# Enhanced Cellular Uptake and Gene Delivery of Glioblastoma with Deferoxamine-coated Nanoparticle/Plasmid DNA/Branched Polyethylenimine Composites

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# Electronic Supplementary Information (ESI)

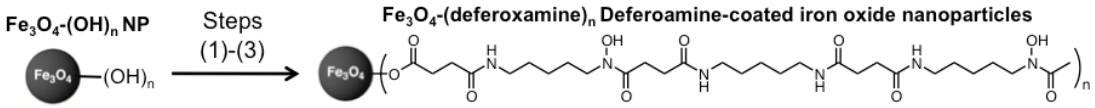
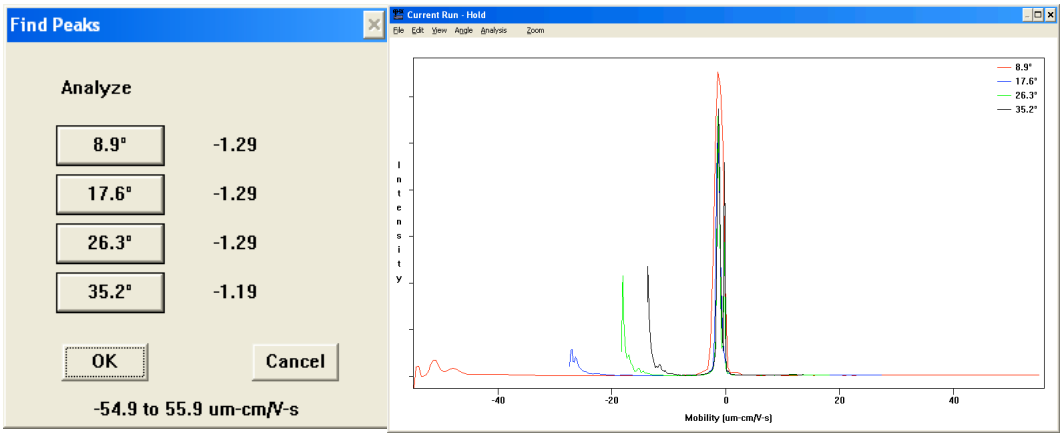



**Fig S1.** Synthetic scheme of deferoxamine-coated superparamagnetic iron oxide nanoparticles. Step (1): succinic anhydride / DMF / rt / 24 h; step (2): *N*,*N*'-dicyclohexylcarbodiimide / *N*-hydroxysuccinimide / THF / rt / 24 h; and step (3): deferoxamine / DMF / rt / 24 h.

**(A)**

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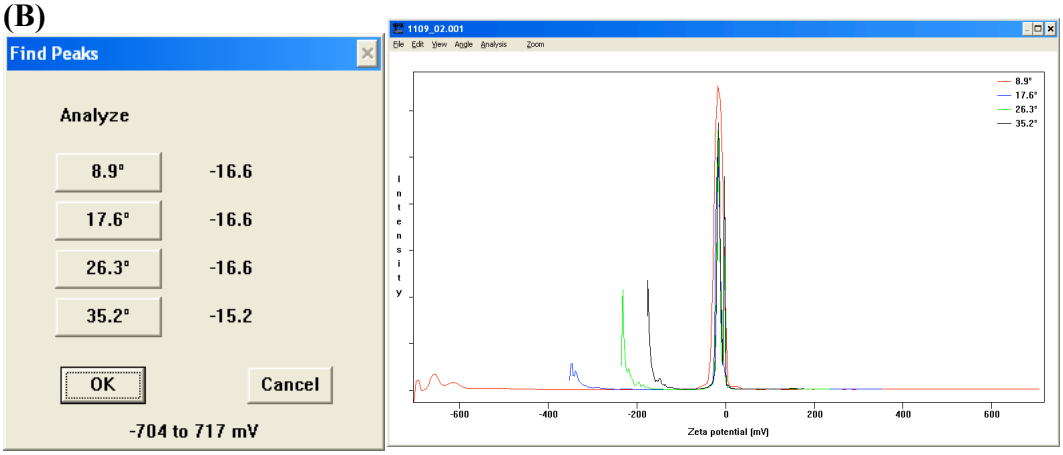
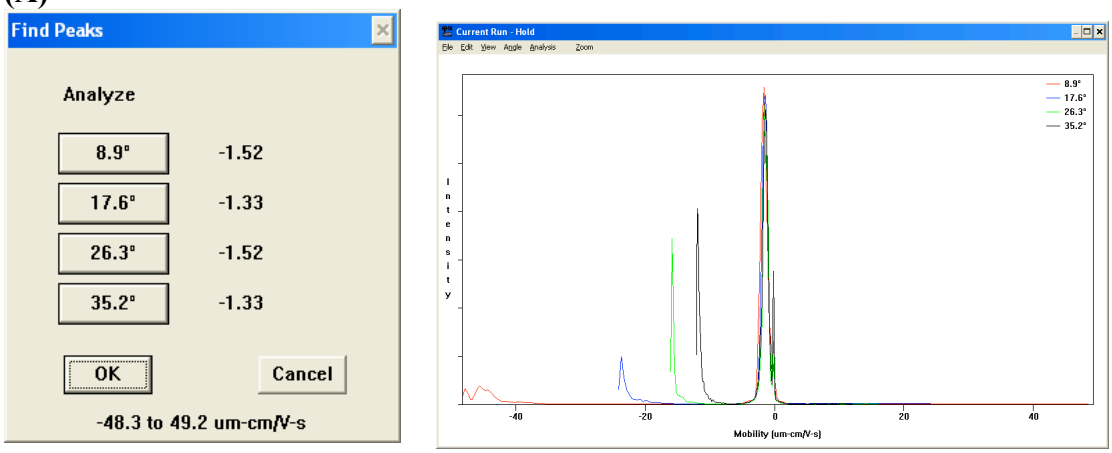



**Fig S2. (A)** Mobility analysis data and **(B)** zeta-potential analysis data (–16.3±0.5 mV) of ternary complex (0.2 μg **PEI**/0.5 μg **pDNA**/0.1 μg **NP**) in PBS (pH 7.4) which was equilibrated for two days.

**(A)**

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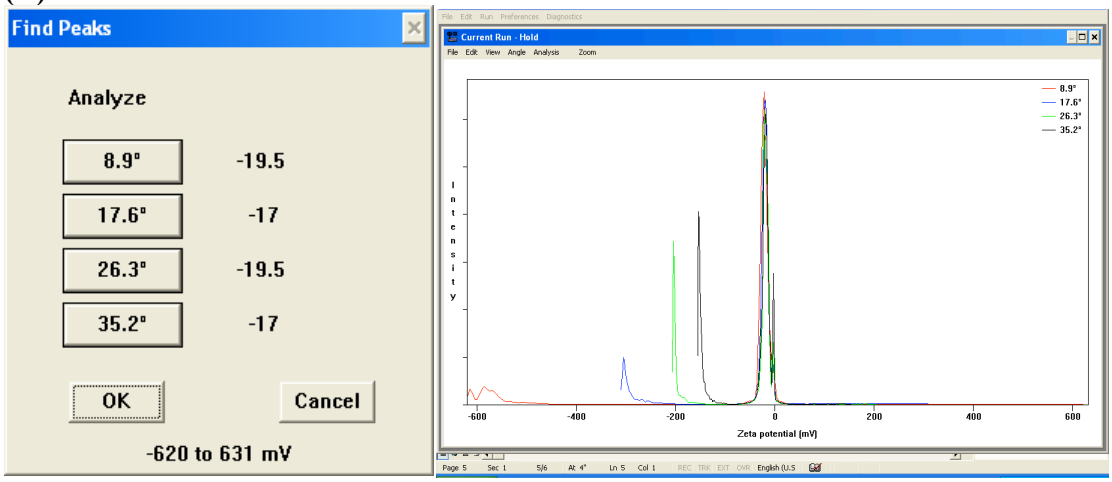


**Fig S3. (A)** Mobility analysis data and **(B)** zeta-potential analysis data (–18.3±1.2 mV) of ternary complex (0.1 μg **PEI**/0.5 μg **pDNA**/0.1 μg **NP**) in PBS (pH 7.4) which was equilibrated for two days.

## Experimental procedures

*General*

Unless otherwise stated, all chemicals were purchased from Aldrich or Acros and used without further purification. All reactions were carried out under high purity (99.9%) nitrogen atmosphere. Deionized water was obtained from Barnstead RO pure system and was bubbled with high purity nitrogen for at least 30 min before use.

*Characterization*

Transmission electron microscopy (TEM) photographs were taken on a FEI CM120 microscope at an accelerating voltage of 120 kV and a high-resolution transmission electron microscope (HRTEM, Tecnai F20, FEI) at an accelerating voltage of 200 kV. Infrared (IR) spectra were recorded in the wavenumbers ranging from 4000 to 500 $cm^{-1}$ with a Nicolet Model 759 Fourier Transform Infrared (FT-IR) spectrometer using a KBr wafer. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was performed on Optima 4300 DV ICP-OES. Samples were dissolved in 2% HCl solution with a few drops of $SnCl_2$ solution. Iron absorption was observed at 238.204 nm. The iron contents in a dispersed nanoparticle solution (in terms of g/mL) and in each nanoparticle (in terms of %) were determined. Zeta (ζ) potentials and mobilities were analysized by a Beckman Coulter Delsa 440SX zeta-potential analyzer. Magnetic resonance relaxometry of the nanoparticles was performed by using a clinical 3.0-T clinical whole-body magnetic resonance unit (Achieva; Philips Medical Systems, in combination with a knee radio frequency coil for excitation and signal reception. $T_2$ relaxation times were measured by using a standard Carr–Purcell–Meiboom–Gill pulse sequence (repetition time [TR] = 2000 ms, echo time [TE] range = 30–960 ms, 32 echoes, field-of-view [FOV] = 134 × 67 $mm^2$, matrix =

128 × 64, slice thickness = 5 mm, number of excitations = 3). $T_2$ relaxation times were calculated by a linear fit of the logarithmic region off interest signal amplitudes versus TE. The $T_2$ relaxivities ($r2$) were determined by a linear fit of the inverse relaxation times as a function of the iron concentrations used.

*Synthesis of deferoxamine-coated $Fe_3O_4$ nanoparticle*

Superparamagnetic iron oxide nanoparticles ($Fe_3O_4$-$(OH)_n$ NPs) with average diameter of 8-10 nm were synthesized according to literature procedures.[S1,S2] These $Fe_3O_4$-$(OH)_n$ NPs were first treated with an excess of succinic anhydride in DMF at r.t. for 24 h with gentle shaking, followed by repeated separation and washing with a magnet. Then, the black residue was then washed with anhydrous THF and redispersed in anhydrous THF with ultrasound. Excess of *N*,*N*'-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were added to the mixture and gently shaked for 24 h at r.t. The nanoparticles were magnetically separated and washed repeatedly with THF twice and DMF once. Subsequently, an excess of deferoxamine was added to the mixture and gently shaked for 24 h at r.t. The nanoparticles were magnetically separated and washed repeatedly with DMF and EtOH. The residue was dried under high vacuum to afford the deferoxamine-coated $Fe_3O_4$ nanoparticles.

*Plasmid DNA*

All the plasmid DNA preparations used in the present study for transfection experiments were prepared using the QIAprep Spin Miniprep Kit (QIAGEN) with $A_{260}/A_{280}$ ratio larger than 1.8. pDNA pEGFP-C1 (~4.7kb) was encoded a reporter of a red-shifted variant of wild-type green fluorescence protein in mammalian cells.

After transfection for 24 h, the green fluorescence of the transfected cells was visualized. pDNA pRL-CMV (~4.0kb) encoded a reporter of *Renilla* luciferase (RLU) in various cell types. After transfection for 24 h with U138MG cells, luminescence was detected by a luminometer. The activity of RLU was normalized against total cellular protein per well and expressed as RLU/mg protein. As markers, the fluorescence or luminescence intensity is directly proportional to the amount of GFP/RLU. By the strong, enhanced and constitutive expression of the reporters, the signals can be easily detected. They are optimized so that the reporters can be expressed in a variety of cell types/lines. By using two signal detection methods, any reduction or enhancement of DNA uptake by nanoparticles can be estimated by different aspects.

*Synthesis of ternary complex*

A stock solution of branched PEI was prepared with a concentration of 0.010 μg/μL in water. By serial dilution, the branched PEI of different concentrations was added to 250 μL medium containing pDNA. After incubation for 1 h, pre-ultrasonicated, deferoxamine-coated nanoparticles of known particle and iron concentrations in water was added to the mixture and further incubated for 2 h to obtain the ternary complex.

*Cell culture*

Human glioblastoma cells (U87MG and U138MG) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured with DMEM (Invitrogen) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and in a humidified 5% $CO_2$ atmosphere.

*Cellular uptake*

About 5,000 U87MG cells were seeded into each well of the 96-well plates (for MTT assay) and about 50,000 U138MG cells were seeded into each well of the 24-well plates (for luciferase). After incubation for 12 h, the culture medium was replaced with the serum-free DMEM containing different ternary complexes. The cells were further incubated for 24 h.

*Prussian blue staining*

After incubation with iron oxide-containing ternary complexes, the cells were washed with PBS to remove any free complexes. Cells were fixed for 40 min using 4% paraformaldehyde. Then, cells were washed with PBS and incubated with freshly prepared Perls' reagent (4% potassium ferrocyanide/12% HCl, 1:1 v/v) for 30 min. The cells were washed three times with PBS, counterstained with neutral red (0.02%), and subsequently observed by an inverted optical microscope.

*Intracellular iron content measurement*

Colorimetric method was used to study the iron concentration for iron oxide nanoparticles or cell samples labeled with ternary complexes. For the intracellular iron content quantification, after the cells were incubated with ternary complex for 24 h, the cells were washed, collected, and counted. After 4500 *g* centrifugation for 5 min, the collected cell pellets were dispensed in 100 μL 12% HCl solution and incubated at 60 °C for 4 h. After incubation, the suspension was centrifuged at 12,000 × *g* for 10 min, and the supernatants were collected for iron concentration quantification. A volume of 50 μL of sample solution was added into the wells of a 96-well plate, and then 50 μL of 1% ammonium persulfate was added to oxidize the

ferrous ions to ferric ions. Finally, 100 μL of 0.1 M potassium thiocyanate was added to the solution and incubated for 5 minutes to form the red color iron-thiocyanate. The absorption was read by a microplate reader (Model 3550; Bio-Rad, Richmond, CA) at a wavelength of 490 nm.

*Biocompatibility assay*

The cytotoxicities of cells incubated with different ternary complexes were examined by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay in U87MG cells. A total of 5,000 U87MG cells were seeded into the wells of a 96-well plate. After incubation for 12 h, the medium in the wells was replaced with 100 μL serum-free DMEM containing different ternary complexes. After incubation for 24 h, cells were washed with PBS once, and 100 μL fresh medium containing 0.5 mg/mL MTT was added into each well. After incubation for 3 h, the medium was removed, and formazan crystals were dissolved with 150 μL dimethyl sulfoxide for 10 min on a shaker. A flat magnet was put under the plate to attract the magnetic particles to the bottom of the well. Then, 100 μL of supernatant was transferred to another 96-well plate. Absorbance of each well was measured by a microplate reader (Model 3550, Bio-Rad, Richmond, CA) at a wavelength of 540 nm. The relative cell viability (%) for each sample related to control well was calculated.

*In vitro MRI*

*In vitro* MRI was performed with U87MG cells incubated with ternary complexes for 24 hours. After washing with PBS, the cells were trypsinized and counted. Different numbers (0, 12.5k, 25k, 50k, 100k, 150k, and 300k) of cells were placed in an Eppendorf tube (1.5 mL) seprarately. After centrifugation at 4000 rpm for 5 min, the

Eppendorf tubes were placed perpendicular to the main magnetic induction field ($B_0$) in a 20 cm × 12 cm × 8 cm water bath. MRI was performed with a 3.0-T clinical whole-body magnetic resonance unit (Achieva; Philips Medical Systems, Best, The Netherlands), using a transmit–receive head coil. The magnetic resonance sequence was a two-dimensional gradient-echo sequence with TR/TE = 400/48 ms, flip angle = 18°, matrix = 512 × 256, resolution = 0.45 × 0.45 mm, slice thickness = 2 mm, and number of excitations = 2. Sagittal images were obtained through the central section of the bottom tips of the Eppendorf tubes. The areas of signal void at the bottom of the Eppendorf tubes due to U87MG cells transfected with superparamagnetic iron oxide nanoparticle-containing ternary complexes from which the nanoparticles are MRI-responsive contrast agents.[S1,S2] Cell pellets were compared visually.

**References**

**S1.** H.-H. Wang, Y.-X. J. Wang, K. C.-F. Leung, D. W. T. Au, S. Xuan, C.-P. Chak, S. K. M. Lee, H. Sheng, G. Zhang, L. Qin, J. F. Griffith and A. T. Ahuja, *Chem. Eur. J.*, 2009, **15**, 12417-12425.

**S2.** X.-M. Zhu, Y.-X. J. Wang, K. C.-F. Leung, S.-F. Lee, F. Zhao, D.-W. Wang, J. M. Y. Lai, C. Wan, C. H. K. Cheng and A. T. Ahuja, *Int. J. Nanomed.*, 2012, **7**, 953-964.