SUPPLEMENTARY INFORMATION

Anti-HER2/neu Peptide-Conjugated Iron Oxide Nanoparticles for Targeted Delivery of Paclitaxel to Breast Cancer Cells

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1. Experimental section

1.1. Materials

The 2-iminothiolane (Traut's reagent) was purchased from Molecular Biosciences (Boulder, CO). NHS-PEG_{12}-maleimide was purchased from Thermo Fisher Scientific (Rockford, IL). PTX was purchased from LC Laboratories (Woburn, MA). AHNP (FCDGFYACYMDV) was synthesized by GenScript Inc. (Piscataway, NJ). Wheat germ agglutinin-Alexa Fluor 555 conjugate was purchased from Life technologies (Grand Island, NY). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

1.2. Synthesis of IONP-PEG-NH_2 and surface functionalization

PEG2000-NH_2 monolayer-coated IONPs (IONP-PEG-NH_2) were synthesized using the method reported previously. To prepare IONP-PTX-AHNP, AHNP was conjugated to IONP-PEG-NH_2 using a heterobifunctional PEG linker. To functionalize IONP-PEG-NH_2 (2 mg), AHNP (368 μg, 2 mg/mL in DI water and diluted with equal volume of PBS) was thiolated by 2-iminothiolane water solution (4.2 μL, 10 mg/mL) for 1 h to form AHNP-SH. Concurrently, solution of NHS-PEG_{12}−maleimide (1.42 μL, 250 mM) was added to IONP-PEG-NH_2 (1 mg of Fe/mL in PBS) and allowed to react for 30 min. Unreacted PEG was removed using a PD-10 desalting column (GE Healthcare, Piscataway, NJ) equilibrated with PBS. PEG−maleimide modified IONP-PEG-NH_2 were then mixed with the AHNP-SH and allowed to react for 30 min before removing unreacted AHNP using S-200 sephacryl resin equilibrated with 10 mM of MES buffer (pH 4.6) to obtain IONP-AHNP. Then the conjugation of carboxymethylated beta-cyclodextrin (CM-β-CD) onto

IONP-AHNP proceeded as follows. First, CM-β-CD was obtained from modifying β-CD using a previously reported method. IONP-AHNP (~2 mg, 1 mg/mL in MES buffer) was then allowed to react with CM-β-CD (1.7 mg) for 4 hrs in the presence of EDC (0.7 mg) and NHS (0.2 mg) before purification with S-200 column equilibrated with distilled water to obtain IONP-CD-AHNP. Finally, PTX was loaded onto IONP-CD-AHNP to obtain IONP-PTX-AHNP. PTX dissolved in DMSO were added into IONP-CD-AHNP (1 mg of Fe/mL in DI water) (final concentrations of PTX was 500 μM), and incubated for 24 hrs with stirring at room temperature. The mixture was then centrifuged to remove large aggregates and filtered through 0.2 μm PTFE syringe filters. NPs were finally purified by S-200 column equilibrated with PBS. For preparation of IONP-AHNP-PTX-Cy5, 2 mg/mL IONP-PEG-NH_2 in PBS was firstly mixed with 1.9 μL NHS-Cy5 (5 mg/mL in DMSO) and reacted for 2 hrs followed by S-200 column purification equilibrated with PBS. The IONP-Cy5 was then conjugated with AHNP and CM-β-CD and loaded with PTX according to aforementioned procedure. For preparation of IONP-AHNP-Cy5.5, AHNP was firstly conjugated onto IONP-PEG-NH_2 followed by reaction with NHS-Cy5.5 (NP/NHS-Cy5.5 = 2 mg/95 μg). IONP-Cy5.5 was prepared by conjugation of IONP-PEG-NH_2 with NHS-Cy5.5 only without AHNP conjugated.

1.3. Characterization of NP conjugates

PTX bound on NPs was extracted by acetonitrile and quantified by HPLC. IONP-PTX-AHNP solution (100 μL) was freeze-dried and resuspended in acetonitrile (100 μL) with brief ultrasonication to extract PTX. The suspension was then centrifuged (15000 g, 2 min) to remove NPs. Supernatant was transferred to a new tube for HPLC analysis. XBridge

BEH C18 column was used (130 Å, 3.5 μm, 4.6 mm × 100 mm) (Waters Corporation, Milford, MA). The A solvent was 5% ACN in 0.1% v/v acetic acid and the B solvent was 95% ACN in 0.1% v/v acetic acid. The gradient was 60–100% B in 4 min. The equilibration time was 3 min at 60% 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 254 nm was used as a detection wavelength. The loading of AHNP onto NPs was quantified by mass spectroscopy (Bruker Esquire ion trap mass spectrometer, positive mode) with integration of extracted ions. Briefly, the concentrations of free AHNP-SH before and after conjugation reaction was quantified using an AHNP-SH standard curve. The amount of conjugated AHNP-SH were obtained by subtracting unreacted AHNP-SH from total AHNP-SH.

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.

1.4. Animal study

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. SK-BR-3 (ATCC) was injected subcutaneously into right flanks of athymic nude mice with 5×10^6 cells per mouse. Tumors were allowed to grow for 9 days before NPs injections. Tumor size was measured by caliper and calculated by the following equation: $V = {ab^2}/{2}$, where *a* is length and *b* is width of tumor. NPs (IONP-AHNP-Cy5.5 or IONP-Cy5.5) were injected intravenously into mice with amount of 0.5 mg iron equivalent. Fluorescence images as well as photographs were taken 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.

1.5. Cell culture

SK-BR-3 and MDA-MB-231 human breast cancer 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.

1.6. Cellular uptake of NPs by flow cytometry analysis

SK-BR-3 or MDA-MB-231 cells were incubated with NPs for 1 hr (40 μg/mL Fe) followed by washing with cold PBS for 3 times. Cells were then trypsinized and resuspended in cold PBS and analyzed by flow cytometry (FACSCanto II, BD Biosciences).

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

SK-BR-3 or MDA-MB-231 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 1 hr. Cells were then fixed with 4% paraformaldehyde for 15 min at 37°C and stained with 5 μg/mL WGA-Alexa Fluor 555 (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).

1.8. Cell viability by Alamar Blue assay

Cells were seeded in a 96-well plate and incubated overnight in the growth conditions described in section 2.4. In the following day, the medium was replaced with a medium containing PTX (PTX was dissolved in DMSO to a concentration of 180 μM as stock solution), IONP-CTX, IONP-PTX-FL or IONP-PTX-FL or with medium control. Nine different drug concentrations (1800, 600, 200, 66.7, 22.2, 7.4, 2.5, 0.82 and 0.27 nM) were used, and samples at each concentration were ran in triplicate. The cells were incubated with PTX, IONP-AHNP, or IONP-PTX-AHNP 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 hrs. 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.

2. Supplementary figures

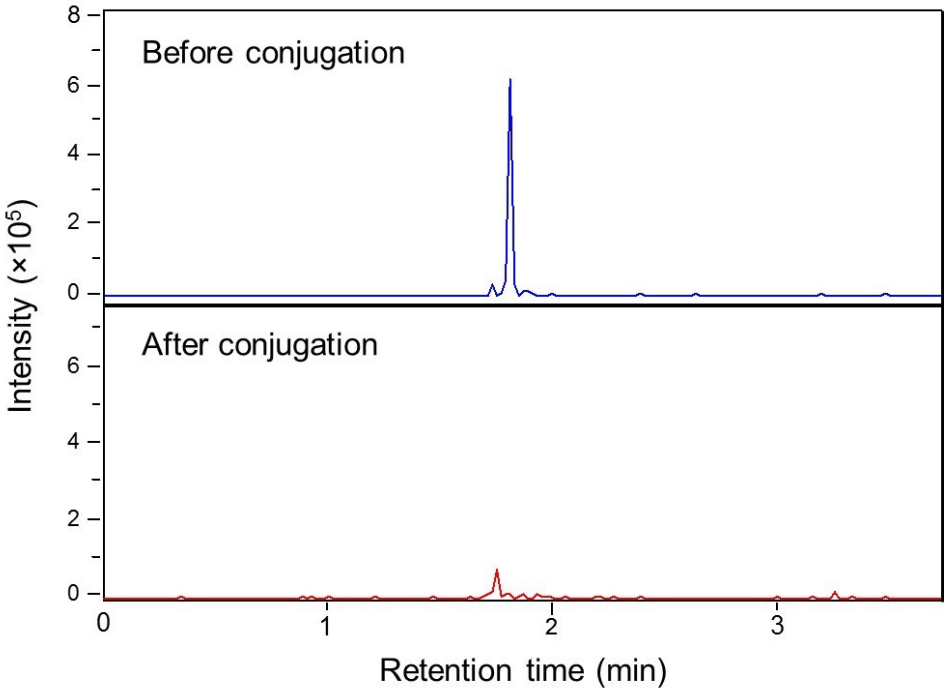

Figure S1. Chromatographs of extracted ions of AHNP-SH with positive mode (m/z = 1539.6) before and after NP conjugation by HPLC-MS. Y-axis indicates mass spectrometry intensities.