SUPPLEMENTARY INFORMATION

Gemcitabine and Chlorotoxin Conjugated Iron Oxide Nanoparticles for Glioblastoma Therapy

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1. Materials

Sodium hyaluronate (HA) (5 kDa) was purchased from Lifecore Biomedical, LLC (Chaska, MN). Gemcitabine hydrochloride (GEM) was purchased from LC Laboratories (Woburn, MA). Cy3-hydrazide and Cy5.5-hydrazide were purchased from Lumiprobe Corporation (Hallandale Beach, Florida). Wheat germ agglutinin-Alexa Fluor 647 conjugate was purchased from Life technologies (Grand Island, NY). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2. Synthesis of IONP-PEG-NH_2

PEG2000-NH_2 monolayer-coated IONPs (IONP-PEG-NH_2) was synthesized following our previous method with minor modification.[1] 50 mg of oleic acid-coated IONP[2] was suspended in 43 ml of anhydrous toluene followed by addition of 50 µl of triethylamine in a three-neck round-bottom flask fitted with a Graham condenser. The flask was sealed with rubber septa and purged with nitrogen. The solution was heated to 100°C and 0.10 ml SATES was added to the flask. 187.5 mg of mPEG2K-NH_2 was dissolved in 7 ml of anhydrous toluene and added to the flask 15 min after the addition of SATES. An additional 50 µl of SATES was injected 1h after the mPEG2K-NH_2 injection, and the solution was reacted for another 6 hours and 45 min. The solution was transferred to a single-neck round-bottom flask and NPs were precipitated with hexane. The NP precipitate was dispersed in THF, sonicated for 10 min, and precipitated with hexane. The resulting NP pellet was suspended in 10 ml anhydrous THF and sonicated for 10 min. 62.5 mg of mPEG2K-NH_2 and 187.5 mg of bis(amine) functionalized PEG (PEG2K-bis(amine), MW 2000) were dissolved in 12 ml anhydrous THF and added to the NP solution. The flask was then sealed with a septum and purged with nitrogen. 12.5 mg of N,N[1]-dicyclohexylcarbodiimide (DCC) was dissolved in 2 ml anhydrous THF and added to the flask, and the reaction solution was placed in a sonication bath at 25°C and allowed to react for 16 h. Fully PEGylated NPs were precipitated with hexane, redispersed in 20 ml ethanol, sonicated for 10 min, and precipitated again with hexane. The pellet was fully dried and dispersed in distilled water with sonication for 10 min. The particles were purified through Sephacryl S-200 size exclusion gel chromatography.

3. Surface functionalization of IONP-PEG-NH_2

To prepare IONP-HA-GEM, GEM (2.5 mg), HA (3.3 mg), EDC (3.2 mg), and NHS (1.9 mg) were dissolved in 0.4 mL MES buffer (pH 4.7). IONP-PEG-NH_2 (1 mg in 0.4 mL MES buffer) was then mixed with above solution and incubated overnight on rocker. S-200 sephacryl column equilibrated with MES buffer was used to purify the IONP-HA-GEM. To prepare IONP-HA-GEM-CTX, the IONP-HA-GEM was then mixed with 143 μg CTX in the presence of EDC (0.4 mg) and NHS (0.45 mg) in MES buffer and incubated overnight. S-200 sephacryl column equilibrated with PBS buffer (pH 7.4) was used to purify the IONP-HA-GEM-CTX. To prepare the IONP-HA-GEM-CTX-Cy3 or IONP-HA-GEM-CTX-Cy5.5, HA (5 mg) was firstly reacted with Cy3-hydrazide or Cy5.5-hydrazide (2.5 μL in DMSO, 50 mM) in the presence of EDC (0.64 mg) in MES buffer overnight. The same amount of HA-Cy3/Cy5.5 used for IONP-HA-GEM-CTX was used for preparation of IONP-HA-GEM-CTX-Cy3 or IONP-HA-GEM-CTX-Cy5.5. NPs were finally purified with S-200 sephacryl column equilibrated with PBS buffer.

4. Characterization of NP conjugates

GEM on NPs was extracted through basic hydrolysis and quantified by HPLC. Briefly, 90 μL of NP solution was added with 10 M NaOH and incubated for 1 h at 40 °C water bath. NPs precipitated during this process. The suspension was briefly vortexed and centrifuged to remove NPs (15000 g, 2 min). Supernatant was transferred to a new tube and neutralized with concentrated HCl and then analyzed by HPLC. XBridge BEH C18 column was used (130 Å, 3.5 μm, 4.6 mm × 100 mm) (Waters Corporation, Milford, MA). The A solvent was water and the B solvent was methanol. The gradient was 10–60% B in 4 min. The equilibration time was 4 min at 10% B. The column temperature was set at 30°C and the flow rate was 0.6 mL/min. The injection volume was set at 10 μL and 260 nm was used as a detection wavelength.

For negative-staining TEM images of NPs, NP solution (4 μL) was transferred onto a TEM grid (copper grid, 300-mesh, coated with carbon and Formvar film) and stained with 5% uranyl acetate. After drying the solution in air using a filter paper, negative-staining TEM images were acquired on a Tecnai G2 F20 electron microscope (FEI,

Hillsboro, OR) operating at a voltage of 200 kV. Regular non-staining images were obtained on grids without uranyl acetate staining. The hydrodynamic size and ζ-potential of IONP were determined using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). The analyses were performed at the room temperature. The pH value of all NP solutions was 7.4. The medium stability test of IONPs was performed by diluting IONPs 10 times in complete DMEM cell culture medium and incubated in a 37°C water bath. The hydrodynamic sizes were measured several times during a two-week incubation.

5. Cell culture

SF-763 and U-118 MG human glioblastoma cells were purchased from American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Life technologies, Grand Island, NY). Cells were cultured in an incubator maintained at 37°C and 5% CO_2 with 95% humidity.

6. Characterization of cellular uptake of NPs by confocal laser scanning microscopy (CLSM)

SF-763 or U-118 MG cells were seeded onto glass cover slips in a 6-well plate. After overnight incubation, cells were incubated with NPs (40 μg/mL [Fe]) for 2 h. Cells were then fixed with 4% paraformaldehyde for 15 min at 37°C and stained with 5 μg/mL WGA-Alexa Fluor 647 (Invitrogen, Carlsbad, CA) for 5 min at 37°C, followed by 3 times of PBS washing (5 min each). Cells were then incubated with DAPI for 5 min at 37°C, followed by PBS washing. After PBS washing, cells were mounted with VECTASHIELD mounting medium (Vector Laboratories, Inc. Burlingame, CA). The images of cells were acquired using a Leica SP8 confocal laser scanning microscope (Leica, Germany).

7. Cell viability by Alamar Blue assay

Cells were seeded in a 96-well plate and incubated overnight in the growth conditions described in section 4. In the following day, the medium was replaced with a medium

containing GEM, IONP-HA-CTX, IONP-HA-GEM-CTX or with medium control. Five different drug concentrations were used for each cell lines (800, 400, 200, 100 and 50 nM for SF-763; 200, 100, 50, 25 and 12.5 nM for U-118 MG), and samples at each concentration were ran in triplicate. The cells were then incubated for 72 h. Cell viability was assessed using the Alamar Blue assay. Briefly, the medium was replaced with cell culture medium containing reagent and allowed to incubate for 2 h. Following the incubation, a microplate reader (SpectraMax i3 multimode microplate reader, Molecular Devices) was used to determine the fluorescence intensity of the dye (550ex/590em). The fluorescence intensity from NP or free drug treated cells was compared to those from untreated control cells to determine percent viability.

8. Blood half-life of IONP-HA-GEM-CTX-Cy5.5 in mice

All animal studies were conducted in accordance with University of Washington's Institute of Animal Care and Use Committee (IACUC) approved protocols as well as with federal guidelines. Female BALB/c mice with 6 weeks old were injected through the tail vein with 100 μL of 2 mg/mL nanoparticle (n = 3). At 1.5, 3, 6, 16, 24 and 48 h after injection, blood (20-50 μL) was collected from tail veins. Each mouse was collected twice at two time points. The total amount of blood withdrawn from mice never exceeded one percent of the total body weight of the animal during this experiment. Whole blood was spun using a benchtop centrifuge for 2 min at 5000 g to separate the plasma. Then, 20 μL of plasma was diluted to 100 μL by PBS and added to a 96-well black plate. The plate was scanned on the SpectraMax i3 plate reader (fluorescence mode) (ex: 673 nm; em = 727 nm) to measure Cy5.5 fluorescence signals.

9. Biodistribution of IONP-HA-GEM-CTX-Cy5.5 in mice

Same mice in pharmacokinetic study were used in this study. Mice were euthanized 3 and 48 h after NP injections after blood drawing was completed. Organs (liver, spleen, kidneys, heart and brain) were collected and fluorescence intensities from these organs were measured by a XENOGEN IVIS 200 imaging system (PerkinElmer Inc.). Imaging parameters: excitation wavelength: 710 nm; emission filter: ICG; exposure time: 1 second; binning factor: 2; f/stop: 4.

10. Blood-brain barrier penetration of IONP-HA-GEM-CTX-Cy5.5

The blood-brain barrier penetration was assessed as previously described.[3] Each animal was euthanized 2 and 6 h post injection, and brain tissues were dissected. Tissues were then embedded in OCT and kept frozen at −80 °C until they were needed. The frozen tissues were sliced in 12 μm thick sections and mounted on glass slides. Slides were then washed with TBS with 0.025% Triton X-100 and blocked in 10% normal serum with 1% BSA in tris(hydroxymethyl)aminomethane-buffered saline (TBS) for 2 h. Slides were then rinsed and stained with rabbit anti-mouse CD31 primary antibody and goat anti-rabbit Fc secondary antibody-FITC conjugate according to instructions provided by Abcam. Coverslips were then mounted on microscope slides using Prolong Gold antifade solution containing DAPI for staining of cell nuclei. Images were acquired on a Leica SP8 confocal laser scanning microscope equipped with a 40× oil immersion lens and appropriate filters.

11. References

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