Supplementary information

Enhancement of Cancer Specific Delivery Using Ultrasound Active Bio-originated Particles

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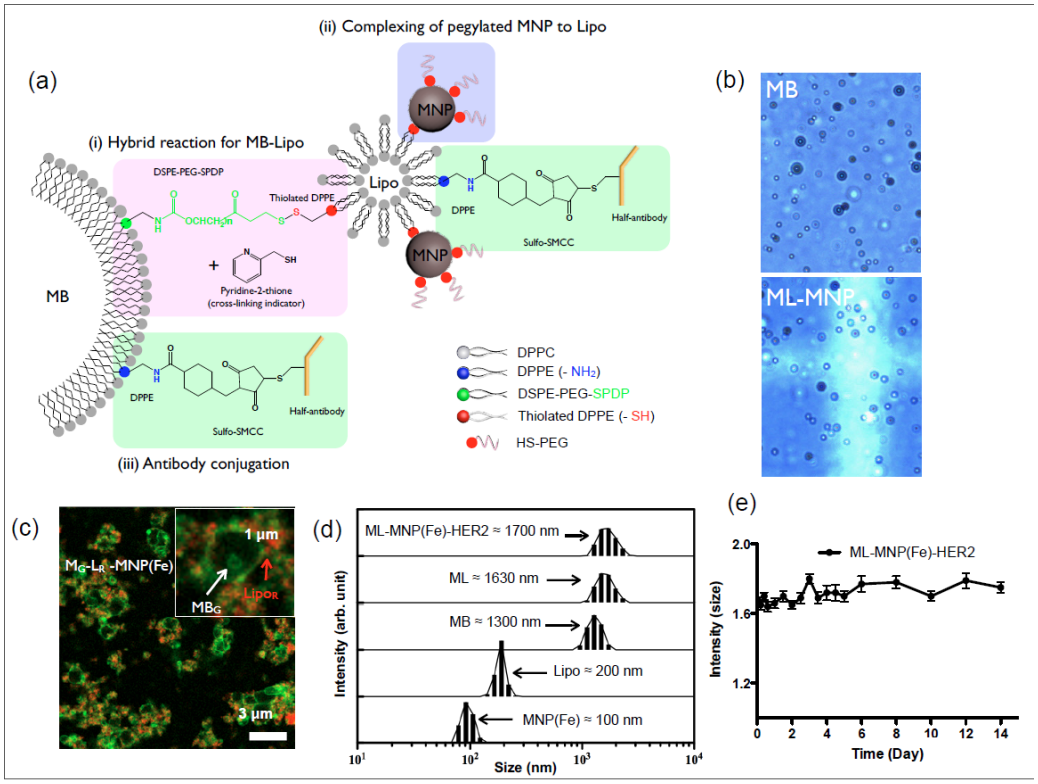

Fig. S1. Preparation of the ML-MNP(Fe)-HER2 hybrid particles; The red and green fluorescent M_GL_R-MNP(Fe)-HER2 particle complexes were synthesized by a three-step chemical reaction; an MB-Lipo hybrid reaction, MNP(Fe) anchoring, and antibody conjugation (a). For the ML preparation (a, i), DSPE-PEG-SPDP in MB was cross-linked with the sulfhydryl groups after the thiolation of Lipo-DPPE, and the complete hybrid reaction was confirmed by the detection of pyridine-2-thione as a leaving chemical group with a UV-Vis spectrometer. MNP(Fe) was attached to the thiolated Lipo (a, ii), which was further treated with HS-PEG chemicals in order to increase the solubility in aqueous environments. Finally, to render specificity to the particles, the HER2 half antibody (HER2) was bound to the maleimide functional group on the MB and Lipo after reaction of the Sulfo-SMCC with DPPE (a, iii). All procedures have been completely described in the experimental section. The MB and final ML-MNP particles were characterized the shape and stability by optical microscope (b). The red and green organic dyes incorporated into the Lipo and MB complexes were characterized with a confocal laser scanning microscope (c). The MB revealed a hollow sphere due to the core SF_6 gas, and the Lipos around the green MB were indicated by small red dots (c, inset). The size distribution of all the prepared particles was determined with a DLS study (d). To realize for stability in solution, the particle was determined the size intensity as a time using DLS study. During the measurement, the particle was stored in a vial with the SF_6 gas full-filled (e).

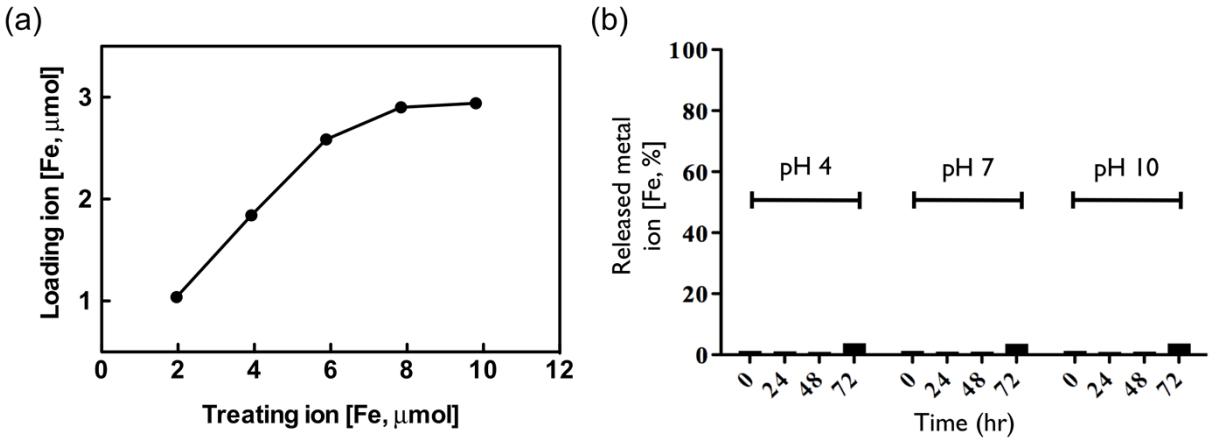

Fig. S2. Chelating capacity of Fe^{3+} ions onto the MNP; To test the chelating capacity of Fe^{3+} ions onto the melanin nanoparticle (MNP), Fe (NO3)3·6H2O was dissolved in distilled H_2O at various concentrations (2–10 μmol), and then mixed with 1 mL of the solution with dispersed MNPs (1 mg/mL). After completion, it was washed out to remove the excess Fe^{3+} ions by precipitation at 13,000 rpm for 10 min. The amount of loaded Fe^{3+} ions was determined directly by ICP-AES analysis (a). The Fe^{3+} ions were maximized to a 3 μmol loading capacity per mg of MNP, while treating with 8 μmol of the Fe^{3+} ion solution. More importantly, the chelated Fe^{3+} ions should not release from the MNP particle at various pH levels that meant sustaining ability for a contrast agent. An extremely low amount of Fe^{3+} ions was detected (< 5 %) after 72 h of stirring at various pH levels (4, 7, and 10), according to the ICP-AES analysis (b).

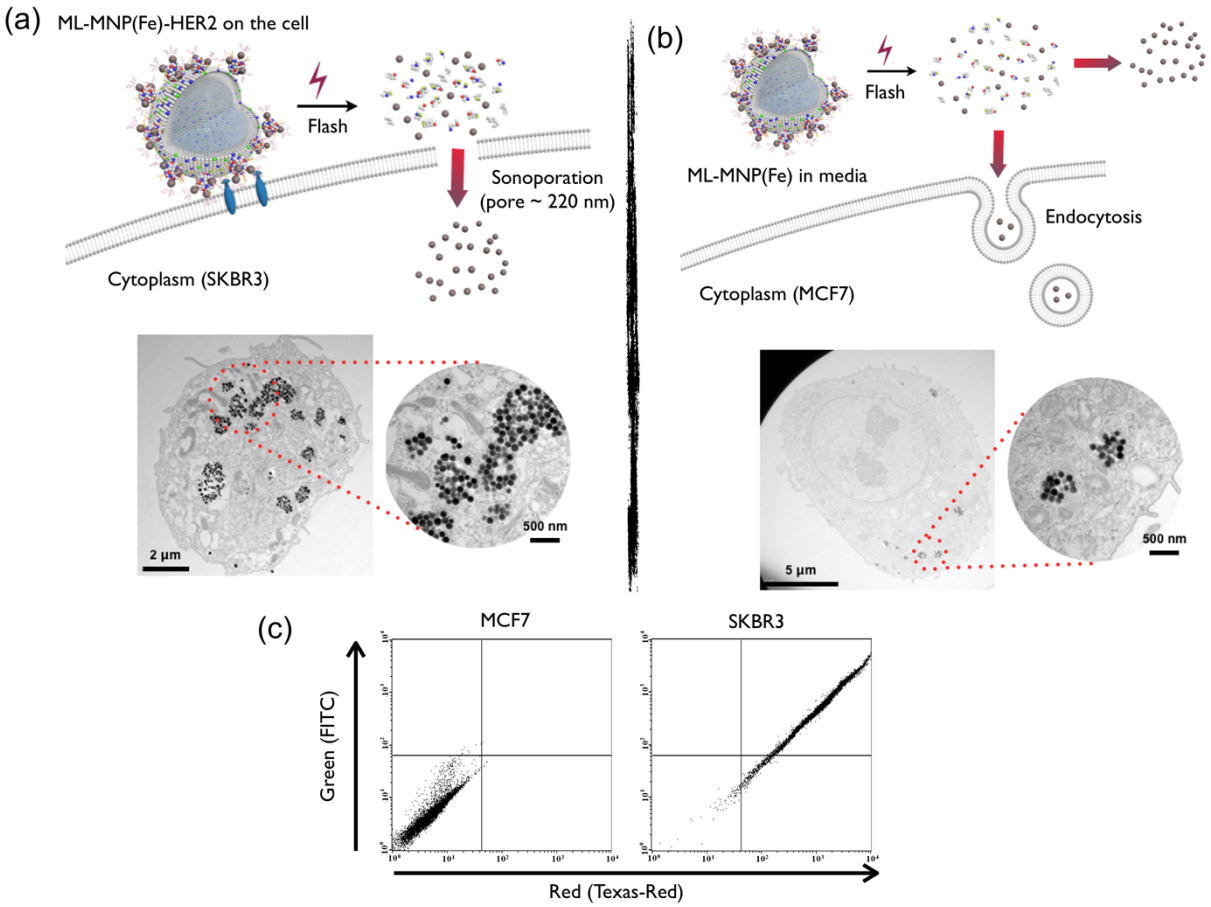

Fig. S3. Enhancement of the uptake efficiency *via* specific targeting based US flash illumination; ML-MNP(Fe)-HER2 particles can exhibit specific targeting to HER2 positive SKBR3 cells and attach onto these cells. After a US flash (MI = 0.61) for 1 min, the MB of the particle was quickly destroyed and it released the Lipo- and MNP(Fe)-linked materials into the cell. Furthermore, the destructive forces of the MB introduced pores (≈ 220 nm in diameter[1]) into the cell membrane, which eventually soaked up the linked material (a). As a control, MCF7 cells expressing very low levels of the HER2 receptor (Fig. 3b) were treated with particles under the same condition. The rare MNP(Fe) particles were found into the cell cytosol owing to non-specific endocytosis (b). The red and green fluorescent organic dyes from MB and Lipo that were located cytosol after exposing flash were quantitatively analyzed using FACS (c).

[1] Zhou Y, Kumon RE, Cui J, Deng CX. "The size of sonoporation pores on the cell membrane" Ultrasound Med. Biol. 2009, 35, 1756-1760.

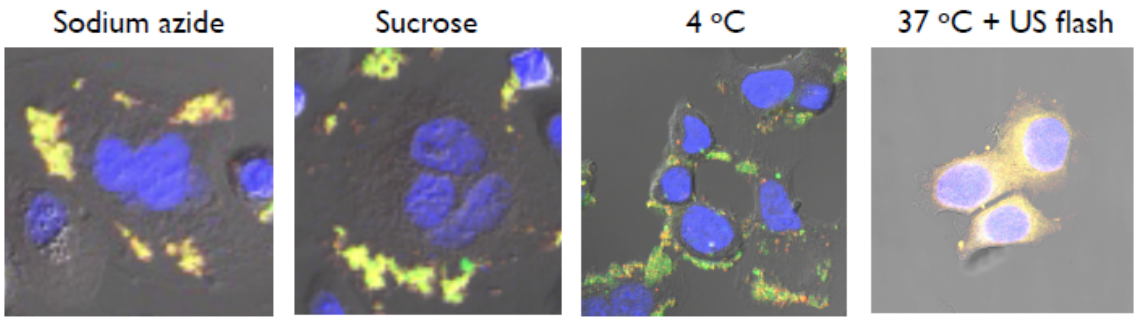

Fig. S4. To determine the mechanism mediating uptake of the organic dyes by flash, the cells were incubated after targeting with M_GL_R-MNP(Fe)-HER2 at different temperatures of 37 °C and 4 °C, respectively. Particles did not absorb well into the cytosol at 4 °C, but were nearly completely absorbed at moderate temperature (37°C). To confirm the involvement of cell metabolism using a specific inhibitor, cells were pretreated with 0.1% sodium azide or 0.45 M sucrose and incubated with the M_GL_R-MNP(Fe)-HER2 at 37 °C for 30 min; this resulted in decreased uptake of the organic dye

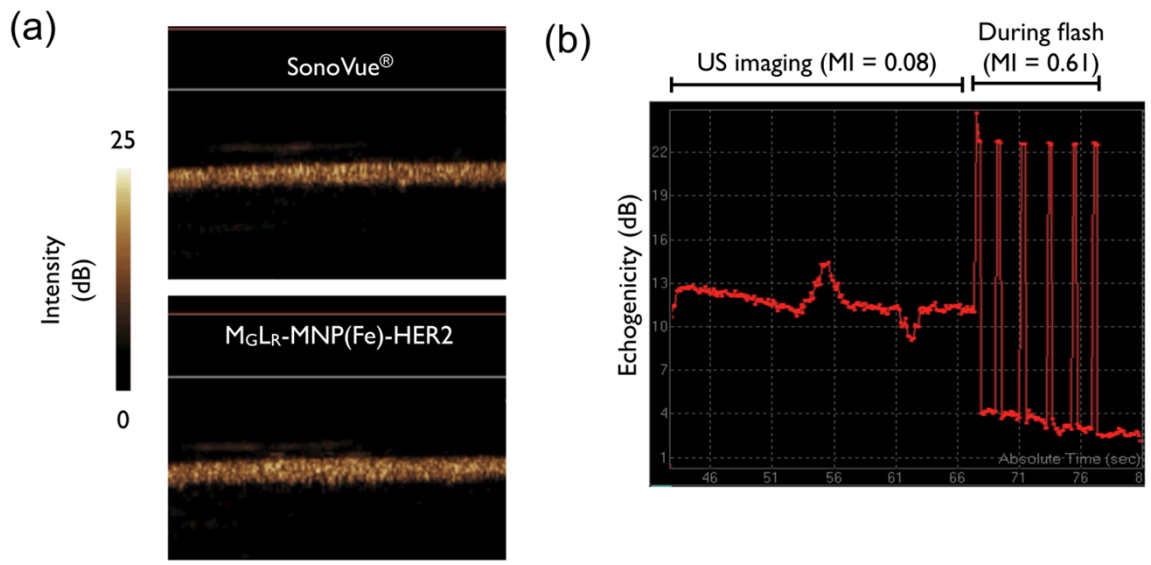

Fig. S5. US phantom study for the comparison of echogenicity of contrast agents and monitoring of MB cavitation under the high acoustical pressure (flash) treatment; After loading the US phantom plastic-tubes, the synthesized M_GL_R-MNP (Fe)-HER2 particle solution displayed an echogenicity and intensity similar to that of a clinically used SonoVue® US agent at the same concentration of phospholipids (DPPC, 10.5 mM) under the US imaging mode (MI = 0.08) (a). The echogenicity disappeared gradually after flash application (MI = 0.61) (b). We found that the prepared particles were destroyed within 6 flash treatments.

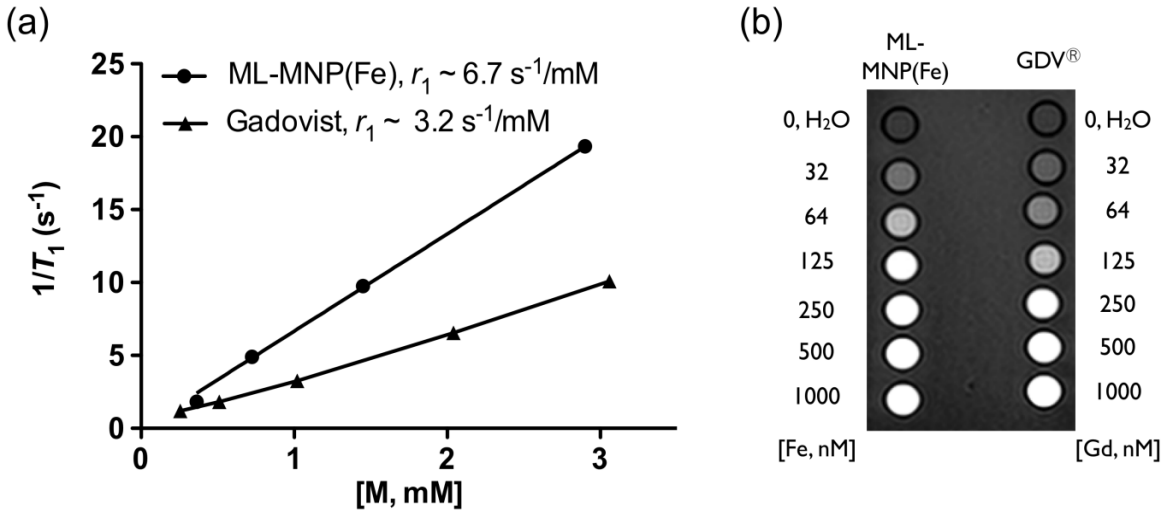

Fig. S6. Measurement of the longitudinal relaxivity (r_1) and T_1-*w* MR in the phantom study; We compared the longitudinal relaxivity value of the prepared ML-MNP(Fe)-HER2 particles and a clinically used T_1 MR agent (Gadovist®) using a 0.47-T magnetic relaxometer (mq20, Bruker). The longitudinal relaxation times were measured at various concentrations of the metal ion, which was also calculated accurately using ICP-AES analysis. The particle solution showed a 2-fold higher r_1 than Gadovist® (a). According to the Solomon-Bloembergen-Morgan (SBM) theory, a second-sphere of water molecules and oxygen atoms in the Fe^{3+} chelated catechol complexes increases r_1, and the nano-particulate character of MNPs exhibits an enhanced r_1 and a restricted rotational mobility. For the MR phantom study with a clinically used MRI instrument (3.0-T, Philips), ML-MNP(Fe)-HER2 particle solution had approximately 2-fold higher white contrast intensity at the same metal ion concentration (b).

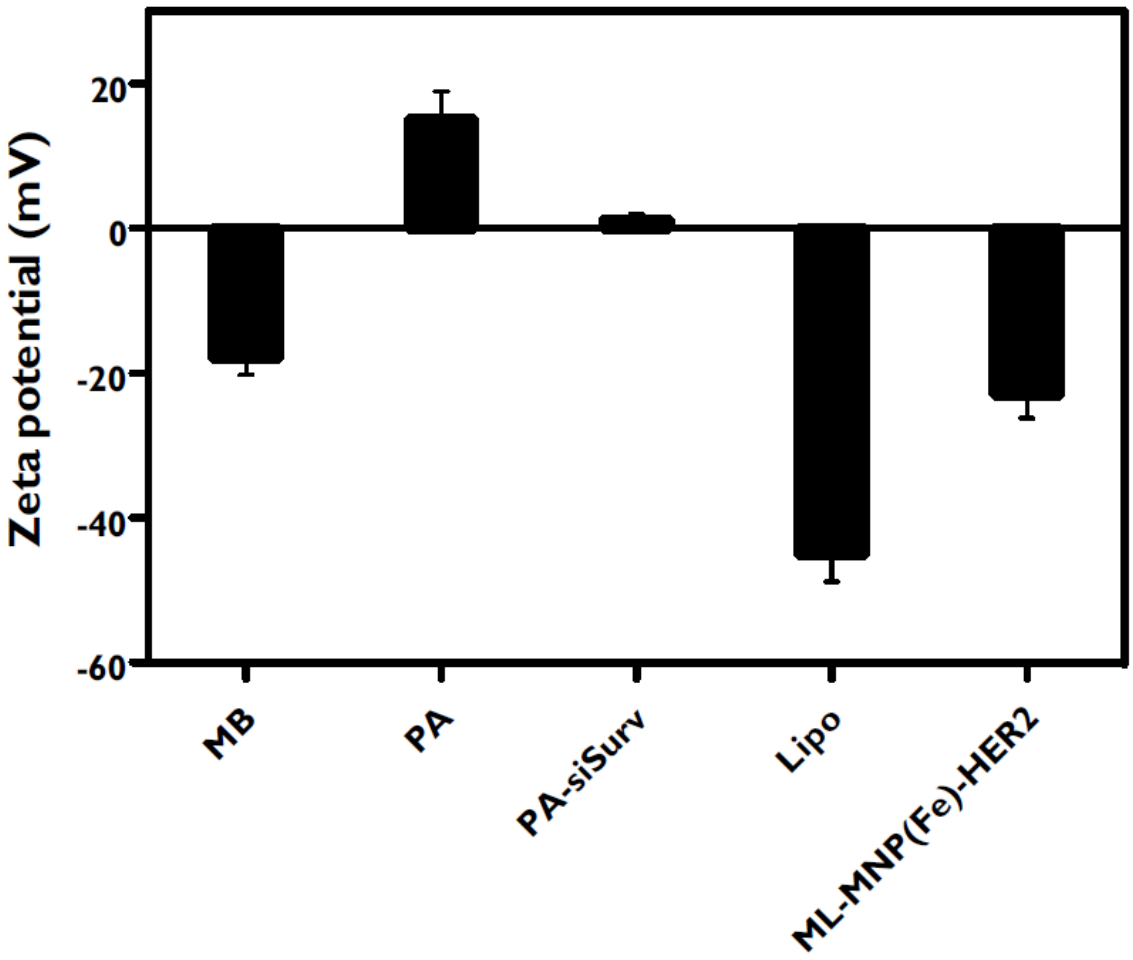

Fig. S7. Measurement of electro-charge density for various compounds.
Protamine (PA) and therapeutic gene (siSurv) were revealed different charge character with 7 mV and -45mV. After complexation of PA-siSurv, the charge was changed to -22 mV. The Liposome particle possessing of PA-siSurv complex was shown to - 32 mV.

Experimental section

Preparation of MB, Lipo, MNP (Fe,) and their complexes:

1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC, 15.4 mg), diacetyl phosphate (DCP, 1.0 mg), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE, 1.2 mg), 1,2-distearoyl-sn-phosphoethanolamine-N-[PDP(polyethylene glycol)-2000] (DSPE-PEG-PDP, 5.0 mg), and cholesterol (3.5 mg) were dissolved in 5 mL chloroform (99.9 %, Sigma-Aldrich, St. Louis, MO, USA). The mixed solution was allowed to evaporate for 5 min at room temperature and was then freeze-dried for 24 h at -45 °C to induce the formation of a phospholipid film. Subsequently, 2 mL of a solution containing glycerin, propylene, and H_2O (volume ratio: 1:2:7) was added to the film and the solution was gently shaken. It was then transferred to a hermetic vial (Wheaton, NJ, USA) and vigorously mixed for 15 s with a mechanical high-speed shaking-device (KIMS, South Korea) during bubbling with SF_6 gas. The prepared MB particle solution was stored in a refrigerator. Lipo was produced from a chloroform solution containing DPPC, DCP, DPPE, and cholesterol. The solution was evaporated and lyophilized as above. After adding 2 mL H_2O, the lipid film underwent sonication for 5 min at 60 °C. The solution was subjected to a freeze-thaw cycle five times, using liquid nitrogen and a water bath. The prepared Lipo dispersion solution was extruded through a 200 nm filter at 60 °C (mini-extruder, Avanti polar lipids). To render green and red fluorescence, fluorescein isothiocyanate (1 mg, Sigma-Aldrich) and Texas-red (1 mg, Sigma-Aldrich) were homogeneously added to the lipid mixed solution before generating a film of MB and Lipo, respectively. The excess organic dyes were continually removed by centrifugation (5 min at 13,000 rpm) and washing with H_2O, until the color of the supernatant disappeared completely.

To fabricate the sulfhydryl functional groups on the Lipo particle, 2 mL amine active Lipo (21 mg), derived from DPPE, was reacted with 5 mg of 2-iminothiolane·HCl (Traut's reagent, Pierce) for 2 h at room temperature, after adjusting the pH to 8.2 with 1 M $NaHCO_3$. The 2 mL thiolated Lipo (10.5 mg/mL) and 1.5 mL MB (13.1 mg/mL) solutions were gently shaken for 2 h at room temperature and the complex reaction was monitored through calculating of the amounts of pyridine-2-thione in supernatant by UV-Vis spectroscopy. To prepare the MNP particles, 180 mg of dopamine hydrochloride was dissolved in distilled water at 50 °C and 780 μL of NaOH solution (1 M) was vigorously stirred. After 6 h, the color turned black and the solution was centrifuged (10 min at 20,000 rpm), and washed several times with distilled water. The MNP particles displayed a characteristic size (diameter = 100 nm), and showed uniform size distribution, as determined by TEM and DLS. In order to coordinate the Fe^{3+} ion, 4 mL of $Fe(NO_3)_3 \cdot 6H_2O$ with the concentration range 2–10 μmol was mixed with 10 mL of the MNP dispersion

solution (1 mg/mL). After stirring for 3 h, the solutions were centrifuged (10 min at 20,000 rpm), and the unbound Fe^{3+} ions in the supernatant solution were quantitatively estimated in order to determine the overall loading capacity. This was achieved with an inductively coupled plasma atomic emission spectrometer (ICP-AES). The thiolated MB-Lipo (called ML) complex solution (30 mg, 15 mg/mL) was homogeneously mixed with 1 mL of the MNP (Fe) solution (1 mg/mL) for 0.5 h at room temperature. The excess MNP (Fe) particles were removed by centrifugation (5 min at 13,000 rpm) and by washing the ML-MNP (Fe) complexes trice with distilled water. To increase the stability in aqueous environments, the resulting complex particle solution was treated with 2 mL of methoxy-poly(ethylene glycol) (PEG-SH, 2 kDa, 0.5 mM) for 1 h at room temperature and the excess PEG-SH was removed by centrifugation (2 min at 13,000 rpm). The size and shape of the complex was characterized using FE-SEM, TEM, and DLS measurements. In our preparation, all phospholipid chemicals were purchased from Avanti Polar Lipids and all other chemical reagents were purchased from Sigma-Aldrich. All chemicals were used without further purification.

HER2 antibody conjugation:
In order to fabricate with an active maleimide functional group, 2 mL of the ML-MNP(Fe) particle solution (14.9 mg/mL) containing an amine functional group was reacted with sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, 5 mg, Sigma-Aldrich) for 3 h, after adjusting the pH to 8.2 with a 1M $NaHCO_3$ solution. The maleimide moiety was then linked to the appropriate HER2 half antibody using a general bio-conjugation procedure. The antibody-harboring particles were quantitatively analyzed using a typical protein estimation assay.

Cell toxicity assay:
A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay kit (Invitrogen) was utilized to evaluate the cell viability in the presence of the ML-MNP(Fe) particles. The SKBR3 cells (ATCC, VA, USA) were cultured in RPMI supplemented with 10% FBS, penicillin, and 1% streptomycin. Cells were maintained at 37 °C in a 5 % CO_2 incubator. The cells were washed, trypsinized, and re-suspended in the culture medium after they achieved the desired 80% confluence. The cells were then seeded at a concentration of 5,000 cells/well in a 96-well tissue culture plate and allowed to grow overnight in a CO_2 incubator. Various concentrations of the ML-MNP(Fe) particles in 1 mL PBS buffer solution were added to cell culture solution and the cells were allowed to grow for another 24, 48, and 72 h, respectively. To determine the cell viability, the culture medium was

replaced with an MTT solution. After 3 h of incubation inside a CO_2 incubator, the MTT solution was added to dissolve the resulting formazan crystals. The cell viability was determined spectrophotometrically at 570 nm, with a background subtraction at 690 nm.

US phantom study:
The echogenicity of ML-MNP(Fe)-HER2 was evaluated using a phantom sample. This solution was made using a plastic connecting tube with an inner diameter of 2 mm, located in a chamber filled with water. 9 mg of the particles or Sonovue® solution in 2 mL PBS were passed through the plastic tube at 0.1 mL/s. An ultrasound scanner (iU22, Philips, Bothell, WA, USA) equipped with a 5–12 MHz broadband linear probe was used for imaging (Mechanical Index, MI = 0.08).

Phantom and in vitro MR study:
For a T_1 weighted (T_1-*w*) MR phantom study, various concentrations (0–1000 nM, [Fe] or [Gd]) of ML-MNP(Fe) particles or Gadovist® were prepared by fusing these into a 0.5% agarose gel (1:1 volume ratio). To test *in vitro* MR imaging, a 3 μM [Fe] concentration of the particles were incubated with HER2-positive SKBR3 and HER2-negative MCF7 cells for 1 h inside a 5 % CO_2 incubator. Subsequently, a US probe was placed in the backside of the well plate and 6 flash pulses (MI = 0.61) were applied for 1 min. The excess particles were removed by washing the cell culture with the culture media, and the treated cells were detached by trypsinization and subsequently centrifuged at 3,000 rpm. All T_1-*w* MR images were acquired on a 3.0-T clinical MR scanner (Philips medical system, Netherland, archieva Release 3.2.1.0 version). Axial and coronal T_1-*w* images were obtained with a TR 400 ms, a TE 10 ms, a 240 × 240 matrix, a flip angle 90 degrees, a slice thickness 3 mm, number of averages with 4, bandwidth 115 Hz/pixel and the FOV of 120 × 120 mm.

siSurv incorporation and cell treatment:
To prepare Protamine (PA, 7.5 kDa) and siRNA complex, the siSurv gene (50 μM) and different concentration of PA solutions (10 ~ 80 μM) were incubated for 1 hr at room temperature using plate shaker. And we optimized that the ratio of PA and siSurv concentration was 40 and 50 μM, respectively. To generate liposome incorporation, the fresh PA and siSurv complex solution was homogenized into lipids films (21 mg, 2 mL) for 5 min at room temperature, using electrophoresis. The Lipo-harboring *siSurv*-PA was washed twice with centrifugation (5 min at 13,000 rpm) to remove the unloaded gene complex and the loading capacity was then estimated with a UV-Vis spectrophotometer.

The 10.5 mg Lipo(PA-siSurv) dissolved in 1 mL aqueous solution was reacted with Traut's agent (5 mg) for thiolation of DPPE and sulfhydryl terminated Lipo complex were mixed with thiol active MB 1 mL aqueous solution (13 mg). And the MLsiSurv solution was progressed to MNP(Fe) and antibody conjugation according to above particle preparation procedure. Subsequently, 3×10^4 cells were seeded onto a 48-well plate (BD Falcon) and ML_{siSurv}-MNP(Fe)-HER2 particles (1 mg) in 0.5 mL PBS were added to the cells in the culture medium containing 10 % FBS. Following 1 h of incubation, the excess particles were washed out with a PBS solution. For flash application, the clinical US scanner probe was placed at the back of the 48-well plate and flash pulses (MI = 0.61) were applied for 1 min. The treated cells were also washed twice with the culture medium, and then sub-cultured for 2 days.