Supporting Information for:

Site-Specific Labeling of Cyanine and Porphyrin Dye-Stabilized Nanoemulsions with Affibodies for Cellular Targeting

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Contents

HER2. (C) The hydrodynamic diameter and T_2 relaxation time of ISCs-HER2 was monitored for 6 days in water at 25°C. (D) Magnetic resonance (MR) relaxometry measurements of ISCs-HER2.

Figure S7. Viability of T617 cells after incubation with increasing concentrations of ISCs-HER2 (A) and PSCs-HER2 (B) for 24 h.

Figure S8. Phase contrast (top row) and fluorescence microscopy (bottom row) images of HER2/neu cells incubated without particles (control) and with ISCs, ISCs-EGFR and ISCs-HER2 for 1h (A). Relaxivity measurements of HER2/neu cells incubated with targeted and non-targeted nanocluster were acquired (B). MR phantom image of targeted and non-targeted ISCs after incubated with HER2/neu cells for 1h (C).

Figure S9. (A) Phase contrast (top row) and fluorescence microscopy (bottom row) images of HER2/neu-positive T617 cells incubated without nanoemulsions (control), with ISCs, ISCs mixed with DBCO-HER2 (ISCs + HER2) or targeted nanoemulsions (ISCs-HER2) for 1h. (B) Relaxivity measurements of T617 cells incubated with the targeted (ISCs-HER2) or non-targeted (control, ISCs, ISCs + HER2) nanoemulsions. (C) Phase contrast (top row) and fluorescence microscopy (bottom row) images of HER2/neu-positive T617 cells incubated without nanoemulsions (control), with PSCs, PSCs mixed with DBCO-HER2 (PSCs + HER2) or targeted nanoemulsions (PSCs-HER2) for 1h. (D) Relaxivity measurements of T617 cells incubated with the targeted (PSCs-HER2) or non-targeted (control, PSCs, PSCs + HER2) nanoemulsions.

S1. Methods and instruments

Synthesis of Azide-Functionalized Indocyanine Green (ICG-N_3) and Protoporphyrin IX (PpIX-N_3).

Materials

Protoporphyrin IX (PpIX), compound 2, azidoethylamine and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Reversed phase HPLC was performed using Agilent Prostar 325 UV/VIS Dual Wavelength Detector (Groton, CT) equipped with model 210/218 solvent delivery module and Agilent Prep C-18, 10 μm, 100 x 30.0 mm column. ^{1}H NMR spectra were acquired with a Bruker Avance-500 spectrometer.

3-(4-azido-3-methyl-3-sulfonyl)-1,1,2-trimethyl-1*H*-benzo[e]indolinium chloride (3). Compound 1 was prepared as reported earlier.[1] Compound 1 (1.0 g; 2.4 mmol) and compound 2 (0.17 g; 0.81 mmol) were heated at 120ºC in 50 mL of 1,2-dichlorobenzene (DCB) for 48 h. The reaction was cooled to room temperature. The precipitate was filtered and washed repeatedly with ether (200 mL). The solid was collected and dried under vacuum to yield brown solid (0.20 g, 60%). ESI-MS: 439 (M+Na). ^{1}H NMR (500 MHz, DMSO-d6): δ = 1.8 (m, 6H), 2.58 (t, 2H), 2.68 (t, 2H), 3.15 (s, 3H), 3.3 (s, 3H), 5.6 (s, 2H), 7.2 (m, 3H), 7.45 (m, 3H).

4-(2-((1*E*,3*E*,5*E*,7*Z*)-7-3-(4-azido-3-methyl-3-sulfonyl)-1,1,2-trimethyl-1*H*-benzo[e]indol-2(3*H*)-ylidene)hepta-1,3,5-trienyl)-1,1-dimethyl-1H-benzo[e]indolinium-3-yl)butane-1-sulfonate (**5**). Compound 3 (0.050 g; 0.12 mmol) and compound 4 (0.065 g; 0.12 mmol) (Toronto Research Chemicals; Toronto, Canada) were heated at 50ºC in 1 mL of pyridine (Py) for 1h. Pyridine was removed under vacuum and the crude product was purified by reversed phase chromatography (Rt = 30 min) to yield blue crystalline solid (0.044 g, 45%). The title compound was eluted with 5 % of CH_3CN (5 min), 5-70% (35 min) and 80-100% (5 min) in water at a flow rate of 4.5 mL min-1. ESI-MS: 412 (M+2). ^{1}H NMR (DMSO-d6, 500 MHz): δ = 1.7 (m, 4H), 1.9 (s, 3H), 2.35 (s, 3H), 2.45 (m, 2H), 2.65 (s, 3H), 2.75 (t, 2H), 3.2 (s, 3H), 3.3 (s, 3H), 3.4 (t, 2H), 4.1 (Brt, 2H), 4.2 (Brt, 2H), 6.45 (d, 1H), 6.6 (m, 1H). 7.4, 7.65, 7.75, 7.8, 8.03, 8.27, (several m, total 17H) (Figure S1).

3-(3-(3-((2-azidoethyl)amino)-3-oxopropyl)-2,8,12,17-tetramethyl-13,18-divinylporphyrin-7-yl)propanoic acid (7). PpIX (0.13 g, 0.23 mmol), N, N-Diisopropylethylamine (DIEA, 0.014 g, 0.11 mmol), 1-Hydroxybenzotriazole hydrate (HOBt, 0.015 g, 0.11 mmol) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, 0.017 g, 0.11 mmol) were all dissolved in 20 mL DMF and stirred at room temperature for 2.5 h. Then the azidoethylamine (0.010 g, 0.12 mmol) was added and stirred for 48h. The solvent was removed under vacuum and the crude product was purified by HPLC (Rt = 22 min) to yield a purple to red solid (0.029 g, 40%). The title compound was eluted with 10 % CH_3CN (5 min), 10 -20% (8 min), 20-80% (27 min) and 80-100% (5 min) in water at a flow rate of 4.5 mL min^{-1}. ESI-MS: 634 (M+1). ^{1}H NMR (DMSO-d6, 500 MHz), δ = 10.36, 10.30, 10.26 (s, 4H), 8.55, 8.25 (m, 2H), 6.45, 6.25 (d, 4H), 4.33, 4.15 (m, 4H), 3.76, 3.75, 3.70,3.65 (s, 12H), 3.55 (t, 2H), 2.90 (t, 2H), 2.35, 2.64 (t, 4H) (Figure S1).

Preparation of Superparamagnetic Iron Oxide Nanoparticles (SPIONs).

SPION were prepared by thermal decomposition as previously described.[2] Briefly, iron(III) acetylacetonate [$Fe(acac)_3$] (2 mmol), 1,2-hexadecanediol (5 mmol), oleic acid (2 mmol), oleylamine (6 mmol), and benzyl ether (20 mL) were stirred and heated under nitrogen at 200 °C for 15 min. Then, the mixture under a blanket of nitrogen was heated to reflux (300 °C) for 1 h and allowed to cool back to room temperature. Nanoparticles were precipitated by adding two volumes of ethanol to the mixture and centrifuged at 5500 × g for 15 min. The nanoparticles were allowed to air dry before dissolving in toluene. Large aggregates were removed via centrifugation at 3000 × g for 15 min.

Preparation of DBCO-Conjugated Affibody.

Anti-HER and Anti-EGFR affibodies were cloned into pSRTA plasmids and was transformed into T7 Expression Crystal Competent Cells (New England Biolabs). Bacterial starter cultures were then grown in 2 mL LB + 100μg/mL ampicillin at 37°C in a shaker overnight. Starter cultures were added at a 1:1000 dilution to Autoinduction Media LB Broth Base Including Trace Elements

(Formedium), 100μg/mL ampicillin. Proteins were expressed at 37°C in shaker for 24h and pelleted by centrifugation.

For lysis, cell pellets were resuspended in Lysis buffer with 200 μg/mL lysozyme, 4μg/mL DNAseI, and 1 cOmplete Mini EDTA-free Protease Inhibitor Tablet (Roche). Resuspended pellets were incubated at room temperature for 30 min and then put in freezer to increase the lysis efficiency. The clarified lysates were incubated with cobalt resin (TALON Metal Affinity Resin, Clontech, 0.5 mL per 100mL expression culture) for 30 min at room temperature for binding. Following the incubation, resin washed by PBS. For labeling affibodies, 500ul PBS + 50μM $CaCl_2$ + 200μM GGGSK(DBCO)NH_2 peptide (NEO Scientific) was added and inoculated for 4 hours at 37°C. Product was purified from access labeling reagent by using 3 kD molecular weight cut-off (MWCO) filter (Amicon Ultra, Milipore, Temecula, CA).

Purified DBCO labelled affibodies were checked by SDS-PAGE gel and concentration was quantified by BCA assay (Pierce, Rockford, IL).

Preparation of Azide-Functionalized Nanocluster.

A mixture of ICG-N_3/ICG (1/20 molar ratio) into dimethyl sulfoxide (DMSO) and SPIO (1.25 mg based on the Fe concentration in toluene) was pipetted into a glass vial containing 4 ml of water, and the sample was sonicated until a homogeneous solution was observed. The toluene was evaporated overnight. Dialysis was performed with 4 L of water to remove DMSO. Azide-functionalized nanoclusters were purified by passing through a MACS (25 LD columns, Miltenyi Biotec, Germany) column. Same method was run to prepare PpIX-N_3/PpIX nanoclusters.

Preparation of Affibody Conjugated Nanocluster (ISCs-HER2 and PSCs-HER2).

To prepare affibody-conjugated nanocluster, 20 uM of azide-functionalized nanoclusters (based on the ICG-N_3 and PpIX-N_3 concentration) and were reacted with 40 uM of DBCO-functionalized affibody in 1 ml of PBS for 12 h at room temperature with shaking. The affibody conjugated nanoclusters were washed with PBS several times by using centrifugal filter (50K, Amicon Ultra) to remove free affibody.

Characterization of ISCs-HER2 and PSCs-HER2.

The diameter and size distributions of the HER2-targeted nanoclusters were measured with dynamic light scattering (DLS, Malvern, Zetasizer, Nano-ZS). The morphology of the nanoparticles was observed using a transmission electron microscope (TEM) (JOEL 1010). T_2 relaxation times were measured using a benchtop relaxometer (Bruker, mq60 NMR analyzer). Iron concentration was quantified by plasma optical emission spectroscopy (ICP-OES) (Spectro Genesis, GMBH).

Cell Viability Assay.

T6-17 cells (1×10^4 cells per well) were seeded in 96-well plates and incubated overnight to allow the cells to attach to the surface of the wells. The cells were then mixed with increasing concentrations of HER2-targeted nanoclusters 24 h, and the cell viabilities were determined according to the supplier's instructions. After 24 h of incubation, 10 ul of MTS reagent was added. After 2 h, 490-nm absorbance was read on a Tecan microplate reader.

Cellular Binding/Uptake Measured by Fluorescence Microscopy.

Fluorescence microscopy was used to determine the cellular binding behavior of ISCs-HER2 and PSCs-HER2 nanoclusters. T6-17 cells were utilized upon seeding in a 12-well plate at a density of 2×10^5 cells per well. The cells were submerged in 2 mL of DMEM cell culture medium and incubated in a 5% CO2 environment at 37 °C for 24 h. After incubation, the original culture medium was removed and incubated with HER2-targeted nanoclusters at ICG and PpIX concentration of 10 uM in newly added DMEM for 1 h. The cells were washed with phosphate buffer saline (PBS) two times to remove excess nanoclusters that were not uptaken by the T6-17 cells. As a negative control, we prepared and used non-targeted nanocluster, ISCs-EGFR and PSCs-EGFR nanoclusters at the same condition. Microscopy images were taken with an Olympus IX81 motorized inverted fluorescence microscope with a back-illuminated EMCCD camera (Andor), an X-cite 120 excitation source, and Sutter excitation and emission filter wheels.

Cellular Targeting.

T6-17 cells were incubated with 100 ug Fe/mL of HER2-targeted nanoclusters for 1 h in full media in triplicate. The media was removed and the cells were washed with PBS two times to remove any unbound nanoclusters. Cells were harvested and counted. Cell suspensions were diluted to 5×10^5 cells/ml and T_2 relaxation times were measured using a benchtop relaxometer (Bruker mq60). Then relaxometry measurements were performed in T_2* mode (Varian, 4.7 T) for cell suspensions. A plastic 384-well plate (MR phantom) was used to test the T_2 hypointensity associated with targeted-nanocluster and non-targeted nanocluster compared to control cells (without nanoparticles) and control media (i.e., water) on a 4.7 T magnet.

References:

(1) Steinmann, J. G.; Phillips, J. H.; Sanders, W. J.; Kiessling, L. L. Synthesis of Cyclic Sulfates by Halocyclization. *Org. Lett.* **2001**, *3* (22), 3557–3559.

(2) Thawani, J. P.; Amirshaghaghi, A.; Yan, L.; Stein, J. M.; Liu, J.; Tsourkas, A. Photoacoustic-Guided Surgery with Indocyanine Green-Coated Superparamagnetic Iron Oxide Nanoparticle Clusters. *Small* **2017**, *13* (37), 1–9.

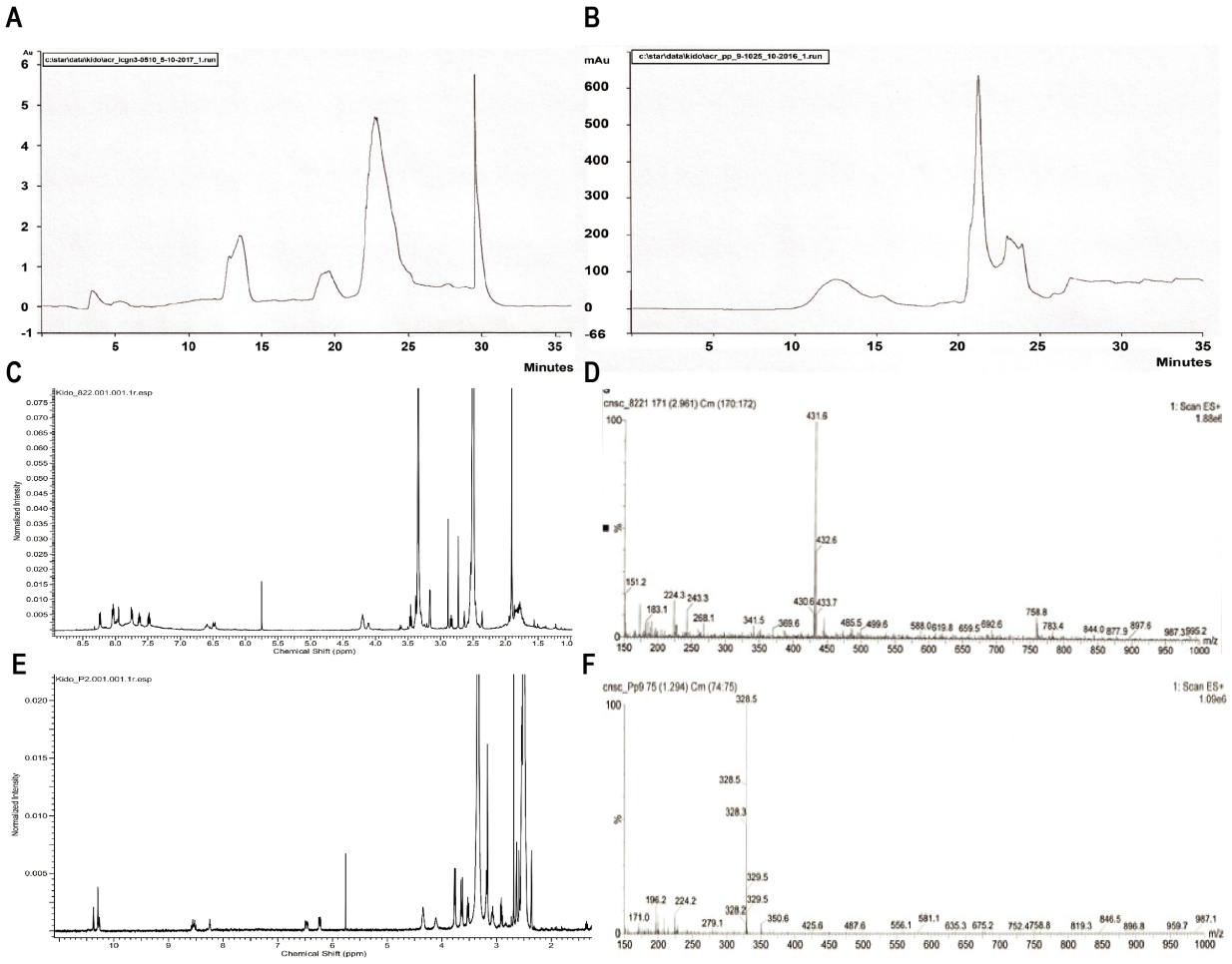

Figure S1. (A) HPLC trace of crude product of compound 5 and (B) compound 7. (C) 1H NMR spectrum and (D) ESI-MS analysis of compound 5. (E) 1H NMR spectrum and (F) ESI-MS analysis of compound 7.

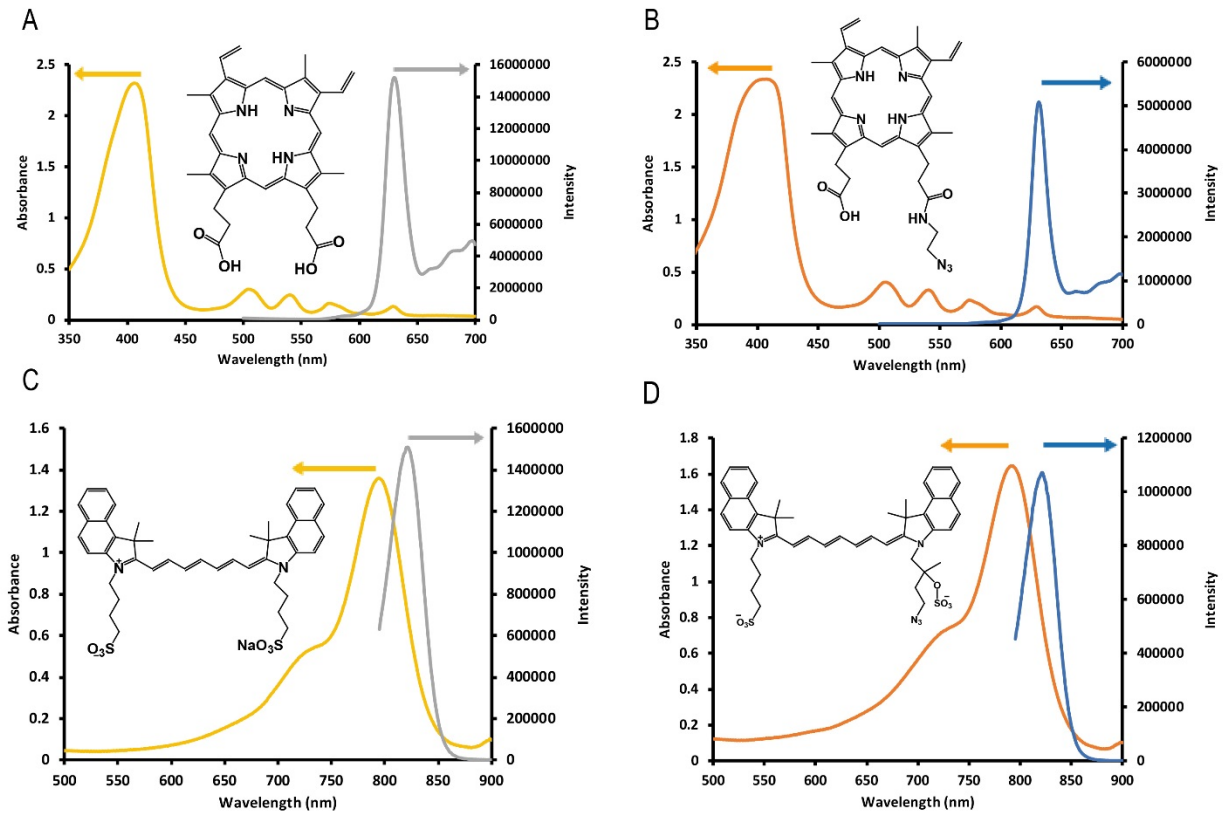

Figure S2. UV-Vis absorbance and fluorescence emission spectra of PpIX (A), PpIX-N_3 (B), ICG (C) and ICG-N_3 (D) in DMSO.

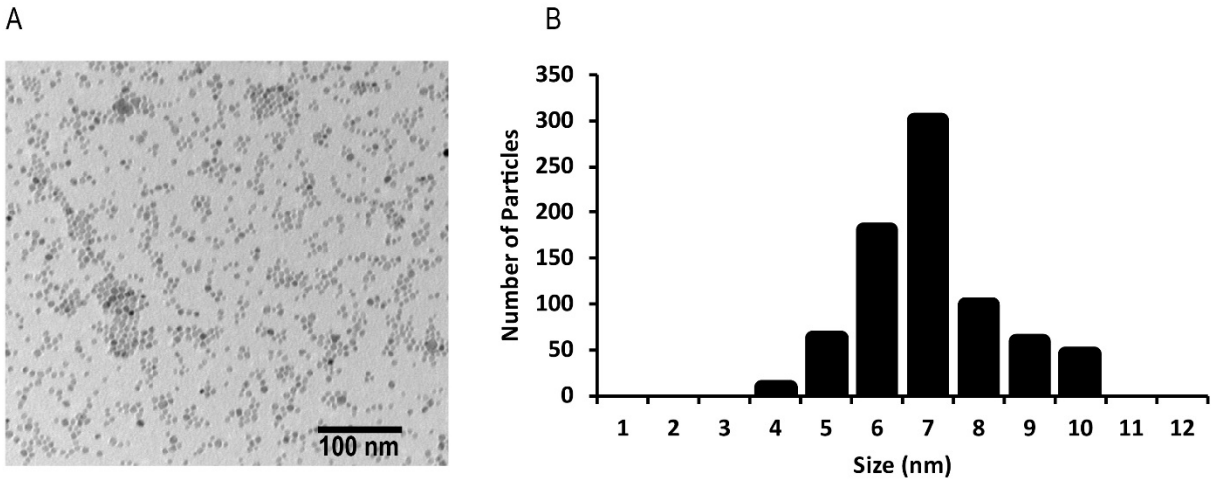

Figure S3. (A) Transmission electron microscopy (TEM) of superparamagnetic iron oxide nanoparticles (SPIONs) following thermal decomposition reaction. Scale bar = 100 nm. (B) Analysis of TEM results demonstrating the size distribution of SPIONs (diameter = 7.6 ± 1.0 nm).

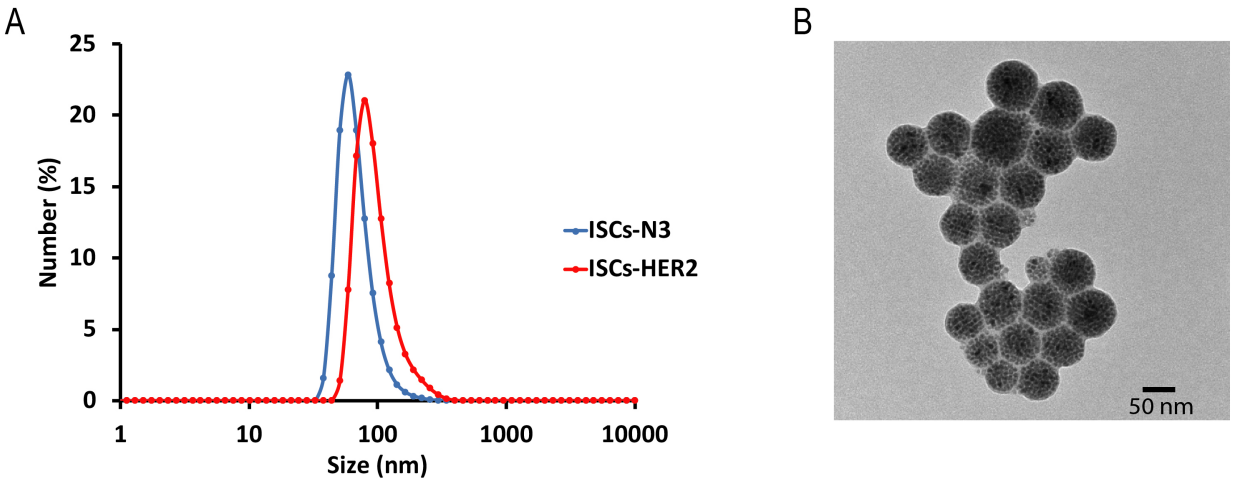

Figure S4. (A) Dynamic light scattering (DLS) of ISCs-N_3 (58 ± 4.3 nm) and ISCs-HER2 (78 ± 5 nm) in water. (B) TEM image of ISCs-HER2 shows tightly packed SPIONs within the nanoemulsions (scale bar: 50 nm).

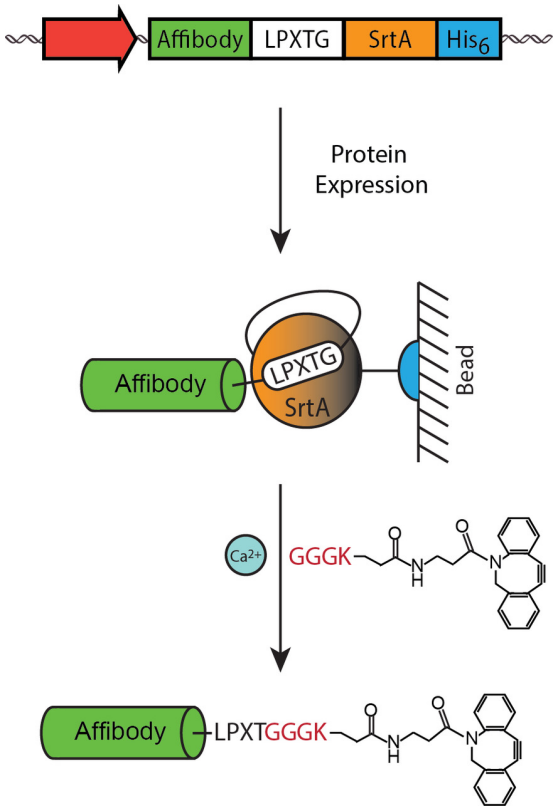

Figure S5. Schematic of Sortase-Tag Expressed Protein Ligation (STEPL).

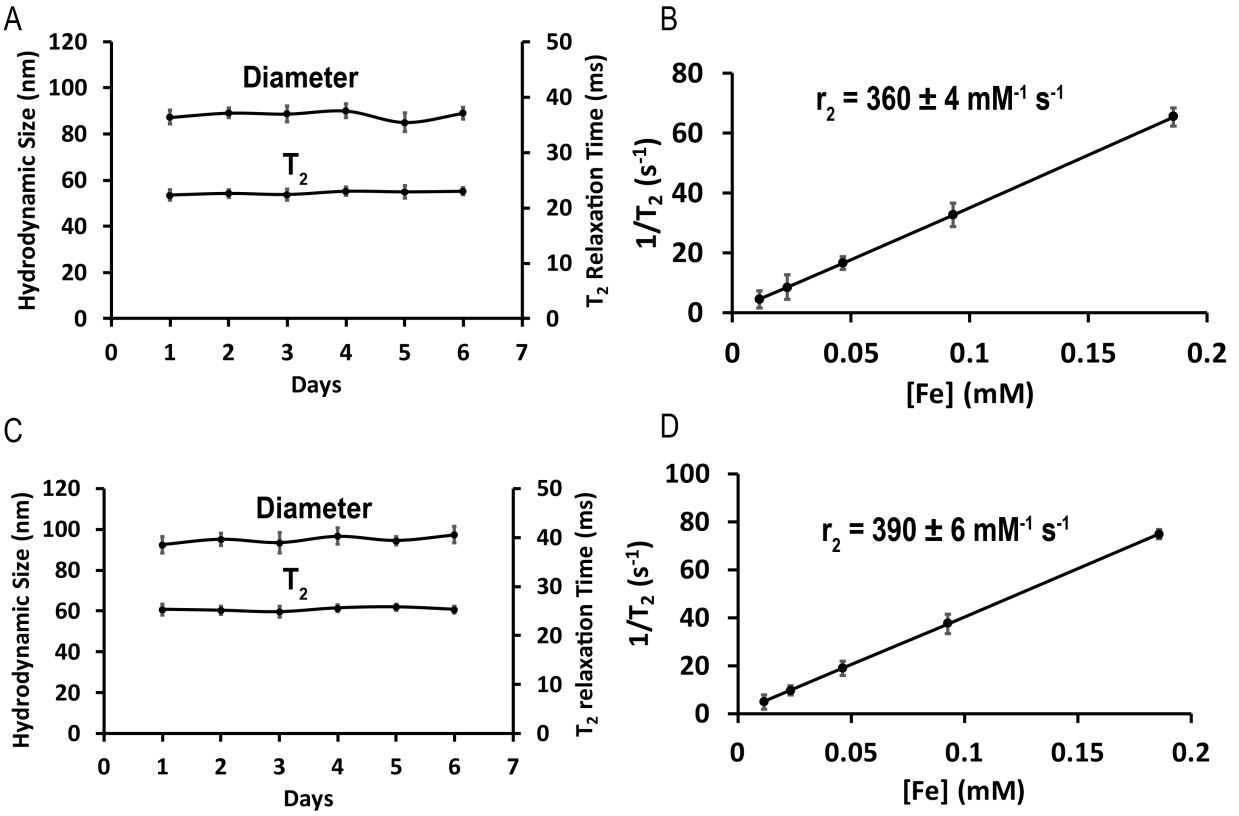

Figure S6. (A) The hydrodynamic diameter and T_2 relaxation time of PSCs-HER2 was monitored for 6 days in water at 25°C. (B) Magnetic resonance (MR) relaxometry measurements of PSCs-HER2. (C) The hydrodynamic diameter and T_2 relaxation time of ISCs-HER2 was monitored for 6 days in water at 25°C. (D) Magnetic resonance (MR) relaxometry measurements of ISCs-HER2.

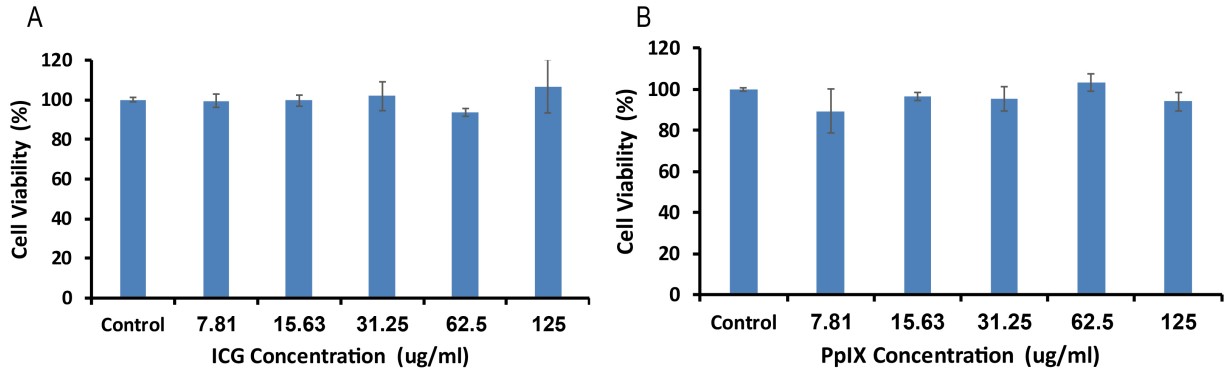

Figure S7. Viability of T617 cells after incubation with increasing concentrations of ISCs-HER2 (A) and PSCs-HER2 (B) for 24 h.

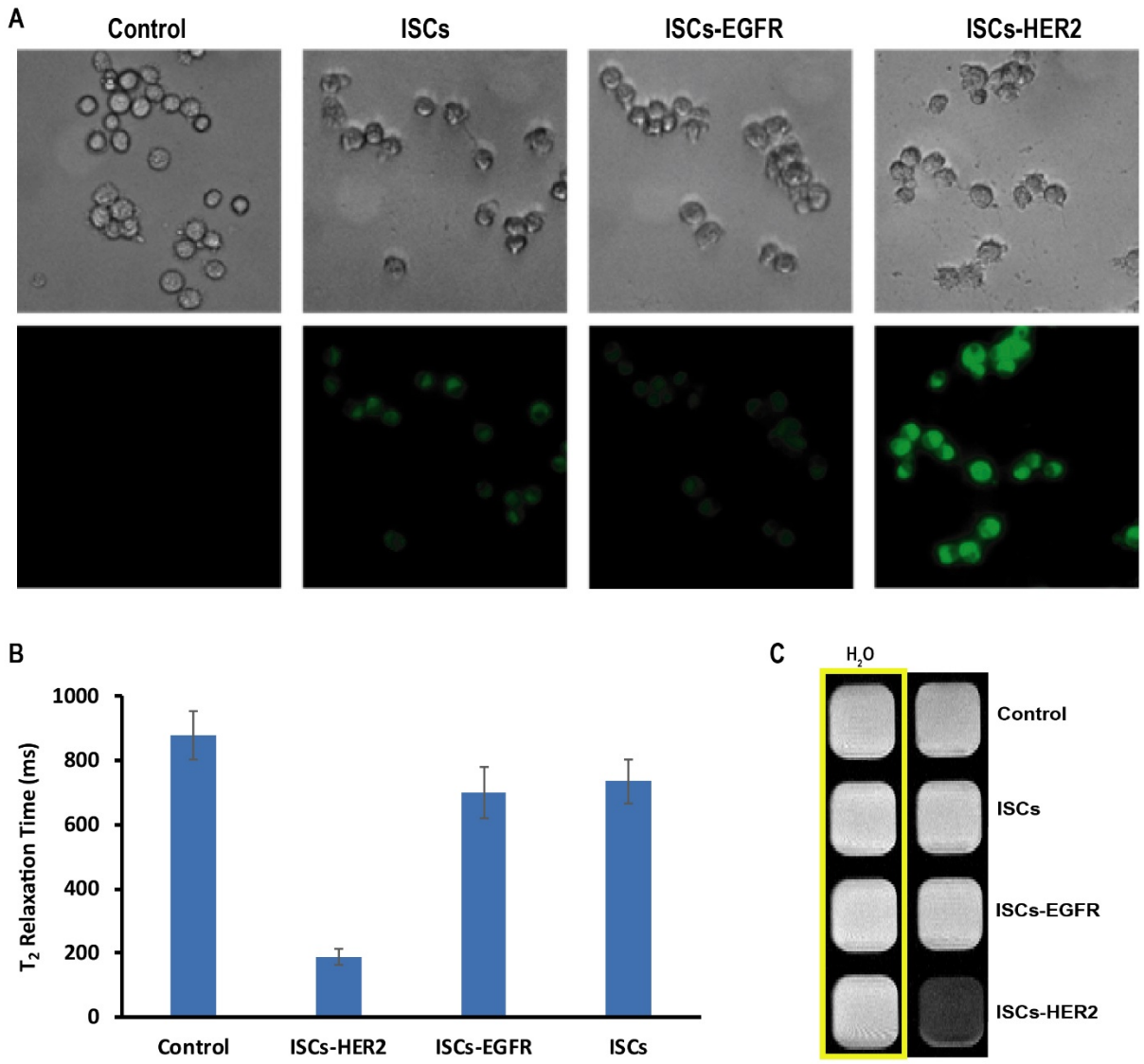

Figure S8. (A) Phase contrast (top row) and fluorescence microscopy (bottom row) images of HER2/neu-positive T617 cells incubated without nanoemulsions (control) and with ISCs, ISCs-EGFR and ISCs-HER2 for 1h. (B) Relaxivity measurements of T617 cells incubated with targeted and non-targeted nanoemulsions. (C) MR phantom image of T617 cells after incubation with targeted and non-targeted ISCs for 1h.

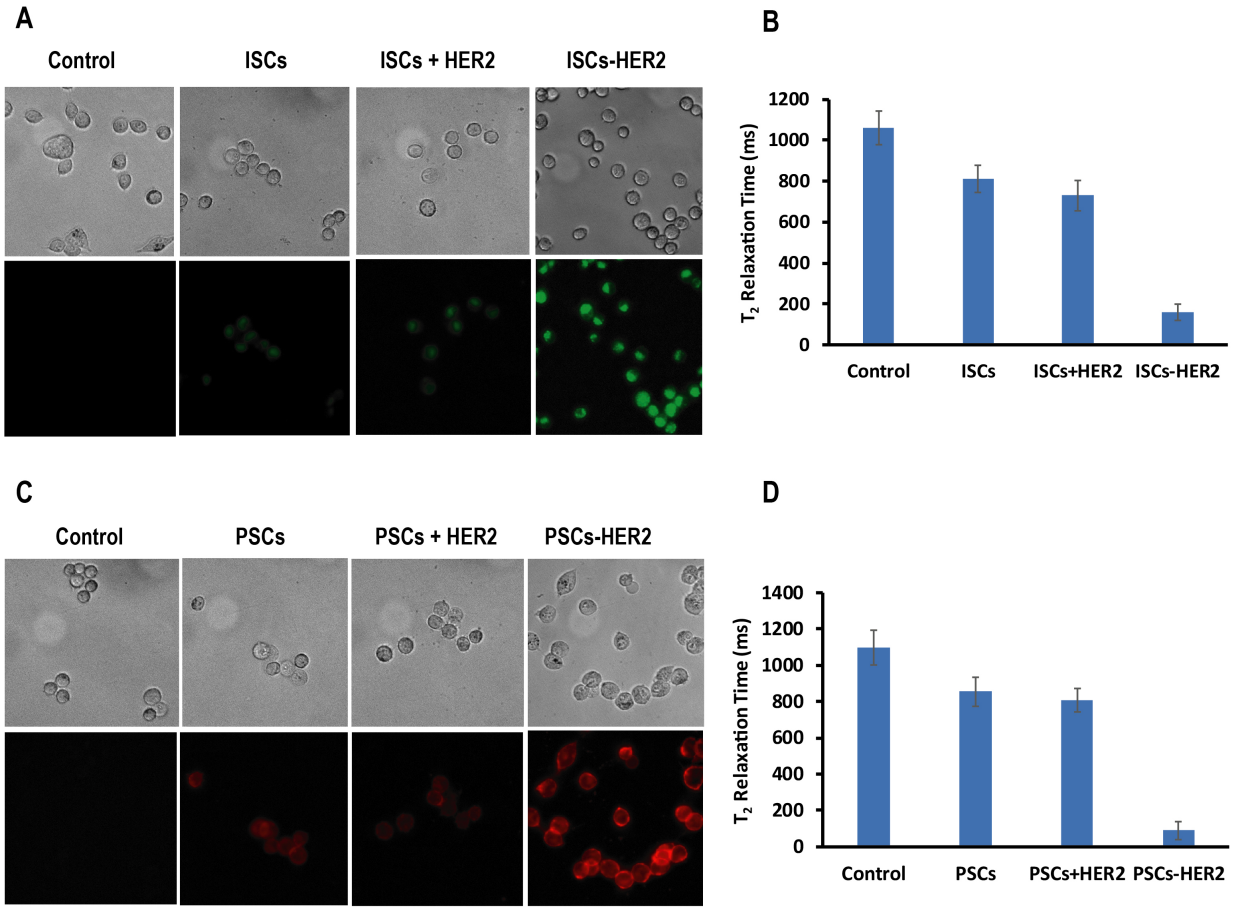

Figure S9. (A) Phase contrast (top row) and fluorescence microscopy (bottom row) images of HER2/neu-positive T617 cells incubated without nanoemulsions (control), with ISCs, ISCs mixed with DBCO-HER2 (ISCs + HER2) or targeted nanoemulsions (ISCs-HER2) for 1h. (B) Relaxivity measurements of T617 cells incubated with the targeted (ISCs-HER2) or non-targeted (control, ISCs, ISCs + HER2) nanoemulsions. (C) Phase contrast (top row) and fluorescence microscopy (bottom row) images of HER2/neu-positive T617 cells incubated without nanoemulsions (control), with PSCs, PSCs mixed with DBCO-HER2 (PSCs + HER2) or targeted nanoemulsions (PSCs-HER2) for 1h. (D) Relaxivity measurements of T617 cells incubated with the targeted (PSCs-HER2) or non-targeted (control, PSCs, PSCs + HER2) nanoemulsions.