Experimental

Materials

The monomer and comonomer, dimethylaminoethyl methacrylate (DMAEMA) and 2-hydroxyethyl methacrylate (HEMA), were purchased from Acros (Morris Plains, NJ, USA). The tetraethylene glycol dimethacrylate (TEGDMA) as a cross-linking agent and hexylamine was obtained from Fluka (St. Louis, MO, USA). The ammonium persulfate, sodium metabisulphite, doxorubicin (Dox), monobasic and dibasic sodium phosphates, 2,2-dimethoxy-2-phenylacetophoenone (DMPAP), potassium tetrachloroaurate ($KAuCl_4$), sodium borohydride ($NaBH_4$), carbon tetrachloride, diethyl ether, hexane, 2,2'-azobisisobutyronitrile (AIBN), sodium bisulfate, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3dimethylaminopropyl)carbodiimide (EDAC), dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF) were purchased from Sigma (St. Louis, MO, USA). Pluronic F68 as a surfactant was kindly provided from BASF Corporation (Mount Olive, NJ, USA). For the synthesis of dT-DMAEMA, 1,4-diisopropenylbenzene was purchased from Tokyo Chemical Industry (TCI Co., LTD., Tokyo, Japan). Poly(lactide-co-glycolide) (PLGA, 50:50, MW 17000-22000) and polyvinyl alcohol (PVA, MW ~25000) were purchased from Polysciences, Inc. (Warrington, PA, USA) and dichloromethane was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). All materials were used without further purification.

Anti HER2/neu antibody (Herceptin®) was kindly provided by Roche Pharmaceutical Ltd. (South San Francisco, CA, USA). SK-BR-3 and MCF-7 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SK-BR-3 cells were grown in McCoy's 5a (ATCC) and MCF-7 in DMEM supplemented with 10% fetal bovine serum and 1%

penicillin/streptomycin (Invitrogen; Carlsbad, CA, USA). Cells were cultured in a humidified incubator containing 5% CO_2 at 30^{o}C. Deionized water (18.2 MΩ) was obtained from a Milli-Q purification system (Millipore Corp., Billerica, MA, USA). All reagents were of analytical grade and were used as received.

Synthesis of dT-DMAEMA

To synthesize dT-DMAEMA, a bisfunctional RAFT agent 1,4-bis(2-(thiobenzoylthio)prop-2-yl)benzene was synthesized. Briefly, dithiobenzoic acid (6.1 g) and 1,4-diisopropenylbenzene (3.0 g) in carbon tetrachloride (50 mL) was heated at 75^{o}C for 20 h, as described previously.[1] The solvent was removed under vacuum and a pink solid was obtained after filtration with 1:2 volume ratio of diethyl ether and hexane. Then, DMAEMA (1 g), AIBN, and 1,4-bis(2-(thiobenzoylthio)prop-2-yl)benzene were added into a 20 mL glass vial in an oil bath at 60^{o}C for 48 h for RAFT polymerization.[2] The obtained α,ω-dithioester-terminated DMAEMA was precipitated by hexane and filtered. Aqueous sodium bisulfate (0.5 mL) was added to α,ω-dithioester-terminated DMAEMA (1 g) in 10 mL THF and 0.5 mL hexylamine. The reaction mixture was stirred for 5 h under a nitrogen atmosphere. The mixture was washed in excess hexane and then filtered. One milliliter of DMSO was added to the product in deionized water and stirred for two weeks. Finally, oxidized dT-DMAEMA was obtained by removing water and precipitating in excess hexane.

Characterization of dt-DMAEMA

^{1}H NMR was used to investigate the structure of dt-DMAEMA. Figure S1 shows the ^{1}H-NMR spectrum of dt-DMAEMA. The signals at 1.7–2.0 ppm are assigned to the methylene

protons in polymer backbone (–CH2–). The signal from the dimethylamino protons of DMAEMA (–NH2) could be clearly visible at δ = 2.2-2.3 ppm. The NMR peaks at δ = 2.6 and δ = 4.2 ppm are assigned to (–O–CH2–C**H**2–) and (–O–C**H**2–CH2–), respectively. The presence of the centrally located phenylene group in dt-DMAEMA is confirmed by the signals at 7.0–8.0 ppm.

Preparation of Dox-loaded DMAEMA/HEMA nanoparticles

The 100 μL of a dT-DMAEMA/HEMA solution with different molar ratios of DMAEMA/HEMA (10/90 and 30/70) and of Dox (0.36 μM) was added to 10 mL of deionized water containing Pluronic F68 (150 mg) and TEGDMA (3 mol%) as a cross-linker and also aqueous solutions of ammonium persulfate (0.5% w/v) and sodium metabisulphite (0.25% w/v) were added as initiators. The solution was immediately sonicated (200 W, 20 kHz; Digital sonifier 250, Branson Ultrasonics Corp., Danbury, CT, USA) in a laminar flow hood over an ice bath for 10 min. The polymerization process was carried out at room temperature for 3 h. dT-DMAEMA/HEMA nanoparticles were collected by high-speed centrifugation at 39000×g for 20 min (Sorvall RC26 Plus, SA-600 rotor; Thermo Fisher Scientific Inc., Waltham, MA, USA). The particles were washed thrice with pH 7.4 phosphate buffer to remove residual surfactant and initiators with same conditions for the centrifuge. Obtained Dox-loaded dT-DMAEMA/HEMA nanoparticles were lyophilized (FTS systems, INC., Stone Ridge, NY, USA) overnight and stored at 4℃ for later use.

Synthesis of Au nanoparticles within Dox-loaded DMAEMA/HEMA nanoparticles

Ten milligrams of Dox-loaded dT-DMAEMA/HEMA nanoparticles were added into 2 mL of 5 mM $KAuCl_4$ for 1 h with gentle stirring and then added 0.5 mL of 50 mM $NaBH_4$ to synthesis Au nanoparticles. Colloidal Au nanoparticles in nanoparticles were obtained by reduction of Au salt at room temperature for 7 min. Finally, Au nanoparticle embedded dT-DMAEMA/HEMA nanoparticles were washed with pH 7.4 phosphate buffer three times. 0.05 wt% aqueous Au-nanoparticle embedding Dox-loaded DMAEMA/HEMA nanoparticle suspension was prepared to determine the size and morphology of nanoparticles by transmission electron microscopy (TEM, JEOL 2100; JEOL Ltd., Tokyo, Japan). Twenty microliters of nanoparticle suspension was placed on a copper grid (300 mesh, TED PELLA Inc., Redding, CA, USA) supporting a thin film of amorphous carbon. The excess liquid was removed with filter paper and the copper grid was dried in the hood. The particle size and size distribution were measured by dynamic light scattering (ZetaPALS; Brookhaven Instrument, Holtsville, NY, USA). The zeta potential of the nanoparticles was analyzed by electrophoresis (Zeta PALS; Brookhaven Instrument, Holtsville, NY, USA). Nanoparticles were diluted to 0.1 mg/mL in a 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) buffer at pH 7.4. The particle size and zeta potential were measured a minimum of three times. The absorbance of multifunctional nanoparticles (HPG-Dox-30D70H) was analyzed by UV-vis spectrometer (SpectraMax Plus384; Molecular Devices Corp., Sunnyvale, CA, USA).

Thermogravimetric analysis

Thermogravimetric analysis (TGA) of nanocomposites was carried on a TGA Q 5000 analyzer (TA Instrument-Waters LLC., New Castle, DE, USA). The samples, which have 20 mg

mass, were scanned with a heating range of 20^{o}C/ min from 20 to 600^{o}C under continuous argon flow.

pH-sensitive swelling and drug release studies

Swelling study of Dox-loaded DMAEMA/HEMA nanoparticles was performed in a buffered solution of known pH (either pH 5.5, 6.5, and 7.4), composition (10 mM phosphate buffer, adjusted with 1 N HCl), and temperature (37 ± 0.5°C). 10 mg of freeze-dried Dox-loaded DMAEMA/HEMA was suspended in 2 mL of different pH phosphate buffers and put into dialysis tubing with MWCO 1,000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Dialysis tubing was immersed in 20 mL of phosphate buffered medium. Samples were placed on a shaker (OS-500 orbital shaker, VWR, West Chester, PA, USA) with a shaking rate of 100 ± 1 rpm in an incubator maintained at 37°C. The average nanoparticle diameter was measured by dynamic light scattering at 0, 1, 2, and 4 h. Controlled release of Dox from Au-nanoparticle impregnated DMAEMA/HEMA nanoparticles was performed same procedure like swelling study. Briefly, 10 mg of freeze-dried Dox-loaded DMAEMA/HEMA nanoparticles were put into the dialysis tubing with 2 mL of different pH phosphate buffers and immersed in 20 mL of same buffer medium. During release experiments, samples were placed in a shaker in an incubator with a shaking rate of 100 ± 1 rpm. Samples were collected once every hour until 6 h and then every 6 h until 24 h. The volume of each collected sample was 350 μL. To maintain a uniform concentration of Dox in the media, fresh buffer was added after sampling during the release studies. The concentration of released Dox was directly determined by a Gemini XPS microplate spectrophotometer (Molecular Devices Corp, Sunnyvale, CA, USA). at an excitation wavelength 485 nm and emission wavelength 590 nm based on a standard fluorescence

concentration calibration curve. To evaluate the encapsulation efficiency of Dox, nanoparticles was put into the pH 2 phosphate buffer titrated by 1 N HCl and vigorously agitated with magnetic stirrer for 24 h and then Dox concentration was determined by a fluorescent microplate reader.

Drug release under near-infrared irradiation

Controlled Dox release from dT-DMAEMA/HEMA nanoparticles was carried out in a buffered medium at pH 5.5, 6.5, and 7.4 phosphate buffers (10 mM) with and without NIR laser irradiation to confirm the pH-sensitivity and thermal effect. Dox-loaded dT-DMAEMA/HEMA nanoparticles (10 mg) were suspended in 2 mL of phosphate buffer and sealed in dialysis tubing with MWCO 1,000 Da (Spectrum Laboratories, Inc., Rancho Dominquez, CA, USA) and placed into a glass jar containing 20 mL of same buffer. During release experiments, the glass jar was placed in a shaker in an incubator with a shaking rate of 100 ± 1 rpm. Samples were collected at every 2 h until 6 h and then every 6 h until 24 h. The volume of each collected sample was 350 μL of sample and same volume of fresh medium was added after sampling to maintain a uniform concentration during the release studies. To demonstrate the drug release pattern of Dox-loaded dT-DMAEMA/HEMA nanoparticles impregnating Au-nanoparticles with thermal effect, nanoparticles (10 mg) were suspended in 2 mL of phosphate buffers (10 mM and pH 5.5, 6.5, and 7.4) and exposed under near infrared laser (810 nm, 5 W/cm^2 for 10 min). Temperature change in nanoparticle solution was measured and visualized with thermal images using a thermal camera (FLIR i5; Extech Instruments, Corp., Waltham, MA, USA). The amount of released Dox was monitored by a Gemini XPS microplate spectrophotometer at an excitation wavelength 485 nm and emission wavelength 590 nm with standard calibration curves. To

investigate the temperature change of medium, thermal camera images were taken at 0, 2, 5, 7, and 10 min irradiation during Dox release study.

Conjugation of antibody with Dox-loaded pH-sensitive nanoparticles

HPG-Dox-30D70H was conjugated with Herceptin® for tumor targeting to metastatic breast cancer cells. The 10 μmol of hetero-functional polyethylene glycol (SH-PEG-COOH) as a linker was bound to the nanoparticle surfaces (10 mg of nanoparticles in 1 mL 10 mM phosphate buffer) using the affinity between Au (or Au^{3+}) in nanoparticles and thiol groups from SH-PEG-COOH molecules (Laysan Bio Inc.; Arab, AL, USA). Centrifugation (15,000 rpm for 10 min) was used to remove unbound PEG from PEGylated nanoparticles. The carboxyl group of nanoparticles was conjugated to the *N*-terminal group of the antibody using a carbodiimide reaction. For PEGylation, 10 mg of nanoparticles was suspended in 10 mL of phosphate buffer (pH7.4). Each 20 μmol of *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3dimethylaminopropyl)carbodiimide (EDAC) was added into particle suspension. And then, 9 nmol of Herceptin® was added into PEGylated nanoparticles solution at 4°C. After 4 h, Herceptin® conjugated nanoparticles were washed out with phosphate buffer three times using centrifugation at 15,000 rpm for 20 min and unbound Herceptin® was removed. The efficiency of antibody conjugation was determined by Bio-Rad DC protein assay kit (Hercules, CA, USA) with the standard curve by bovine serum albumin. Similarly, as a control, the immunoglobulin G (IgG) antibody was also conjugated with HPG-Dox-30D70H in the same method.

Targeting efficiency using different cancer cell lines

The breast carcinoma cell lines SK-BR-3 and MCF-7 were obtained from the American Tissue Type Culture (ATCC) and cultured under recommended conditions. Both cells were seeded in a 6-well plates at 3.0×10^5 cells/well density and allowed to grow in a humidified chamber at 37°C with 5% CO_2 for 24 h. Herceptin®-conjugated and IgG-conjugated nanoparticles (0.5 mg/mL) including antibody-free nanoparticles as a control were suspended in a serum free media and added into each well. After 30 min incubation, cells were washed with PBS three times and fresh growth media were replaced. After additional 24 h incubation, both cells were washed with PBS, collected using a trypsin/EDTA solution and transferred to polystyrene culture tube (BD, Franklin Lakes, NJ, USA). Cells were washed with PBS three times and suspended in a 500 μL phosphate buffer solution. Targeting efficiency was determined by the LSRII flow cytometry (BD Immunocytometry Systems, San Jose, CA, USA) and analyzed with WEASEL software developed by WEHI (Parkville, Australia). The result was determined by dividing the mean fluorescence intensity for nanoparticles conjugated either Herceptin® or IgG by that of non-conjugation.

Cell viability

The cellular cytotoxicity of free Dox and Dox-loaded nanoparticles against SK-BR-3 and MCF-7 cells was evaluated by calcein AM staining and resazurin-based cell toxicology assay kit (Sigma, St. Louis, MO, USA). Cells were seeded in 96-well plates at a seeding density of 1.0×10^4 cells/well and grown for 24 h. Dox free or Dox-loaded nanoparticle samples (0.1 mg/mL) were suspended in serum free media, added into each well in culture dishes, and incubated at 37°C for 30 min. Cells were washed with PBS three times, replaced with the same volume of

fresh growth medium, and incubated for additional 24 h. After NIR irradiation at 810 nm for 10 min to induce photothermal cell damage, cell viability was assessed by using calcein AM (1 μM). Cells showing green fluorescence, which are considering live cells, were taken images using an Axiovert 200 inverted fluorescent microscope (Carl Zeiss Inc., Thornwood, NY, USA) equipped with a Hamamatsu CCD camera (Bridgewater, NJ, USA). To quantify the cell damage by photothermal effect, resazurin-based cell toxicology assay kit was also used. Resazurin dye solution (10 μL) was added to each well (100 μL of growth medium) in 96-well plate and the plate was incubated in a humidified chamber at 37^{o}C with 5% CO^{2} for 4 h. 100 μL of sample was taken from each well and transferred to 96-well UV plate (Corning Inc., Acton, MA, USA). The number of viable cells in each well was determined using a Gemini XPS microplate spectrophotometer at an excitation of 560 nm and an emission of 590 nm by comparison to a standard curve. The results represented by the differences of cell viability between nanoparticle-treated and non-treated samples.

To investigate the cytotoxicity of Dox-free DMAEMA/HEMA (30/70, mol/mol, 30D70H) and Au-nanoparticle impregnating DMAEMA/HEMA nanoparticles (Au-30D70H), resazurin-based cell toxicology assay was also performed with SK-BR-3 and MCF-7 cells for 24 h incubation. As a control, PLGA nanoparticles, which are commonly used as drug carriers, were also performed cell toxicology assay for comparison. PLGA nanoparticles with average diameter 167.8 ± 13.2 nm were prepared by an oil-in-water (O/W) emulsion and solvent evaporation method. Briefly, 50 mg of PLGA was dissolved in 5 mL of dichloromethane and organic phase was mixed with 10 mL of a 1% (w/v) aqueous polyvinyl alcohol solution and sonicated in a laminar flow hood for 5 min at 200 W output. The formed emulsion was evaporated overnight under magnetic stirring to remove the organic solvent. The sample was

collected by centrifugation (15,000 rpm) for 20 min. The particles were washed with deionized water three times. The nanoparticles were resuspended with 3 mL deionized water and freeze-dried overnight. All cell viability assays were performed three times.

To investigate cell viability of SK-BR-3 and MCF-7 cells, Dox at different concentrations was added into both cell lines and cell viability assay after 24-h incubation was performed as described above.

Statistical analysis

Unless otherwise mentioned, triplicate data were obtained and presented as mean ± standard deviation. Statistical difference was analyzed using analysis of variance with Student's *t*-test on the significance level of $p < 0.05$.

References

[1] D. L. Patton, M. Mullings, T. Fulghum, R. C. Advincula, *Macromolecules* **2005**, *38*, 8597.

[2] J. Chiefari, Y. K. Chong, F. Ercole, J. Krstina, J. Jeffery, T. P. T. Le, R. T. A. Mayadunne, G. F. Meijs, C. L. Moad, G. Moad, E. Rizzardo, S. H. Thang, *Macromolecules* **1998**, *31*, 5559.

[3] Y. Z. You, D. S. Manickam, Q. H. Zhou, D. Oupicky, *J. Controlled Release* **2007**, *122*, 217.

[4] K. Shroff, E. Kokkoli, *Langmuir* **2012**, *28*, 4729.

Figure S1

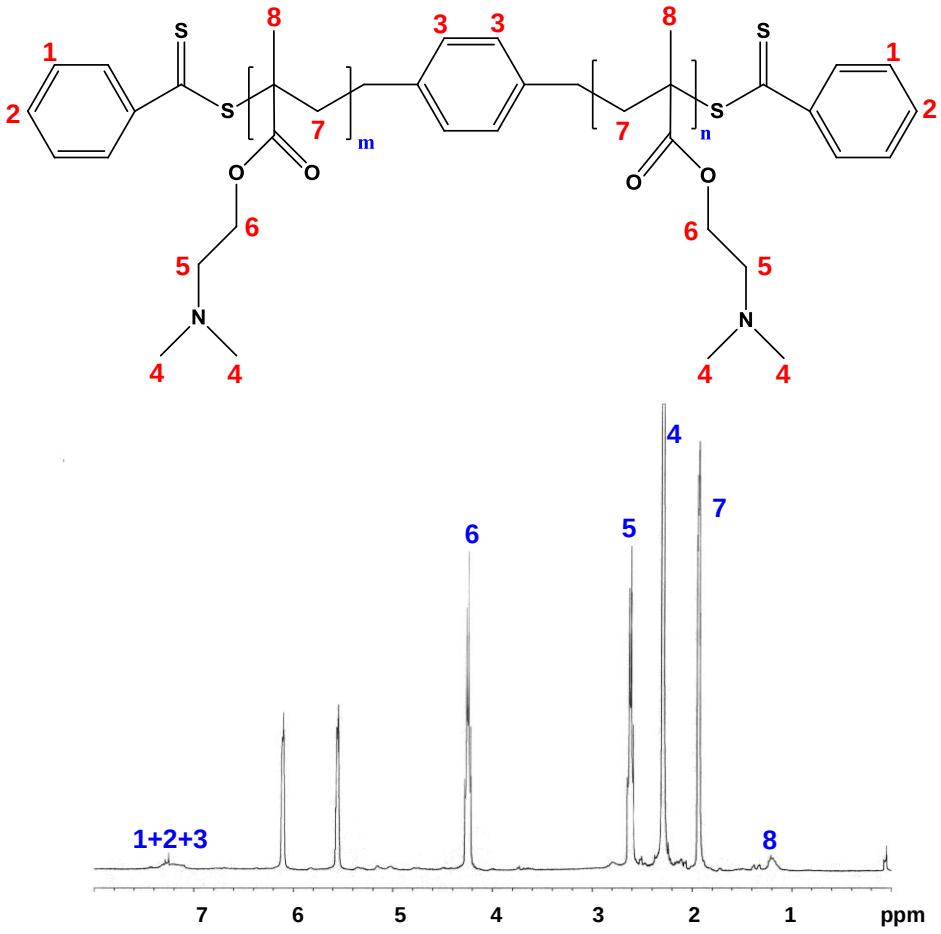

Figure S1. ^{1}H-NMR spectrum of α, ω-dithioester-terminated DMAEMA.[3]

Figure S2

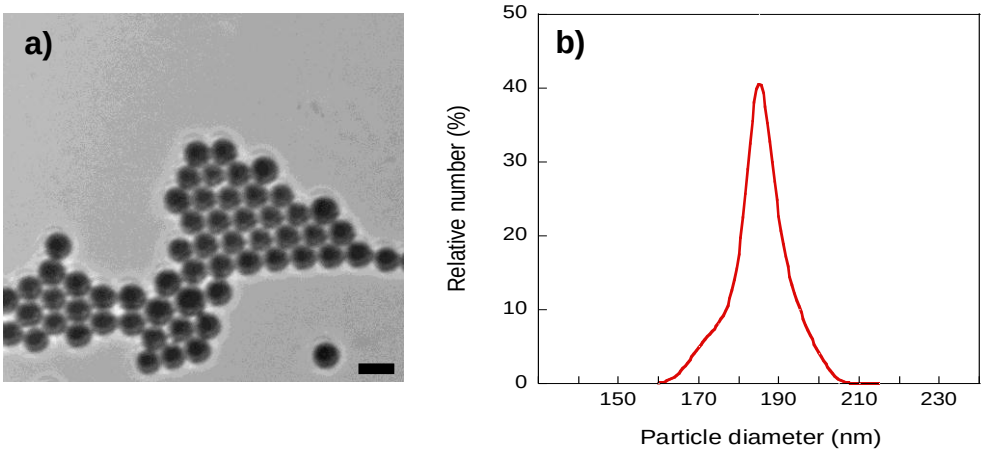

Figure S2. a) TEM image of Dox-loaded pH-sensitive DMAEMA/HEMA nanoparticle impregnating Au-nanoparticles (HPG-Dox-30D70H), scale bar = 200 nm. b) Particle size distribution of HPG-Dox-30D70H by a dynamic light scattering.

Figure S3

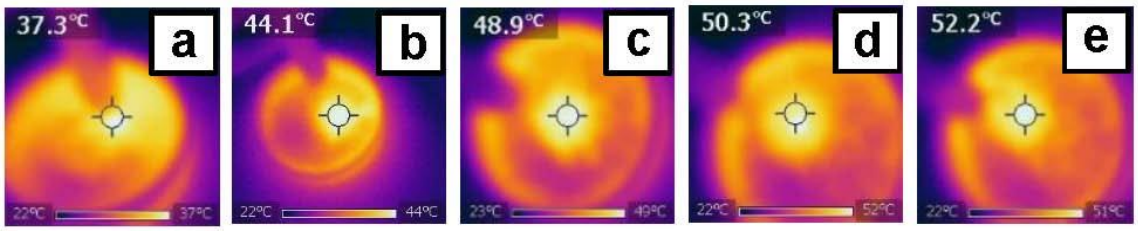

Figure S3. Thermal camera images of HPG-Dox-30D70H nanoparticles in medium (5 mg/mL) at (a) 0, (b) 2, (c) 5, (d) 7, and (e) 10 min near infrared laser irradiation (820 nm) during Dox release study. The temperature of the well increased from 37.3 to 52.2^{o}C after 10 min irradiation.

Figure S4

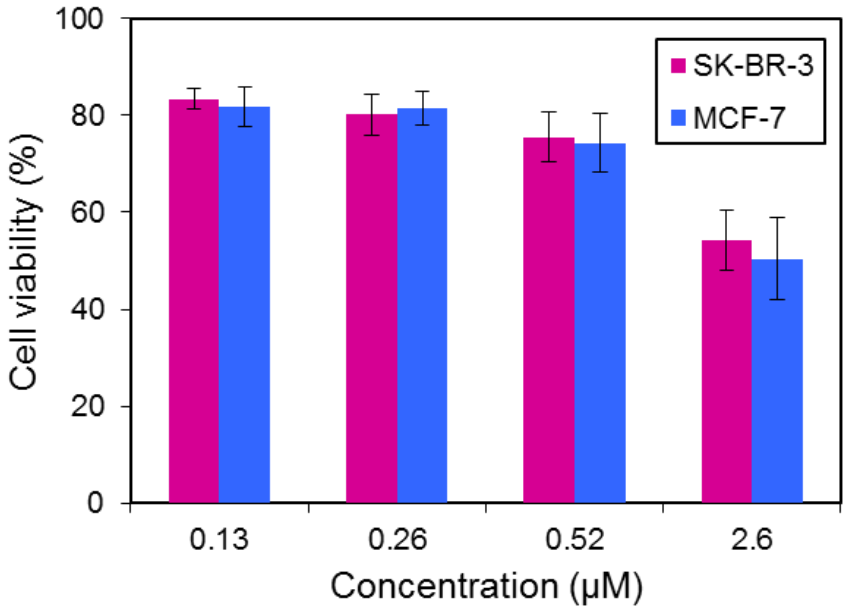

Figure S4. Cell viability of SK-BR-3 and MCF-7 cells as a function of Dox concentration after 24-h incubation. The half maximal inhibitory concentration (IC50) of Dox is 2.6 μM for SK-BR-3 and MCF-7 cells, as confirmed by other reports.[4]

Figure S5

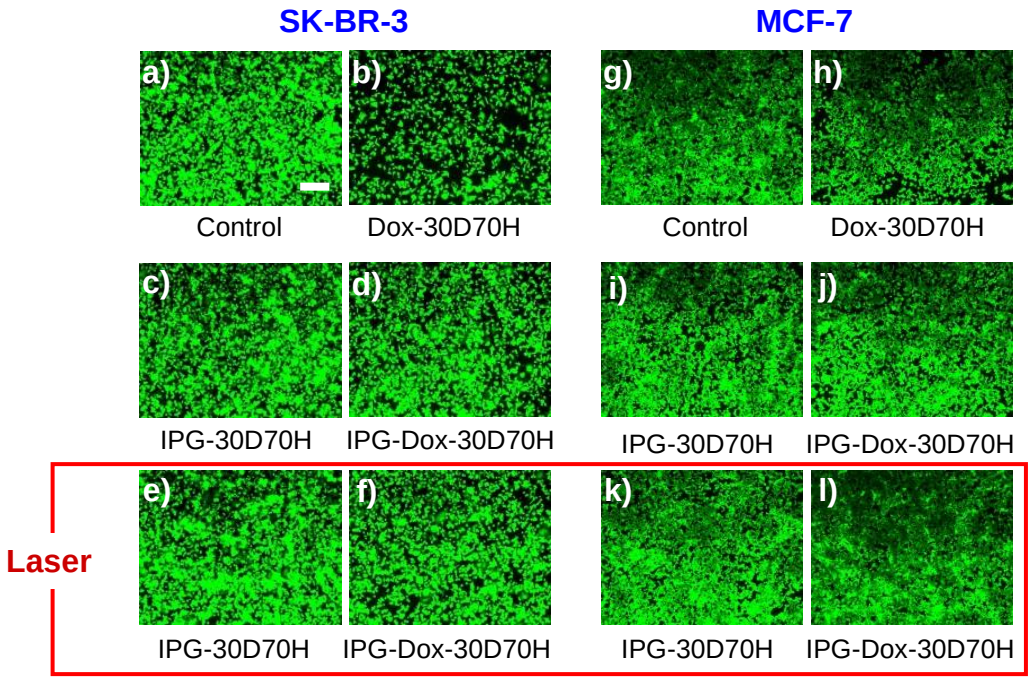

Figure S5. Fluorescent microscopy images of cell viability assay for 24 h incubation after with and without NIR laser irradiation (810 nm and 5 W/cm^2 for 10 min). SK-BR-3 and MCF-7 cells were treated with IPG-30D70H and IPG-Dox-30D70H under NIR laser exposure (e-f and k-l) and no laser exposure (c-d and i-j). As controls, fluorescent microscopy images with particle-free (a and g) and antibody-free (b and h) cells were taken. Scale bars = 200 µm.

Figure S6

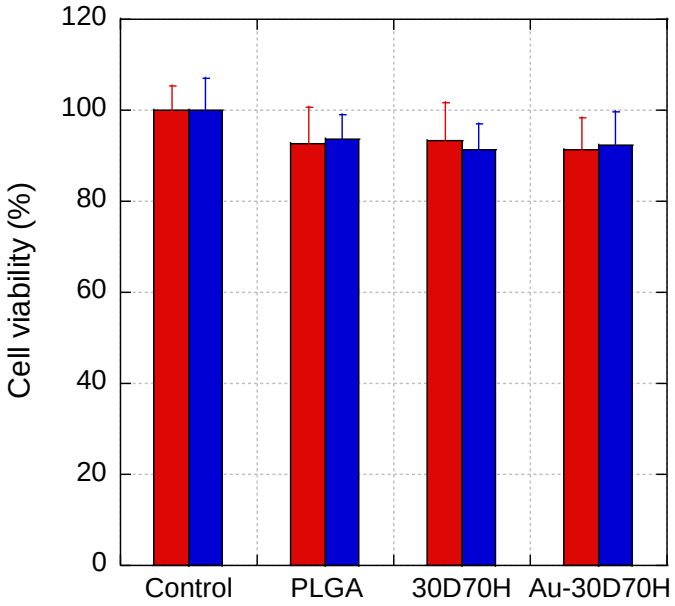

Figure S6. Cell viability assay. Dox-free pH-sensitive DMAEMA/HEMA nanoparticles (30D70H; 30/70, mol/mol) and Au-impregnated pH-sensitive DMAEMA/HEMA nanoparticles (Au-30D70H) were incubated with SK-BR-3 (red) and MCF-7 (blue) cells for 24 h. For comparison, PLGA nanoparticles were also performed cell viability. The error is the standard deviation from the mean, where $n = 3$.

Table S1. Particle size and zeta potential analysis of HPG-Dox-DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA in pH 7.4 10 mM TES buffer.

	Diameter* (nm)	ζ Potential* (mV)
HPG-Dox-10D90H	172.8 ± 17.2	-18.9 ± 2.4
HPG-Dox-20D80H	177.4 ± 21.3	-12.4 ± 1.8
HPG-Dox-30D70H	186.4 ± 18.6	-7.3 ± 2.1

* Data were performed in triplicate.

Table S2. Antibody binding density on PG-30D70H and PG-Dox-30D70H nanoparticles

	Herceptin® (molecule/particle)	IgG (molecule/particle)
PG-30D70H	116.3± 6.8	123.7 ± 7.9
PG-Dox-30D70H	117.5 ± 10.7	115.8 ± 12.3

* Data were performed in triplicate.