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Electrostatically Assembled Biocompatible Polymer Nanoparticles for MR/Optical Dual-modality Imaging Nanoprobes†

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Synthesis of γ-PGA[Gd-DTPA]. Poly(γ-glutamic acid) (γ-PGA, MW 50 kDa) was obtained from Bioleaders Corporation (Daejeon, Korea). γ-PGA(100 mg) in 10 ml of 50 mM $NaHCO_3$ were mixed with DTPA (110 mg, p-NH_2-Bn-DTPA, MW 644.3 g mol^{-1}, Macrocyclics), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) (76.7 mg), and N-hydroxysuccinimide (NHS)(57.6 mg). The reaction mixture was stirred at room-temperature for 3 hr. The resulting solution was filtered through 0.22 μm membrane filters. The reaction solution was dialyzed against water (MWCO 10,000) and the sample was dried in vacuo to allow IR and NMR analysis. Fourier transform infrared (FT-IR) spectra were recorded on a Perkin Elmer FT-IR spectrometer using KBr pellet. We averaged 1000 scans to yield the spectra at a

resolution of 4 cm^{-1}. IR spectrum of the sample showed absorbance band of aromatic backbone (C=C) at around 1400 cm^{-1} and C-N stretching bands at around 1260 cm^{-1}, respectively (Figure S1). ^{1}H-NMR spectra were recorded on a Bruker DPX300 Avance instrument at 300 MHZ (scan number = 1000 with classical acquisition parameters). The relative area ratio between N-H resonance at 8.0 ppm C-H resonance at 4.1 ppm associated with γ-PGA was 1.085, while that associated with γ-PGA[Gd-DTPA] was 1.236. The result of this increment in area ratio value between N-H resonance and C-H resonance suggest that DTPA molecules were well conjugated on the γ-PGA by amide bond (i.e. 15.1 % of carboxyl group was reacted) (Figure S2). To chelate with Gd^{3+}, 370 μl of $GdCl_3 \cdot 6H_2O$ in 100 mM sodium acetate (pH 5.5) (100 mg/ml) was added to the aqueous solution of γ-PGA[DTPA] in 100 mM sodium acetate (90 mg, 9 mg/ml). The reaction solution was dialyzed against water (MWCO 10,000) until no free gadolinium was detected in the receiving medium and the dialysate was finally lyophilized. To analyze the amount of chelated Gd, elemental analysis was performed by Inductively Coupled Plasma Atomic Emission Spectrophotometer (Optima 4300 DV, PerkinElmer) (Table S1).

Synthesis of Chitosan[IRDye800]. IRDye800CW-N-hydroxysuccinimide ester (LI-COR, Lincoln, NE) was used conjugated with chitosan (Sigma-Aldrich, St. Louis, MO, USA, 50 kDa) with the following scheme. The NHS group of IRDye800CW-NHS allows a covalent bond formation with an amine group of chitosan. IRDye800-NHS dissolved in DMSO (1 mg/ml) was reacted with a 10 mg/ml chitosan solution for 3 h at room temperature. The chitosan[IRDye800] was separated from free IRDye800-NHS by a gel chromatography (Sephadex G-25, Amersham-Pharmacia Biotech, Piscataway, NJ,

USA) with dH_2O as the eluent. The sample was dried in vacuo to allow NMR analysis. ^{1}H-NMR spectra were recorded on a Bruker DPX300 Avance instrument at 300 MHz (scan number =1000 with classical acquisition parameters). The chemical up-field shift at δ (7.6-7.7 ppm) suggest that new N-H stretch (amide bond) was generated by the conjugation between amine moiety of chitosan and NHS ester part of IRDye800 (Figure S3). For quantification of conjugated dye, the absorbance (at 771 nm) of IRDye800 conjugated on chitosan was measured by spectrophotometer (Table S1) and IRDye800 concentration was calculated from a previously determined calibration using standard solutions of IRDye800 (Table S1).

Preparation of γ-PGA[Gd-DTPA]/chitosan[IRDye800] Nanoparticles and Antibody Conjugation. γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles were prepared using a simple ionic-gelation method under magnetic stirring at room temperature. In brief, an aqueous γ-PGA[Gd-DTPA](10 mg/ml, 2 ml) was added by flush mixing with a pipet tip into an aqueous chitosan[IRDye800](0.5 mg/ml, 40 ml, in dH_2O). To increase the stability of nanoparticles at various pH and salt (NaCl) concentrations, 1 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) (16.5 mg) was added for the crosslinking of carboxyl group in γ-PGA and amine group in chitosan. The γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles were collected by centrifugation at 15, 000 rpm for 10 min. Supernatants were discarded and nanoparticles were re-suspended in dH_2O. For antibody conjugation, 1 ml of anti-HER2 (200 μg mL^{-1}, Santa Cruz Biotechnology, Inc.) was reacted with γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles (5 mg) in 1 ml PBS, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) (16.5 mg),

and N-hydroxysuccinimide (NHS)(14.7 mg). The resulting γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles conjugated with anti-HER2 were collected by centrifugation at 15,000 rpm, washed several times with DI water and dried under reduced pressure. The amount of anti-HER2 bound to γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles was quantified using the Easy-Titer Human IgG assay kit (Pierce). After three-times washing, a sample of γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles conjugated with anti-HER2 was appropriately diluted and used in the assay. The absorbance at 405 nm was measured with SpectraMax 340PC microplate spectrophotometer (Molecular Devices) and anti-HER2 concentration was calculated from a previously determined calibration using standard solutions of anti-HER2. According to the assay, the amount of conjugated anti-HER2 on the surface of (γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles was shown to be approximately [14.37 ± 2.13 μg (anti-HER2)/ mg (γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles)] (mean ± SD; N=3).

Characterization γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs. The mean particle sizes (and their distribution) and zeta potential values of NPs were measured by a dynamic light scattering technique using an electrophoretic light scattering photometer (ELS-Z, Otsuka Electronics, Osaka, Japan)(Figure S4). Scanning electron microscope was used to characterize the morphology of the synthesized γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs. The samples were prepared by the following protocols. After the NPs were lyophilized, the dried NPs were mounted onto silicon-wafer plate followed by coating with platinum using a Technics Hummer II sputter coater during 120 s at 30 mA. The surface morphology of the NPs was then visualized

under Field Emission-Scanning electron microscopy (FE-SEM, SIRIONTM, FEI Company, Hillsboro, OR, USA). Spectroscopic observations were performed by UV-Vis-NIR spectroscopy (Beckman Coulter, DU 800 spectrophotometer). NIR fluorescence images of NPs were acquired using home-made NIR imaging instrument that is equipped with a 760 nm LED light source as an excitation light source and an 845/55 emission filter. T_1-weighted images and relaxation time of γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs were acquired using a Bruker Biospin 4.7 T scanner (Bruker BioSpin, Ettlingen, Germany) with a 200 mT m^{-1} gradient system equipped with a home-made surface coil. The stability of fabricated γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles was also examined at various conditions. For serum stability test, the fabricated γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles were treated with 10% (v/v) FBS in RPMI at 37 ^{0}C for 24 h. The particle diameter was measured by DLS measurements. There was no significant variation in average diameter (Figure S5-a), which means they were stable in serum condition. We have also added the stability data of γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles by incubating them various salt concentration (Figure S5-b). If we consider that the salt concentration at physiological condition is around 0.15 M NaCl, the experimental results suggest excellent stability of γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles under physiological conditions.

Cell imaging. RAW264.7 (murine macrophage cells; ATCC) and DC2.4 cells (murine dendritic cells) were cultured in 10 mm dishes (1×10^7/dish) in Dulbecco's modified Eagle Medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% heatinactivated FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin. For fluorescence

imaging, RAW264.7, DC2.4 cells were incubated with 50 μl/ml of crosslinked γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs for 24 hr at 37 °C. After washing with PBS, the labeled cells were fixed with Cytofix/Cytoperm solution and stained with DAPI in PBS. To detect intracellular localization of QDs to lysosomes, macrophage cells were stained with the lysosomal marker, Lysosome-Associated Membrane Glycoprotein-1 (LAMP-1) by incubating with FITC-conjugated rat anti-LAMP-1 monoclonal antibody (1D4B) (BD PharMingen) for 30 min at room temperature, and then washed (Figure S6). Fluorescence image was obtained by using a Deltavision RT (Applied Precision Technologies, Issaquah, WA, USA), and then near-infrared (NIR) fluorescence image was obtained by using a filter set (excitation: 775/50, emission: 845/55; Omega Optical, Brattleboro, VT, USA).

SKBR3, a breast carcinoma cell line, was obtained from the ATCC (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 2 mM glutamine, antibiotics (100 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin), and 10% (v/v) fetal bovine serum (FBS, Gibco) at 37°C under 5% (v/v) CO_2. SKBR3 cells were plated at 2×10^5 cells/well into 48-well culture plates and incubated for 1 day. The SKBR3 cells (2.5 x 10^5/0.5 ml) were also prepared in 24-well plates. Five hundred μL of DPBS (Dulbecco's Phosphate Buffered Saline, 1×, Gibco) was used to wash SKBR3 cells before experiments. γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs conjugated with anti-HER2 (350 μL) were then added to cells and the mixture were incubated at 37°C for 1 hr. For control experiment, γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs (without anti-HER2) were also incubated with SKBR3 cells at the same condition (Figure S8). After washing with 500 μL DPBS three times to remove unattached nanoparticles, 300 μL McCoy's 5A medium

(containing 10% [v/v] FBS and antibiotics, Gibco) was added. Fluorescence image was obtained by using a Deltavision RT (Applied Precision Technologies, Issaquah, WA, USA), and then near-infrared (NIR) fluorescence image was obtained by using a filter set (excitation: 775/50, emission: 845/55; Omega Optical, Brattleboro, VT, USA). NIR fluorescence images of NPs were acquired using home-made NIR imaging instrument that is equipped with a 760 nm LED light source as an excitation light source and an 845/55 emission filter. T_1-weighted images of SKBR3 cells targeted with γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs conjugated with anti-HER2 were acquired using a Bruker Biospin 4.7 T scanner (Bruker BioSpin, Ettlingen, Germany) with a 200 mT m^{-1} gradient system equipped with a home-made surface coil. The T1 weighted image parameters were as follows: TR = 500 ms, TE = 13 ms; Section thickness = 2 mm; Matrix = 192 × 256; number of acquisitions = 6, FOV = 100 × 100.

Cytotoxcity Assays. For cytotoxicity, MTT assay was performed. Briefly, DC2.4 cells were treated with γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs at a various concentration and incubated for 24 hr. Subsequently, cells were replaced with culture medium containing 0.5mg/ml MTT reagent (Sigma-Aldrich, St. Louis, MO, USA), and incubated for an additional 2 hr. After incubation, cells were replaced with DMSO to dissolve the formazan crystals. Finally, the absorbance at 570 nm was measured using a VICTOR3™ microplate reader (PerkinElmer, Waltham, MA, USA) (Figure S7).

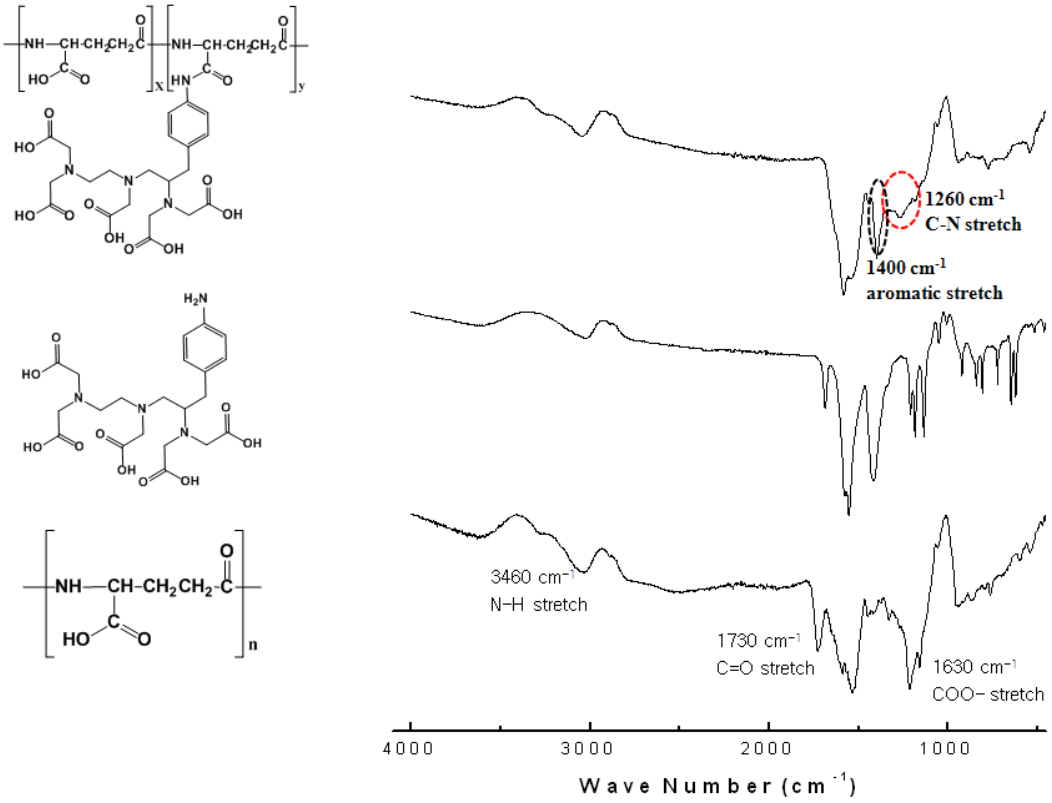


Figure S1. FT-IR spectra of γ-PGA, [Gd-DTPA], and γ-PGA[Gd-DTPA]. IR spectrum of the sample showed absorbance band of aromatic backbone (C=C) at around 1400 cm^{-1} and C-N stretching bands at around 1260 cm^{-1}, respectively.

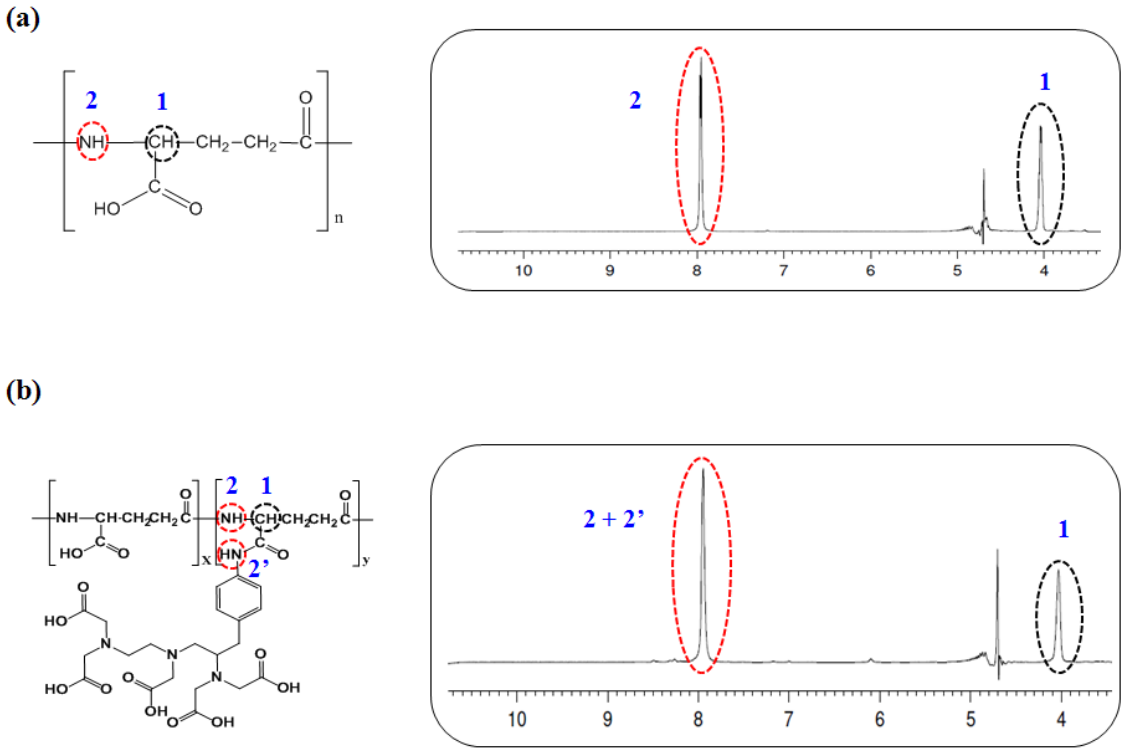


Figure S2. NMR spectra of (a) γ-PGA, and (b) γ-PGA[Gd-DTPA]. The relative area ratio between N-H resonance at 8.0 ppm C-H resonance at 4.1 ppm associated with γ-PGA was 1.085, while that associated with γ-PGA[Gd-DTPA] was 1.236. The result of this increment in area ratio value between N-H resonance and C-H resonance suggest that DTPA molecules were well conjugated on the γ-PGA by amide bond (i.e. 15.1 % of carboxyl group was reacted).

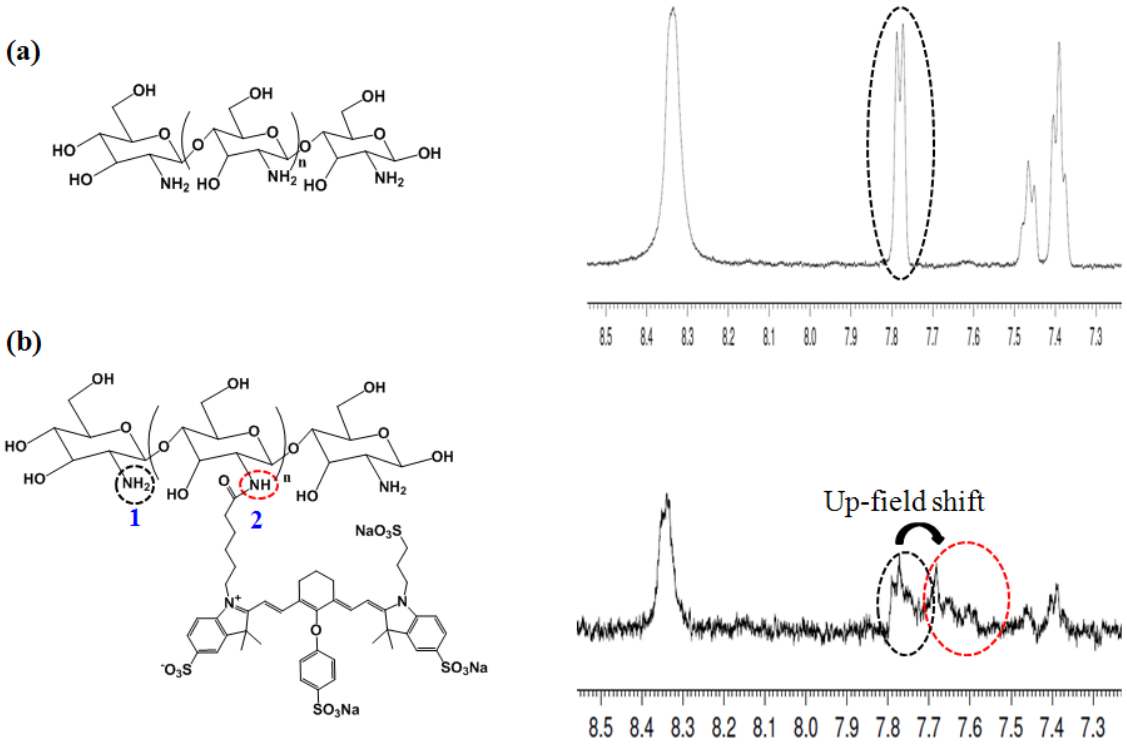
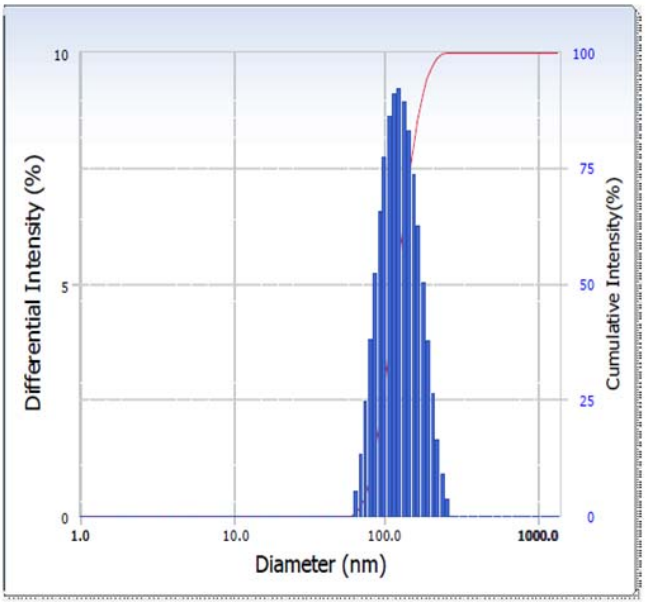


Figure S3. NMR spectra of (a) chitosan, and (b) chitosan[IRDye800]. The up-field chemical shift at δ (7.6-7.7 ppm) suggest that new N-H resonance (amide bond) was generated by the conjugation between amine moiety of chitosan and NHS ester part of IRDye800.

Table S1. Physicochemical properties of γ-PGA[Gd-DTPA] and chitosan[IRDye800]

Property	**γ-PGA[Gd-DTPA]**	**Chitosan[IRDye800]**
% Gd (w/w)[1]	11.45	-
Relaxivity (r1) (mM^{-1} s^{-1})[2]	8.72	-
% IRDye800 (w/w)[3]	-	3.9
[1]Percentage of Gd by weight was measured by elemental analysis [2]All data were obtained at a Bruker Biospin 4.7 T scanner in PBS [3]Percentage of IRDye800 was measured by spectrophotometrically		



Intensity Distribution

Peak	Diameter (nm)	Std. Dev.
1	131.1	38.6

Figure S4. Size distribution of γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles measured by dynamic light scattering.

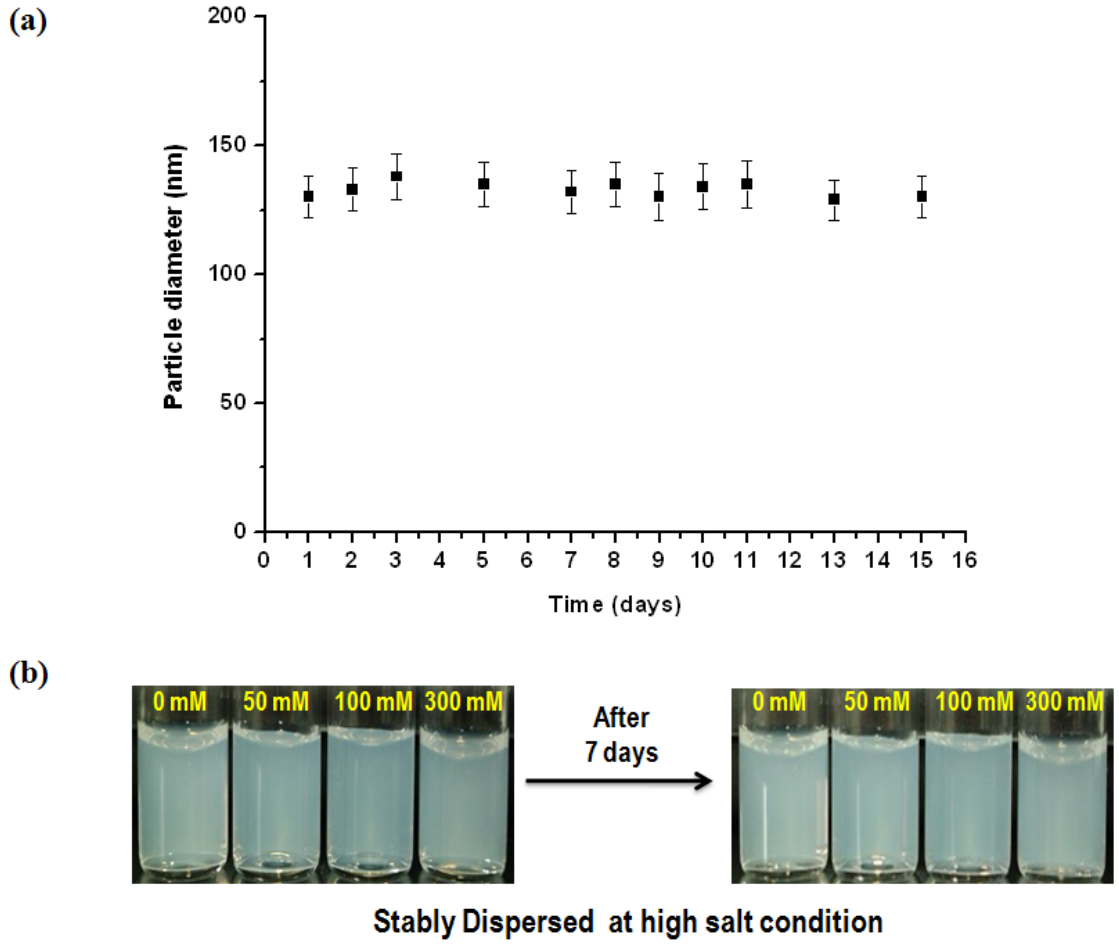


Figure S5. Stability of γ-PGA[Gd-DTPA]/chitosan[IRDye800] nanoparticles (a) at 10% (v/v) FBS in RPMI at 37 ^{0}C for 24 h, (b) at various salt (NaCl) concentration. The particle diameter was measured by DLS measurements.

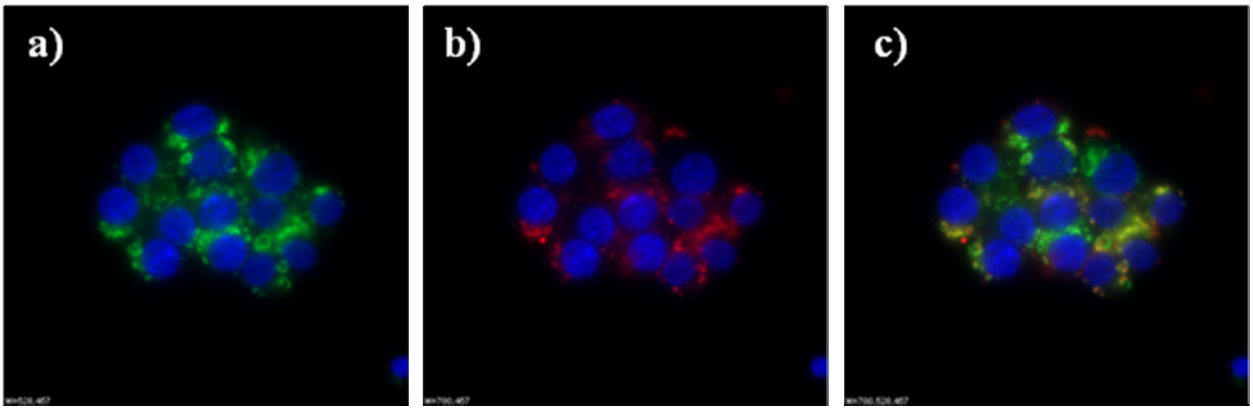


Figure S6. (a) Localization of lysosomes (green) and (b) γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs (red, pseudo-color, b), and (c) merged images obtained by fluorescence microscopy. Macrophage cells (RAW264.7) were stained with the lysosomal marker, Lysosome-Associated Membrane Glycoprotein-1 (LAMP-1) by incubating with FITC-conjugated rat anti-LAMP-1 monoclonal antibody (1D4B) (BD PharMingen).

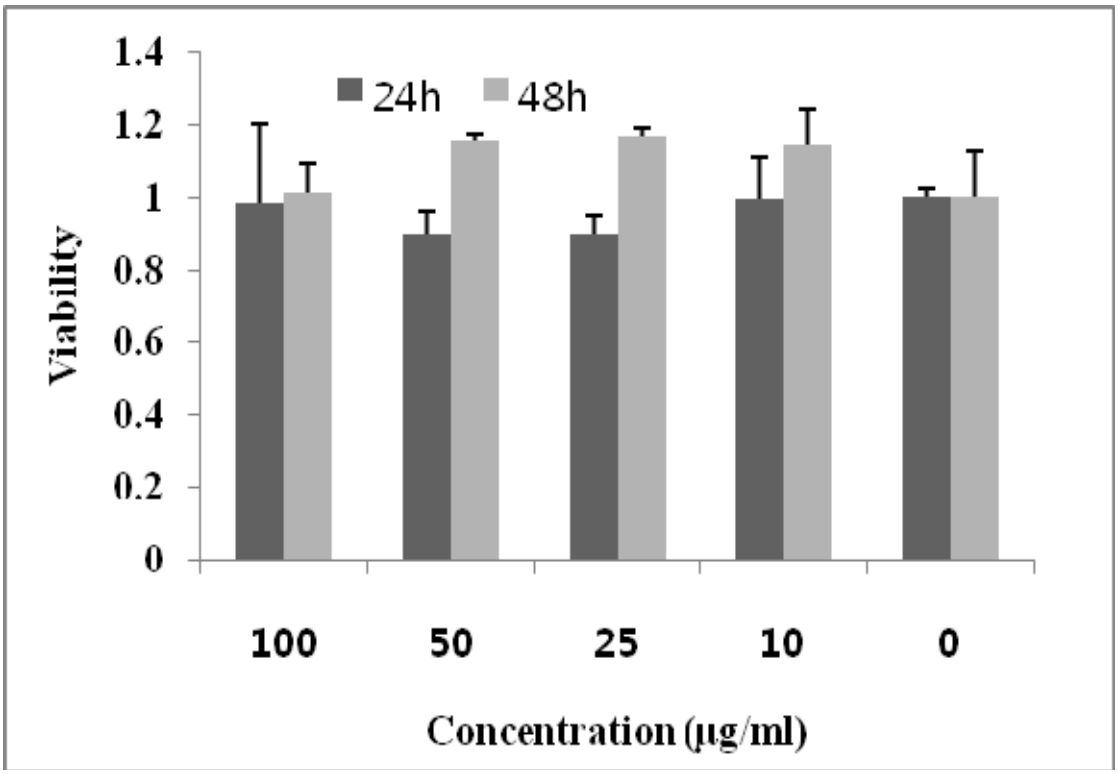


Figure S7. Cell viability of macrophage cells (RAW264.7) treated with various concentration of γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs.

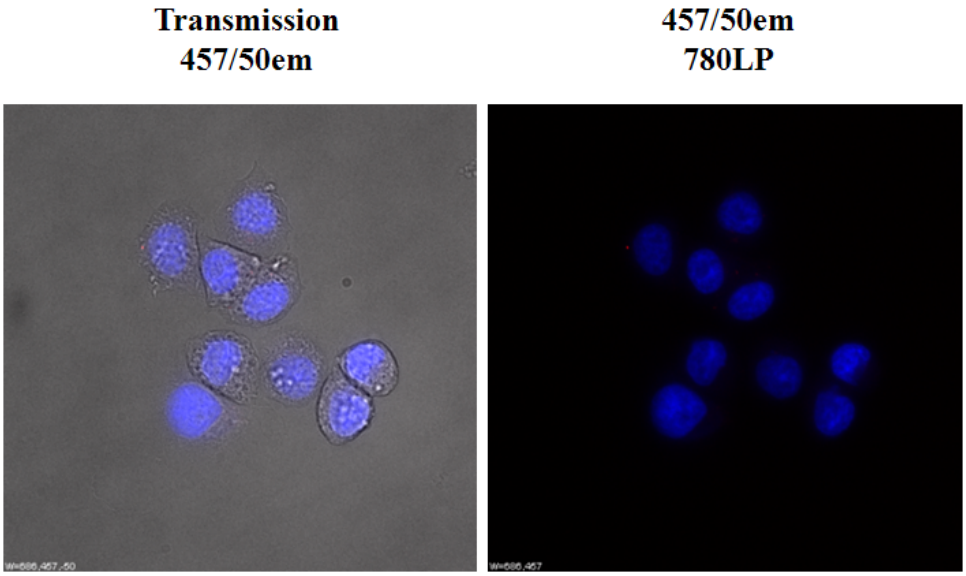


Figure S8. Fluorescence microscopy images of SKBR3 cells treated with γ-PGA[Gd-DTPA]/chitosan[IRDye800] NPs (without anti-HER2).