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## HER2-specific Aptide Conjugated Magneto-nanoclusters for Potential Breast Cancer Imaging and Therapy

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## Methods

### Materials.

All aptides, including the HER2 aptide, APT<sub>HER2</sub>, were chemically synthesized and purchased from AnyGen Co. (Gwangju, South Korea). Recombinant HER2 extracellular domain protein was from BenderMedSystems (Vienna, Austria); streptavidin was from New England BioLabs (Hertfordshire, UK); and human tumor necrosis factor (TNF)- $\alpha$  was from R&D Systems (Minneapolis, MN). Maleimide forms of the fluorescent dyes Alexa488 and Alexa594 were obtained from Life Technologies (Carlsbad, CA). Cell culture reagents (RPMI-1640, high-glucose Dulbecco's modified Eagle medium [DMEM], Opti-MEM reduced serum medium, fetal bovine serum [FBS]) were purchased from Life Technologies (Carlsbad, CA). Hybri-Care medium was purchased from the American Type Culture Collection (ATCC; Manassas, VA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide-(polyethylene glycol)2000] (Mal-PEG<sub>2000</sub>-DSPE), 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)2000] (MeO-PEG<sub>2000</sub>-DSPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-DMPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

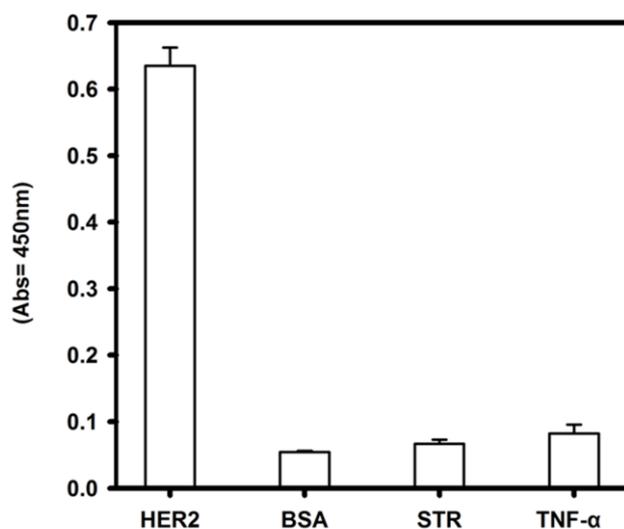
### Selection of APT<sub>HER2</sub>-displaying phages and ELISA analysis.

At final round following the rapid increase in titer ratio, a sufficient number of phage particles of each clone for subsequent analyses was prepared by first inoculating each of the 20 selected colonies into 1 mL of LB medium containing 50  $\mu$ g/mL ampicillin, 10  $\mu$ g/mL kanamycin and EX12 helper phages, and then culturing with vigorous shaking at 37 °C for 2 days. Cultures were centrifuged and the cleared supernatants were used for phage ELISA employing the target protein HER2; BSA was used as a control. For this ELISA, proteins (10  $\mu$ g/mL in PBS) were immobilized on a 96-well plate by incubating overnight at 4 °C, after which the plate was incubated for 2 h at 25 °C in blocking solution (2% skim milk in PBS). The 50  $\mu$ L of cleared supernatant was added to each well and plate was incubated for 2 h at 37 °C. After washing each well eight times with 0.1% PBST, HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, Piscataway, NJ), diluted in 0.1% PBST, was added and plates were incubated for an additional 1 h at 37 °C. After washing three times with 0.1% PBST, TMB substrate (BD Biosciences, San Diego, CA) was added for color formation, and color intensity was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Phage clones showing a high ELISA signal ratio (Signal<sub>HER2</sub>/Signal<sub>BSA</sub>) were evaluated further by analysis of DNA sequences.

### Specificity of APT<sub>HER2</sub>-displaying phage.

Using the selected *E. coli* clone harboring APT<sub>HER2</sub>-displaying phage DNA, a sufficient amount of APT<sub>HER2</sub>-displaying phage was obtained. Procedures of phage production and

purification were described above. The specificity of the APT<sub>HER2</sub>-displaying phage for HER2 was tested by ELISA. In addition to BSA, streptavidin and human TNF- $\alpha$  were used as controls. Proteins (all at 10  $\mu$ g/mL in PBS) were immobilized as described above (tested in quadrants). Purified, APT<sub>HER2</sub>-displaying phages ( $2 \times 10^{10}$  cfu), preincubated in 2% skim milk at 25 °C for 15 min, were loaded into each well, and ELISAs were carried out as described.



**Fig. S1.** Testing the specificity of APT<sub>HER2</sub>-displaying phages. Phage ELISA was carried out against human HER2, BSA, streptavidin (STR), and human TNF- $\alpha$ .

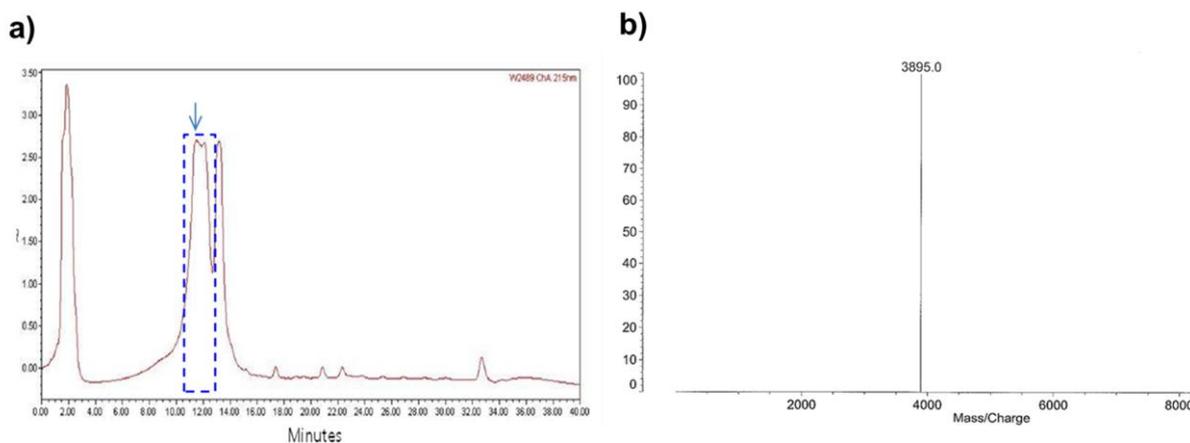
#### **Affinity measurement of APT<sub>HER2</sub> for recombinant HER2 protein.**

The affinity of APT<sub>HER2</sub> for HER2 protein was estimated by surface plasmon resonance analysis. Recombinant HER2 protein and BSA (control) were immobilized on different channels in a CM5 chip (GE Healthcare) using a conventional EDC/NHS conjugation method. Different concentrations of APT<sub>HER2</sub> (250, 500, 750, 1000 nM) dissolved in PBS containing 0.1% NaN<sub>3</sub> were applied to the CM5 chip installed in a BIAcore X instrument (GE Healthcare). All kinetic values, including affinity ( $K_d$ ), of the interaction were evaluated using BIA Evaluation 3.1 software (GE Healthcare).

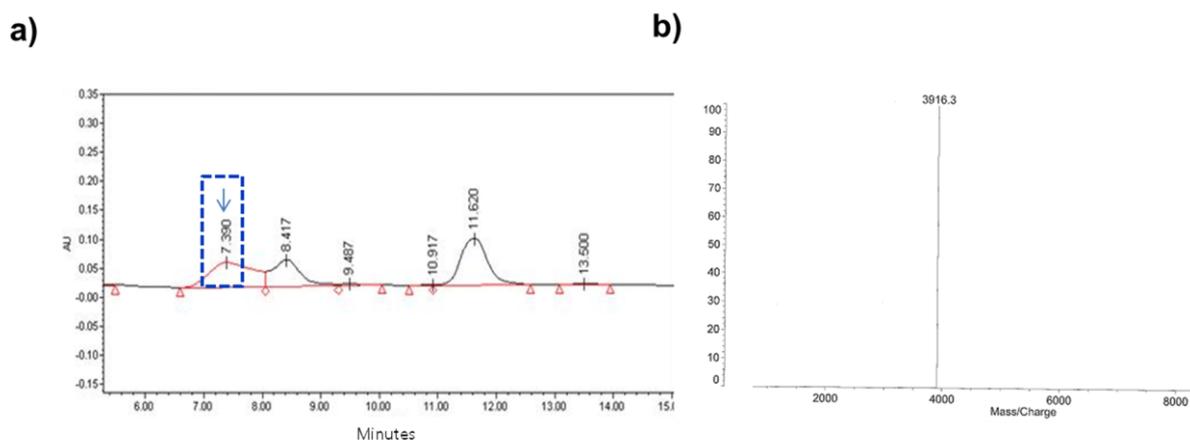
#### **Preparation of dye-labeled APT<sub>HER2</sub> and APT<sub>scrambled</sub>.**

For convenient conjugation of fluorescent dye without severely affecting affinity, a cysteine residue was introduced to the amine group of lysine in the middle of the tryptophan zipper structure. The resulting cysteinylated APT<sub>HER2</sub> (PFTPLAGSWTWENGK(*cys*)WTWKGHYRGKT) as well as cysteinylated APT<sub>scrambled</sub> (HASDRNGSWTWENGK(*cys*)WTWRGLHEQSD) were chemically synthesized. Alexa488-maleimide and each cysteinylated aptide were mixed in anhydrous dimethyl sulfoxide (DMSO) at a dye-to-aptide molar ratio of 1.5:1, and the reaction mixture was vortexed for 24 h at 25 °C. Alexa488-conjugated APT<sub>HER2</sub> and Alexa488-conjugated APT<sub>scrambled</sub> were

purified using a C18 high-performance liquid chromatography (HPLC) column and Agilent 1100 series HPLC system (Agilent, Santa Clara, CA), and the masses of HPLC peaks were analyzed using MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry (Bruker Co, Fremont, CA), as shown in Fig. S2 and S3. Alexa594-conjugated APT<sub>HER2</sub> was prepared using the same procedure.



**Fig. S2.** a) Purification of Alexa488-APT<sub>HER2</sub> (HPLC histogram). b) MALDI-TOF-MS spectrum of Alexa488-APT<sub>HER2</sub>.

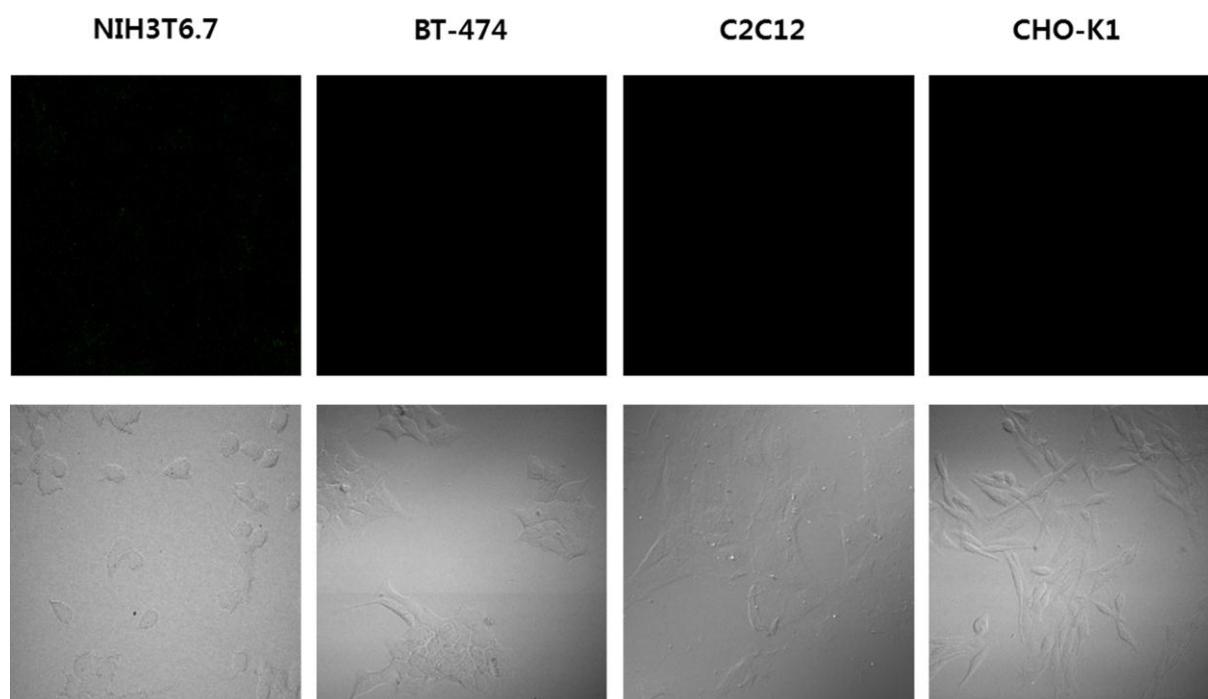


**Fig. S3.** a) Purification of Alexa488-APT<sub>scrambled</sub> (HPLC histogram). b) MALDI-TOF-MS spectrum of Alexa488-APT<sub>scrambled</sub>.

### Cell culture.

Parental NIH3T3 cells and NIH3T6.7 cells, an NIH3T3 cell line overexpressing human HER2, were grown as a monolayer in high-glucose DMEM. CHO-K1 (Chinese hamster ovary cell line) and C2C12 (mouse myoblast cell line) cells were grown as monolayers in RPMI-1640 medium. BT-474 (human breast carcinoma cell line; ATCC) cells were grown in Hybri-Care medium. All media were supplemented with 10% (v/v) fetal bovine serum (FBS)

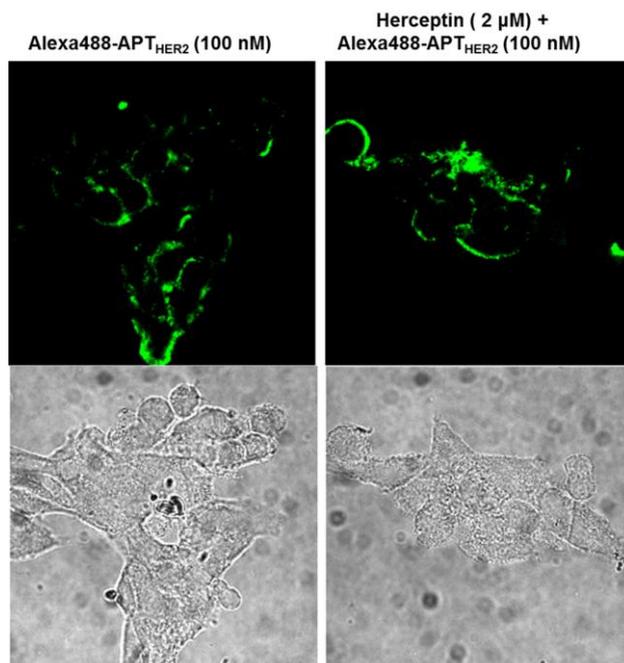
and antibiotics (100 IU/mL penicillin and 100 IU/mL streptomycin). Cells were incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.



**Fig. S4.** Confocal laser scanning microscope images taken after treatment of Alexa488-APT<sub>scrambled</sub>. HER2-overexpressing cell lines: BT-474 and NIH3T6.7; Negative control cell lines: CHO-K1 and C2C12.

#### Competition assay between Herceptin and Alexa488-APT<sub>HER2</sub>

NIH3T6.7 cells were subcultured on coverslip ( $\Phi = 18$  mm) for 30 h before treatment with either Alexa488-APT<sub>HER2</sub> or Herceptin plus Alexa488-APT<sub>HER2</sub>. For competition with Herceptin, cells were treated with serum-free DMEM containing 2  $\mu$ M of Herceptin for 1 h and subsequently treated with 100 nM of Alexa488-APT<sub>HER2</sub> for 30 min. Cells on coverslips were mounted with glycerol mounting medium after washing with PBS (x3). Only Alexa-APT<sub>HER2</sub> (100 nM)-treated cells were prepared and compared.



**Fig. S5.** Confocal laser scanning microscope images of NIH3T6.7 cells treated with either Alexa488-APT<sub>HER2</sub> alone or Herceptin (2 μM) plus Alexa488-APT<sub>HER2</sub> (100 nM).

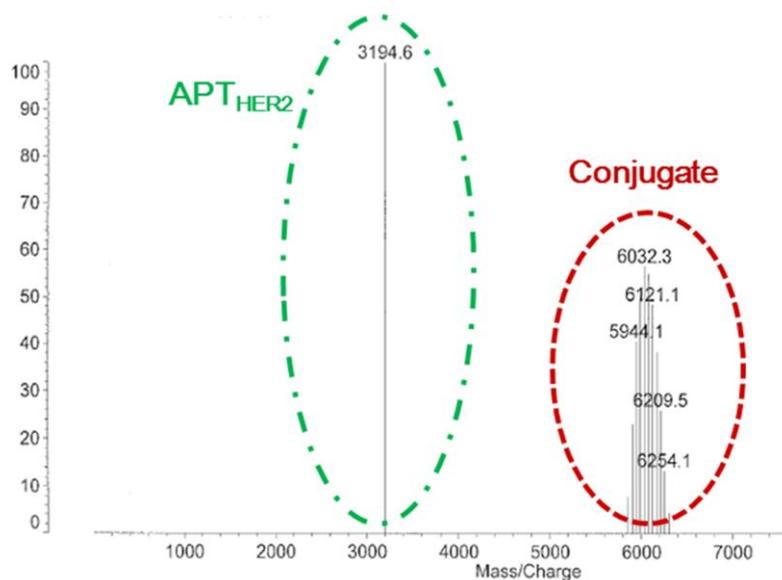
#### **Confocal imaging of tissues with dye-labeled APT<sub>HER2</sub>.**

A xenograft tumor model was created by subcutaneously injecting Balb/c nude female mice (6–7 wk old) with  $5 \times 10^5$  HER2-overexpressing NIH3T6.7 cells. An NIH3T6.7 cell-derived tumor ( $\sim 360 \text{ mm}^3$ ) was obtained 14 d after injection. As controls, liver and spleen tissue were also incised from the same mouse. Each tissue was molded with FSC22 frozen section compound (Leica Microsystems, Wetzlar, Germany) and placed at  $-80 \text{ }^\circ\text{C}$ . Frozen tissue molds were sliced in 20-μm thick sections using a LEICA CM1850 cryostat (LEICA, Wetzlar, Germany). Tissue sections were placed on glass slides and fixed by incubating with 4% PFA for 15 min. After washing sections three times with PBS, blocking solution (PBS containing 3% BSA) was applied for 2 h at  $25 \text{ }^\circ\text{C}$ . The excess BSA was removed by washing twice with PBS, and 50 nM Alexa594-APT<sub>HER2</sub> in 3% BSA was applied to tissue sections and incubated at  $25 \text{ }^\circ\text{C}$  for 2 h. Nonspecifically bound Alexa594-APT<sub>HER2</sub> was removed by washing four times with PBS. Air-dried tissue sections were mounted with fluorescence mounting medium and analyzed with an Olympus FluoView FV1000 fluorescence microscope.

#### **Synthesis of APT<sub>HER2</sub>-PEG<sub>2000</sub>-DSPE.**

Cysteinyllated APT<sub>HER2</sub> (0.5 mg) was dissolved in anhydrous DMSO (40 μL) and was dropped slowly into Mal-PEG<sub>2000</sub>-DSPE (1 mg) in chloroform (80 μL) solution under atmospheric conditions, and the mixture was gently vortexed for 12 h. After the reaction, organic solvents were evaporated using a speed vacuum concentrator (Hanil, Seoul, South

Korea), after which the aptide-conjugated phospholipid was analysed by MALDI-TOF-MS spectrometry (Shimadzu, Kyoto, Japan).



**Fig. S6.** MALDI-TOF-MS spectrum of APT<sub>HER2</sub> and APT<sub>HER2</sub>-PEG<sub>2000</sub>-DSPE conjugate.

#### ***In vitro* MRI phantom assay.**

NIH3T6.7 cells and parental NIH3T3 cells were grown to ~80% confluence in 100-mm cell culture dishes. HER2-overexpressing NIH3T6.7 cells were incubated with APT<sub>HER2</sub>-MNCs (Fe concentration, 200  $\mu$ M) at 37 °C in a humidified 5% CO<sub>2</sub> environment for 60 min, with and without pretreatment with free APT<sub>HER2</sub> (50  $\mu$ g/mL) for 30 min. HER2-negative parental NIH3T3 cells were treated with APT<sub>HER2</sub>-MNCs only for 60 min. Cells were washed three times with PBS, after which they were collected using a cell scraper and fixed with 4% formaldehyde. Next, T2-weighted, fast spin-echo images of cells were obtained by using a 3T MR scanner under the same conditions in previous reported article.<sup>1</sup>

#### **Cytotoxicity assay.**

NIH3T6.7 cells were seeded into 96-well plates at  $5 \times 10^3$  cells/well and incubated under standard conditions. Free DTX dissolved in DMSO diluted 100-fold with Opti-MEM at a relevant concentration and was put in wells separately. DTX@ APT<sub>HER2</sub>-MNCs at a same DTX concentration and APT<sub>HER2</sub>-MNCs (Fe concentration, 200  $\mu$ M) were dissolved in Opti-MEM and then added to each well. After co-incubation for 48 hr, samples were removed and then 100  $\mu$ L of E-Z cytotoxicity kit reagent (iTSBiO, Seoul, South Korea), which estimates the reduction of tetrazolium to formazan by the mitochondria of live cells, was added to each well of the plate. After 4 h, cytotoxicity was determined by measuring the absorbance of the solution at 570 nm using a microplate reader (FL600; Bio-TekInc., Winooski, VT).

## References

1. J. Park, S. Kim, P. E. Saw, I.-H. Lee, M. K. Yu, M. Kim, K. Lee, Y.-C. Kim, Y. Y. Jeong and S. Jon, *J. Control Release*, 2012, **163**, 111-118.