Bispecific Antibody Conjugated Manganese-Based Magnetic Engineered Iron Oxide for Imaging of HER2/neu- and EGFR-Expressing Tumors

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SUPPORTING INFORMATIONS

Synthetic procedures

Synthesis of MnMEIO-mPEG NPs

MnMEIO NPs were prepared according to the literature report [S1] using iron acetylacetonate and manganese chloride as the iron and manganese precursor respectively, oleic acid and oleylamine as the ligands, and benzyl ether as the solvent. MnMEIO-mPEG NPs were obtained by ligand exchange reaction. [S2] In the typical ligand exchange reaction, synthesized MnMEIO NPs (56 mg, 0.24 mmol) were dispersed in toluene (20 mL) in a round-bottom flask, followed by the addition of silane-NH_2-mPEG (600 mg, 0.24 mmol). The resulting solution was sonicated for 6 hrs at 50 °C. The silane-NH_2-mPEG-coated NPs were precipitated by addition of hexane and isolated via centrifugation (1,000 × g). After decanting the supernatant, the precipitate was re-dispersed in hexane for three times. Then, the particle was dispersed in deionized water and purified by dialysis against deionized water (M.W. = 50 kDa). Selected IR bands (ν in cm^{-1}): 3100-3700 (-OH & -NH_2), 2884 (-CH_2), 2820 (-CH_3), 1693 (C=O), 1410 (CH_2-COO^-), 1247 (-CH_2 & Si-C), 1202 (-OCH_3), 1177 (C-N), 1170-1000 (Si-O-R), 1110 (C-O-C), 1034 (C-O-C), 592 (Fe-O).

Synthesis of CyTE777

The CyTE777 dye was prepared according to the literature report. [S3] To a solution of IR-783 (250 mg, 0.33 mmol) in 6 mL of anhydrous DMF, 3-mercaptopropionic acid (40.7 μL, 0.47 mmol) and TEA (65.3 μL, 0.47 mmol) were added. The green solution was allowed to stir in the dark for 21 hrs, and the completion of the reaction was monitored by HPLC. A green solid was isolated by precipitation with cold ether and washed with cold ether (3 mL). The precipitations were dissolved in water and dried under vacuum (214 mg, 81.5% yield). 1H NMR

(300 MHz, CD3OD) δ (ppm): 1.76 (s, 12H), 2.00-1.92 (m, 10H), 2.56 (t, 2H, J = 6.9 Hz), 2.71 (t, 4H, J = 5.9 Hz), 2.90-2.87 (m, 4H), 3.10 (t, 2H, J = 7.0 Hz), 4.20 (t, 4H, J = 6.7 Hz), 6.30 (d, 2H, J = 14.3 Hz), 7.26 (t, 2H, J = 7.5 Hz), 7.33 (d, 2H, J = 7.8 Hz), 7.41 (t, 2H, J = 7.6 Hz), 7.48 (d, 2H, J = 7.4 Hz), 8.87 (d, 2H, J = 14.2 Hz). ESI-MS m/z calcd for $C_{41}H_{51}N_2O_8S_3$ 795.3, found 795.5.

Production of recombinant bispecific antibody

The recombinant tetravalent bispecific antibody (Bis) was obtained by stable transfection of the expression constructs in CHO-RD cells and reseeded in semi-solid medium for second run of stable clone selection. After 10~14 days of culture, colonies were imaged and picked using an automated robotic colony picker. A stable clone with high bispecific antibody expression was cultured in a shaker flask (5×10^5 cells/mL) after two runs of selection. The bispecific antibody was purified using protein A affinity chromatography.

Determination of bispecific antibodies binding affinity

The binding affinity of bispecific Ab for EGFR and HER2/neu was measured in enzyme-linked immunosorbent assay (ELISA) experiments. Soluble EGFRv3-ECD-hFc and Her2-ECD-hFc were coating on ELISA plates at 10 μg/mL and binding of bispecific antibodies was detected via a horseradish peroxidase-conjugated rabbit anti-c-myc antibody. The binding affinity (KD value) of bispecific antibodies was ~10 and ~3 nM for soluble EGFRv3-ECD-hFc and Her2-ECD-hFc, respectively, assayed in three times independent experiments. Data were analyzed through GraphPad Prism software.

Synthesis of MnMEIO-CyTE777-(Bis)-mPEG NPs and control NPs (MnMEIO-CyTE777-mPEG NPs, MnMEIO-CyTE777-(Her)-mPEG, MnMEIO-CyTE777-(Erb)-mPEG NPs, and MnMEIO-CyTE777-(Erb+Her)-mPEG NPs)

CyTE777 (0.8 mg, 1.00 μmol), EDC (0.38 mg, 1.98 μmol), and NHS (0.46 mg, 4 μmol) were dissolved in DMSO (5 mL) and allowed to react for 20 min at room temperature. Subsequently, MnMEIO-mPEG NPs (100 mg) were dispersed in deionized water (5 mL), added into the mixture, and shaken vigorously for overnight at 4 °C in the dark. Excess EDC was removed by centrifugation (1,000 × g) and dialyzed (M.W. = 12 kDa~14 kDa) against deionized water to yield MnMEIO-CyTE777-mPEG NPs. After that, the -COOH moiety of antibody (20 μL of bispecific (Bis) (1 mg/mL)) was activated by EDC/NHS. MnMEIO-CyTE777-mPEG NPs (100 mg) were dispersed in PBS buffer (5 mL), mixed with activated antibody solution, and reacted for overnight at 4 °C in the dark. After reaction, the crude mixture was purified by dialysis in PBS buffer (M.W. = 50 kDa) to yield MnMEIO-CyTE777-(Bis)-mPEG NPs. The control NPs (MnMEIO-CyTE777-(Her)-mPEG, MnMEIO-CyTE777-(Erb)-mPEG and MnMEIO-CyTE777-(Erb+Her)-mPEG NPs) were synthesized by following procedures as mentioned above by using Herceptin (Her), Erbitux (Erb), and both Erbitux and Herceptin (Erb+Her) antibody, respectively, with yield of MnMEIO-CyTE777-(Her)-mPEG, MnMEIO-CyTE777-(Erb)-mPEG and MnMEIO-CyTE777-(Erb+Her)-mPEG NPs. Solid contents of nanoparticles were evaluated by drying the solution (200 μL) at 40 °C for 3 days. [S4] Iron contents of nanoparticles were estimated by dissolving the dried samples in 70% nitric acid (50 μL), and subjecting to ICP-MS analysis. [S5]

Synthesis of MnMEIO-FITC-(Bis)-mPEG NPs and control NPs (MnMEIO-FITC-mPEG, MnMEIO-FITC-(Her)-mPEG, MnMEIO-FITC-(Erb)-mPEG, and MnMEIO-FITC-(Erb+Her)-mPEG NPs)

MnMEIO-FITC-(Bis)-mPEG NPs and control NPs (MnMEIO-FITC-mPEG, MnMEIO-FITC-(Her)-mPEG, MnMEIO-FITC-(Erb)-mPEG, and MnMEIO-FITC-(Erb+Her)-mPEG NPs) were synthesized by the procedures mentioned above with CyTE777 replaced by FITC for flow cytometry and confocal fluorescent microscopy. NHS-FITC (0.01 mg, 0.03 μmol) and EDC (0.38 mg, 1.98 μmol) were dissolved in DMSO (1 mL). Subsequently, NPs (100 mg) were dispersed in deionized water (5 mL) in a reaction tube, added into the mixture, and shaken vigorously for overnight at 4 °C in the dark. Subsequently, the free NHS-FITC and DMSO were removed by dialysis (M.W. = 50 kDa) against deionized water. Solid contents of nanoparticles were evaluated by drying the solution (200 μL) at 40 °C for 3 days. [S4]

Blocking study

All tumor cells (SKBR-3, A431, Colo-205, MCF-7, MDA-MB-231 cells) were were plated in 96-well plate at a density of 1×10^4 cells/well for 24 hrs. These tumor cells were pretreated with with excess Herceptin, Erbitux, and both antibodies, respectively, for 1 hr at 37 °C, following by incubated with MnMEIO-CyTE777-(Bis)-mPEG NPs for 1 hr. After incubation, the supernatants were removed and cells washed three times with PBS. The optical imaging was then acquired using an IVIS spectrum system. The emission at 820 nm was measured with an optimal excitation wavelength of 745 nm (1 sec, F-stop = 2 and small binning). Data is represented as mean ± SD (n = 4).

Supporting data

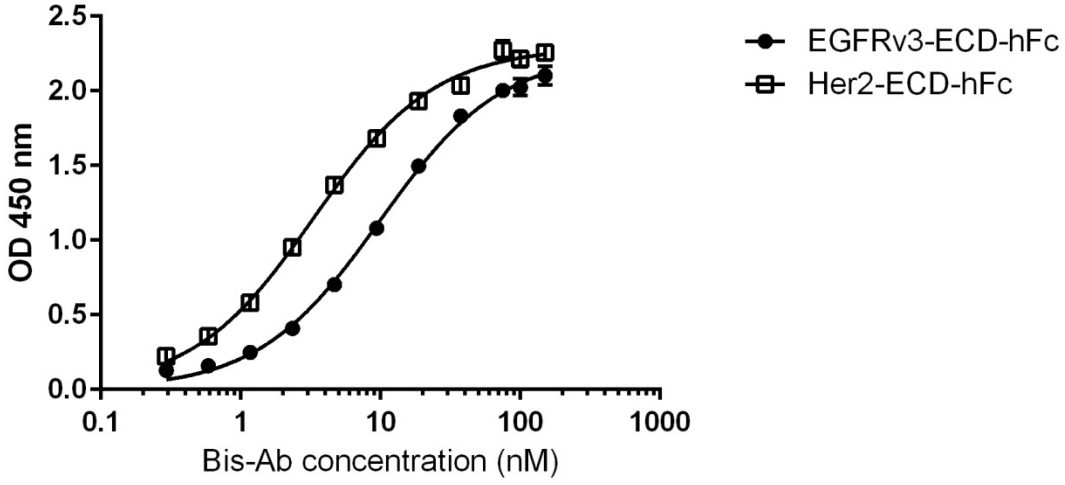

Fig. S1 The binding affinity study of bispecific antibody for EGFRv3-ECD-hFc and Her2-ECD-hFc.

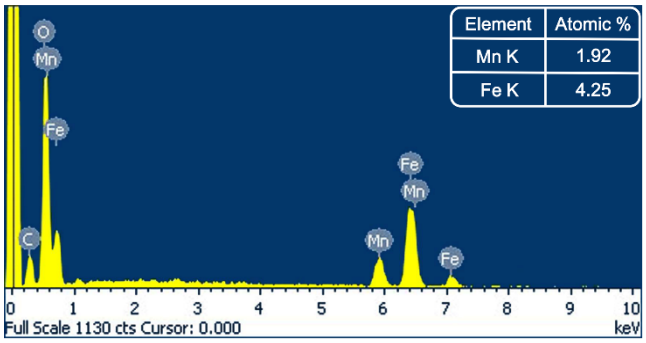

Fig. S2 EDS spectra of as-synthesized MnMEIO.

Table S1 Hydrodynamic size, zeta potential, relaxivities (r_1 and r_2), and r_2/r_1 ratio of MnMEIO-CyTE777-(Bis)-mPEG NPs, and the control NPs (MnMEIO-CyTE777-mPEG, MnMEIO-CyTE777-(Her)-mPEG, MnMEIO-CyTE777-(Erb)-mPEG, and MnMEIO-CyTE777-(Erb+Her)-mPEG NPs)

	Hydrodynamic size (nm)	Zeta potential (mV)	r_1 ($mM^{-1}s^{-1}$)	r_2 ($mM^{-1}s^{-1}$)	r_2/r_1
MnMEIO-CyTE777-mPEG	**27.4 ± 2.1**	**+ 15.2 ± 1.6**	**42.1 ± 1.7**	**293.5 ± 6.2**	**6.9**
MnMEIO-CyTE777-(Bis)-mPEG	**42.5 ± 1.8**	**- 3.8 ± 0.3**	**33.7 ± 1.2**	**229.8 ± 3.9**	**6.8**
MnMEIO-CyTE777-(Her)-mPEG	**45.1 ± 1.4**	**- 1.9 ± 0.2**	**32.2 ± 1.3**	**214.3 ± 3.7**	**6.7**
MnMEIO-CyTE777-(Erb)-mPEG	**38.7 ± 0.8**	**- 1.3 ± 0.1**	**37.6 ± 0.7**	**238.7 ± 4.1**	**6.3**
MnMEIO-CyTE777-(Erb+Her)-mPEG	**43.2 ± 0.4**	**- 3.1 ± 0.2**	**38.9 ± 0.1**	**241.8 ± 2.3**	**6.2**

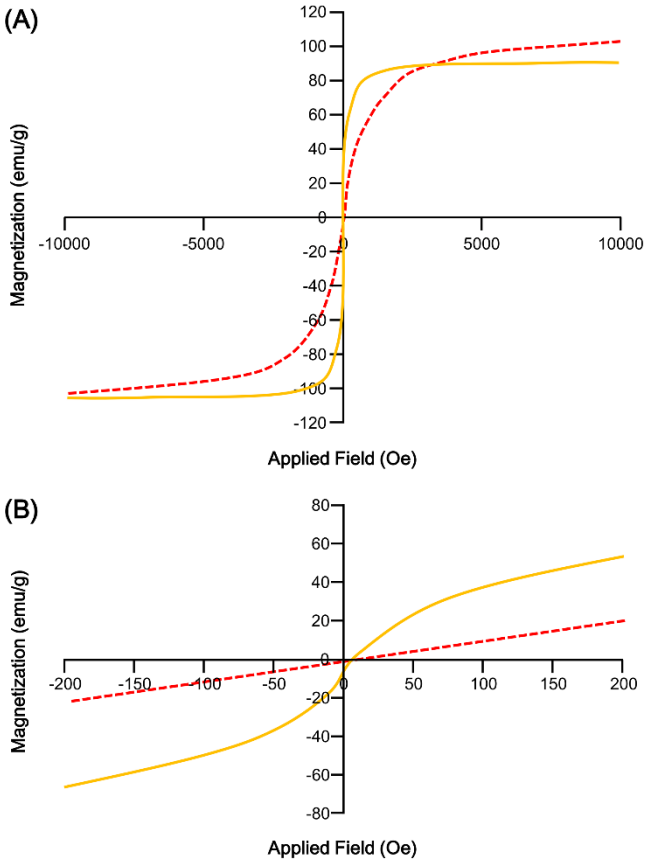

Fig. S3 (A) The hysteresis loop and magnetic susceptibility of MnMEIO-CyTE777-mPEG NPs (102.8 emu/g) and MnMEIO-CyTE777-(Bis)-mPEG NPs (83.3 emu/g). (B) Coercivity of MnMEIO-CyTE777-mPEG NPs (1.5 Oe) and MnMEIO-CyTE777-(Bis)-mPEG NPs (6.6 Oe).

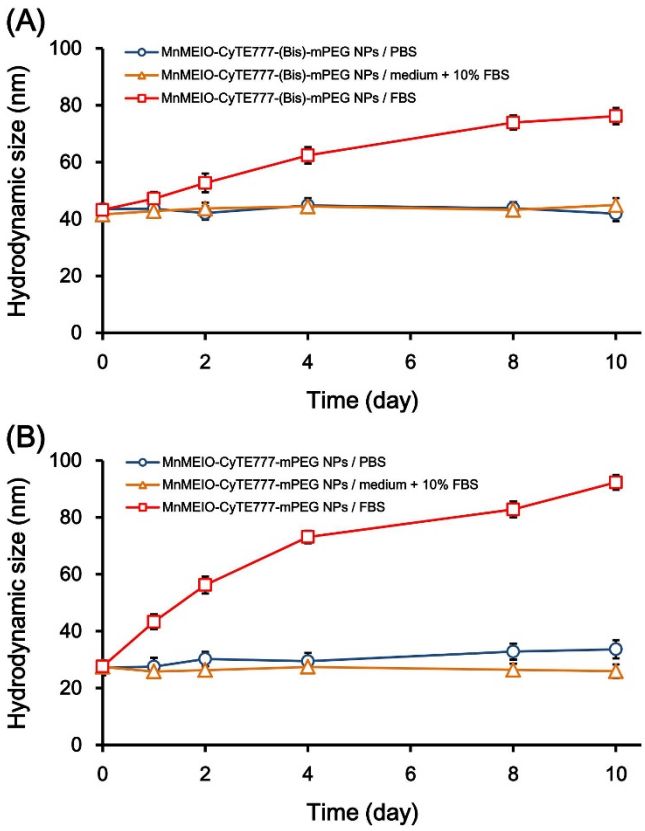

Fig. S4 Stability test of MnMEIO-CyTE777-(Bis)-mPEG and MnMEIO-CyTE777-mPEG NPs in PBS buffer, medium + 10% FBS, and FBS for ten days.

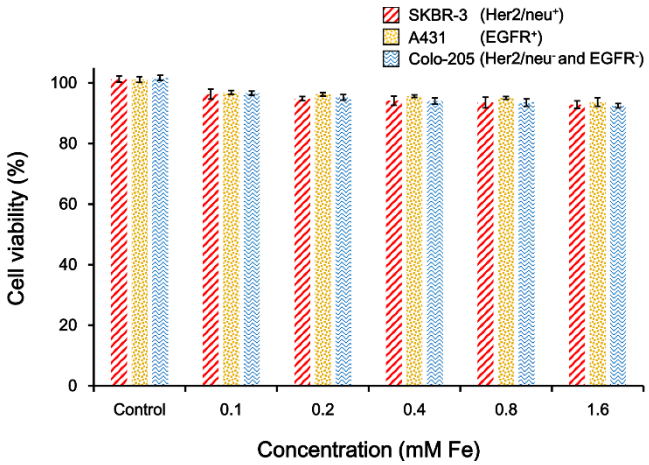

Fig. S5 *In vitro* cell viability of tumor cells upon MnMEIO-CyTE777-(Bis)-mPEG treatment at different Fe concentration in MTT assay.

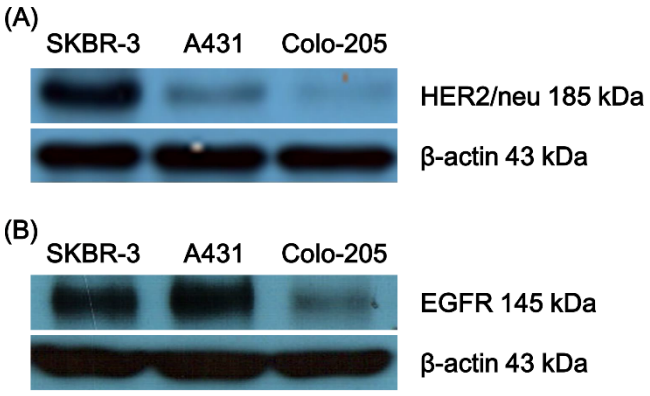

Fig. S6 Western blotting assays of (A) HER2/neu and (B) EGFR protein expression levels in SKBR-3, A431 and Colo-205 tumor cells.

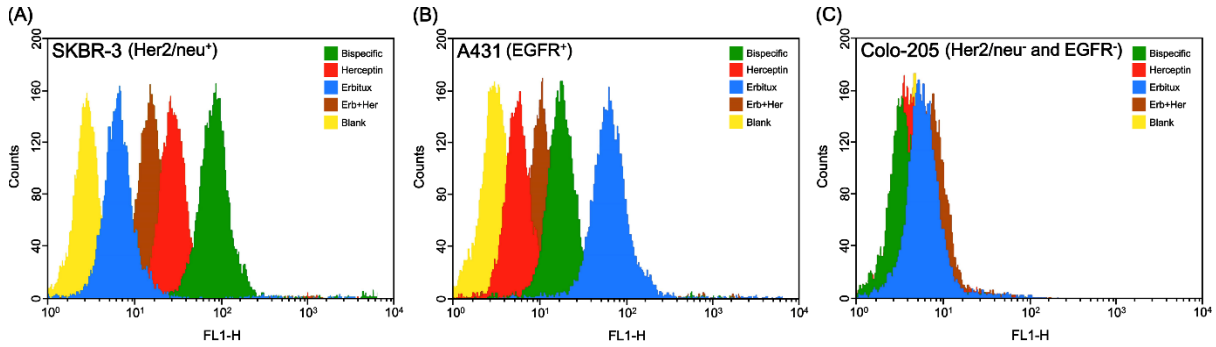

Fig. S7 Binding specificity of MnMEIO-FITC-(Bis)-mPEG NPs, and the control NPs (MnMEIO-FITC-mPEG (Blank), MnMEIO-FITC-(Her)-mPEG, MnMEIO-FITC-(Erb)- mPEG and MnMEIO-FITC-(Erb+Her)-mPEG NPs) with (A) SKBR-3, (B) A431, and (C) Colo-205 cells. In these experiments NPs (10 μg/mL) were incubated with cells for 1 hr at 4 °C. Cell-associated fluorescent intensities were analyzed with a FACScan flow cytometer.

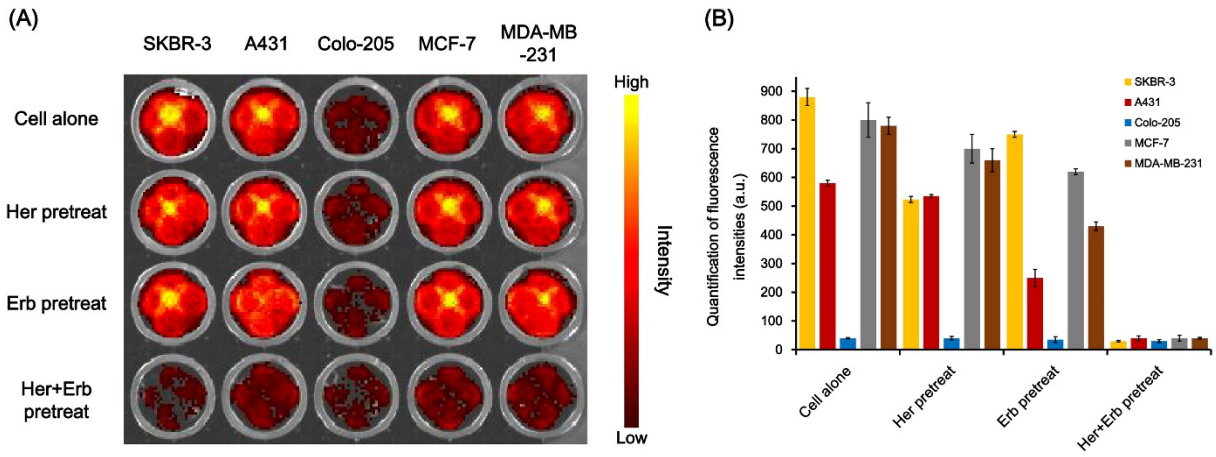

Fig. S8 Blocking study of SKBR-3, A431, Colo-205, MCF-7, and MDA-MB-231 cells pretreated with excess Herceptin, Erbitux, and both antibodies, respectively, for 1 hr at 37 °C, following by incubated with MnMEIO-CyTE777-(Bis)-mPEG NPs (10 μg/mL) for 1 hr.

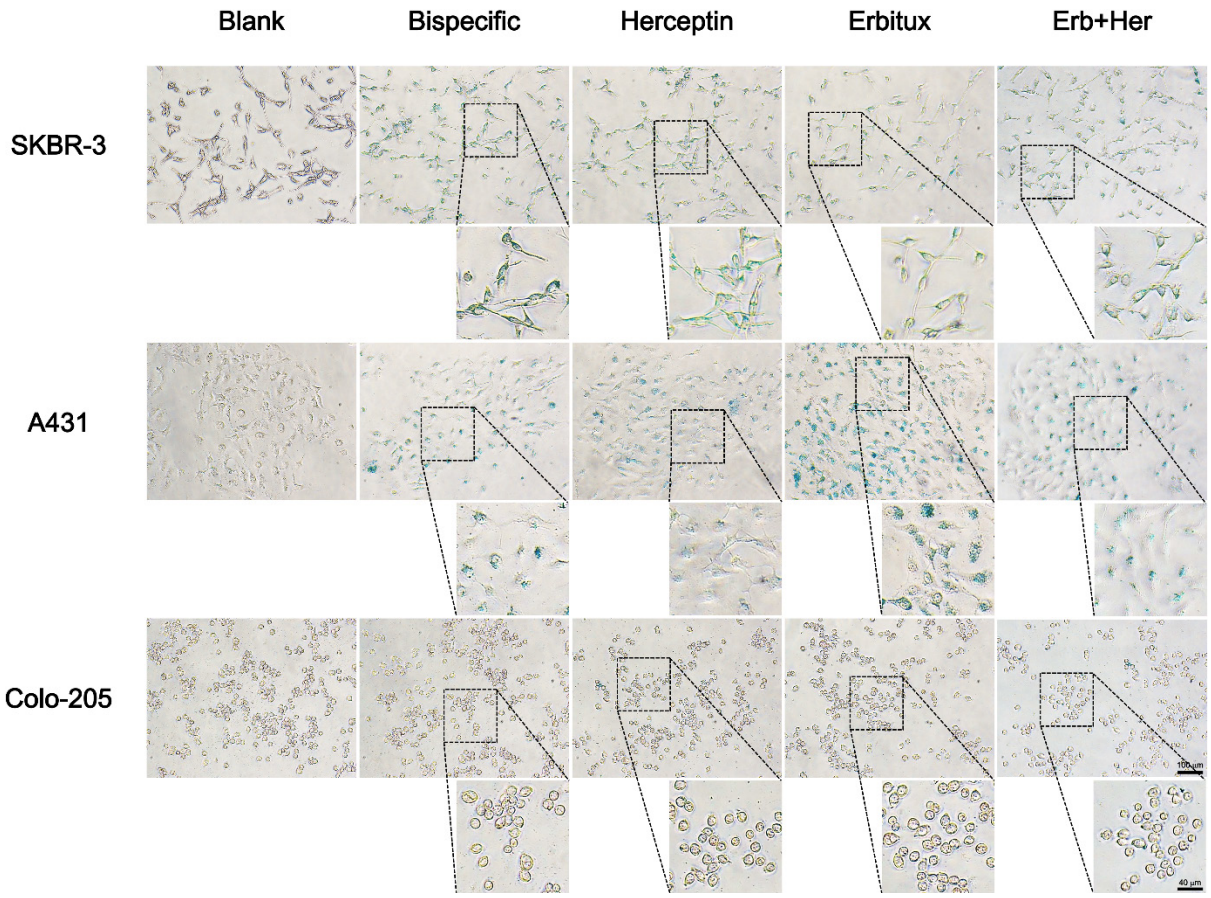

Fig. S9 Prussian blue staining of SKBR-3, A431, and Colo-205 cells treated with MnMEIO-CyTE777-(Bis)-mPEG NPs, and the control NPs (MnMEIO-CyTE777-mPEG (Blank), MnMEIO-CyTE777-(Her)-mPEG, MnMEIO-CyTE777-(Erb)-mPEG, and MnMEIO-CyTE777-(Erb+Her)-mPEG NPs) (10 μg/mL) for 1 hr at 37 °C.

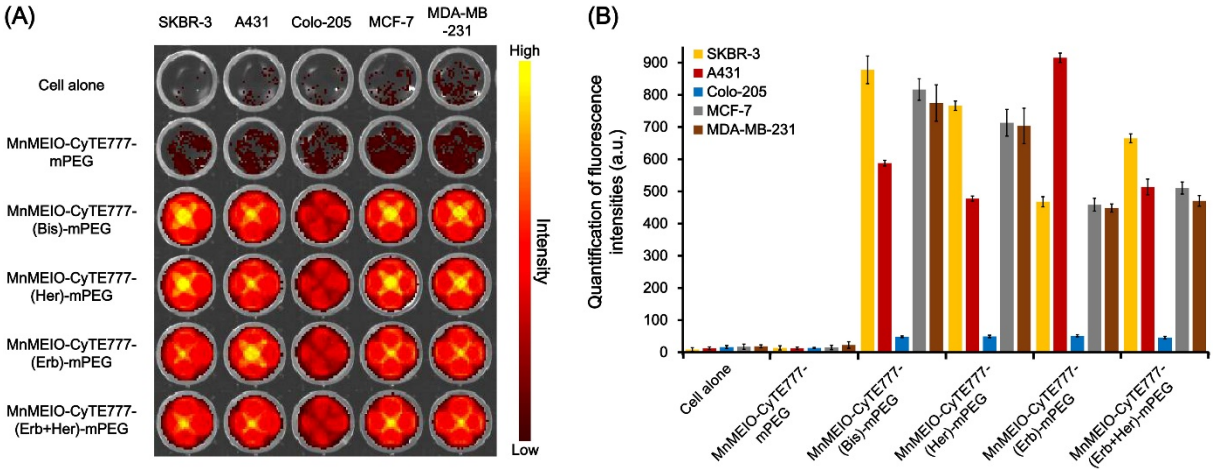

Fig. S10 *In vitro* optical imaging of SKBR-3, A431, Colo-205, MCF-7, and MDA-MB-231 tumor cells after the treatment with MnMEIO-CyTE777-(Bis)-mPEG NPs and control NPs (MnMEIO-CyTE777 -mPEG, MnMEIO-CyTE777-(Her)-mPEG, MnMEIO-CyTE777-(Erb)-mPEG, and MnMEIO-CyTE777-(Erb+Her)-mPEG NPs). In these experiments NPs (10 μg/mL) were incubated with cells for 1 hr at 37 °C. (A) Fluorescence images in a multi-well format were obtained by the IVIS spectrum system; (B) Quantitative analysis of fluorescence intensities of (A).

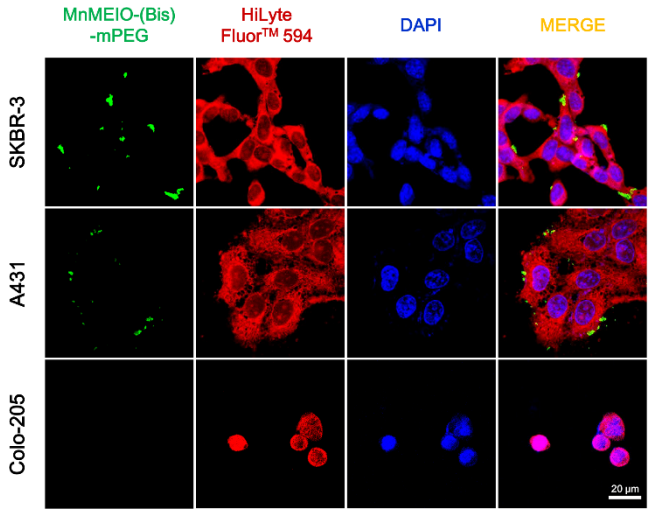

Fig. S11 Confocal microscopy images of SKBR-3, A431, and Colo-205 tumor cells incubated with MnMEIO-FITC-(Bis)-mPEG NPs (10 µg/mL) for 1 hr at 4 °C. HiLyte FluorTM 594 stained the cytoplasm in red and DAPI stained the nuclei in blue.

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