Supporting Information

Cationic Polymer Modified Mesoporous Silica Nanoparticles for Targeted SiRNA Delivery to HER2+ Breast Cancer

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Reagents and siRNAs

TEOS, CTAC, $NaH_2PO_4 \cdot H_2O$, Na_2HPO4 and TEA were obtained from Sigma Aldrich (MO). Branched-PEI (1.8 or 10 kDa) was obtained from Alfa Aesar (MA). Maleimide-PEG(5kDa)-NHS was obtained from JenKem Technology USA (TX). Trastuzumab, Rituximab, Abraxane and Feraheme were obtained from the OHSU pharmacy. PBS (pH 7.2) was obtained from Life Technologies (CA). Desalting columns (MW 40 kDa), RNase free water, Traut's reagent, DSP, ethanol, concentrated HCl, sodium hydroxide and DharmaFECT #1 were obtained from Thermo Fisher Scientific (MA). All reagents are of highest purity grade available.

siHER2 and siSCR (without fluorescent dye tag) were custom synthesized (*in vivo* HPLC grade) by Dharmacon, Thermo Scientific (CO). siSCR with Alexa-488 dye was synthesized by Qiagen (CA). siLUC was custom synthesized by Life Technologies (CA). The sequences are summarized as follows.

SiRNA sequences

siRNA	siRNA sequence
siHER2	Sense: 5' CACGUUUGAGUCCAUGCCCAAUU 3' Antisense: 5' UUGGGCAUGGACUCAAACGUGUU 3'
siLUC	Sense: 5' CGGAUUACCAGGGAUUUCAtt 3' Antisense: 5' UGAAAUCCCUGGUAAUCCGtt 3'
siSCR	Sense: 5' UGGUUUACAUGUCGACUAA 3' Antisense: 5' UUAGUCGACAUGUAAACCA 3'

Cell lines and media recipes

Cell lines	Cell media recipe
BT474, HCC1954	RPMI-1640 + 10% FBS
BT474-R	RPMI-1640 + 10% FBS + 1 μM Lapatinib
JIMT1, MCF7, MDAMB231, MDAMB468, and HepG2	DMEM + 10% FBS
SKBR3	McCoy5A + 10% FBS
MCF10a	Ham's F-12:DMEM + 5% horse serum + 10 μg/mL insulin + 100 ng/mL cholera toxin + 20 ng/mL EGF +

Supplemental Methods

Immunofluorescence staining and microscopy

Cells were washed two times with dPBS (*Dulbecco's Phosphate Buffered Saline*, Life Technologies), fixed with 100% ice-cold methanol for 15 minutes at room temperature, and washed two times with dPBS. Then, cells were washed three times with 1X TBST (*Tris Buffered Saline with Tween 20*, Cell Signaling), two times with blocking buffer (1X PBS + 5% Goat Serum) and incubated for one hour in the blocking buffer at room temperature. Blocking buffer was then removed and 50 μL of primary antibodies (anti-HER2 (1:200, Abcam)) in blocking buffer solution was added and incubated overnight at 4 °C. Following a

series of PBS washes (5 times), blocking buffer was added and incubated for 10 minutes. Blocking buffer was removed and 50 µL of secondary antibodies (Alexa-488 goat anti rabbit (1:500, Life Technologies)) were incubated at 37 °C for 30 minutes. Cells were then washed five times with blocking buffer. Cell nuclei were visualized by DAPI (Life Technologies) following manufacturer's protocol. Fluorescence images were obtained with the EVOS FL cell imaging system (Life Technologies). All images were processed for signal intensity by CellProfiler image analysis software.

For tumor tissue immunofluorescence study, tumors were harvested and paraffinized until the time of analysis. At the time of analysis, the tumor sections were deparaffinized and incubated with antibodies under the same conditions as entailed above. Fluorescence images were obtained and analyzed the same way.

Blood compatibility of nanoparticles: hemolysis, thrombogenesis and platelet aggregation

Hemolysis. *In vitro* hemolytic property of the nanoparticles was evaluated with some modifications from other works.[51] Briefly, human blood was collected in the presence of EDTA and serum was removed. Red blood cells were suspended at $1x10^9$ cells per mL and exposed to nanoparticle (final concentrations of 70 or 350 µg/mL for 1X or 5X, respectively) for 4 hours and 37° C. Following centrifugation, absorbance of hemoglobin in the supernatants (at 542 nm) was measured and used to quantify percent hemolysis. Abraxane (Celgene) at 94 µg/mL for 1X and 470 µg/mL for 5X was used as the FDA approved nanoparticle based drug benchmark.

Coagulation (thrombogenesis) assay**.** Platelet-poor plasma (PPP) was obtained following a two-step centrifugation of isolated blood (diluted in 3.2% sodium citrate, 1:10). After the first spin at (2150 g, 10 min), the top portion of plasma (~75% of the total volume) was collected without disturbing the plasma at the bottom. The collected portion was centrifuged again at the same speed for 10 minutes, and the top portion (~75% of the total

volume) was collected as PPP. Nanoparticles were mixed with 0.15 mL PPP at the final concentration of 70 or 350 μg/mL nanoparticles. The tubes were incubated for 30 minutes at 37 ^{o}C. After 30-minute incubation, 0.05 mL of APTT-xl reagent was added and incubated for 3 minutes in the Trinity Biotech KC-4 coagulation analyzer. After which, 8.3 mM $CaCl_2$ was added and the time until the onset of coagulation was recorded. Abraxane at 1X and 5X (same dose as the above, see *hemolysis* section) was used as the FDA approved nanoparticle based drug benchmark. Likewise, Feraheme (AMAG Pharmaceuticals), another FDA approved nanoparticle based contrast agent, at 102 μg/ml for 1X and 510 μg/ml for 5X, was also compared in parallel.

Platelet aggregation assay. Platelet-rich plasma (PRP) was obtained following centrifugation of isolated blood (diluted in 3.2% sodium citrate, 1:10). The isolated blood was centrifuged at 200g for 20 minutes. The supernatant (which contains PRP) was collected and maintained at room temperature prior to treatment. Following a 1-min incubation at 37^{o}C (baseline), reactions were initiated by addition of nanoparticle (70 or 350 μg/mL) or collagen related peptide (CRP; 100 μg/ml) and monitored for three minutes for optical density via an aggregometer (Chrono-log Corp). Abraxane at 1X and 5X as entailed above was also used as a benchmark.

Animal studies: mouse ectopic tumor model.

For preliminary *in vivo* study with O-87 materials, ectopic mouse tumor model was used. 2.5 x 10^6 cells of HCC1954 were implanted subcutaneously into the flank of 5-week-old nu/nu mice (Charles River) and allowed to grow to an average size of ~200. Mice were then grouped and proceeded to be intravenously administered with nanoparticle with siHER2 or siSCR at 5 mg siRNA/kg or 2.5 mg siRNA/kg, as specified in Figure S11A (NP/siRNA mass ratio = 25).

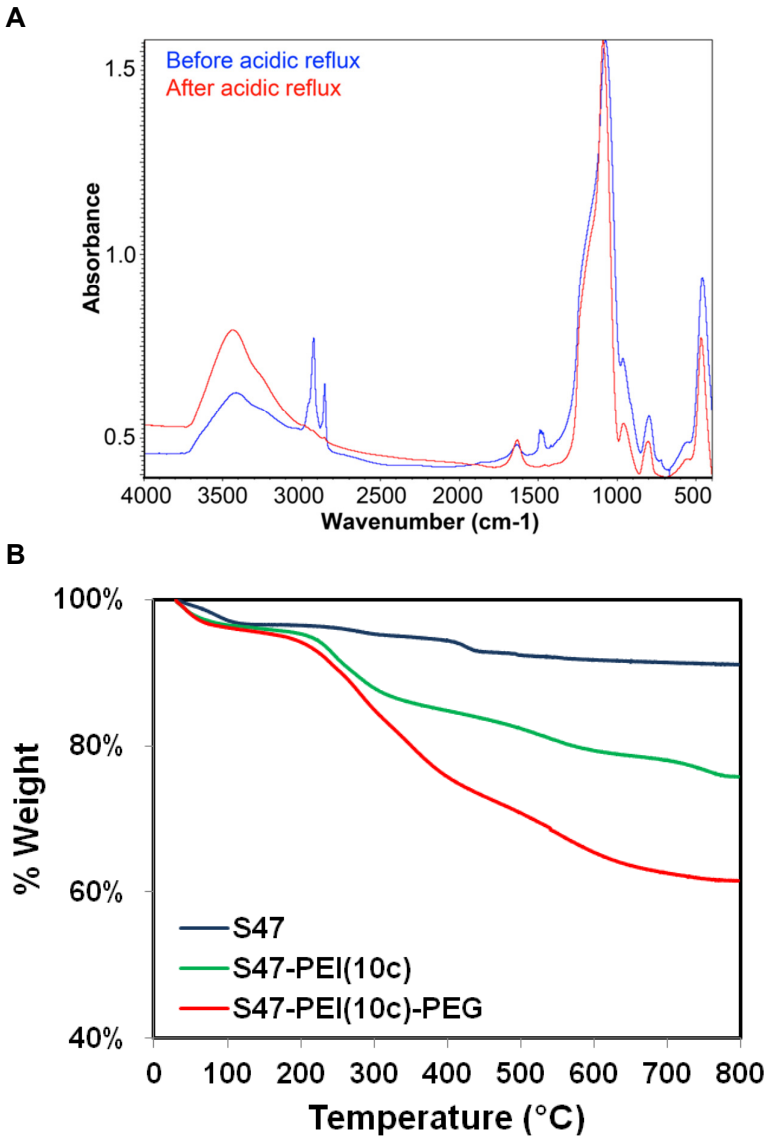


Figure S1. **Nanoparticle characterization. (A)** FT-IR spectra of MSNP before and after surfactant removal by acidic reflux showing disappearance of surfactants associated peaks at 2960, 2870, and 1460 cm^{-1} after the reflux. **(B)** Weight versus temperature profile of S-47 modified with PEI-PEG, measured by TGA (Q50, TA Instruments).

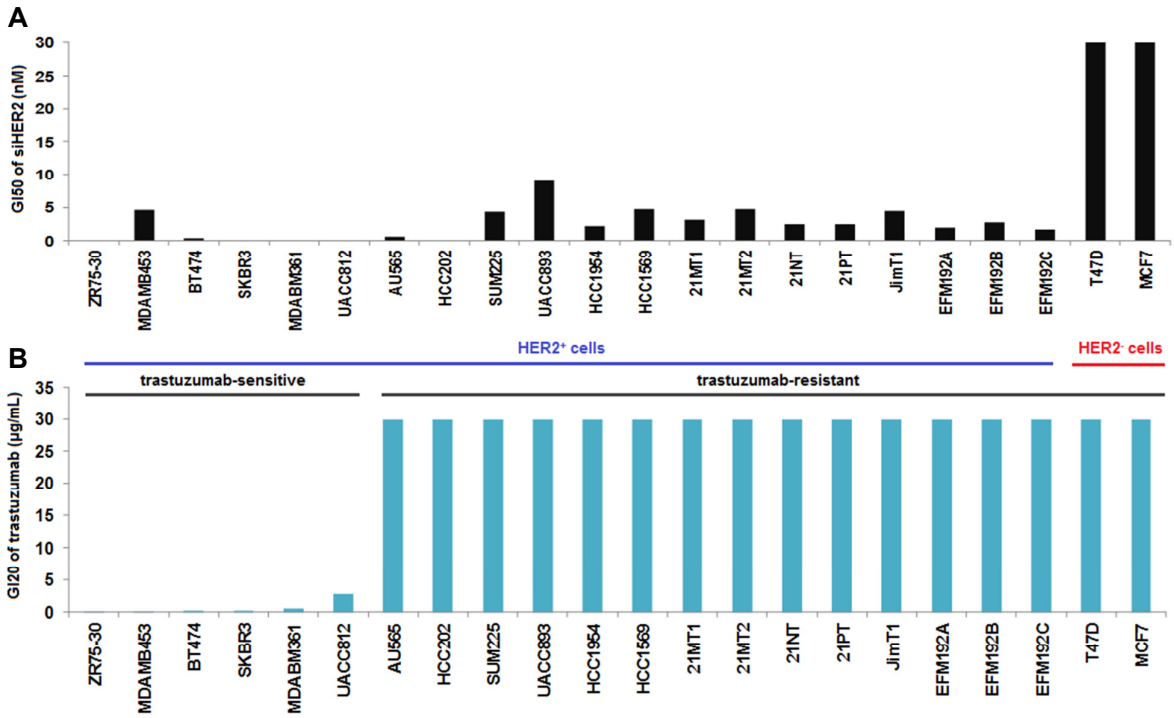


Figure S2. SiHER2 and trastuzumab response of 20 HER2$^+$ cell lines and 2 HER2$^-$ cell lines. Cells were treated with siHER2 in a dose range of 0-30 nM or trastuzumab in a dose range of 0-30 µg/ml. Cell viability was measured 5 days after treatment. **(A)** GI50 values (dose required to achieve 50% inhibition of cell growth) of siHER2 across the cell lines are reported. **(B)** GI20 values (dose required to achieve 20% inhibition of cell growth) of trastuzumab across the cell lines are reported. If required growth inhibition was not achieved, 30 nM and 30 µg/ml were reported for siHER2 and trastuzumab, respectively.

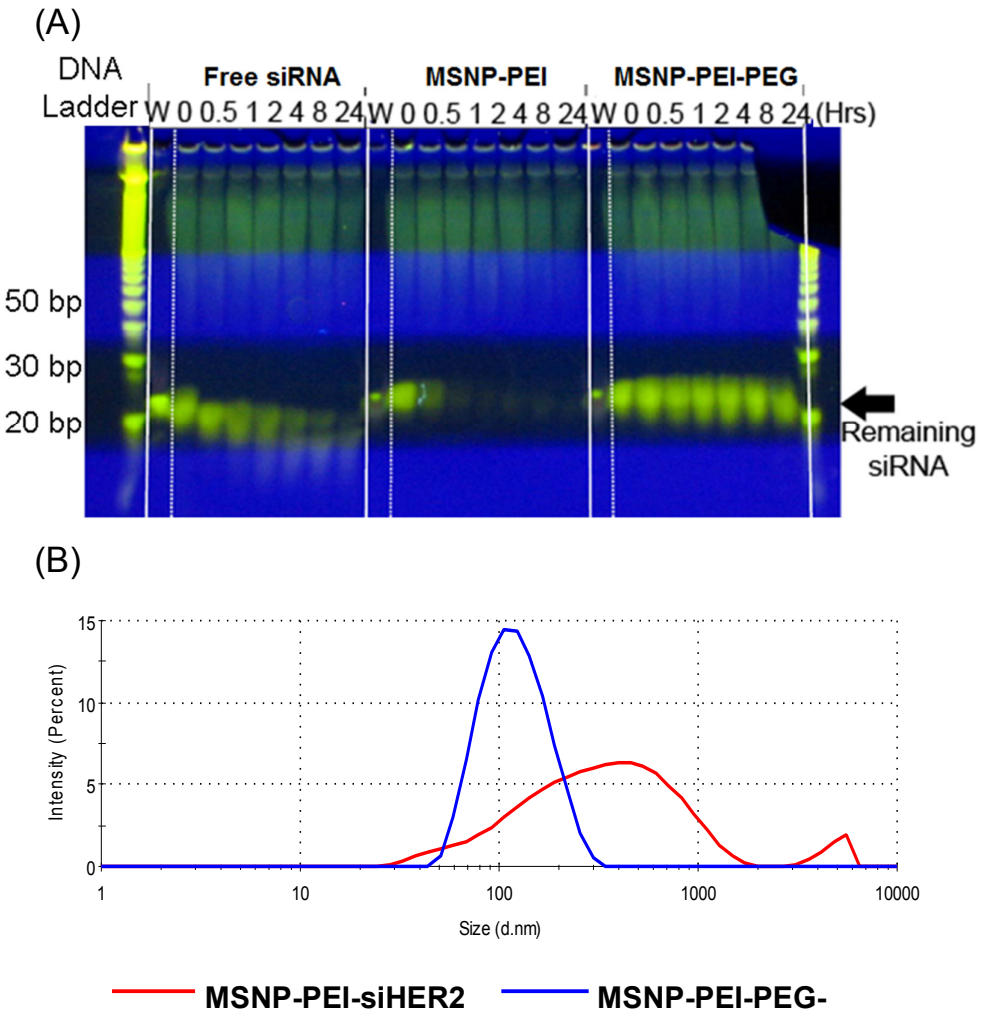


Figure S3. **Importance of PEG in protecting siRNA from serum enzymatic degradation and preventing aggregation upon siRNA binding. (A)** SiRNA against luciferase (siLUC) was loaded on PEI-coated MSNP without PEG (MSNP-PEI) and with PEG (MSNP-PEI-PEG) and was subjected to serum enzymatic degradation essay in the same fashion as Figure 3. PEI was 10-kDa. **(B)** Nanoparticle without PEG is shown to aggregate upon siHER2 loading (NP/siRNA of 50), while those with PEG did not show significant size increase.

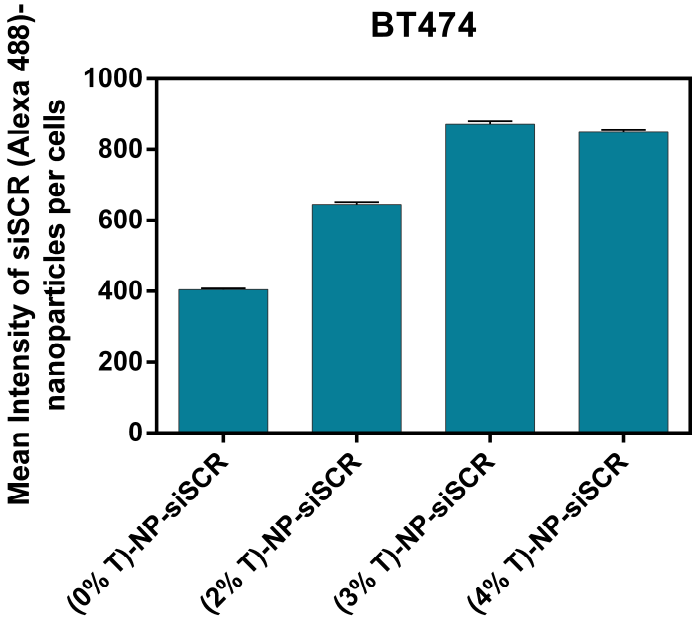


Figure S4. Effect of trastuzumab (T) loading on cellular uptake of siRNA-nanoparticles. Data are reported as mean intensity (per cell) of Alexa 488-tagged scrambled siRNA (siSCR)-nanoparticles (NP10C), and conjugated with varied percentage of trastuzumab (T). Experiment was conducted in the same manner as Figure 4.

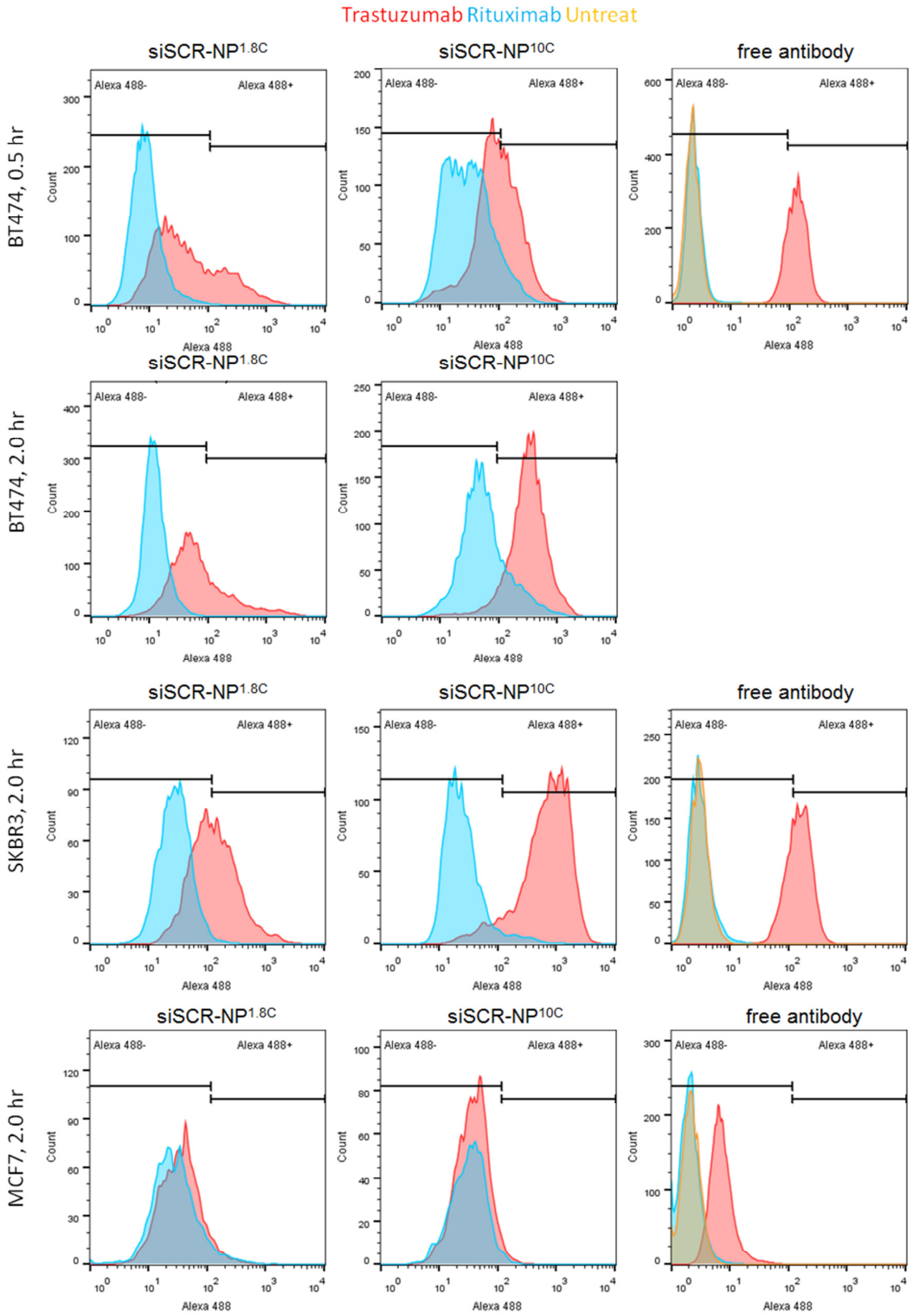


Figure S5. Flow cytometry histograms demonstrating the uptake of fluorescent-labeled siRNA-nanoparticles in breast cancer cells. The corresponding bar chart was presented in Figure 4.

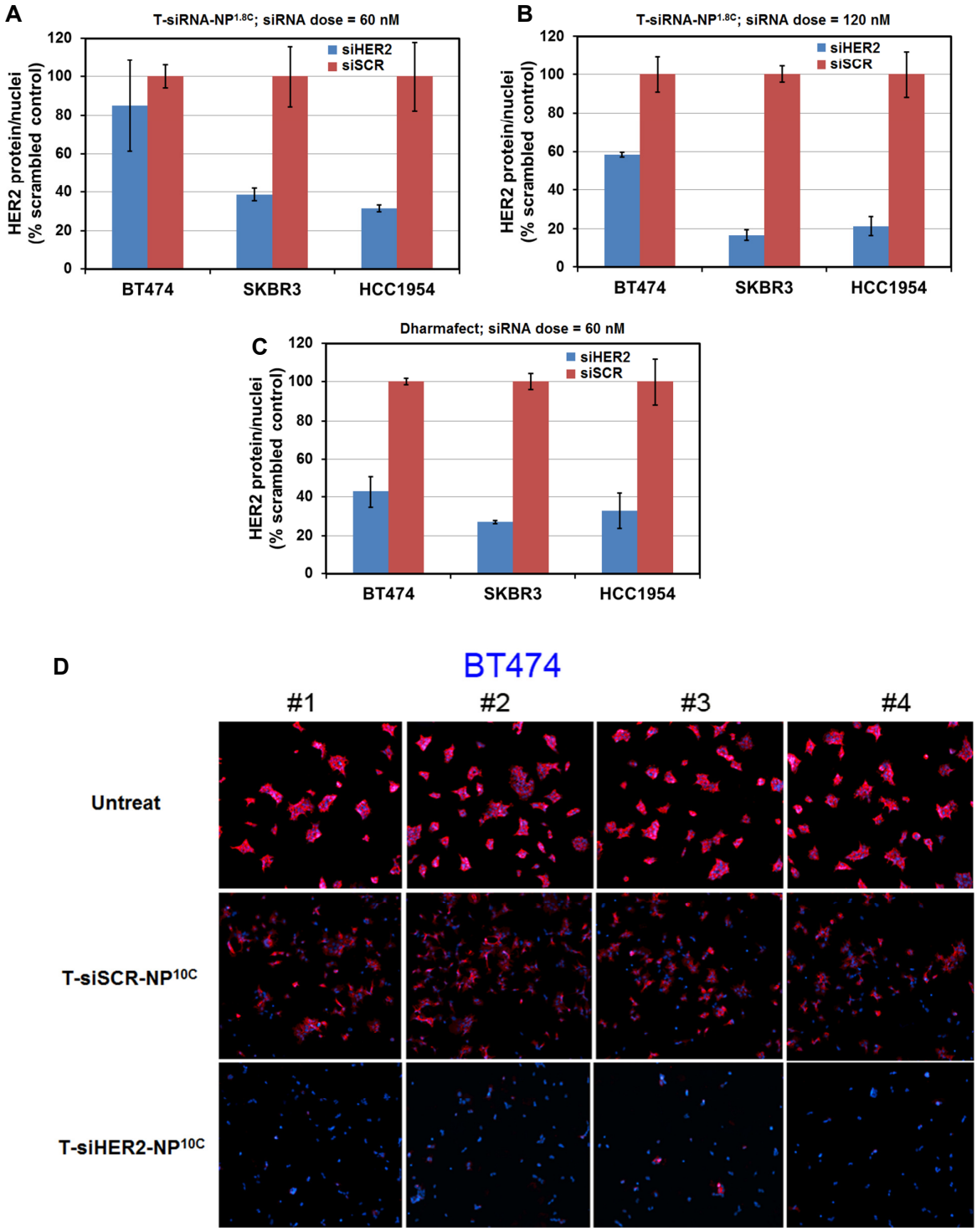


Figure S6. HER2 protein reduction analyzed by Immunofluorescence imaging. HER2 level per cell from three HER2^{+} cell lines after treatment with siHER2 or siSCR on nanoparticles with cross-linked 1.8-kDa PEI **(A-B)**, or DharmaFECT **(C)**. Experiment was

conducted in the same manner as Figure 5A. All values were normalized with scrambled siRNA control. **(D)** Representative immunofluorescent images of HER2 (red) and nuclei (blue, DAPI stain) of BT474 cells treated with 60 nM of siHER2 or siSCR, delivered by the most optimal nanoparticles (T-siRNA-NP^{10C}, Figure 5A), compared to the untreated control. All were performed in 4 replicates (wells). Cells were fixed and stained for IF analysis at 72 hours after treatment (nanoparticle exposure was the first 20 hours). Signal intensity was processed by CellProfiler image analysis software. Only HER2 (red) signal associated with nuclei was accounted for. HER2 signal was reported per number of cells (nuclei).

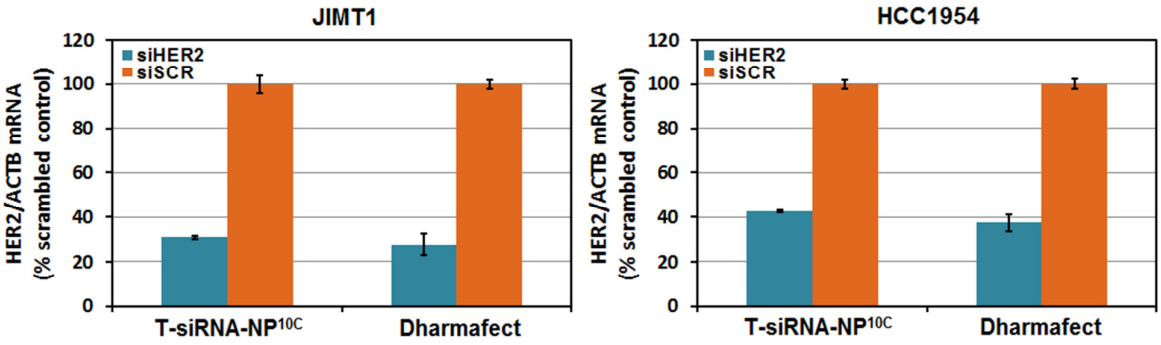


Figure S7. HER2 mRNA reduction in JIMT1 and HCC1954. HER2 mRNA level (normalized by β-actin mRNA) of JIMT1 **(A)** and HCC1954 **(B)** after treatment with siHER2 or siSCR on nanoparticles with cross-linked 10-kDa PEI (T-siRNA-NP^{10C}). Experiment was conducted in the same manner as Figure 5B. All values were normalized with scrambled siRNA control.

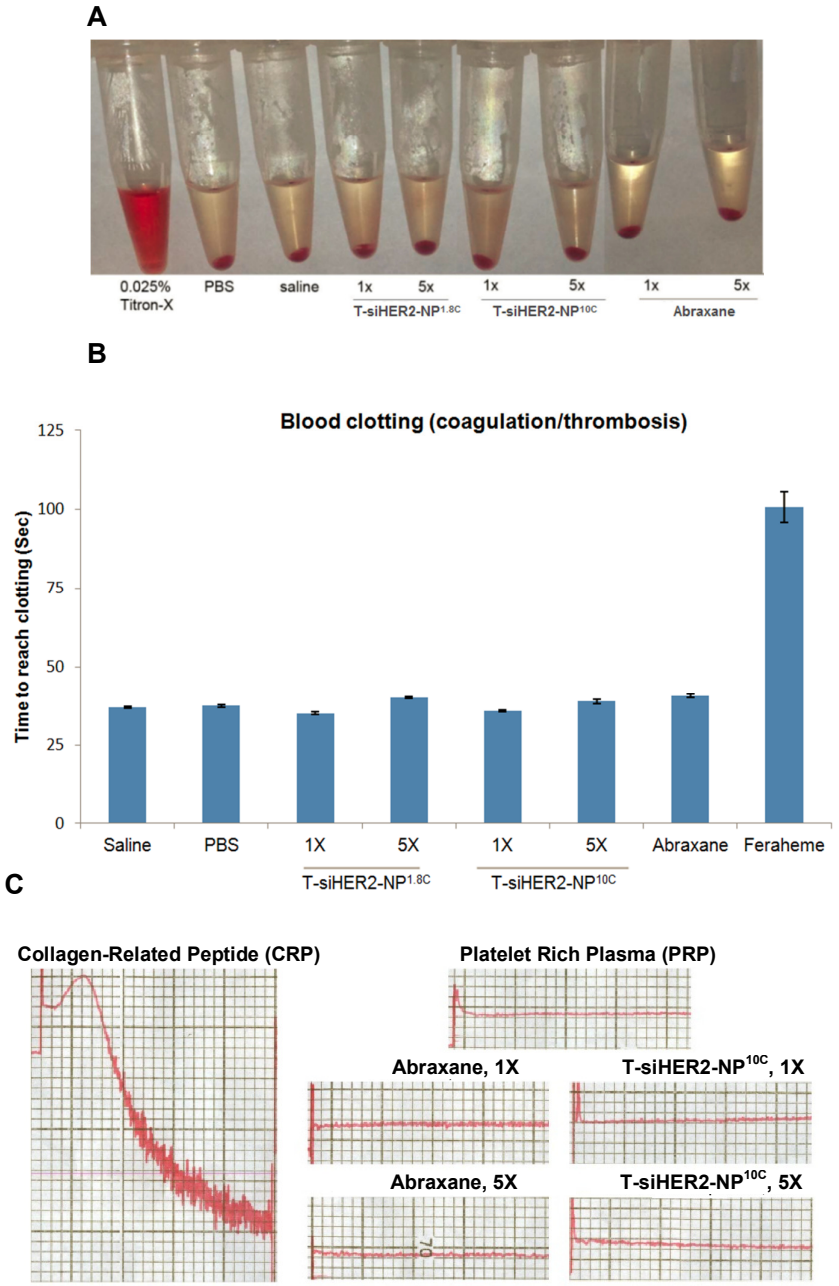
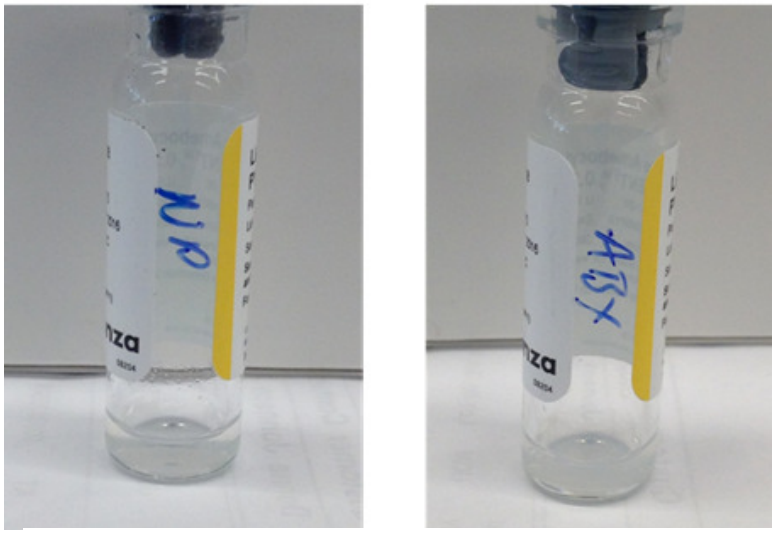


Figure S8. Blood compatibility of siRNA-nanoparticles. **(A)** Hemolysis assay: siRNA-nanoparticles were incubated with $1x10^9$ red blood cells/mL for four hours at 37°C. At the end of incubation, red blood cells were pelleted down and supernatant was analyzed for lysed hemoglobin (Triton-X as positive control). **(B)** Coagulation time: siRNA-nanoparticles were incubated in Platelet Poor Plasma (PPP) for 30 minutes at 37 °C. Clotting time was measured upon addition of APTT-xl reagent and $CaCl_2$. **(C)** Evaluation on platelet aggregation of

materials incubated in Platelet Rich Plasma (PRP) with collagen related peptide (CRPs) as positive control.



T-siHER2-NP^{10C} (5X) **Abraxane (5X)**

Figure S9. Images from LAL gel-clot assay on our nanoparticles (T-siHER2-NP^{10C}), and benchmarked with Abraxane; both at 5X concentration (similar to Figure 7). Both are negative for endotoxin according to manufacturer's protocol, "A negative test is characterized by the absence of solid clot after inversion. The lysate may show an increase in turbidity or viscosity. This is considered a negative result."

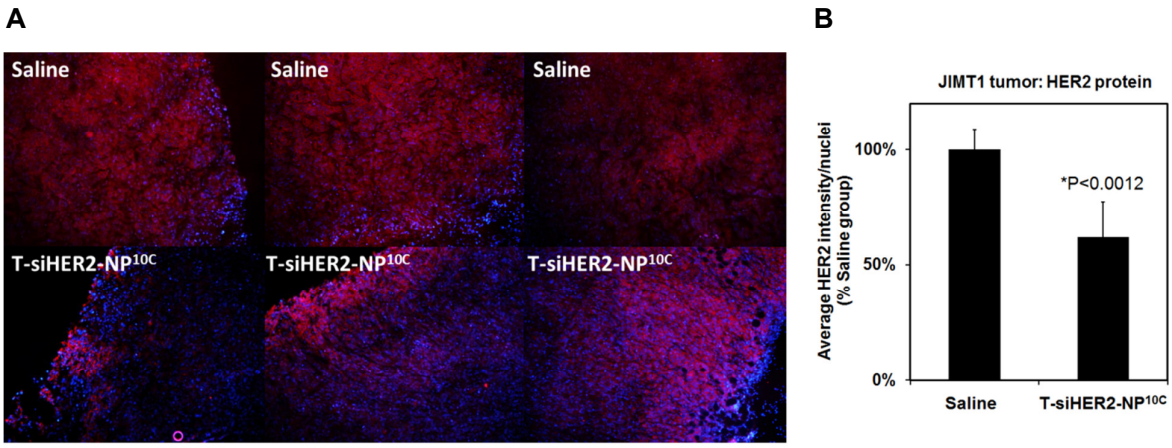


Figure S10. ***In vivo*** **delivery of siHER2-nanoparticles to JIMT1 tumors. (A)** Representative immunofluorescence images. Tumor tissues from animals treated with saline (negative control) or nanoparticles loaded with siHER2 (1.25 mg siRNA/kg). The bar graph **(B)** shows the intensity of HER2 signal from tissue images analyzed by CellProfiler; red = HER2 protein; blue = DAPI staining cell nuclei. P-values are against saline treated group.

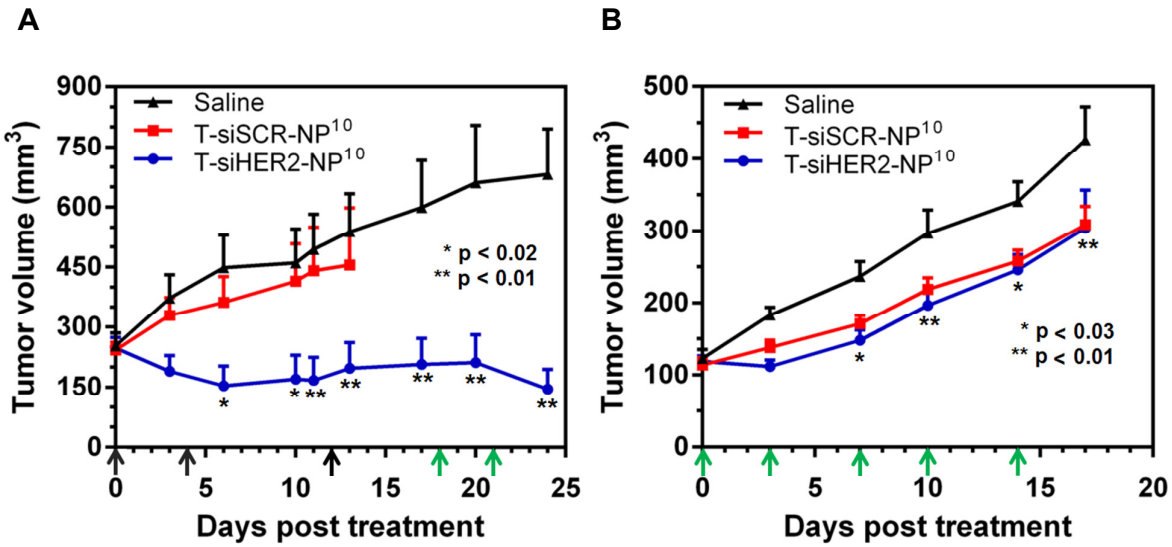


Figure S11. ***In vivo*** **delivery of T-siHER2-NP[10] (based on O-87 core) to HCC1954 tumors.** Treatments include nanoparticles loaded with siHER2 or siSCR (given via tail vein injections). Arrows indicate days of injection: 5 mg siRNA/kg (black) and 2.5 mg siRNA/kg siRNA (green). Specified p-values are against saline control. Tumor inhibition was evaluated in **(A)** HCC1954 ectopic (flank) tumor model (n=5/group) and **(B)** HCC1954 orthotopic tumor model (n=11/group).

A

Batch	MSNP core	T-NP^{10C}		
	Hydrodynamic Size Z-average ± SD [nm]	Hydrodynamic size Z-average ± SD [nm]	Zeta Potential Average ± SD [mV]	Luc silencing efficacy [% siSCR]
1	61.1 ± 0.7	115.8 ± 4.0	25.0 ± 0.1	75.7 ± 4.0
2	58.1 ± 0.6	117.4 ± 0.5	24.9 ± 0.1	80.5 ± 2.8
3	59.7 ± 0.5	114.5 ± 7.1	25.0 ± 0.1	76.1 ± 2.4
4	57.7 ± 0.9	123.8 ± 3.3	25.0 ± 0.1	76.6 ± 3.9
5	60.8 ± 0.8	113.2 ± 2.3	25.0 ± 0.1	76.2 ± 2.8
6	58.8 ± 0.3	115.6 ± 1.3	25.0 ± 0.1	77.0 ± 2.2
Average	**59.4**	**116.7**	**25.0**	**77.0**
% Relative SD	**2.4**	**3.2**	**0.2**	**2.3**

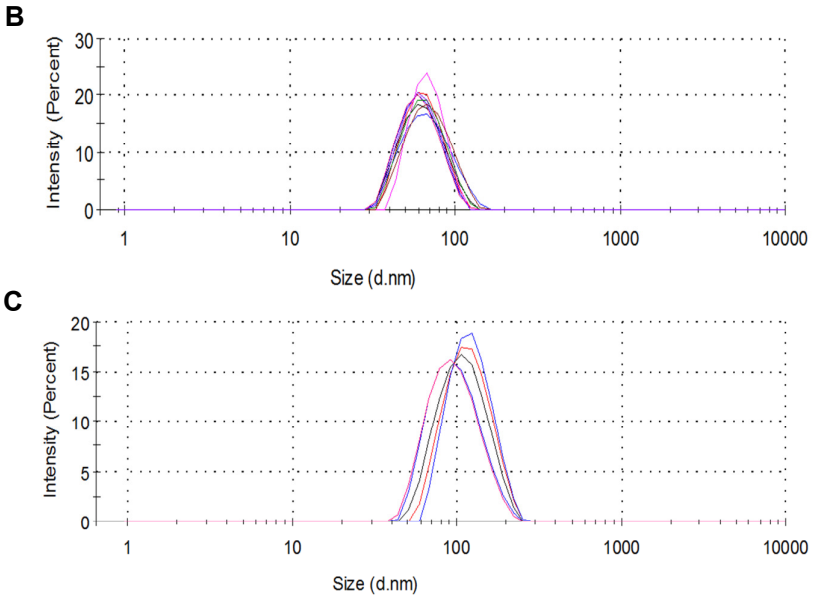


Figure S12. Reproducibility of nanoparticle synthesis. (A) Summary of sizes (before and after surface modification), charge, and silencing efficacy of six individual batches of mesoporous silica nanoparticles (47 nm core). Hydrodynamic size of MSNP **(B)** before and **(C)** after surface modification.

Table S1. HER2 knockdown by siHER2 on two nanoparticles (vs. siSCR control) and DharmaFECT. Cells were treated with nanoparticles for 20 hours and HER2 quantitation with IF was performed at 72 hours post treatment. Data were tabulated from those in Figure 5A and S5, which were processed from IF images.

Materials	siRNA [nM]	HER2 knockdown (Average ± SD) [% siSCR control]		
		BT474	SKBR3	HCC1954
T-siRNA-NP^{10C}	60	80.5 ± 11.7	82.2 ± 12.1	92.6 ± 8.7
T-siRNA-NP$^{1.8C}$	60	15.2 ± 14.8	61.3 ± 12.2	64.9 ± 8.6
	120	41.6 ± 9.4	83.3 ± 5.0	78.6 ± 12.8
DharmaFECT	60	57.1 ± 8.2	72.9 ± 4.2	67.0 ± 15.0