Supporting Information

Redox-Responsive Magnetic Nanoparticle for Targeted Convection-Enhanced Delivery of O\textsuperscript{6}-Benzylguanine to Brain Tumors

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Supporting Results and Discussion

NPCP cross-linking optimization

Cross-linking of the NPCP polymer coating via disulfide bond formation was utilized to further stabilize the NPCP for subsequent conjugation of BG, fluorescent probes and CTX, as well as provide a mechanism for release of drug within target cells. To optimize cross-linking, amine reactive Traut’s reagent was reacted with NPCPs at 10:1, 5:1 and 2.5:1 weight ratios of Traut’s reagent to iron. The stability of the cross-linked NPCPs were compared to non-cross-linked NPCPs in PBS (Supporting Information Figure 1a) in terms of hydrodynamic size change over time. The cross-linking dramatically stabilized the nanoparticle at all three Traut’s reagent ratios. Since the reaction with Traut’s reagent consumes amines that are necessary for subsequent conjugations, we quantitated the number of reactive amines of NPCP and cross-linked NPCPs produced at the three cross-linking ratios (Supporting Information Figure 1b). These results show that at the 2.5:1 ratio, only a small fraction of available amines are consumed by Traut’s reagent, indicating that few disulfide bonds could be formed at this ratio. Both the 5:1 and 10:1 ratios show a significant drop in the number of amines indicating a greater number of thiols for disulfide bond formation, yet maintain an adequate number of amines for further conjugation. Since there was not a significant gain in free thiols by increasing the Traut’s reagent ratio from 5:1 to 10:1 and further experiments showed that the 2.5:1 ratio was not consistent in stabilizing NPCPs, the 5:1 ratio was determined to be optimal.
Supporting Information Figure 1. Cross-linking optimization of NPCP polymer coating. a) Stability over 5 days of NPCP and NPCP cross-linked at 10:1, 5:1 and 2.5:1 weight ratio of Traut’s reagent to iron in PBS. b) Number of reactive amines on the polymer coating of NPCP and NPCP cross-linked at 10:1, 5:1 and 2.5:1 weight ratio of Traut’s reagent to iron.

NPCP-BG-CTX characterization

Unmodified BG contains no reactive handle for conjugation to NPs, and therefore, bromination of BG (BG-Br) at the C8 position was necessary for conjugation of BG to NPCP. BG-Br was then coupled to the amines of the chitosan backbone through nucleophilic substitution. Bromination of BG was confirmed by tandem liquid chromatography-mass spectrometry (LC-MS) (Supporting Information Figure 2a). Extracted ion chromatography (EIC) was used to extract data for ions with specific mass to charge ratios (m/z) allowing for easy identification of analytes. The chromatographic peak at 13.1 min (242 m/z) in the blue trace corresponds to unmodified BG, whereas the peak at 16.2 min (321-323 m/z) in the yellow trace corresponds to BG-Br. Integration of these peaks showed ~60% yield of BG-Br from the reaction mixture.
The hydrodynamic size of NPCP-BG-CTX determined by DLS in HEPES buffer pH 7.4 was 76 ± 4 nm, and the NPs maintained stability in serum containing media for at least 10 days (Supporting Information Figure 2b-c). The zeta potential of NPCP-BG-CTX in HEPES buffer pH 7.4 was 4 ± 7.4 mV (Supporting Information Figure 2d).

**Supporting Information Figure 2**: LC-MS analysis of BG-Br and physicochemical characterization of NPCP-BG-CTX. a) Extracted ion chromatogram of reaction mixture of BG and N-bromosuccinimide in methanol, analyzed by LC ESI TOF MS. The blue trace corresponds to unmodified BG (242 m/z) and the yellow trace corresponds to BG-Br (321-323 m/z). b)
Intensity based hydrodynamic size distribution of NPCP-BG-CTX in 20mM HEPES, pH 7.4 as determined by DLS. c) NPCP-BG-CTX stability in biological fluid (DMEM containing 10% FBS). d) Zeta potential distribution of NPCP-BG-CTX in 20mM HEPES, pH 7.4

To accurately determine the number of BG and CTX molecules per NP, the NP molecular weight was determined by measuring the mean core diameter of NPs from transmission electron microscopy (TEM) images (Supporting Information Figure 3a-b). The mean core diameter of NPCP-BG-CTX was 7.5 ± 1.3 nm which corresponds to an iron based molecular weight of ~600,000 g/mol.

Supporting Information Figure 3: Determination of NP core size. a) Representative TEM image of NPCP-BG-CTX. Scale bar corresponds to 50 nm. b) Distribution of NP core diameters yielding a mean of 7.5 ± 1.3 nm determined from 200 independent core measurements using ImageJ software.

NPCP-BG-Cy5.5-CTX in vivo BBB permeability
To evaluate BBB permeability of NPCP-BG-Cy5.5-CTX, analysis was performed on mouse brain sections three hours after NP administration. Gross examination was first performed on 12 µm thick brain sections using odyssey scanner assays described in the main text to monitor biodistribution (Supporting Information Figure 4a). Images obtained from three mice show distribution of nanoparticles throughout the entire brain with markedly higher intensities noticeable in blood vessels. Brain tissue from mice receiving no injection showed no signal from the nanoparticles. The extravasation of nanoparticles from blood vessels was further confirmed through immunohistological analysis of mouse brain sections (Supporting Information Figure 4b). The presence of fluorescent signal in the brain’s extracellular matrix in wild-type mice further supports the ability of these particles to escape the neural vasculature.
Supporting Information Figure 4: Evaluation of BBB permeability of NPCP-BG-Cy5.5-CTX using a fluorescence-based assay. Shown are representative images of brains of wild-type mice receiving no-injection or three hours after tail vein injections of NPCP-BG-Cy5.5-CTX. a) Fluorescence image of 12-µm sections of mice brain and 100× dilution of blood in 96 well plate scanned using an Odyssey imaging system. b) Histological examination of nanoparticle permeability across the BBB. Cell nuclei (blue; DAPI) and endothelial cells (green; FITC-PECAM-1) were stained to visualize the localization of nanoparticles (red) within the brain tissue. The scale bar in the confocal images corresponds to 20 µm.

In vitro GBM targeting

Flow cytometry was used to determine the effectiveness of CTX as a targeting ligand for the NP system. Supporting Information Figure 5 shows uptake of targeted (NPCP-BG-CTX) and control NP (NPCP-BG) by target cells (SF767) and evaluated against a control cell line, HFF, which are not upregulated in MMP-2 or Annexin A2 expression. Here, SF767 cells treated with the CTX-enabled NP showed significantly increased internalization levels when compared with cells exposed to the non-functionalized NP. Conversely, the HFF cells showed little interaction with both NP types, indicating selective binding of the NP presenting the CTX peptide. All cell lines were incubated with NP formulations at a 50 µg of Fe/ml dose. At this dose there was an ~2 fold increase in uptake of CTX-modified NP by SF767 cells compared with control NP. Furthermore, there was a four-fold improvement in uptake of the targeted NPCP-BG-CTX NPs by SF767 cells compared to HFF cells.
Supporting Information Figure 5: Flow cytometry analysis of NP internalization by SF767 (GBM cell line), and control HFF cells 2 hours post treatment with either NP-BG or NP-BG-CTX; also shown is the result for cells receiving no nanoparticle treatment (UT) as a reference.

CTX was further evaluated in vitro as a targeting ligand for primary GBM6 cells. Supporting Information Figure 6 shows GBM6 uptake of targeted CTX-AF647 and free AF647 control. Cells were incubated with CTX-AF647 or free AF647 at a 4 μM fluorophore concentration. At this concentration there was a nearly 3-fold increase of CTX-AF647 uptake as compared to free AF647, demonstrating ligand specific targeting of GBM6 cells by CTX.
Supporting Information Figure 6: Flow cytometry analysis of AF647 internalization by primary GBM6 cells, 2 hours post treatment with either free AF647 or CTX-AF647; also shown is the result for cells receiving no treatment (Untreated) as a reference.

Histological analysis of tissue specific toxicity

Histological analysis of kidney, spleen, liver, and brain of mice injected with NPCP-BG-CTX was performed to evaluate acute toxicity. Tissues were harvested from mice 120 h after receiving nanoparticle injection, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) as described in the method section of the manuscript. No noticeable differences were observed between tissue collected from PBS injected mice and those injected with NPCP-BG-CTX (Supporting Information Figure 7).
Supporting Information Figure 7. Representative H&E stained tissue sections of mouse liver, kidney, spleen, and cerebellum obtained from PBS injected animals and from those injected with NPCP-BG-CTX. The scale bar corresponds to 150 μm.

Supporting Methods

Characterization of NPCP crosslinking

Hydrodynamic sizes of NPCP and NPCP-crosslinked at 10:1, 5:1 or 2.5:1 weight ratio of 2-Iminothiolane to iron was analyzed at 100 μg/mL in PBS (pH 7.4) using a DTS Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

Quantification of the number of amine groups immobilized on the surface of NPCP and crosslinked NPCP was performed by reaction of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (4.3 mg) to NPCP (2 mg) for 2hrs at room temperature to produce pyridyldithiol-activated NPCP. Pyridyldithiol-activated NPCP was purified using size exclusion chromatography with S-200 resin into 100mM boric acid buffer, pH 7.4. Purified pyridyldithiol-activated NPCP was then mixed with tris-(carboxyethyl)phosphine hydrochloride (TCEP) at a final TCEP concentration of 50mM. The NPCP/TCEP solution was reacted on a rocker for 30
minutes at room temperature. Cleaved pyridine-2-thiol (P2T) was separated from the reaction mixture (0.5 mL) using a 30 kDa MW cut off Amicon Ultra centrifugal filter (Millipore, Billerica, MA) centrifuged at 12,000 rcf for 10 minutes. The UV-vis absorbance spectra of the P2T rich supernatant was measured at 343 nm and quantified using extinction coefficient of 8080 cm$^{-1}$. The concentration of P2T is equivalent to the concentration of reactive amines. The number of amines/NPCP was determined by measuring Fe concentration of the reaction mixture and calculating the molar concentration of NPCP assuming the nanoparticle has a core diameter of 7.5 nm and the density of bulk magnetite.

**Characterization of brominated BG**

To quantitate BG-Br yield from the reaction mixture a Hewlett Packard 1100 Liquid Chromatography (LC) system (Palo Alto, CA, USA), with autosampler, coupled to a Bruker Esquire ion trap mass spectrometer (Billerica, MA, USA) with electrospray ionization (ESI) source was utilized. The BG/N-bromosuccinimide reaction mixture was separated with an Agilent Zorbax narrow bore C18 column that was 100 mm × 2.1 mm i.d. with 3.5 µm particle size (Agilent, Santa Clara, CA, USA). A binary mobile phase system of solvent A (water with 5% acetonitrile and 1% acetic acid) and solvent B (acetonitrile with 1% acetic acid) provided the best separation at 30 °C and a flow rate of 200 µL/min with the following gradient: B increased from 0% to 50% over 15 min followed by an increase to 100% B for 16.5 min. A sample volume of 1 µL was injected into the column.

Analytes were ionized for mass spectrometric detection by positive ion ESI with the following conditions: spray voltage, 3 kV; drying gas temperature, 350°C; drying gas flow rate, 10 L/min; nebulizer, 30 psi; capillary voltage, 4 kV. MS data were collected in full scan mode over the
mass range 50–2200 m/z with a scan resolution of 13,000 m/z/sec. Ion optic voltages were as follows: skimmer 1, 30 V; skimmer 2, 6 V; octopole, 3 V; octopole RF, 100 Vpp; octopole Δ, 2 V; lens 1, −5 V; lens 2, −60 V. Bruker Daltonics DataAnalysis software, version 3.0, was used for data acquisition and analysis.

**Nanoparticle size and zeta potential characterization**

The hydrodynamic size and zeta potential of NPCP-BG-CTX were analyzed at 100 µg/mL in 20 mM HEPES buffer (pH 7.4) using a DTS Zetasizer Nano (Malvern Instruments, Worcestershire, UK). NPCP-BG-CTX stability in biological fluid was analyzed at 100 µg/mL in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic.

**TEM analysis of NP core diameter**

TEM images were acquired with an FEI TECNAI F20 TEM (Hillsboro, OR) operating at 200 kV. NP core diameters were analyzed with ImageJ software and the size distribution, mean diameter and standard deviation was calculated from 200 NP measurements.

**In vitro GBM targeting**

SF767 cells were maintained at 37°C in 95%/5% humidified air/CO2 in DMEM containing 10% FBS and 1% antibiotic-antimycotic. For targeting experiments, 50,000 cells were plated in 24-well plates the day before NP or CTX-AF647 treatment. NP and CTX-AF647 treatments were performed in fully supplemented culture medium at 50 µg/mL NPs and 4 µM CTX-AF647. After a 2-hr treatment, cells were washed thrice before preparation for detection of NP or CTX-AF647 labeling. Cells were washed with PBS, and detached using TrypLE Express (Invitrogen,
Carlsbad, CA), and suspended in PBS containing 2% FBS. At least 10,000 cells were then analyzed using a BD FACSCanto flow cytometer (Beckton Dickinson, Franklin Lakes, NJ) and data analyses were performed using the FlowJo software package (Tree Star, Ashland, OR).