SUPPORTING INFORMATION for

Effect of ligand density, receptor density, and nanoparticle size on cell targeting

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SUPPLEMENTARY METHODS:

Materials

Azido-dPEG$_4$-NHS ester was purchased from Quanta BioDesign Ltd. (Powell, OH). The SPIO coating material, dextran T10, was purchased from Pharmacosmos (Denmark). Human breast carcinoma (MDA-MB-361) and human breast adenocarcinoma (BT-20) cells were obtained from the American Type Culture Collection (Manassas, VA). NIH/3T3 cells that were engineered to stably express the Her2/neu receptor (T6-17) were kindly provided by Dr. Mark Greene, MD/PhD (University of Pennsylvania). The 35 mm volume coil used for radiofrequency transmission and reception was purchased from Insight Neuroimaging Systems, LLC (Worcester, MA). All other reagents were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise noted.

SPIO NPs Synthesis and Amination

SPIO NPs were prepared by chemical coprecipitation, as previously described [1]. Briefly, 0.7313 g $FeCl_2$ and 1.97 g $FeCl_3$ were each dissolved in 12.5 mL diH_2O and added to 25g dextran T10 in 50 mL diH_2O, which was placed on ice. Ammonium hydroxide (15 mL) was slowly added to this mixture, turning the light yellow–colored solution black. This NP slurry was then heated to 90°C for 1 hour and cooled overnight. Purification of SPIO NPs was accomplished by ultracentrifugation of the mixture at 20,000 relative centrifugal force (RCF) for 30 minutes. Pellets were discarded, and the supernatant was subjected to diafiltration against greater than 20 volumes of 0.02 M citrate, 0.15 M sodium chloride buffer using a 100 kDa cutoff membrane filter (GE Healthcare). The purified particles were then cross-linked by reacting the particles (10 mg Fe/mL) with 25% (v/v) 10 M NaOH and 33% epichlorohydrin. After mixing for 24 hours, the particles were briefly dialyzed and then functionalized with amines by adding 25% ammonium hydroxide. This reaction was allowed to continue for another 24 hours followed by diafiltration as described above.

To synthesize 50 nm SPIO NPs, 0.7313 g $FeCl_2$ and 1.97 g $FeCl_3$ were each dissolved in 12.5 mL diH_2O and added to 25g dextran T10 in 50 mL diH_2O at 4°C. The rate of addition of ammonium hydroxide (15 mL) was halved, but still added until the light yellow–colored solution turned black. All other subsequent steps were carried out in an identical fashion.

SPIO Characterization

The hydrodynamic diameter of SPIO NPs was measured by dynamic light scattering using a Zetasizer Nano-z (Malvern Instruments, Malvern, UK). SPIO NPs were diluted in phosphate-buffered saline (PBS) to a concentration of approximately 0.5 mg Fe/mL and read in triplicate.

The values reported are the intensity peak values. The longitudinal (R1) and transverse (R2) relaxivity of each particle was calculated as the slope of the curves 1/T1 and 1/T2 against iron concentration, respectively. T1 and T2 relaxation times were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz). T1 measurements were performed by collecting 12 data points from 5.0 ms to 1000 ms. T2 measurements were made using τ = 1.5 milliseconds and two dummy echoes and fitted assuming monoexponential decay.

ADIBO Modification of SPIO NPs

Surface amines on SPIO NPs were reacted with the amine-reactive ADIBO-dPEG$_4$-NHS in 0.1 M sodium phosphate buffer, pH 9. Specifically, a 138 mM stock of ADIBO-dPEG$_4$-NHS was diluted 100-fold into a 50 μM solution of SPIO NPs. All nanoparticle solutions were mixed overnight at room temperature. SPIO NPs were purified via superdex 200 chromatography columns (GE Healthcare, Piscataway, NJ). The resulting ADIBO-SPIO NPs were incubated with 100 times molar excess of succinic anhydride to convert all remaining amines to carboxyl groups. ADIBO-SPIO NPs were subsequently purified on superdex200 chromatography columns, equilibrated with PBS.

Cloning of HER2-Affibody/AHNP recombinant protein into pTXB1 vector

Complementary oligonucleotides comprising the HER2-Affibody or AHNP coding sequence flanked at both ends by 15 base sequences homologous to the desired restriction sites of the destination vector were ordered from Integrated DNA Technologies (Coralville, IA) .To improve subsequent affinity column cleavage, an additional 9 base pairs encoding a “MRM” amino acid sequence were included in the oligonucleotides at the C-terminal end of both sequences. The full

nucleotide and amino acid sequence for the HER2-Affibody and AHNP can be found below (Figure S9 and S10, respectively). Oligonucleotides were incubated together at a final concentration of 5 μM and hybridized at room temperature for 30 minutes. The resulting sequence was agarose gel purified and directly ligated with gel-purified *Nde*I-*Xho*I double digested pTXB1 vector (New England Biolabs, Inc) via the CloneEZ kit (Genscript). Insertion of the HER2-Affibody and AHNP sequences was verified by DNA sequencing using the T7 promoter as the sequencing primer.

Expression and Purification of HER2-Affibody recombinant protein

The pTXB1-HER2-Affibody vector was transformed in Rosetta™ 2(DE3)pLysS Competent Cells (Novagen). Bacterial cell cultures were initially grown overnight in an air shaker (225 rpm) at 37 °C in 3 mL of LB medium. Cultures were scaled up to fifty mL of LB medium and grown overnight under the same conditions, and then inoculated into 1 L LB containing 50 mg/L of ampicillin. At $OD_{600\ nm}$ = 0.6, IPTG was added at a final concentration of 0.5 mM to induce T7 RNA polymerase-based expression. Cultures were allowed to express for 2 hours at 37 °C. Bacterial cultures were centrifugally pelleted at 10,000 x g for 5 minutes, resuspended in 5 mL of column buffer (20 mM Na-HEPES, 0.5 M NaCl, 1 mM EDTA, pH 8.5) containing 0.75 g/L lysozyme and 50 mM phenylmethylsulfonyl fluoride. Cells were lysed by pulse sonication on ice. Cells were centrifuged at 15,000 g for 30 minutes at 4 °C. Supernatant was collected and stored at -20 °C. For the following purification steps, all procedures were run at 4 °C. One mL of the supernatant was incubated for 10 minutes in a 10 mL Poly-Prep chromatography column (Bio-Rad, Hercules, CA) packed with 1 mL of chitin beads (New England Biolabs, Inc). Supernatant was allowed to pass through the column and chitin beads were washed with 50 mL

of column buffer at a flow rate of approximately 2 mL/min. Three mL of 50 mM MESNA was quickly passed through the column in order to evenly distribute the MESNA throughout the chitin beads, and flow was stopped. The column was incubated for 16 hours at 4 °C. HER2-Affibody proteins, now containing a C-terminal thioester, were eluted from the column in a total 4 mL buffer (0.1 M Tris-HCl, pH 8.5) and concentrated to a volume of 500 μL using an Ultracell 3,000 (Millipore, Billerica, MA). An analogous experimental protocol was used for the production and purification of AHNP peptides, with the exception of the IPTG concentrations used for induction, which were lowered from 0.5 mM to 0.35 mM final concentration.

Expressed Protein Ligation

Expressed protein ligation was carried about between the thioester containing HER2-Affibody and an azido-fluorescent peptide (AzFP) with an N-terminal cysteine. The sequence of the AzFP was NH_2-CDPEK(5-FAM)DSGK(N3)S-OH. The K(5-FAM) represents a lysine with a fluorescein covalently attached to its ε-amino group and the K(N3) represents a lysine with an azido group attached to its ε-amino group. The AzFP (0.1 mM) was incubated with approximately 0.01 mM HER2-Affibody or AHNP. The EPL reaction was mixed overnight at room temperature. For the HER2-Affibody, the EPL product and excess AzFPs were separated on a Superdex 30 chromatography column. For the AHNP-peptide, several rounds of washing using Ultracell 3,000 filtration columns were used to remove unreacted AzFP peptides.

Copper-Free Click Conjugation

ADIBO-SPIO NPs (1 mg/mL) were mixed with increasing concentrations of HER2-AzFP ligand (2.5 μM to 40 μM) in PBS, pH 7.4 at a final volume of 50 μL. Reactions were mixed overnight

at room temperature and then purified on Superdex 200 chromatography columns equilibrated with PBS. The extent of labeling was readily assessed by measuring the absorbance of the attached dye. These studies were initially carried out using an AzFP labeled with the near infrared fluorescent (NIRF) dye Hylite-750 (Figure S11). Utilization of the molar extinction coefficient of the NIRF dye allowed for calculations of the average number of ligands per SPIO NP. Analogous studies were carried out using an AzFP labeled with fluorescein (FAM5); however, since the natural absorbance of the SPIO NPs overshadowed the absorbance of the fluorescein dye on the AzFP, it was not possible to directly quantify the number of HER2 per SPIO in this case. Therefore, it was assumed that the reaction efficiencies between the HER2-AzFP (FAM5) and SPIO NPs were the same as when HER2-AzFP (Hylite-750) was used.

Cell Culture

T6-17 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37°C and 5% CO_2. BT-20 cells were cultured and maintained in Eagles Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1 penicillin/streptomycin at 37°C and 5% CO_2. KB cells were cultured and maintained in folate-free RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Flow Cytometric Analysis

Cells (T6-17s or BT-20s) were dissociated from culture flasks using PBS-based enzyme free dissociation buffer, washed, resuspended in media containing 10% FBS and transferred to sterile 96-well plates at a final concentration of 50,000 cells per well. HER2-SPIO conjugates were

added to the wells for 30 minutes at 37°C at a final concentration 20 μg Fe/mL for T6-17 cells and 50 μg Fe/mL for BT-20 cells. For competitive inhibition assays, the same parameters were maintained while adding ~500 μM of free HER2-Affibody to each well. Cells were transferred to 1.5 mL centrifuge tubes and washed in triplicate by pelleting cells at 1000 RCF for 3 minutes and then resuspending in PBS. Cells were resuspended in 250 μL of PBS and transferred to a 96-well plate (50,000 cells per well) and analyzed using a Guava Easycyte Plus system (Guava Technologies, Hayward, CA). Flow cytometry data were analyzed using *FlowJo* software (TreeStar Inc., San Francisco, CA). Measurements of mean fluorescent intensity (MFI) were adjusted for differences in the fluorescence intensity of the different HER2-SPIO formulations. Adjusted measurements were further normalized to span 0 to 1, where unlabeled cells had a value of 0 and maximally labeled cells had a value of 1.

HER2/neu Positive Cell Relaxation Studies

T6-17 cells were dissociated using PBS-based enzyme free dissociation buffer and transferred to sterile 48-well plates at a concentration of 4 x 10^6 cells per well. HER2-SPIO and AHNP-SPIO conjugates were incubated with these cells in the 48-well plate at a final concentration of 100 μg Fe/mL for 1 hour at 37°C (n=3 for each targeting agent). For competitive inhibition assays, the same parameters were maintained while adding ~500 μM of free HER2-Affibody or AHNP to each well. Cells were transferred to 1.5 mL centrifuge tubes and washed in triplicate by pelleting cells at 1 RCF for 3 minutes and then resuspending in PBS. Cells were suspended in a final volume of 300 μL PBS and T2 measurements were taken using the benchtop relaxometer.

Folate Receptor Cell Relaxation Studies

KB cells were grown in 6-well plates for targeted studies. When cells had grown to 80% confluency, FA-SPIO nanoparticles were added at a final concentration of 50 μg Fe/mL and allowed to target for 24 hours at at 37°C (n=3 for each targeting agent). For competitive inhibition assays, the same parameters were maintained while adding 1 mM of free folic acid to each well. Following the 24 hour incubation, flash trypsinization was used to dissociation the cells from the well bottoms. Cells were transferred to 1.5 mL centrifuge tubes and washed in triplicate by pelleting cells at 1 RCF for 3 minutes and then resuspending in PBS. Cells were suspended in a final volume of 300 μL PBS and T2 measurements were taken using the benchtop relaxometer.

Cell Pellet MR Imaging

Following relaxation measurements, cell samples used for the triplicate measurements were combined and centrifugally pelleted. Cells were then transferred to a 384-well plate that had been altered to properly fit the volume coil for MR imaging (125 mm x 70 mm, 12 x 10^6 cells per well) The 384-well plate was centrifugally spun at 2,000 RCF for 2 minutes and the supernatant was carefully removed from each cell pellet. The cells were then imaged on 9.4-T magnet interfaced to a Varian INOVA console using a 35 mm inner diameter volume coil for radiofrequency transmission and reception. T2*-weighted gradient echo (GRE) MR images were collected using parameters as follows: repetition time (TR) = 200 ms, echo time (TE) = 5 ms, flip angle = 20°, slice thickness = 0.5 mm, number of acquisitions = 8.

SUPPLEMENTARY FIGURES

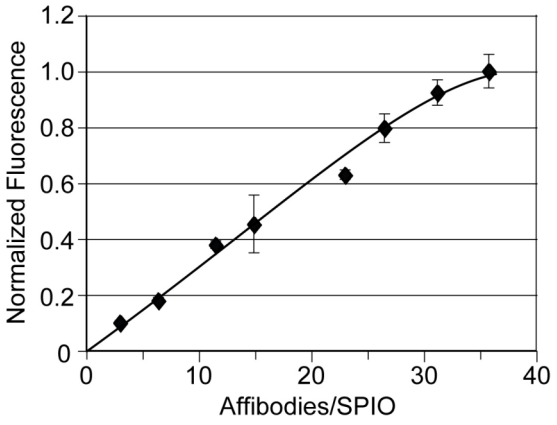

Figure S1. Fluorescence measurements of SPIO following bioconjugation with HER2-AzFP. A fixed concentration of 26nm ADIBO-modified SPIO NPs (1 mg Fe/mL) were labeled with increasing concentrations of HER2-AzFP using the EPL-Click conjugation strategy. Fluorescent measurements of each conjugate were acquired following removal of unbound affibody.

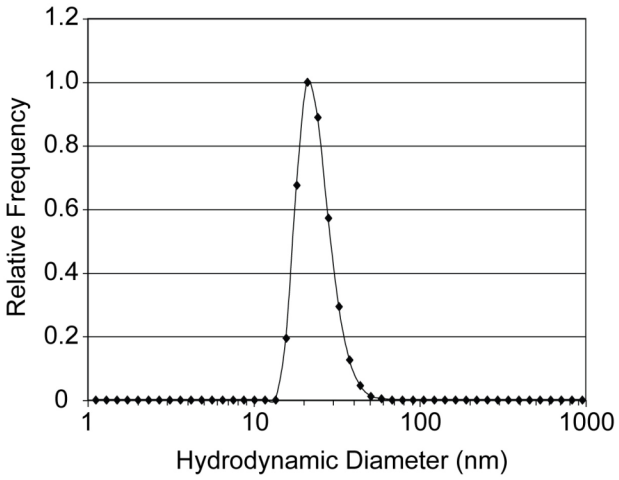

Figure S2. Size distribution of 26 nm SPIO nanoparticles. The intensity-weighted hydrodynamic diameter was determined by dynamic light scattering (DLS). The measuring angle of the DLS was 90°.

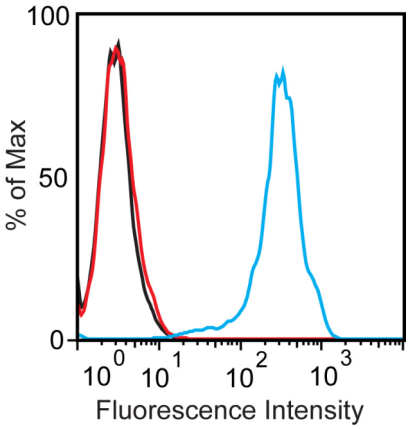

Figure S3. Flow cytometric analysis of cells labeled with aminated SPIO (NH_2-SPIO) and ADIBO-modified SPIO. T6-17 cells were incubated with 26nm NH_2-SPIO (25 ug/mL; blue) or ADIBO-modified SPIO (100 ug/mL; red) for 30 minutes at 37°C and analyzed by flow cytometry. Unlabeled cells are also shown (black). ADIBO-modified SPIO were prepared by reacting NH_2-SPIO with ADIBO-dPEG$_4$-NHS and subsequently blocking any remaining free amines with succinic anhydride. The resulting negatively charged ADIBO-modified SPIO showed little cell binding, even at 4-times higher concentrations than the NH_2-SPIO, which exhibit high non-specific cellular binding.

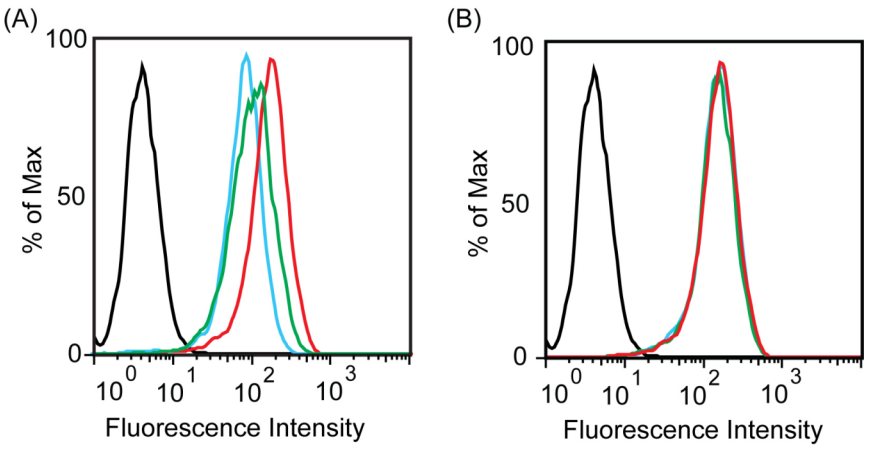

Figure S4. Representative raw flow cytometry data of T6-17 cells following incubation with 26 nm HER2-SPIO. (A) Flow cytometry histograms of unlabeled T6-17 cells (black) and T6-17 cells following incubation with 26 nm SPIO nanoparticles functionalized with an average of 11.5 (blue), 23.0 (red), or 35.8 (green) HER2-affibodies per SPIO. The flow data has not been adjusted to account for differences in fluorescence per nanoparticle. (B) Flow cytometry histograms of unlabeled T6-17 cells (black) and T6-17 cells following incubation with 26 nm SPIO nanoparticles functionalized with an average of 23.0 HER2-affibodies per SPIO. This experiment was performed in triplicate. Each histrogram (red, green, and blue) represents a different sample.

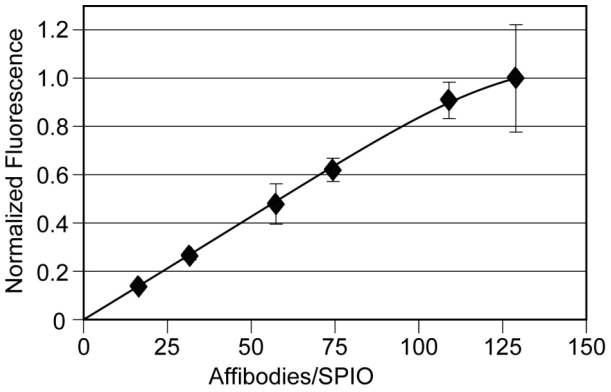

Figure S5. Controlled labeling of 50nm nanoparticles with HER2 affibody. (A) A fixed concentration of 50nm ADIBO-modified SPIO NPs (1 mg Fe/mL) were labeled with increasing concentrations of HER2-AzFP using the EPL-Click conjugation strategy. Fluorescent measurements of each conjugate were acquired following removal of unbound affibody.

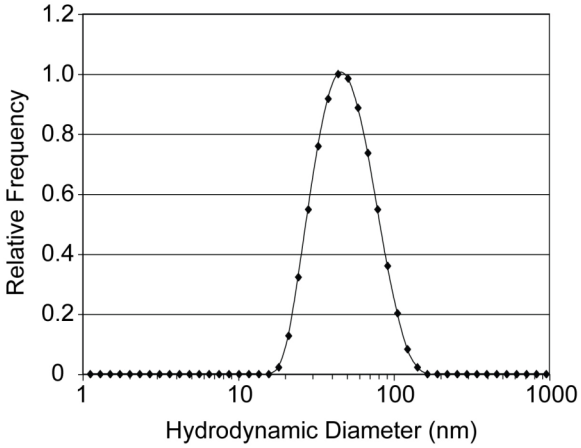

Figure S6. Size distribution of 50 nm SPIO nanoparticles. The intensity-weighted hydrodynamic diameter was determined by dynamic light scattering (DLS). The measuring angle of the DLS was 90°.

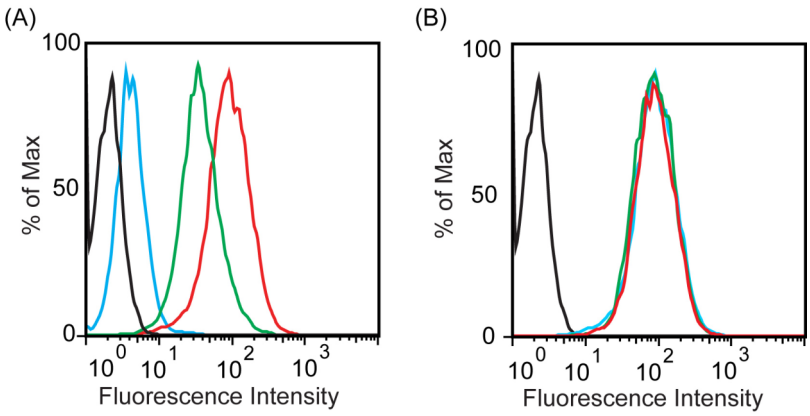

Figure S7. Representative raw flow cytometry data of T6-17 cells following incubation with 50 nm HER2-SPIO. (A) Flow cytometry histograms of unlabeled T6-17 cells (black) and T6-17 cells following incubation with 50 nm SPIO nanoparticles functionalized with an average of 16.5 (blue), 57.4 (red), or 129 (green) HER2-affibodies per SPIO. The flow data has not been adjusted to account for differences in fluorescence per nanoparticle. (B) Flow cytometry histograms of unlabeled T6-17 cells (black) and T6-17 cells following incubation with 50 nm SPIO nanoparticles functionalized with an average of 57.4 HER2-affibodies per SPIO. This experiment was performed in triplicate. Each histrogram (red, green, and blue) represents a different sample.

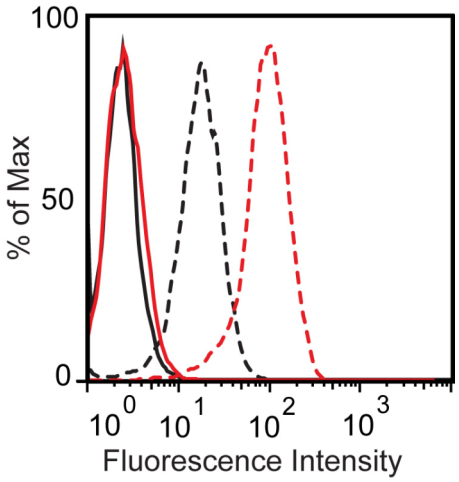

Figure S8. Flow cytometry histrogram of T6-17 cells and BT-20 cells following incubation with 26 nm HER2-SPIO. T6-17 cells (red dash) were incubated with 26nm NH_2-SPIO (23 HER2-affibody/SPIO, 20 ug/mL) for 30 minutes at 37°C and analyzed by flow cytometry. Unlabeled T6-17 cells are also shown (red solid). Similarly, BT-20 cells (black dash) were also incubated with 26nm NH_2-SPIO (23 HER2-affibody/SPIO, 50 ug/mL) for 30 minutes at 37°C and analyzed by flow cytometry. Unlabeled BT-20 cells are represented by the red solid line.

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GTG GAT AAC AAA TTT AAC AAA GAA ATG CGC AAC GCG TAT TGG GAA ATT
Val Asp Asn Lys Phe Asn Lys Glu Met Arg Asn Ala Tyr Trp Glu Ile

GCG CTG CTG CCG AAC CTG AAC AAC CAG CAG AAA CGC GCG TTT ATT CGC
Ala Leu Leu Pro Asn Leu Asn Asn Gln Gln Lys Arg Ala Phe Ile Arg

AGC CTG TAT GAT GAT CCG AGC CAG AGC GCG AAC CTG CTG GCG GAA GCG
Ser Leu Tyr Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala

AAA AAA CTG AAC GAT GCG CAG GCG CCG AAA ATG CGC ATG
Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Met Arg Met
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Figure S9. Nucleotide and corresponding amino acid sequence of the HER2-Affibody. The additional base pairs added to improve affinity column cleavage are shown in bold.

TTT TGC GAT GGC TTT TAT GCG TGC TAT ATG GAT GTG **ATG CGC ATG**
Phe Cys Asp Gly Phe Tyr Ala Cys Tyr Met Asp Val **Met Arg Met**

Figure S10. Nucleotide and corresponding amino acid sequence of the Anti-HER2/neu peptide (AHNP). The additional base pairs added to improve affinity column cleavage are shown in bold.

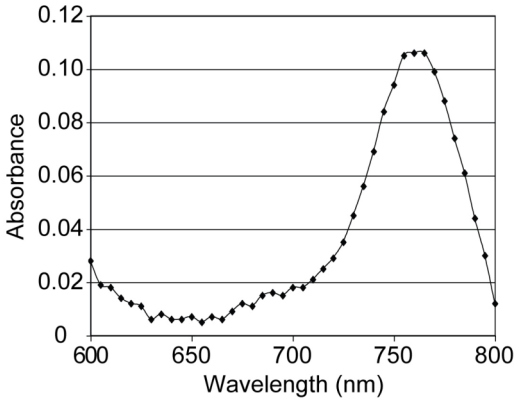

Figure S11. Representative absorbance measurements of SPIO following bioconjugation with HER2-AzFP. The AzFP was labeled with the near infrared fluorescent dye HyLite-750. Absorbance measurements were acquired following removal of unbound affibody.

REFERENCE:

1. Thorek DL, Tsourkas A. Size, charge and concentration dependent uptake of iron oxide particles by non-phagocytic cells. Biomaterials 2008;29:3583-90.