SUPPLEMENTARY INFORMATION

Engineered Nanoparticles for Systemic siRNA Delivery to Malignant Brain Tumors

Johan Karlsson,[a,b,c] Yuan Rui,[a,b] Kristen L. Kozielski,[a,b] Amanda L. Placone,[b,c] Olivia Choi,[a,b] Stephany Y. Tzeng,[a,b] Jayoung Kim,[a,b] Jamal J. Keyes,[a,b] Max I. Bogorad,[b,c] Kathleen Gabrielson,[d] Hugo Guerrero-Cazares,[e] Alfredo Quiñones-Hinojosa,[e] Peter C. Searson,[b,c*] and Jordan J. Green[a,b,c,f,g,h,*]

[a.] Department of Biomedical Engineering and the Translational Tissue Engineering Center, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA
[b.] Institute for Nanobiotechnology, Johns Hopkins University, Baltimore, MD 21218, US
[c.] Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA
[d.] Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA
[e.] Department of Neurosurgery, Mayo Clinic Florida, Jacksonville, FL 32224, USA
[f.] Departments of Neurosurgery, Oncology, and Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA
[g.] Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21231, USA
[h.] Sidney Kimmel Comprehensive Cancer Center and the Bloomberg~Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA

*Corresponding authors:
Peter C. Searson, searson@jhu.edu, 3400 N Charles Street, 100 Croft Hall, Baltimore, MD 21218, USA
Jordan J. Green, green@jhu.edu, 400 N Broadway, Smith Bldg, Room 5017, Baltimore, MD 21231, USA

Experimental

Syntheses of base monomer BR6 and polymers

For the synthesis of BR6, bis(2-hydroxyethyl) disulfide (15.4 g, 10 mmol) and triethylamine (TEA, 37.5 mL, 300 mmol) were dissolved in 450 mL tetrahydrofuran (THF). The solution was subsequently flushed with N_2 for 10 min and kept under a N_2 environment for the rest of the reaction time. Into the same flask, a solution of 24.4 mL of acryloyl chloride (300 mmol) dissolved in 50 mL dried THF was added dropwise for 2 h while shaking was applied, and the reaction was allowed to proceed for 24 h at room temperature. The TEA HCl precipitate was thereafter removed by filtration and rotary evaporation. The obtained product was dissolved in 200 mL dichloromethane (DCM), washed five times with 0.2 M Na_2CO_3 and three times with distilled water, and subsequently dried with Na_2SO_4 and rotary evaporation was applied to remove DCM from the prepared BR6 monomer. The diacrylate backbone monomer, BR6, was polymerized to the side chain monomer, 4-amino-1-butanol (S4), using Michael Addition as synthesis method at a ratio of 1.01:1 (BR6:S4) in dimethyl sulfoxide (DMSO) at 90 °C for 24 h. As a second step, the diacrylate-terminated base polymer (BR6-S4) were end-capped in DMSO at 100 mg/mL with 0.2 M of the end-capping monomers using either 2-(3-(aminopropyl)-amino)methanol (E6) or 1-(3-aminopropyl)-4-methylpiperazine (E7). This reaction was carried out for 1 h at room temperature while shaking was applied for proper mixing. The formed PBAEs R646 (M_n = 2984 and M_w = 5360) and R647 (M_n = 2474 and M_w = 4001) were stored at – 20 °C in small aliquots until use.

In vitro blood-brain barrier permeability

Human brain microvascular endothelial cells (hBMECs) were used as monolayers in the *in vitro* permeability assay. hBMECs were derived from BC1 human induced pluripotent stem cells

(iPSCs), where iPSCs were cultured for four days on 40 μg/mL Matrigel-treated (Fisher Scientific, Pittsburgh, PA, USA) tissue culture dishes (Corning, Corning, NY, USA) in TeSR-E8 medium (Stem Cell Technologies, Vancouver, BC, Canada) which were changed daily. The formed cell colonies were thereafter placed in unconditioned medium (UM/F-, without basic fibro growth factor, bFGF) which included Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Life Technologies) supplemented with 20% KOSR (Life Technologies), 1% non-essential amino acids (Life Technologies), 0.5% L-glutamine (Sigma), and 0.836 μM Beta-Mercaptoethanol (Life Technologies).

The cells were cultured in UM/F- medium for six days, and thereafter switched to endothelial cell (EC) serum-free medium (Life Technologies) supplemented with 10 μM all-trans retinoic acid for two days. Cells were cultured in T75 flask (BD Falcon, Bedford, MA, USA) containing 12 mL of medium. The culture plates with the transwells were incubated at 37 °C with 5% CO_2 during the experiments. **Figure S1** shows a TEM image of a cross-section of the transwell visualizing that the dhBMEC monolayer is formed on the apical side of the porous membrane.

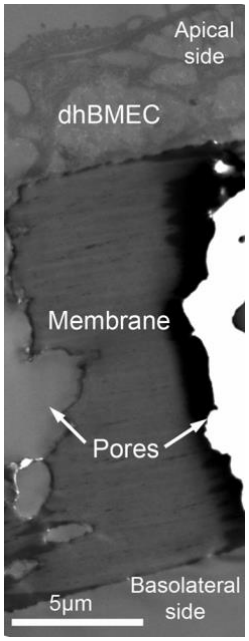

Figure S1. Cross-sectional transmission electron microscopy (TEM) image of the transwell. Induced pluripotent stem cells (iPSC)-derived human brain microvascular endothelial cells (dhBMECs) are seeded at the apical side of the porous membrane.

***In vitro* siRNA delivery to patient-derived glioblastoma cells**

Prior to seeding of GBM 612 cells, each well was coated with laminin (Sigma) that were dissolved in PBS for 2 h prior to experiments to obtain a laminin surface coverage of 1 μg/cm^2. The GBM cells were thereafter seeded at a density of 10,000 cells/well (96-well culture plates) or 60,000 cells/well (24-well culture plates) in DMEM-F12 medium (Life Technologies) with 2% (v/v) B-27 Serum-Free Supplement (Invitrogen), 1% (v/v) Antibiotic-Antimycotic, and 20 μg/mL each of epidermal growth factor (PeproTech, Rocky Hill, NJ, USA) and basic fibroblast growth factor (PeproTech). The cells were subsequently allowed to adhere for two days prior to experiments. The nanoparticles were prepared in the same manner as described above. Prior to adding nanoparticles into the culture plates with cells, the culture medium was replaced with medium containing 10% FBS. In the method with transwells was both the medium in apical and basolateral compartment replaced. In the first method without transwells, the nanoparticles were added at a 1:6 (v/v) ratio to the culture medium where the final concentration of the polymer was 180 μg/mL and for siRNA 10, 60, or 120 nM. In the method with transwells, the nanoparticles were added to the apical compartment again at a 1:6 (v/v) ratio to obtain a polymer concentration of 180 μg/mL and siRNA concentration of 10 nM. To evaluate the siRNA delivery efficacy, the GBM 612 cells were fixed in 3.7% formalaldehyde and stained with a 10 μg/mL DAPI (Invitrogen) solution, hence cell death mediated by the death siRNA molecules was determined. Fluorescence images were acquired using a Nikon TiE microscope (Nikon Instruments, Melville, NY, USA) equipped with a Photometrics camera using NIS elements software. Cell counting for the different conditions was performed using the software ImageJ to evaluate cell death mediated by the death siRNA molecules.

Results

PBAE/siRNA nanoparticle biodistribution in orthotopic human brain tumors *in vivo*

The nanoparticle formulation R646-10, which showed the highest permeability in the *in vitro* BBB model, was chosen for the *in vivo* studies. To analyze the biodistribution of this nanoparticle formulation, we used siRNA labeled with an IR-fluorescent dye. The biodistribution 24h post systemically injected nanoparticles obtained by LI-COR imaging is demonstrated in **Figure S2A**. Representative images of the organs for a control and for an animal with nanoparticle treatment is shown in **Figure S2B** and **S2C**, respectively. The obtained result of the biodistribution experiment showed that at 24 hours post-administration, there was siRNA nanoparticle accumulation only in the brain compared to control ($p = 0.028$; $n = 3$-4).

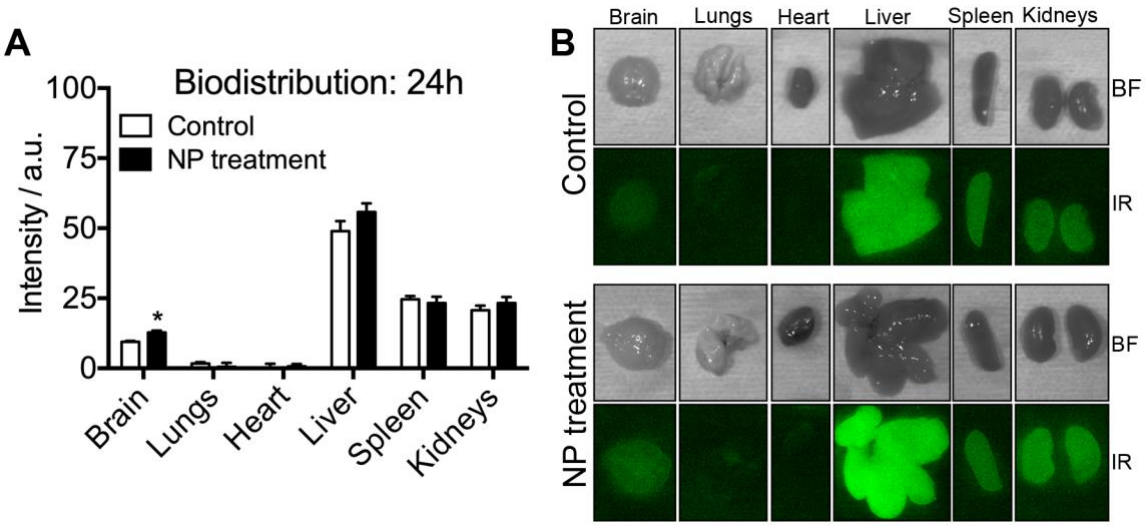

Figure S2. The *in vivo* biodistribution of the nanoparticle-siRNA (NP) formulation in the harvested organs 24h post intravenous injections based on LI-COR imaging ($n = 3$-4). (**A**) A statistically higher IR intensity was observed in the brain for the animals with NP treatment. (**B**) Representative LI-COR images, both in brightfield (BF) and IR-fluorescence (IR), for the harvested organs from a control and from an animal that received NP treatment. The error bars correspond to standard error of the mean (SEM).

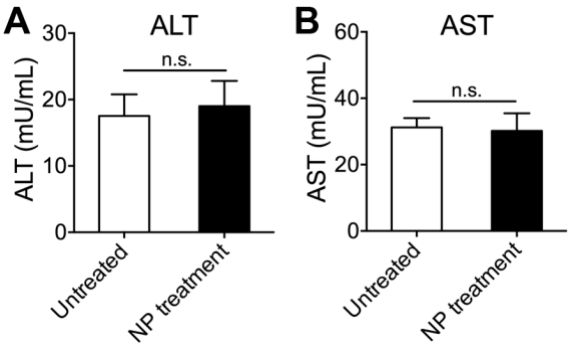

Figure S3. Injected nanoparticles do not cause liver toxicity. Blood analysis of nude mice 48 h post-intravenous injection of R646-10 nanoparticles (NPs) compared to without treatment (n = 3). (A) Alanine aminotransferase (ALT) activity and (B) Aspartate aminotransferase (AST) activity in serum is normal. The error bars correspond to standard error of the mean (SEM).

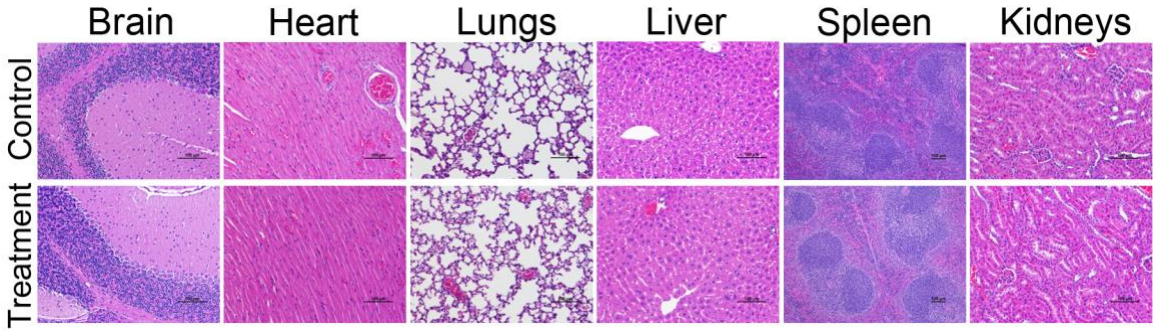

Figure S4. Injected nanoparticles do not cause tissue toxicity. Representative histopathological images (scale bars: 100 μm) of H&E stained tissue sections of organs from nude mice for the treatment group two weeks after receiving R646-10 nanoparticles *via* intravenous injections and the control group without treatment (n = 3).