signaling Technologies, Danvers, MA, USA).

GNP cell loading and mixed culture experiment

U87-mCherry cells were incubated with 6000 GNPs/cell for 24 hours using either mPEG12-SERS420 or α EGFR-SERS440. The GNP loaded U87-mCherry cells were mixed with naïve U87-GFP cells in a 1:1 ratio and plated onto glass coverslips. Fluorescence cell imaging was performed as described in Supplementary Methods. Serial images were obtained for 1 to 2 min periods in multiple fields at 5 hours and 24 hours after cell mixing. At least ten 20X fields were photographed for counting in each condition. The number of green and red cells, the number of green cells showing at least 1 intracellular GNP and the number of extracellular GNPs in the field were counted. Counts were averaged for three independent experiments and compared between groups by student's t-test with a P-value of 0.05 as significant.

Fluorescence assisted cell sorting of GNP loaded cells

Cells were washed with PBS, pelleted, and fixed with cytofix/cytoperm fixation buffer (BD Biosciences, Bedford, MA, USA) for 30 min at 20°C then washed with BD wash buffer (BD Biosciences) and stored in staining buffer (3% FBS in PBS). The cell suspension was filtered through a 40 μ m nylon cell strainer (BD Biosciences). A total of 10,000 cells were sorted for each cell line and experimental condition and each experiment was performed in triplicate. An LSR II analyzer (Becton Dickinson, Franklin Lakes, NJ, USA) was used with FACSDiva software. Photomultiplier voltage was set to achieve a median fluorescence intensity of 641 for 0.04% relative intensity 6 μ m fluorescent microspheres (LinearFlow™ Deep Red Intensity Calibration kit, Life Technologies, Inc., Ontario, Canada). Data analysis was conducted with FlowJo (Tree Star Inc., Ashland, OR, USA).

Electron Microscopy

Cells were grown with a ratio of 1000 GNPs : 1 cell in a total volume of 5 mL (GNP concentration of 660 pM) for 22 hours. Dissociated cells were fixed in 250 μ L of 2% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.3 for 1 hour at room temperature. Samples were post-fixed in 1% osmium tetroxide in buffer, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in Quetol-Spurr resin. Sections 100 nm thick were cut on an RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate and viewed in an FEI Tecnai 20 TEM (Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto, Ontario).

Multimodality GBM cell tracking using anti-EGFR functionalized SERS GNPs

U87-mCherry cells were incubated with 3 pM (~3900 particles per cell) α EGFRSERS440 for 24 hours. A cranial window was made over the right frontal lobe of NOD/SCID male mice³⁰ and 400,000 cells were injected into the right frontal lobe. One day after tumor cell injection the site of cell implantation was imaged in anaesthetized live mice (n=5) through the cranial window. A tail vein injection of Alexa-488-conjugated 10 kDa Dextran (Invitrogen, Carlsbad, CA, USA) at a dose of 0.6 mg/kg was administered in order to visualize cerebral vasculature. The animals were then inverted onto the imaging platform of a two-photon laser confocal microscope (Leica LSM510 2Photon Laser Confocal). Using 5X, 10X and 20X long working distance air objectives, images were obtained at the site of tumor cell implantation. The animals were euthanized by CO₂ chamber and the brains were excised, washed in PBS, and placed in 3.7% formaldehyde. Brains were sectioned in axial, sagittal and coronal planes along the injection tract and mounted on a quartz slab (SPI supplies, PA, USA) submerged in PBS for ex-vivo fluorescent and Raman spectral imaging as described in Supplementary Methods.

TcMRgFUS-mediated nanoparticle delivery

9L tumor cells (3.2×10^5) were injected into the right frontal lobe of Wistar rats (Charles River, Sherbrook, QC, Canada). These cells were selected for their highly proliferative and infiltrative phenotype, ability to grow in immunocompetent rats, and extensive use in multiple brain tumor therapy studies.³¹ Brain imaging 8 days after tumor cell implantation was performed under isofluorane gas anaesthesia in a 1.5 T MRI (Signa 1.5 T, General Electric) using imaging parameters described previously.³² PEG-coated 50 nm GNPs were concentrated to a 10 mg/mL suspension by centrifugation. The suspension was administered 8 min prior to focused ultrasound by tail-vein catheter at a dose of 17 mg HAuCl₄/kg body weight and immediately followed by 0.5 mL 0.9% NaCl. Control animals received GNPs but no focused ultrasound. Transcranial focused ultrasound was applied to four points at the tumor periphery using a 551.5 kHz transducer with a 10 ms burst, 1 Hz pulse repetition frequency (PRF), 2 min duration (estimated *in situ* pressure ~ 0.23 MPa). At the start of sonication, 0.02 mL/kg Definity microbubbles was administered. Animals were euthanized at 2 hours (n=6), 30 min (n=3), or when moribund from tumor growth at 7 days (n=6) post-sonication and the brains excised and fixed in 3.7% formaldehyde. Brains were embedded in paraffin, sectioned, and stained by silver enhancement followed by hematoxylin and eosin (H&E).

Rats bearing 9L gliosarcoma tumors and having implanted common carotid artery catheters were imaged before sonication on a 7T MRI (Bruker Corporation, MA, USA; imaging parameters in Supplementary Methods). Infusion of α EGFR-SERS440 was performed at a rate of 0.1 mL/min in common carotid catheters (1.2×10^{11} GNPs per animal, n=3; 6.4×10^{11} GNPs per animal, n=6) or administered by tail-vein as a bolus (1.2×10^{11} , n=6). The α EGFR-SERS440 GNPs were suspended in a total volume of 500 μ L 0.9% NaCl with 5 units/mL Heparin for carotid delivery or in 20 mM MOPS pH7.5 with 0.1% BSA for intravenous delivery. Two of the animals receiving intravenous administration of GNPs received 4 μ L/g liposomal clodronate (17 mM clodronate disodium salt, 24 mM L- α -phosphatidylcholine, 11 mM cholesterol; Encapsula NanoSciences LLC, Nashville, TN, USA) 48 hours before the FUS procedure to deplete liver associated macrophages.³³ With the start of the carotid infusion, or immediately after the intravenous bolus, sonication of four points at the tumor periphery was performed. A hydrophone in the transducer assembly recorded the microbubble emissions during each ultrasound burst. The spectral information from the microbubble response was used to control the power output of the transducer in order to prevent vascular damage, as described by O'Reilly and Hynynen.³⁴ The mean peak pressure reached was 0.33 ± 0.06 MPa, with a drop to 50% of the peak pressure on detection of ultraharmonic signal content. The rats were imaged after FUS administration and allowed to recover from anesthetic to ensure no gross neurological deficits were present. Brain, liver, and spleen were excised and fixed in 3.7% formaldehyde for ex-vivo fluorescent and Raman spectral imaging. Alternatively, the tissues were embedded in OCT (Tissue-Tek; Sakura Finetek USA) and frozen in dry-ice chilled ethanol, followed by liquid nitrogen. Frozen sections were cut at 10 μ m and 80 μ m thickness and air-dried or fixed in ethanol or 3.7% formaldehyde followed by staining with H&E prior to bright light and Raman spectral imaging.

Results

Targeted delivery of polyethylene glycol (PEG) (5000 MW) coated 50 nm GNPs to intra-axial brain tumors and their margins was performed using TcMRgFUS. The disruption of the BBB at tumor margins was confirmed by the presence of increased Gadodiamide enhancement at the tumor periphery (Figure 1). Opening of the BBB with fixed acoustic power resulted in extravasation of circulating 50 nm GNPs adjacent to the infiltrating tumor margin observed by silver enhancement histology (Figure 1). No parenchymal distribution of 50 nm GNPs was found in control brains that did not receive targeted transcranial focused ultrasound after tail-vein administration of GNPs. Among animals that received TcMRgFUS for delivery of 50 nm GNPs to intra-axial brain tumor margins no gross neurological deficits were observed after recovery from anaesthesia. Furthermore, these animals remained alive for 7 days after sonication and were euthanized for lethargy or limb weakness related to tumor growth.

The production of SERS GNPs was undertaken in order to apply Raman confocal microscopy to assess nanoparticle internalization by GBM cells. A 50 nm PEG-coated crystal violet adsorbed particle (cvGNP) was used to determine if particles that can be delivered to the tumor periphery by transcranial focused ultrasound could also be used for SERS imaging. We confirm the uptake of cvGNP by A172 human GBM cells at 24 hours in

culture using Raman confocal spectral microscopy (Supplementary Figure 1). The disadvantage of using an unprotected adsorbed reporter is the possibility of reporter displacement or alteration in SERS signal in the presence of serum proteins or change in SERS characteristics due to pH shifts in intracellular structures. Therefore, we used a SiO₂-encapsulated gold nanoparticle to which a Raman reporter molecule has been adsorbed to further study internalization of functionalized GNPs in GBM cells *in-vivo* and *in-vitro* using Raman microscopy. Since GBM cells express abundant epidermal growth factor receptor protein (Supplementary Figure 2), we developed a SERS nanoparticle to target EGFR expressing cells using the monoclonal antibody Panitumumab. This human IgG2 antibody recognizes two overlapping epitopes in the extracellular domain 3 of human EGFR.³⁵ Panitumumab and Cyto647 fluorophore were crosslinked to the silica shell of 60 nm spherical gold core nanoparticles containing Trans-1,2-Bis(4-pyridyl)-ethylene adsorbed to the gold surface (α EGFR-SERS440, Supplementary Figure 3). GNPs with 4-4'dipyridyl adsorbed to the core and silica shell capped with Methyl-PEG₁₂ and Cyto647 fluorophore (mPEG12-SERS420) were used as a non-antibody control. We also synthesized control GNPs carrying d8-4,4'dipyridyl as the reporter molecule and capped with human immunoglobulin G and Cyto647 fluorophore (IgG-SERS421). The synthesis products were monodisperse, had a peak absorbance at 544 nm with no red shift on UV-Vis spectrophotometry compared to starting reagent, and demonstrated unique SERS440, SERS420, and SERS421 Raman spectra (Figure 2, Supplementary Figures 4, 5). The mean hydrodynamic diameters were 152 ± 52 nm (SD) for mPEG12-SERS420, 144 ± 37 nm (SD) for α EGFR-SERS440, and 170 ± 65 nm SD for IgG-SERS421 as determined by nanoparticle motion tracking. Zeta-potentials were -40.0 ± 0.7 mV (SD) for mPEG12-SERS420, -36.8 ± 0.7 mV (SD) for α EGFR-SERS440, and -30.7 ± 1.0 mV (SD) for IgG-SERS421.

Intracellular uptake of α EGFR-SERS440 and mPEG12-SERS420 was observed by fluorescence and Raman confocal microscopy in normal fetal human astrocytes, three different GBM cells lines (A172, U87, U251), and two glioma primary adherent stem cell cultures (GLINS1 and BT2012035) (Figure 2, Supplementary Figure 6). The intracellular distribution of α EGFR-SERS440 was noted to be peri-nuclear at 24 hours of incubation; while that of mPEG12-SERS420 appeared to be random. In order to determine if α EGFR-SERS440 particles were taken up by cells through the same pathway as EGFR trafficking, we determined the co-localization of the Cyto647 fluorescence signal with that of immunolabeled CD63, a protein marker for late endosomes/multivesicular bodies (LE/MVB).^{36, 37} We found that Panitumumab functionalized GNPs showed greater co-localization with CD63 compared to mPEG functionalized GNPs in U251, U87, and A172 cells (Supplementary Figure 7). Transmission electron microscopy showed the subcellular localization of α EGFR-SERS440 and mPEG12-SERS420 within endosomes in U87 human GBM cells and 9L rat gliosarcoma cells (Supplementary Figure 8). Human GBM cells showed a higher density of intracellular nanoparticles on TEM when exposed to an equal nanoparticle-to-cell ratio and extracellular concentration of α EGFR-SERS440 GNPs or mPEG12-SERS420 for 24 hours (Supplementary Figure 8). The perinuclear distribution of α EGFR-SERS440 observed under fluorescence microscopy is not appreciated in the TEM

images due to the small cross-section of the cell visualized in TEM compared to confocal fluorescence microscopy.

Processing of nanoparticles by endocytosis and pinocytosis may take different routes with variable release characteristics that are nanoparticle dependent.^{38, 39} Therefore, we investigated the release capacity of α EGFR-SERS440 and mPEG12-SERS420 nanoparticles after GBM cell uptake and their capacity to be exchanged into neighboring GBM cells. By mixing naïve GFP expressing U87 cells with mCherry expressing U87 cells loaded with a fixed number of α EGFR-SERS440 or mPEG12-SERS420 we show by live-cell video microscopy and fluorescence microscopy the exchange of nanoparticles from loaded cells into non-loaded cells (Supplementary Figure 9 and Video 1). The release rate of mPEG12-SERS 420 is faster than that of α EGFR-SERS440. Also, α EGFR-SERS440 GNPs are preferentially distributed inside cells versus the extracellular space, which is not the case for mPEG12-SERS420 GNPs over a 30-hour period.

Cellular uptake of α EGFR-SERS440 GNPs was observed to be concentration dependent in both normal fetal human astrocytes and A172 human GBM cells by microscopy (Figure 3). We further confirmed a linear relationship between extracellular concentration of α EGFR-SERS440 and mPEG12-SERS420 GNPs and nanoparticle uptake, as measured by intracellular fluorescence intensity in U87 cells (Figure 3, Supplementary Figure 10). Panitumumab functionalized GNPs showed enhanced uptake in U87 cells compared to mPEG covered GNPs. Enhanced uptake of α EGFR-SERS440 GNPs was also observed in U87 cells (high EGFR expression) when compared to 9L gliosarcoma cells (low EGFR expression). GNPs functionalized with non-specific human IgG were internalized by U87 cells to the same extent as anti-EGFR functionalized GNPs (Supplementary Figure 11) and we were not able to block the uptake of anti-EGFR functionalized GNPs with either free Panitumumab or human IgG at a concentration of 80 μ g/mL (Supplementary Figure 11).

Since U87 cells were demonstrated to avidly internalize α EGFR-SERS440 particles, we noted that intracellularly localized nanoparticles could be used as molecular tags for these tumor cells. In order to demonstrate that the internalized nanoparticles could be used to track brain tumor cells in-vivo, we directly implanted U87 with internalized α EGFR-SERS440 particles into the frontal lobe of nude mice. The cells and α EGFR-SERS440 signal were visualized under live cranial window fluorescence microscopy (Figure 4). The α EGFR-SERS440 nanoparticle signal could be visualized in freshly isolated brain imaging using fluorescence microscopy and Raman spectral microscopy (Figure 4). We demonstrated the capacity to image α EGFR-SERS440 signal within tumor cells residing in normal brain tissue by performing Raman spectral microscopy after H&E staining of formalin-fixed paraffin embedded tissue (Figure 4). These observations enabled us to proceed with delivery of α EGFR-SERS440 particles to intra-axial brain tumor margins and to image these particles by Raman spectral microscopy or fluorescence microscopy.

The 9L gliosarcoma orthotopic xenograft model was used for targeted BBB disruption at the intra-axial tumor margin using MRI-guided focused ultrasound. We administered α EGFRSERS440 particles by automated infusion into the common carotid artery through an indwelling cannula directed cephalad immediately before sonication. Alternatively,

nanoparticles were administered as a single bolus via tail vein catheter. Animals tolerated the procedure and recovered after anaesthetic with no evidence of hemorrhage or stroke on MRI (Supplementary Figure 12). Raman spectral imaging on freshly isolated brain maintained in formalin for less than 24 hours revealed a double-peak between 1590 and 1650 cm^{-1} which did not match spectral peaks for Evans Blue, Definity microbubbles, or Gadodiamide (Figure 5, Supplementary Figure 13). These peaks overlapped with the expected peaks of $\alpha\text{EGFR-SERS440}$; however, the Raman signal observed was weak and did not define the entire region of MRI contrast extravasation seen on post sonication imaging (Figure 5). Vascular delivery of $\alpha\text{EGFRSERS440}$ in both the tail-vein and carotid catheter animals was confirmed by visualization of GNPs on Raman mapping of H&E stained sections of liver in these animals (Supplementary Figure 14). We did not observe the SERS440 spectral signature on Raman mapping in the tumor centre or periphery after prolonged formalin fixation (>48 hrs) of brains from rats that received $\alpha\text{EGFR-SERS440}$ by tail-vein or carotid catheter (Supplementary Figure 14).

We found that exposure of $\alpha\text{EGFR-SERS440}$ to formalin resulted in a time dependent degradation of the SERS signal which affected our ability to visualize the nanoparticles in the brain specimens by Raman microscopy. In addition, we postulated that a higher circulating concentration of GNPs would allow better definition of the SERS signal in regions of blood-brain barrier disruption. Therefore, we performed another set of delivery experiments with higher intravascular nanoparticle concentration with or without macrophage depletion using clodronate liposomes administered 48 hours prior. Frozen brain sections were imaged after drying or fast (<2 hr) formalin fixation and stained with H&E. Using this technique we were able to confirm the targeted delivery of $\alpha\text{EGFR-SERS440}$ nanoparticles to the regions of BBB disruption and the absence of $\alpha\text{EGFR-SERS440}$ nanoparticle biodistribution to non-sonicated tumor or brain parenchyma (Supplementary Figure 15, Figure 6). In addition, $\alpha\text{EGFR-SERS440}$ nanoparticle BBB transgression was observed in the region targeted by FUS (Figure 6)

Discussion

Multiple approaches to the delivery of nanoparticles to brain tumors have been described recently including convection enhanced direct tumor delivery,⁴⁰ focal radiation,³ and focused ultrasound BBB disruption combined with magnetic steering.⁴¹ We have shown in this study that TcMRgFUS can be used to target the delivery of GNPs with the capacity for cellular internalization and SERS detection to the invasive margin of a malignant brain tumor model. TcMRgFUS offers a radiation-free and reversible method of BBB disruption as well as the specificity for targeting multifunctional nanoparticle deposition to sites of important biological activity in malignant brain tumors regardless of the baseline vascular permeability of the region. Previously, only 12 nm PEG-coated GNPs have been reported to cross the BBB in a mouse brain tumor model after focal radiation-induced BBB disruption.³ Delivery of 50 nm and 120 nm GNPs to the tumor periphery was achieved safely in our model with the use of controller modified acoustic pressure to prevent vascular damage. Furthermore, the transit of antibody functionalized SERS GNPs outside of capillaries in the region of sonication means that this potential therapeutic carrier and sensitive imaging tag can be targeted to deposit in the perivascular niche in the zone of tumor invasion, which is

the location of brain tumor initiating stem cells that are the drivers of tumor therapeutic resistance and recurrence.⁴²

While GNPs can be delivered to the invading tumor margin, tuning the nanoparticle specificity for GBM cells remains a challenge. We show that both mPEG and anti-EGFR targeting antibody coated GNPs are internalized by GBM cells and fetal human astrocytes. Fetal astrocytes show EGFR protein expression, which would explain the internalization of α EGFR-SERS440. However, mPEG12-SERS420 GNPs are also internalized by fetal astrocytes over 24 hours. The anti-EGFR antibody (Panitumumab) and IgG functionalized GNPs are preferentially taken up by GBM cells over non-targeted mPEG12-SERS420 GNPs, suggesting an antibody-mediated mechanism of uptake. Our results implicate multiple mechanisms contributing to nanoparticle internalization by GBM cells. We have found that Panitumumab-coated particles distributed more abundantly to multivesicular bodies than PEG-coated particles and that mPEG particles are able to more rapidly exchange between GBM cells and between the intracellular and extracellular environment. These results suggest differential intracellular processing pathways depending on the surface coating of SERS GNPs. The observation that nanoparticles continue to reside within tumor cells after transplanting α EGFR-SERS440 loaded human GBM cells into the mouse brain, despite the exchange of nanoparticles between the intracellular and extracellular environment, further supports the hypothesis that these GNPs will be preferentially taken up by GBM cells within the brain parenchyma.

Large diameter (>50 nm) nanoparticle uptake by GBM cells in our study is striking in that it was previously reported that nanoparticles greater than 50 nm in diameter would be inefficiently endocytosed by tumor cells.⁴³⁻⁴⁵ Nanoparticle uptake by cells may vary according to cell type, nanoparticle size, nanoparticle surface features such as charge and hydrophobicity, and cell surface receptor binding. Cell type dependent nanoparticle internalization is consistent with the observation of specific accumulation of nanoparticles in Kupffer cells of the liver⁴⁶ as well as monocytes and granulocytes⁴⁷ in living animals. The avid uptake of α EGFR-SERS440 nanoparticles and their retention in xenografted cells suggests that multifunctional nanoparticles could be used for GBM cell labeling. One potential use of this nanoparticle labeling strategy is the optimization of GBM resection, whereby tumor cell labeling could be used to guide microscopic tumor cell resection using wide-field Raman optics. The feasibility of SERS imaging in the clinical setting has been demonstrated using endoscopes⁴⁸ as well as a microscope based Raman imaging of freshly isolated brain tissue.⁴⁹ Multifunctional nanoparticles with SERS capability would offer several advantages over fluorescent dyes as optical tracers in the operative theatre. Unlike fluorescent dyes, SERS nanoparticles have enhanced photostability that benefits prolonged surgery.⁵⁰ SERS nanoparticles also allow increased multiplexing capacity allowing for labeling of multiple molecular or tissue substrates to guide surgical resection.⁵⁰ The use of NIR excitation with the SERS reporter allows for acquisition of signal from tissue at greater depth from the imaging surface,²² which could enhance the completeness and safety of resection. NIR SERS nanoparticles further enhance the sensitivity of detection as an optical tag in the brain because the use of NIR excitation reduces background signal from brain autofluorescence.

Kircher *et al.*²⁵ recently reported on a molecular imaging strategy using MRI-photoacoustic-Raman nanoparticles in a xenograft model of GBM, whereby silica-encapsulated gold nanoparticles were injected intravenously and subsequently imaged in intracranial tumors without any specific disruption of the BBB. Focal delivery of α EGFR-SERS440 GNPs to the brain parenchyma at tumor margins was achieved in our study with intravenous administration of 6.4×10^{11} particles, which is approximately 20-fold fewer nanoparticles (per gram body weight) than previously used to visualize tumor in mouse brains by Kircher *et al.*²⁵ Our study is unique in that TcMRgFUS allowed multifunctional nanoparticle delivery in a specific manner to the brain tumor invasive front. We were able to deliver a large-sized (120 nm) multifunctional nanoparticle across the BBB, which opens the possibility for the targeted delivery of a wide spectrum of therapeutic and diagnostic nanoparticles. The influence of nanoparticle size in determining brain biodistribution has been extensively studied, with a size exclusion limit of about 15 nm.^{8, 9} We have not evaluated smaller diameter NIR-SERS GNPs as these are presently not commercially available. Furthermore, the elimination routes of silica encapsulated or PEG-coated gold nanoparticles after entry to the brain parenchyma are unknown. Varying SERS nanoparticle size, surface coating, and composition will be important for optimizing tumor cell uptake, minimizing the loss of nanoparticles to the reticuloendothelial system, and achieving eventual elimination from the body or biodegradation if desirable for a given brain tumor theranostic nanoparticle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Targeted delivery of GNPs across the blood-brain barrier at tumor margins using transcranial focused ultrasound. (A) T1W Gadodiamide-enhanced MRI (7T field strength) showing a right frontal enhancing intra-axial brain tumor established from 9L gliosarcoma cells before sonication (left) and increased peritumoral enhancement after sonication (right). Red arrows indicate the ultrasound target points. The lower panel shows a magnification of the region of contrast enhancement before and after sonication. (B) H&E histology of tumor margins in animals that received intravenous PEG-coated 50 nm gold nanoparticles (iv GNP) in the presence or absence of focused ultrasound (FUS). Arrows indicate the location of nanoparticles. Scale bars 100 μ m.

Figure 2.

Panitumumab-functionalized NIR-SERS capable GNPs are internalized by GBM cells. (A) UV-Vis spectrum of bare and functionalized SERS gold nanoparticles. (B) Representative size distribution graphs and laser reflectance images for bare SERS 440 and Panitumumab functionalized SERS 440 nanoparticles. (C) Raman spectral maps after 24 hours of incubation with αEGFR-SERS440 in normal human fetal astrocytes, U251 GBM cells, and BT2012036 oligodendroglioma stem cell line showing internalization of SERS gold nanoparticles. The white boxes indicate the mapped region of interest. Crosshairs indicate the pixel used to generate the Raman spectra shown below the image. Grey-scale image background is from F-actin labeling by Phalloidin-Alexa Fluor 488. Scale bars 50 μm.

Figure 3.

Cellular uptake of Panitumumab-functionalized NIR-SERS capable gold nanoparticles is concentration dependent and cell-type specific. (A) Confocal fluorescence microscopy of A172 GBM cells showing increasing αEGFR-SERS440 GNP uptake with increasing extracellular concentration over a fixed 24 hour incubation period. Yellow- Cyto647 fluorescence from αEGFR-SERS440 GNPs. Gray – Phalloidin-Alexa 488 labeling of F-actin. Scale bars 25 μm. (B) Confocal fluorescence microscopy showing the intracellular distribution and concentration dependent uptake of αEGFR-SERS440 GNP and mPEG-SERS420 GNP in fetal human astrocytes. Yellow- Cyto647 fluorescence from GNPs. Gray– Phalloidin-Alexa 488 labeling of F-actin. Scale bars 25 μm. (C) The geometric mean fluorescence intensity (MFI) in U87 (high EGFR expression) compared to 9L (low EGFR expression) cells after 24 hours of incubation with varying concentrations of αEGFR-SERS440 or mPEG-SERS420 GNPs. * P<0.05 for comparison between U87 αEGFR-SERS440 and U87 mPEG-SERS420 as well as for comparison between U87 αEGFR-SERS440 and 9L αEGFR-SERS440. No difference in MFI between 9L EGFR-SERS440 and 9L mPEG-SERS420 observed.

Figure 4.

Optical tracking of GBM cells using internalized NIR-SERS gold nanoparticles. (A) In-vivo cranial window imaging of α EGFR-SERS440 nanoparticles (arrows, blue) inside and outside of U87-mCherry cells (red) implanted at injection site (I). Cerebral microvasculature in the top image is defined by Dextran-Alexa 448 (green). Images obtained at 5X (top) and 10X (bottom) magnification. (B) Ex-vivo whole mouse brain sagittal mount Raman spectral map overlay (red) onto autofluorescence image generated by 470/50 nm excitation light with a 525/50 nm emission filter (grey-scale) showing dispersion of α EGFR-SERS440 loaded U87-mCherry cells after parenchymal injection. Needle entry site in the cortex indicated by the white arrow. Individual Raman spectra from regions positive and negative for α EGFR-SERS440 signal are demonstrated. (C) Overlay of Raman spectral map on 40X bright light microscopy of H&E stained section at site of α EGFR-SERS440 loaded U87-mCherry implantation. Note the definition of GBM tumor cells from surrounding gliosis and normal brain parenchyma. Raman spectrum from the site defined by the crosshairs is demonstrated. Mapped region indicated by white box outline. Scale bars 20 μm .

Figure 5.

Delivery of Panitumumab-functionalized NIR-SERS GNPs across the blood-brain barrier using transcranial focused ultrasound. (A) Raman spectral map of a region demonstrating Evans Blue extravasation in the right frontal lobe of a rat brain after peritumoral opening of the blood-brain barrier with MRI guided focused ultrasound immediately after administering α EGFR-SERS440 nanoparticles through the tail-vein. The mapped region is indicated by the red box and is overlaid on a specimen mount fluorescence image generated by 688 nm laser excitation (bright areas indicate Evan's Blue staining). Crosshairs demonstrate the pixel used to generate the Raman spectrum shown to the right of the image. The Raman spectral map with boundaries indicated by the orange box was generated using peak to baseline measurements of intensity between wave numbers 1590 and 1650 cm^{-1} . (B) Raman spectral map in the liver with overlay on specimen fluorescence imaged under 688 nm laser light in the same animal showing α EGFR-SERS440 nanoparticle uptake in the liver and associated spectral signature.

Figure 6. Visualization of Panitumumab-functionalized NIR-SERS GNPs in the brain parenchyma after focused ultrasound disruption of the blood-brain barrier

Raman spectral mapping on a 10 μm H&E stained sections under 100X oil immersion lens. Simple mapping acquisition used with 1-second integration time per pixel. The spectral map was generated using signal to baseline intensity measurements for the double-peak at 1590-1650 cm^{-1} characteristic of the SERS440 reporter. Yellow colour assigned to indicate peak intensity. αEGFR -SERS440 GNPs are identified within the lumen (I) and not outside (II) of the cerebral blood vessel in the absence of FUS. When transcranial FUS is applied in a macrophage depleted animal, αEGFR -SERS440 GNPs can be visualized inside (III) and outside (IV) the cerebral blood vessel. Representative spectral graphs at the locations indicated. Scale bar 10 μm .