Supporting Information

Effects of Polymer 3D Architecture, Size and Chemistry on Biological Transport and Drug Delivery In Vitro and in Orthotopic Triple Negative Breast Cancer Models

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Figure S1. Representative ^{1}H NMR of top: redox-responsive hyperbranched polymer (HB-SS-HH) and bottom: non-degradable hyperbranched HPMA (HB-HPMA-S).

Figure S2. ^{1}H NMR spectra of top) Star-SS-L, middle) Linear-SS and bottom) Micelle-SS.

Figure S3. Critical micelle concentration (CMC) plots for the linear, micelle and star pHPMAs.

Figure S4. Stability of non-degradable and redox-responsive pHPMAs in cell culture DMEM over 48 h incubation at 37 ºC

Figure S5. Stability of pHPMAs to protein corona binding assayed by DLS at A) 0.2 wt% BSA and B) 1 wt% BSA.

Figure S6. In vitro cell viability for all pHPMA materials in triple negative breast cancer cells.

Figure S7. In vitro data depicting A) metabolic activity and B) LDH release of pHPMAs after 24h incubation with RAW 264.7 macrophages

Figure S8. Macrophage uptake data showing A) influence of size on uptake (4h), B) influence of zeta-potential on uptake (4h), C) zeta-potential vs size of nanoparticles and D) ratio of zeta-potential / size vs uptake.

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Figure S11. ^{1}H NMR spectra of a representative pHPMA before and after boc-deprotection.

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Figure S17. Spheroid cytotoxicity assay of the blank Cy5 labelled polymers to confirm no impact on spheroid growth from the blank nanoparticles.

Figure S18. Growth of MDA-MB-231 spheroids over the 3-day treatment period expressed as top) volume on each day and bottom) volume comparison between day 4 and 7.

Figure S19. Fluorescence images of Cy5-labelled nanoparticles captured 24h after injection into MDA-MB-231 tumor-bearing mice.

Figure S20. Luminescence images of MDA-MB-231 tumor-bearing mice at day 17 (pre-treatment) and prior to termination (Day 45) for the control and treatment groups.

Figure S21. ^{1}H NMR spectra of HPMA

Figure S22. ^{1}H NMR spectra of EDMA

Figure S23. ^{1}H NMR spectra of tBHM

Figure S24. ^{1}H NMR spectra of CBMA

Figure S25. ^{1}H NMR of CADB-SS-CADB

Figure S26. ^{13}C NMR spectra of CADB-SS-CADB

Experimental

Materials

1-Amino-2-propanol (>99%), methacryloyl chloride (97%), 4,4′-azobis(4-cyanovaleric acid) (V-501, >97%), 1-Bromobenzene (99%), cysteamine (>98%), pentafluorophenol (>99%), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (98%), magnesium turnings (98%), 4-(dimethylamino)pyridine (98%), carbon disulphide (99%), iodine (99.99%), sodium thiosulfate (98%), methacrylic anhydride (94%), ethylenediamine (>99%), pyridine (99.8%), triethylamine (TEA, >98%), Bovine Serum Albumin (BSA, >98%) and Lactic Dehydrogenase (LDH) Toxicology Assay Kit (TOX7 kit) were purchased from Sigma-Aldrich (Poole, U.K.). Cyanine5 (Cy5) amine fluorescent dye was purchased from Lumiprobe. AlamarBlue™ Cell Viability Reagent, PrestoBlue® Cell Viability Reagent and Potassium hexacyanoferrate(III) (98%) were purchased from Thermo Fisher Scientific. All other chemicals and solvents were analytical or HPLC grade and purchased from Fisher Scientific. Methacryloyl chloride was distilled under Ar flow before use. All other chemicals were used as received unless otherwise stated.

Characterization

^{1}H NMR, ^{13}C NMR, ^{19}F NMR spectra were recorded on a Bruker DPX 400 UltraShield™ Spectrometer (400 MHz) using deuterated solvents from Aldrich. Spectra were processed with MestReNova 9.0.1 software. All chemical shifts are reported in ppm (δ) relative to the chemical shifts of deuterated solvents. Multiplicities are described with the following abbreviations: s = singlet, br = broad, d = doublet, t = triplet, m = multiplet. Mass spectrometry was carried out using a Micromass LCT ToF with electrospray ionization and OpenLynx software. Samples were prepared in MeOH. Size exclusion chromatography-multi-angle static light scattering (SEC-MALS) was carried out using a Wyatt Dawn 8+ 1260 Infinity II series in a system equipped with a Polymer Labs aquagel-OH guard column (50 x 7.5 mm, 8 µm) followed by a PL aquagel-OH MIXED-H analytical column. The mobile phase was 0.1 M $NaNO_3$ with a flow rate of 1 mL min^{-1}. Number average molecular weight (Mn) and dispersity (Đ) were calculated

using a Wyatt DAWN® HELEOS® II MALS, using a dn/dc of 0.158. The resulting chromatograms were analyzed using ASTRA® software, V.6.1.2.84 (Wyatt Tech Corp). Dynamic Light Scattering (DLS) measurements were conducted in triplicate using a Malvern Zetasizer Nano ZS at 25ºC (scattering angle 173º, laser of 633 nm) or a Viscotek 802 DLS with a laser wavelength of 830 nm at 20 ºC. Polymer solutions were prepared as 1 mg/mL in PBS. Data was analyzed using OmniSIZE software. A minimum of 10 measurements were collected per sample. The Zeta Potential of nanoparticles was evaluated according to the electrophoretic mobility of the particles and calculated by the Helmholtz-Smoluchowsky equation. All measurements were performed in triplicate. Transmission electron microscopy (TEM) images were acquired using a JEOL 2000-FX TEM with a tungsten source operating at an accelerating voltage of 80 kV equipped with a Gatan Orius SC1000 camera. To prepare the samples for imaging, each polymer was dissolved in DI H_2O at a concentration of either 0.1 or 0.2 mg/mL and then passed through a 0.22 μm PES filter onto carbon coated Formvar copper grid and allowed to dry. Fluorescence microscopy images were acquired on an inverted Nikon Eclipse TE 300 microscope on bright-field, DAPI and Cy5 filters. Images were merged using ImageJ software (1.50i). The *in vivo* data was obtained using an IVIS® Spectrum imaging system (Perkin Elmer) and data analysis was performed using the Living Image Software (Perkin Elmer).

Synthesis

Synthesis of N-(2-hydroxypropyl)methacrylamide (HPMA)

HPMA was synthesized as previously reported.[26,46] Methacryloyl chloride (14.9 mL, 152.44 mmol) in 40 mL acetonitrile was added dropwise over an hour to a solution of 1-amino-2-propanol in 85 mL acetonitrile at 0 °C under vigorous stirring, and the reaction mixture was stirred for a further 30 min. The precipitated 2-hydroxypropylammonium chloride was removed by filtration and the solvent removed under reduced pressure. The product was

purified by repeated crystallization from acetone (mp = 70 °C) and stored at 8 °C (20.5 g, 140 mmol, 71 % yield). ^{1}H NMR (400 MHz, C*D*Cl$_3$) δ 6.35 (s, 1H), 5.69 (s, 1H), 5.41 – 5.21 (m, 1H), 4.03 – 3.76 (m, 1H), 3.77 – 3.53 (m, 1H), 3.43 (ddd, *J* = 13.9, 6.5, 3.1 Hz, 1H), 3.12 (ddd, *J* = 13.3, 7.6, 5.1 Hz, 1H), 1.92 (t, *J* = 1.2 Hz, 3H), 1.15 (d, *J* = 6.3 Hz, 3H). ESI-TOF-MS: m/z: [M + H] + calcd, 143.09; found, 144.15.

(4-cyanopentanoic acid)-4-dithiobenzoate (CADB)

CADB was synthesized based on a modified procedure of Moad and coworkers.[93] 1-Bromobenzene (8.6 g, 55 mmol) was introduced over 15 min to a round bottomed flask containing anhydrous tetrahydrofuran (150 mL), magnesium turnings (1.4 g, 57 mmol), and a crystal of iodine. The reaction mixture was heated to 80 ºC and allowed to reflux for 2 h, after which time no magnesium metal could be observed. The reaction mixture was then cooled to 0 °C and carbon disulfide (8.3 g, 109 mmol) introduced dropwise, via a degassed syringe. A color change from clear to deep orange was observed, and the reaction mixture was allowed to reach room temperature. The crude magnesiumbromo dithiobenzoate was converted to dithiobenzoic acid with addition of conc. HCl (7.0 mL) and isolated by liquid extraction with diethyl ether (3 x 100 ml). The dithiobenzoic acid was then converted to sodium dithiobenzoate and extracted to the aqueous layer with a 2M NaOH solution (3 x 50 ml). The sodium dithiobenzoate was reduced to S,S'-bisdithiobenzoate with dropwise addition of potassium hexacyanoferrate (15.0 g in 150 mL of water) over 30 minutes, and the crude product dried overnight.

S,S'-Bisdithiobenzoate (3.06 g, 10 mmol) and 4'4'-azobis(4-cyanovaleric acid) (4.2 g, 15 mmol) was added to a round-bottomed flask containing ethyl acetate (100 mL) and refluxed for 18 h. The reaction mixture was concentrated on a rotary evaporator and purified by silica flash column chromatography using 70:30 hexane: ethyl acetate as mobile phase (5.2 g, 20 mmol, 42 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (s, 1H), 7.96 – 7.89 (m, 2H), 7.74

– 7.66 (m, 1H), 7.52 (t, *J* = 7.8 Hz, 2H), 2.60 – 2.51 (m, 2H), 2.43 (d, *J* = 3.2 Hz, 2H), 1.92 (s, 3H). ESI-TOF-MS: m/z: [M + H] + calcd, 263.01; found, 264.02.

Ethylene Dimethacrylamide (EDMA)

A solution of methacrylic anhydride (18 mL, 119.7 mmol) in DCM was added dropwise under vigorous stirring to an ice-cold suspension of ethylenediamine (2 mL, 29.9 mmol) and pyridine (4.8 mL, 59.83 mmol) in 10 mL DCM over 1 h. The reaction mixture was stirred overnight at room temperature. The solid was filtered off and the organic phase washed with deionized water (x3), acidic water (10 %) (x3), deionized water (x3), a solution of sodium bicarbonate in water (x3) and deionized water (x3). The solvent was removed under reduced pressure using a rotary evaporator and the resultant oil was kept at -20 °C until a precipitate formed. The product was further purified via precipitation from ethyl acetate (3.5 g, 18 mmol, 60 % yield. ^{1}H NMR (400 MHz, C*D*Cl$_3$) δ 6.62 (s, 2H), 5.74 (s, 2H), 5.35 (s, 2H), 3.55-3.47 (m, 4H), 1.96 (s, 6H). ESI-TOF-MS: m/z: [M + H] + calcd, 196.12; found, 197.13.

Synthesis of tert-butyl 2-methacryloylhydrazinecarboxylate (t-butoxycarbonyl (boc)-protected hydrazide methacrylate (tBHM))

Tert-butyl carbazate (5 g, 37.8 mmol) and pyridine (5.98 mL, 75.6 mmol) were added to DCM (5 mL) on ice. Methacrylic anhydride (8.41 mL, 56.7 mmol) was added dropwise slowly over half an hour. The solution was then warmed to room temperature and stirred for 24 hours.

The reaction was washed with 10% HCl (2 x 50 mL), distilled water (2 x 50 mL) and $NaHCO_3$ (2 x 50 mL). The organic phase was collected and dried with anhydrous $MgSO_4$ and concentrated. The product was recrystallised three times from hexane:EtOAc (4:1 v/v) (2.45 g, 12 mmol, 33 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.66 (2, 1H), 8.72 (s, 1H), 5.71 (s, 1H), 5.42 (s, 1H), 1.86 (d, *J* = 1.2 Hz, 3H), 1.41 (s, 9H). ESI-TOF-MS: m/z: [M + H] + calcd, 200.12; found, 200.97.

Synthesis of N,N-Cystaminebismethacrylamide (CBMA)

Cystamine dihydrochloride (9.0 g, 40 mmol) was dissolved in 80 mL of water. An aqueous solution of sodium hydroxide (16 mL, 10 M) was added into the above solution, and then the mixture was stirred at 0 ºC for 20 min. Methacryloyl chloride (8.36 g, 80 mmol, in 10 mL of dichloromethane) was added dropwise into the above mixture at 0 °C, while a white precipitate was formed. The reaction mixture was stirred for a further 1 h, and then the mixture was filtered, and the solid was collected and then washed with deionized water (x3). The product was obtained by crystallization from ethyl acetate (7.2 g, 25 mmol, 62.5% yield). ^{1}H NMR (400 MHz, C*D*Cl$_3$) δ 6.54 (s, 1H), 5.77 (t, *J* = 1.1 Hz, 2H), 5.38 (q, *J* = 1.5 Hz, 2H), 3.67 (q, *J* = 6.1 Hz, 4H), 2.91 (t, *J* = 6.4 Hz, 4H), 2.02 – 1.97 (m, 6H). ESI-TOF-MS: m/z: [M + H] + calcd, 288.42; found, 289.52.

Synthesis of disulfide-linked bis-CADB (CADB-SS-CADB)

CADB RAFT agent (300 mg, 1.1 mmol), pentafluorophenol (217 mg, 1.2 mmol) and 4-dimethylaminopyridine (26 mg, 0.2 mmol) were stirred in dichloromethane (3 mL) at 0 ºC for 10 min. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 200 mg, 1.3 mmol) in 3 mL dichloromethane was added dropwise over 30 mins. The reaction was stirred at 0 ºC for a further 2 hours, and then at room temperature overnight.

The crude product was washed with acidic water (20 mL), distilled water (20 mL) and brine (20 mL). The organic phase was dried with $MgSO_4$ and the solvent removed by a rotary evaporator to yield a pink oil. The product was purified by column chromatography using chloroform as eluent, with the product eluting as the first fraction.

Cystamine dihydrochloride (46 mg, 0.2 mmol) was dissolved in 5 mL DMF. TEA (41 mg, 0.4 mmol) was slowly added, and the reaction stirred for 10 minutes. CPTPA-PFP (200 mg, 0.45 mmol) was dissolved in 1 mL DMF and added dropwise to the cystamine solution and stirred at room temperature overnight. The product was purified by column chromatography

(Hexane:Ethyl Acetate 70:30) with the product eluting as the first fraction (290 mg, 0.43 mmol, 43% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (ddt, J = 18.1, 12.1, 5.5 Hz, 2H), 7.98 – 7.87 (m, 4H), 7.72 – 7.60 (m, 2H), 7.60 – 7.46 (m, 4H), 3.37 (d, J = 3.3 Hz, 4H), 2.78 (t, J = 6.7 Hz, 4H), 2.51 – 2.33 (m, 4H), 2.13 (dddd, J = 36.9, 14.3, 9.3, 4.5 Hz, 4H), 1.91 (d, J = 1.9 Hz, 6H). ESI-TOF-MS: m/z: [M + H] + calcd, 674.10; found, 675.11.

Synthesis of Poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA) in varying architectures by RAFT Polymerization

Non-degradable hyperbranched polymers

RAFT polymerizations of hyperbranched HPMA were carried out following a known standard protocol.[64] Briefly, HPMA (188 mg, 1.3 mmol) was dissolved in milliQ water (360 µL) along with CADB RAFT agent (18.4 mg, 0.066 mmol), EDMA (12.9 mg, 0.066 mmol) and V-501 (3.7 mg, 0.013 mmol) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 24 h. The reaction mixture was then precipitated with acetone and unreacted monomer and impurities removed by dialysis in pure water for 48 hours. The polymer was analyzed by ^{1}H NMR and SEC and labelled as HB-HPMA-S. ^{1}H NMR (400 MHz, DMSO) δ 7.33-7.04 (s, 1H), 4.90-4.60 (s, 1H), 3.82-3.57 (s, 1H), 3.10-2.73 (s, 2H), 2.05-0.42 (broad, m, 8H, CH_2 and CH_3 of polymer backbone and $CHCH_3$).

Chain extension of non-degradable hyperbranched polymer

To produce a larger sized particle, HB-HPMA was chain extended with additional HPMA at a target DP of 150. HB-HPMA (50 mg, 0.0025 mmol), HPMA (54 mg, 0.38 mmol) and ACVA (0.07 mg, 0.25 µmol) were dissolved in milliQ water (375 µL) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 6 h and purified as above. The resultant polymer was labelled as HB-HPMA-L.

Redox-responsive hyperbranched polymer architectures

A hyperbranched polymer with 20 mol% content of a hydrophobic drug loading monomer was synthesized as follows. HPMA (150 mg, 1.1 mmol) was dissolved in distilled water (600 µL) along with CADB RAFT agent (37 mg, 0.13 mmol), tBHM (53 mg, 0.26 mmol), CMBA (38 mg, 0.13 mmol) and V-501 (7.3 mg, 0.026 mmol) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 24 hours. The reaction mixture was then precipitated with acetone and unreacted monomer and impurities removed by dialysis for 48 hours. The polymer was analyzed by ^{1}H NMR and SEC and labelled HB-SS-HH. ^{1}H NMR (400 MHz, DMSO) δ 7.33-7.04 (s, 1H), 4.90-4.60 (s, 1H), 3.82-3.57 (s, 1H), 3.10-2.73 (s, 2H), 2.05-0.42 (broad, m, 8H, CH_2 and CH_3 of Polymer backbone and $CHCH_3$).

A hyperbranched polymer with a lower hydrophobic content of 5 mol% was synthesized as above with the modifications of HPMA (179 mg, 1.25 mmol) and tBHM (13 mg, 0.066 mmol) as the monomer ratios. The resultant polymer was labelled as HB-SS-LH.

Redox-responsive star architecture

Star architecture polymers were achieved through chain extension of a HB core polymer with two different target DPs (50 and 100) of HPMA to form two sizes of larger branched molecule. Briefly, HB-SS-HH (100 mg, 0.0167 mmol) was dissolved in distilled water (900 µL) along with HPMA (119 mg, 0.834 mmol, Star-SS-S) or (239 mg, 1.67 mmol, Star-SS-L) and V-501 (0.47 mg, 0.0017 mmol) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 6 hours. The resultant polymers were purified and characterized as above.

Redox-responsive linear architecture

A high molecular weight linear random copolymer was synthesized with a single disulfide bridge utilizing the bis-RAFT synthesized above. Briefly, HPMA (572 mg, 4 mmol), tBHM (43 mg, 0.4 mmol), CADB-SS-CADB RAFT agent (3.4 mg, 0.005 mmol) and V-501 (0.14 mg,

0.5 μmol) were dissolved in milliQ water (4 mL) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 6 hours. The resultant polymers were purified and characterized as above.

Redox-responsive micellar architecture

A HPMA macro-RAFT agent was synthesized as follows. HPMA (258 mg, 1.8 mmol), CADB RAFT agent (3.4 mg, 0.012 mmol) and V-501 (0.34 mg, 0.0012 mmol) were dissolved in milliQ water (1.8 mL) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 4 hours. The reaction mixture was then precipitated with acetone three times and the resultant polymer was dried under vacuum prior to the next step.

Disulfide crosslinked micelles were synthesized through RAFT aqueous dispersion polymerization as follows. HPMA macro-CTA (200 mg, 0.01 mmol), tBHM (60 mg, 0.3 mmol), CBMA (29 mg, 0.1 mmol) and V-501 (0.3 mg, 0.001 mmol) were dissolved in milliQ water (2.5 mL, 10% w/w solids) in a reaction flask and sealed with a rubber septum reinforced with a cable tie. The solution was bubbled with argon for 30 minutes and then stirred at 70 °C for 8 hours. The crosslinked micelles were isolated by precipitation in acetone, followed by dialysis for 48 hours.

Labelling of pHPMA with Cy5

All pHPMA architectures were labelled with Cy5-amine for imaging *in vitro* and *in vivo* through an amidation reaction onto the terminal RAFT agent carboxylic acid groups at a 1:0.5 ratio of polymer:dye as follows. HB-SS-HH (20 mg, 5 μmol), pentafluorophenol (1 mg, 5 μmol) and DMAP (0.1 mg, 1 μmol) were dissolved in DMSO/H_2O (50% v/v) on ice and stirred for 30 minutes. EDC (1 mg, 6 μmol) in the same solvent was added dropwise to the reaction, and stirred over ice for a further 2 hours. The reaction was then warmed to room temperature

and stirred overnight. The reaction mixture was dried under high vacuum, and redissolved in DMF without purification. Cy5 amine (1.5 mg, 2.5 µmol) was dissolved in DMF (1 mL) and TEA (0.2 mg, 2.5 µmol) was slowly added. The solution was stirred for 10 minutes, and then added dropwise to the pHPMA-PFP solution. Polymers were purified by precipitation in acetone, followed by dialysis in pure water for 48 hours until the dialysis water was clear and the SEC trace of the labelled polymer showed a single peak detected by UV at 650 nm.

Boc-deprotection

Two candidate polymer architectures were selected for drug delivery efficacy investigations, HB-SS-HH and Star-SS-L. A predetermined amount of polymer (150 mg) was dissolved in dry MeOH (2 mL). TFA (0.5 mL) was slowly added to the polymer mixture and let stirring for 1 hr. Polymers were purified by precipitation into cold acetone, washed once and analyzed by ^{1}H-NMR.

Doxorubicin conjugation

HB-SS-HH or Star-SS-L (30 mg) were dissolved in 1mL dry methanol in a glass vial equipped with a stirrer bar. DOX.HCl (1.2x excess) was added and the reaction stirred overnight at 35 °C. The reaction was purified by precipitation into cold acetone 3x following by dialysis in PBS for 24 hours. DOX attachment was characterised by ^{1}H NMR and UV–Vis microscopy (ex = 480 nm).

Critical Micelle Concentration studies

In order to determine the critical micelle/aggregation concentrations of the pHPMA materials, a DLS method was utilized. From a stock solution of polymer (500 µg/mL) in PBS, a multipipettor was employed to dilute to predetermined concentrations (100, 50, 25, 15, 10, 5, 2.5, 1, 0.5, 0.25 µg/mL). The count rate of each dilution was measured using DLS, and plotted

as a function of the polymer concentration. A straight line was fitted to each linear segment and the CMC/CAC was determined as the point at which the two lines intersected.

Stability studies

To determine the stability of pHPMAs under physiologically relevant conditions, each polymer architecture was dispersed in DMEM cell culture media at 500 µg/mL and incubated at 37 °C for different times. Size measurements of the samples were taken at 0 h, 24 h and 48 h of incubation. To determine the stability of the redox-responsive pHPMAs under reductive conditions, each polymer was dispersed in PBS 10 mM pH 7.4 enriched with GSH (10 mM) at 500 µg/mL and incubated at 37 °C for different times. Size measurements of the samples were taken by dynamic light scattering on a Zetasizer after 1 h, 24 h and 48 h of incubation.

Protein binding assay

To determine protein corona association to the pHPMAs, binding studies were undertaken using bovine serum albumin (BSA) as the model protein. Each polymer architecture (1 mg/mL in PBS) was sized by DLS at a fixed attenuation (11), and then BSA was added at low (0.2 wt%, equivalent to DMEM+10% FBS) or high (1 wt%) concentrations. The samples were incubated at 37 °C and size measurements of the samples were taken by dynamic light scattering on a Zetasizer after 1 h and 24 h.

In vitro buffer release study

Phosphate buffer solutions at pH 5.5 and 7.4 were used to simulate endosomal and physiological pH. A calibration curve of DOX. HCl was prepared by measuring seven concentrations in pH 7.4 phosphate buffer in a 96 well plate. The excitation and emission wavelengths were 480 nm and 580 nm respectively. The samples were measured in a Tecan plate reader at a depth of 19 mm with 10 flashes and an integration time of 20 µs.

The following release study was performed in triplicate to account for statistical variation between subsets. 1.0 mg of polymer prodrug was dissolved in 500 µL pH 7.4 phosphate buffer. The dissolved polymer was added into a Slide-A-Lyzer™ MINI Dialysis Device (3.5K MWCO) and the receptor tube was filled with 15 mL of either pH 7.4 or 5.5 phosphate buffer. All samples were stirred at 37 °C for 72 h. Aliquots of the dialysis buffer (1 mL) were removed at various time points, replacing with fresh buffer. The samples were measured on the plate reader using the same settings as the standards.

Cell culture

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia) and used in a passage window of 10. MDA-MB-231 cells and MDA-MB-468 cells were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia) and used in a passage window of 16. Cells were cultured in DMEM (Sigma-Aldrich) or RPMI (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich), and at 37 °C with 5% CO_2.

Cytocompatibility evaluation in breast cancer cells

The cytotoxicity of the pHPMA materials was assessed using an AlamarBlue™ Cell Viability assay. MDA-MB-231 and MDA-MB-468 cells were seeded into a 96 well-plate at a density of 4 x 10^3 cells per well, and were allowed to adhere in growth media overnight at 37 °C and 5% CO_2. Cell media were discarded and replaced with fresh media (100 µL) containing different concentrations of pHPMA (100, 250, 500, 750 and 1000 µg/mL) for 48 hours. Cell viability was then assessed by addition of 10 µL alamarBlue to each well, with incubation for a further 4 hours. Fluorescence readings were measured using a Tecan plate reader at an excitation/emission of 530-560⁄590 nm. Fluorescence readings of blank wells (no cells) were

subtracted from all readings and then cell viability values were determined as a ratio to cells grown in the absence of test compounds.

For DOX-conjugated pHPMAs, cytocompatibility assays were conducted as follows. The AlamarBlue™ Cell Viability assay was performed to assess the effects of free DOX and pHPMA-DOX in MDA-MB-231 cells. The cells were seeded at $1x10^4$ cells per well in 96 well plates and cultured for 24 hours prior to assaying. Cell media was discarded and replaced with fresh media (100 µL) containing different concentrations of free DOX or pHPMA-DoX for 48 hours. Triton X-100 applied at 1% (v/v) was used as a cell death (positive) control and a vehicle control containing no nanoparticles used as a negative control. Cells were then washed twice with warm PBS and 100 µL 10% (v/v) AlamarBlue reagent diluted in medium was applied per well, with incubation for a further 4 hours. Fluorescence readings were measured using a Tecan plate reader at an excitation/emission of 530-560⁄590 nm. Relative metabolic activity was calculated by setting values from the negative control as 100% and positive control values as 0% metabolic activity.

Cytocompatibility evaluation in macrophages

The lactate dehydrogenase (LDH) release assay and PrestoBlue™ Cell Viability assay were performed to assess nanoparticle cytotoxicity. RAW 264.7 cells were seeded at $1x10^4$ cells per well in 96 well plates and cultured for 24 hours prior to assaying. Nanoparticles were exposed to cells for 24 hours and applied in 100 µL phenol red free DMEM containing 10% (v/v) FBS and 2 mM L-glutamine. Triton X-100 applied at 1% (v/v) applied in phenol red free medium was used as a cell death (positive) control and a vehicle control containing no nanoparticles used as a negative control. Following exposure, 50 µL of supernatant was collected per well for analysis of LDH content. Cells were then washed twice with warm PBS and 100 µL 10% (v/v) PrestoBlue reagent diluted in phenol red free medium applied per well for 60 minutes. The resulting fluorescence was measured at 560/600 nm ($\lambda_{ex}/\lambda_{em}$). Relative metabolic activity

was calculated by setting values from the negative control as 100% and positive control values as 0% metabolic activity. Assessment of LDH release was performed according to the manufacturer's instructions and involved adding 100 µL LDH detection reagent to the collected supernatant samples and incubating at room temperature shielded from light for 25 minutes. Absorbance was then measured at 492 nm. Relative LDH release was calculated with the negative control absorbance at 492 nm taken as 0%, and the positive control, assumed to cause total cell lysis, as 100%.

Macrophage uptake of nanoparticles assessed by spectrophotometry

RAW 264.7 macrophages were plated in 12 well plates at a seeding density of $1.2x10^5$ cells per well and cultured for 48 hours. Culture medium was then removed and 1 mL of nanoparticles applied in phenol red free medium. The time-dependence of uptake was assessed with a nanoparticle concentration of 50 µg/mL. Additionally, the concentration-dependence of uptake was investigated at a fixed time point of 2 hours. Following exposure, nanoparticle solutions were removed and cells washed three times with ice cold PBS. 500 µl of 1% (v/v) Triton X-100 solution applied in PBS was then added per well for 10 minutes at 37 °C. To assess nanoparticle uptake, 100 µl of the resulting solution was collected per well and transferred to 96 well plates for the measurement of fluorescence at 640/680 nm ($\lambda_{ex}/\lambda_{em}$). Quantification of nanoparticles uptake was achieved via calibration curves of known nanoparticle concentrations diluted in 1% (v/v) Triton X-100 in PBS solution. Values were normalized to viable cell number per well determined by the trypan blue exclusion test and cell counting on a haemocytometer.

Macrophage uptake determined by fluorescence imaging

RAW 264.7 macrophages were seeded at a density of $1x10^4$ cells per well in 96 well plates and cultured for 48 hours. 100 µl of 50 µg/ml nanoparticle solutions were applied to cells for

2 hours, followed by removal and three washes with ice cold PBS. 100 µl of Hoechst 33342 (10 µg/ml) (ThermoFisher) diluted in PBS was then applied to cells for 10 minutes at 37°C with 5% CO_2. Staining solution was removed and cells washed twice with PBS prior to the addition of 100 µl Fluorobrite DMEM (ThermoFisher) for imaging. Cells were imaged on an inverted Nikon Eclipse TE 300 microscope on bright-field, DAPI and Cy5 filters. Images were merged using ImageJ software (1.50i).

Breast cancer cell uptake determined by fluorescence imaging

MDA-MB-231 cells were seeded at a density of 1 x 10^5 cells per well in 12 well plates and cultured for 24 hours. 1 mL of culture medium containing free DOX, blank pHPMA or pHPMA-DOX was applied to cells for 1, 2 and 4 hours, followed by removal and three washes with ice cold PBS. Cells were then fixed in 4% paraformaldehyde (PFA) solution (0.5 mL) for 10 minutes. The PFA was then removed and the cells washed twice with PBS, followed by staining of the nuclei with 0.5 mL of Hoechst 33342 (10 µg/ml) (ThermoFisher) diluted in PBS for 10 minutes at 37°C with 5% CO_2. Staining solution was removed and cells were imaged on a Nikon Ti Eclipse inverted microscope with bright-field, DAPI, tdTomato and Cy5 filters. Images were merged using ImageJ software (1.50i).

In vitro 3D cell viability assays

Corning 7007 Ultra-low attachment (ULA) 96-well round bottom plates were used to culture the 3D spheroids. 80% confluent MDA-MB-231 monolayer cells were detached, collected and the cell number determined using the Biorad TC20 automated cell counter. A single-cell suspension was diluted in culture medium with the addition of Cultrex basement membrane extract (Cultrex-BME, 100 µg/mL) and cells were seeded at 1000 cells/well to generate spheroids (final volume of cell suspension in each well was 100 µL). The plates were then centrifuged at 300 RCF for 5 min and cultured for 3 days until visible spheroid formation.

Free DOX, blank pHPMAs and pHPMA-DOX were prepared as solutions in media at 2x the concentration of 2D cell culture. 100 µL of sample was added to each well which already contained a single spheroid in 100 µL of medium (making the total volume 200 µL) and the plate incubated for 72 h (n = 4). Images were taken immediately prior to treatment and every day thereafter for 3 days for spheroid volume analysis. Images of the spheroids were taken with a Nikon Ti Eclipse inverted microscope.

The volume of the spheroids was analyzed by measuring their cross-sectional area using the in-house open source macro for the FiJi distribution of ImageJ written by Ivanov.[111] The macro determines the area of each spheroid using an established threshold algorithm.[112] This automated process is robust with less than 5% variation found between the algorithm and manual measurement. The measured area (S) from the macro data of the 2D projection of the spheroid was then used to calculate the radius, $\left(r = \sqrt{\frac{S}{\pi}}\right)$ and subsequently, the volume, $\left(V = \frac{4}{3}\pi r^3\right)$ of an equivalent sphere.[113]

At 72 h post treatment of the spheroids, cell viability was measured using AlamarBlue™ Cell Viability assay. 100 µL of cell culture medium was removed from each well and replaced with 100 µL 10% (v/v) AlamarBlue reagent. The contents of the plate were mixed vigorously for 5 minutes to destroy the spheroid structure, and the plate was incubated for 4 hours. Fluorescence readings were measured using a Tecan plate reader at an excitation/emission of 530-560⁄590 nm.

Statistical Analysis

Values obtained from the experiments were analyzed using one-way ANOVA. In all cases, differences were considered significant when $p^{***} < 0.001$, $p^{**} < 0.01$, and $p^{*} < 0.05$.

In vivo biodistribution of Cy5-labelled pHPMA

In vivo biodistribution experiments were performed in order to assess distribution, organ accumulation and clearance of Cy5-labelled pHPMAs of different architectures. The experiments were conducted under the UK Home Office Licence number PPL P435A9CF8. LASA good practice guidelines, FELASA working group on pain and distress guidelines and ARRIVE reporting guidelines were also followed.

8-9 week old female immunodeficient CD-1 NuNu mice were purchased from Charles River UK. Mice were maintained in Individually Ventilated Cages (Tecniplast UK) within a barriered unit, illuminated by fluorescent lights set to give a 12 hour light-dark cycle (on 07.00, off 19.00), as recommended in the guidelines to the Home Office Animals (Scientific Procedures) Act 1986 (UK). The room was air-conditioned by a system designed to maintain an air temperature range of 21 ± 2 ºC and a humidity of 55% ± 10%. Mice were housed in social groups, 3 per cage, during the study, with irradiated bedding and autoclaved nesting materials and environmental enrichment (Datesand UK). Sterile irradiated 5V5R rodent diet (IPS Ltd, UK) and autoclaved water was offered *ad libitum.* The condition of the animals was monitored throughout the study by an experienced animal technician. After a week's acclimatization, the mice were randomly allocated by weight to the study groups of 6 mice per polymer type.

After warming the mice in a thermostatically controlled heating box (Datesand UK), they were injected intravenously via the tail vein with 100 µl of a 500 µM solution of the selected polymer type in PBS. No adverse effects were observed following the injections or for the duration of the study. The concentration delivered was determined from a balance of non-toxicity from *in vitro* analysis as well as sufficient fluorescent intensity for imaging *in vivo*. Prior to the study time points, the mice were anaesthetized with an injectable anesthetic combination (Anaestemine [ketamine]/Sedastart [medetomadine], Animalcare Ltd. UK) before being placed in the imaging system. Images were taken at 1h, 4h and 24h post-injection, and two mice in each group were culled by cervical dislocation, organs were dissected out and were

imaged ex vivo at these time points, with the other mice being allowed to recover from the anesthetic with appropriate post procedural monitoring and therapy, including placing mice on a heat pad and providing fluid replacement via wet mash once awake. The organs excised and imaged were the kidneys, liver, spleen, pancreas, lung, heart, bladder, brain and lymph nodes (subiliac). A urine sample (25 µl) was also collected and imaged.

All images were collected using the IVIS® Spectrum imaging system, PerkinElmer (MA, USA) and fluorescent signals were quantified using Regions of Interest (ROIs) and quantified as photons emitted using Living Image/Igor Pro Software (Caliper Life Sciences).

In vivo efficacy and biodistribution of Cy5-labelled pHPMA-DOX conjugates

In vivo experiments in MB-MDA-231 fLuc breast cancer tumor bearing mice were performed in order to assess efficacy, distribution and organ accumulation Cy5-labelled pHPMAs of different architectures. The experiments were conducted under the UK Home Office Licence number PPL P435A9CF8. LASA good practice guidelines, FELASA working group on pain and distress guidelines and ARRIVE reporting guidelines were also followed.

50 6-7 week old female immunodeficient CD-1 NuNu mice were purchased from Charles River UK. Mice were maintained in Individually Ventilated Cages (Tecniplast UK) within a barriered unit, illuminated by fluorescent lights set to give a 12 hour light-dark cycle (on 07.00, off 19.00), as recommended in the guidelines to the Home Office Animals (Scientific Procedures) Act 1986 (UK). The room was air-conditioned by a system designed to maintain an air temperature range of 21 ± 2 ºC and a humidity of 55% ± 10%. Mice were housed in social groups, 3 per cage, during the study, with irradiated bedding and autoclaved nesting materials and environmental enrichment (Datesand UK). Sterile irradiated 5V5R rodent diet (IPS Ltd, UK) and autoclaved water was offered *ad libitum.* The condition of the animals was monitored throughout the study by an experienced animal technician. After a week's acclimatization, the mice were initiated with tumors as follows.

The cells were maintained *in vitro* in RPMI culture medium (Sigma, UK) containing 10% (v/v) heat inactivated fetal bovine serum (Sigma, Poole, UK) & 2 mM L-glutamine (Sigma, UK) at 37^0C in 5% CO_2 and humidified conditions. Cells from sub-confluent monolayers were harvested with 0.025% EDTA, washed in culture medium and counted. Cells with viability of >90% were re-suspended and seeded at $2x10^6$ cells per T150 flask and incubated for 48 hours. On day of initiation cells were harvested from semi-confluent monolayers with 0.025% EDTA, washed twice in the culture medium and counted 3 times as above. Cells with viability of >90% were re-suspended for *in vivo* administration in standard or growth factor reduced matrigel at $2x10^6$/ml for injection of 100 µl into the left mammary fat pad just inferior to the nipple. Tumor establishment and growth was monitored during the experiment by 2D optical imaging, carried out under anesthesia in an IVIS Spectrum detailed later weekly, and were also measured by Vernier calipers (Camlab) twice weekly and animals weighed weekly. The tumor volumes were calculated using a standard volume formula as previously reported (tumor volume = ½ ($length \times width^2$). Dosing commenced on day 17 when the tumors had reached a suitable size as established by caliper measurement and bioluminescent imaging, average diameters around 6 mm. Animals were randomized by volume and bioluminescence intensity, with group numbers of 10 mice per group. Injections were delivered on days 1, 3, 5, 8, 10, 12 or days 1, 3, 7, 11, 18 by the intravenous route. After warming the mice in a thermostatically controlled heating box (Datesand UK), they were injected intravenously via the tail vein with 100ul of a 500uM solution of the selected polymer type in PBS. No adverse effects were observed following the injections or for the duration of the study. The concentration delivered was determined from a balance of non-toxicity from *in vitro* analysis as well as sufficient fluorescent intensity for imaging *in vivo*. Imaging was carried out weekly as follows: the mice were anaesthetized with an injectable anesthetic combination (Anaestemine [ketamine]/Sedastart [medetomadine], Animalcare Ltd. UK) before being placed in the

imaging system. At the pre-determined scientific end point (28-29 days) animals were culled by cervical dislocation, tissues were dissected out and were imaged *ex vivo*. The tissues excised and imaged were the tumor, kidneys, liver, spleen, pancreas, lung, heart, bladder, brain and lymph nodes (subiliac). A urine sample (25 µl) was also collected and imaged. In addition, the tumor, spleen and heart were weighed prior to preservation. All organs were preserved by snap freezing and fixation (NBF) 50:50. All images were collected using the IVIS® Spectrum imaging system, PerkinElmer (MA, USA) and fluorescent signals were quantified using Regions of Interest (ROIs) and quantified as photons emitted using Living Image/Igor Pro Software (Caliper Life Sciences).

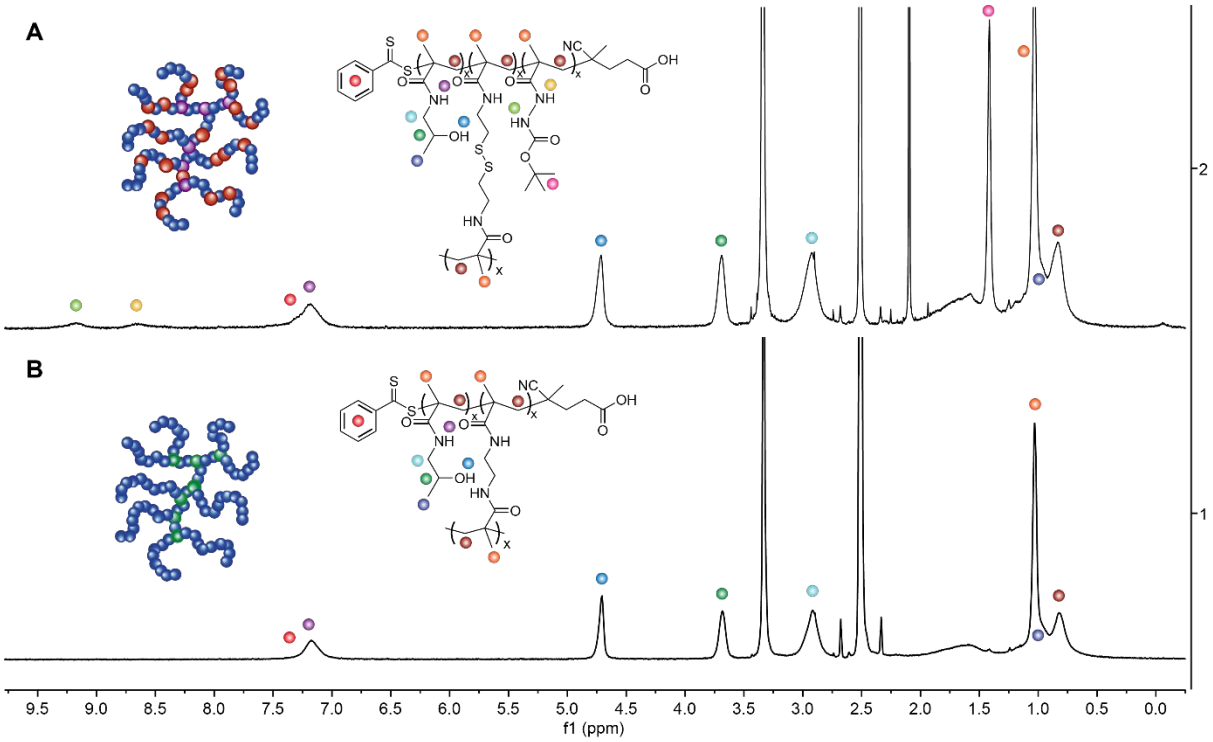

Figure S1. Representative ^{1}H NMR of top: redox-responsive hyperbranched polymer (HB-SS-HH) and bottom: non-degradable hyperbranched HPMA (HB-HPMA-S). The diagnostic resonances for molecular weight characterization have been highlighted.

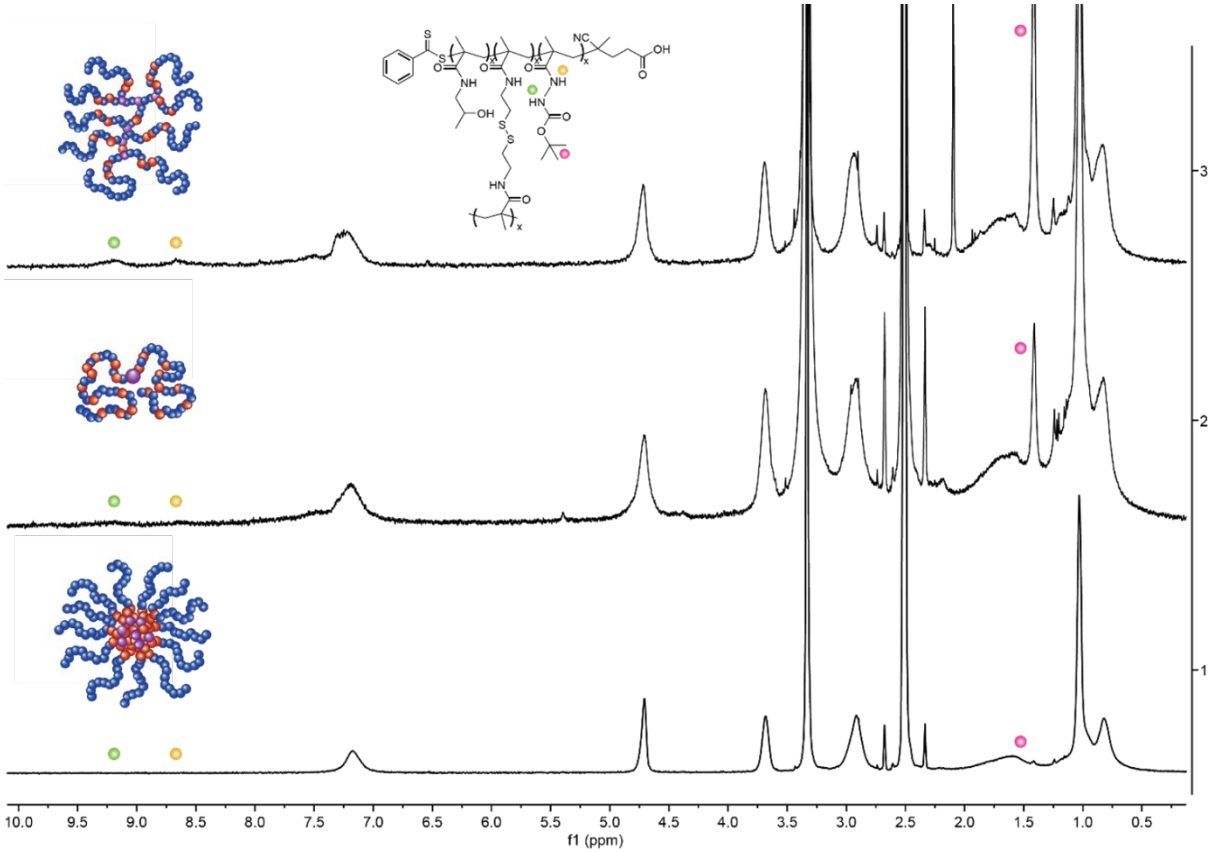

Figure S2. [1]H NMR spectra of top) Star-SS-L, middle) Linear-SS and bottom) Micelle-SS. The resonances of the boc-protected hydrazide monomer have been highlighted. Note: the NMR visibility of the proton signals is disappeared for the micelle due to the self-assembly.

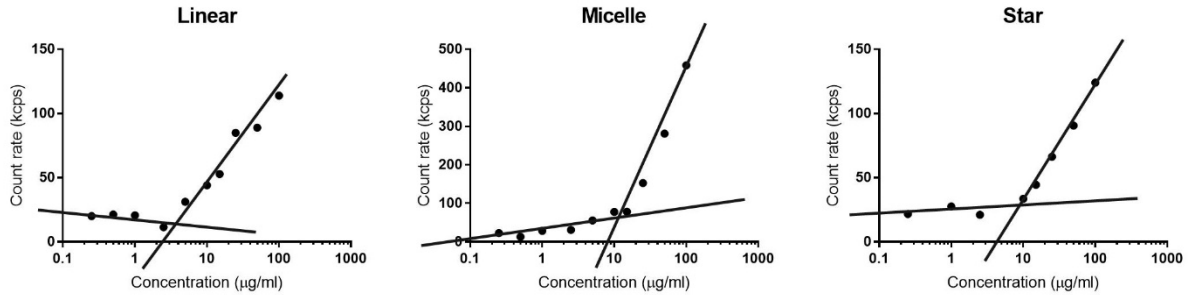

Figure S3. Critical micelle concentration (CMC) plots for the linear, micelle and star pHPMAs.

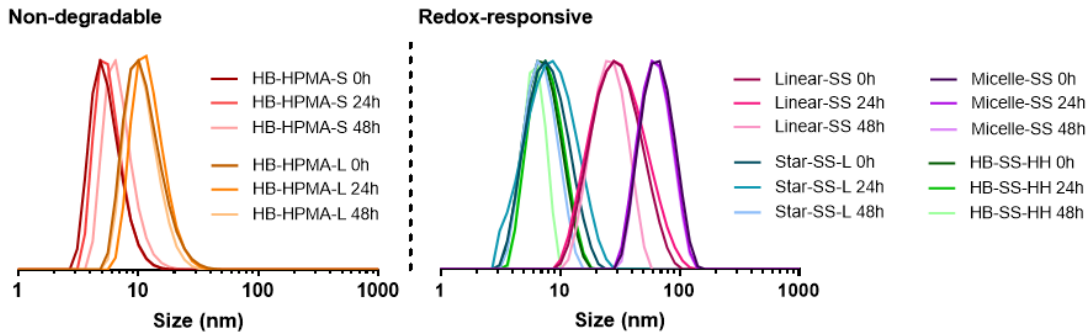

Figure S4. Stability of non-degradable and redox-responsive pHPMAs in cell culture DMEM over 48 h incubation at 37 ºC.

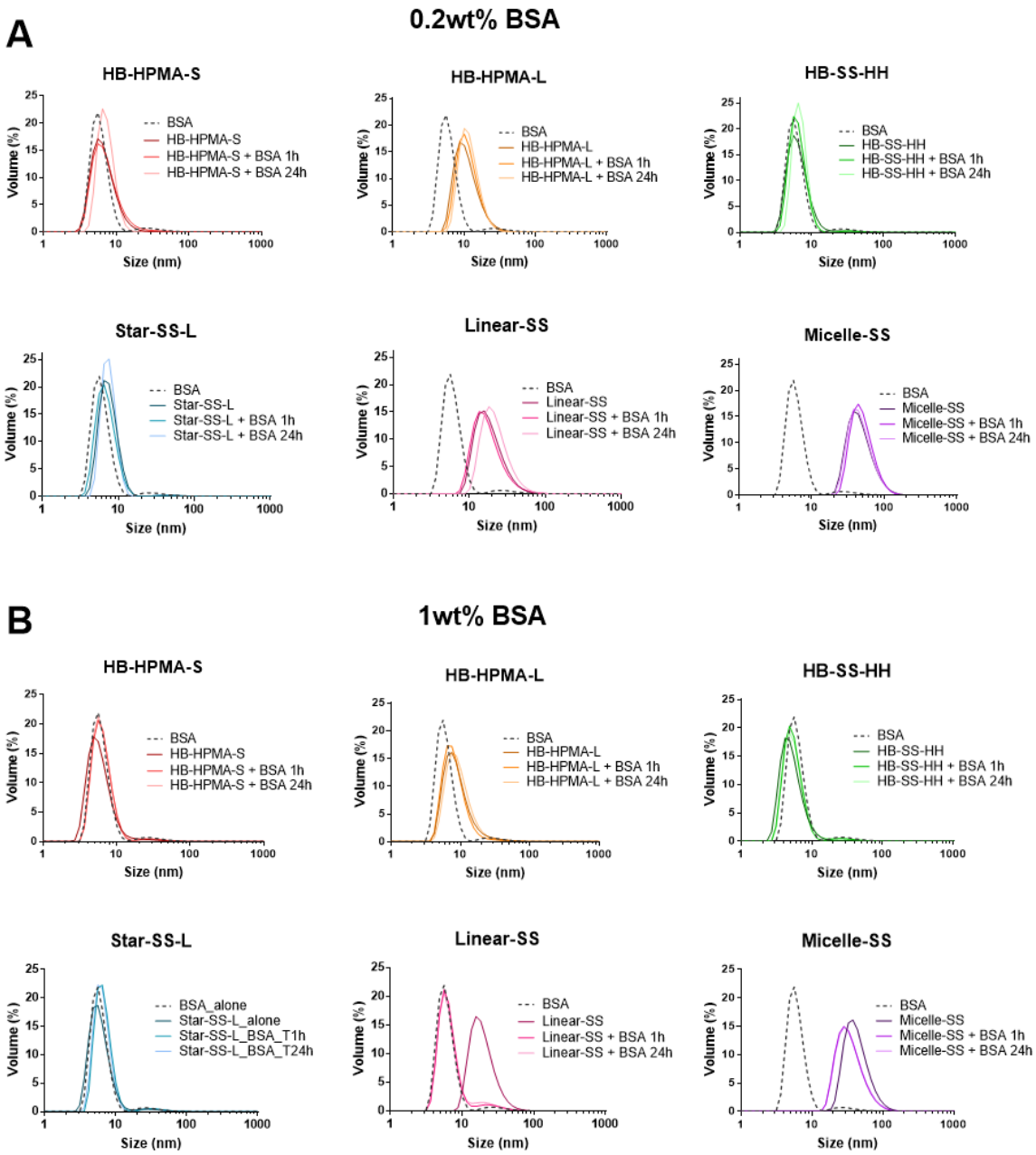

Figure S5. Stability of pHPMAs to protein corona binding assayed by DLS at A) 0.2 wt% BSA and B) 1 wt% BSA.

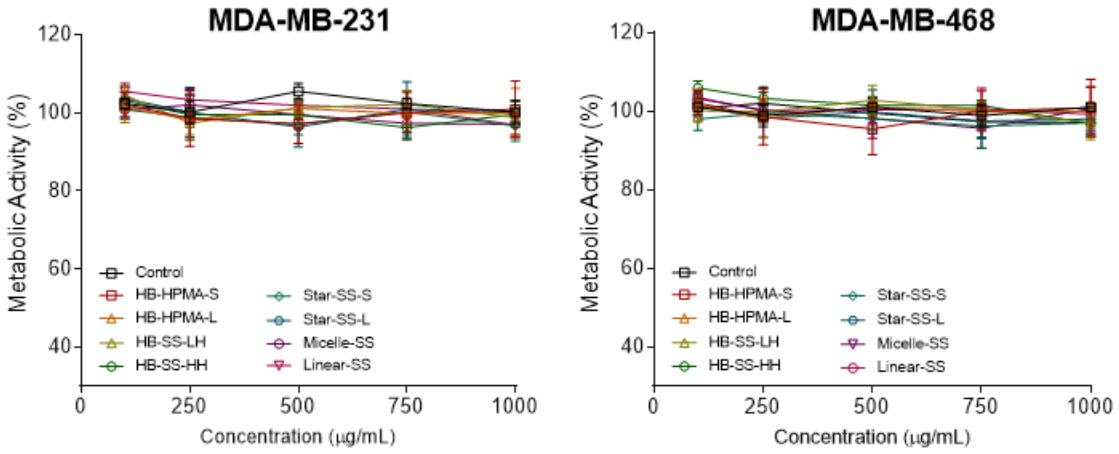

Figure S6. *In vitro* cell viability for all pHPMA materials in triple negative breast cancer cells.

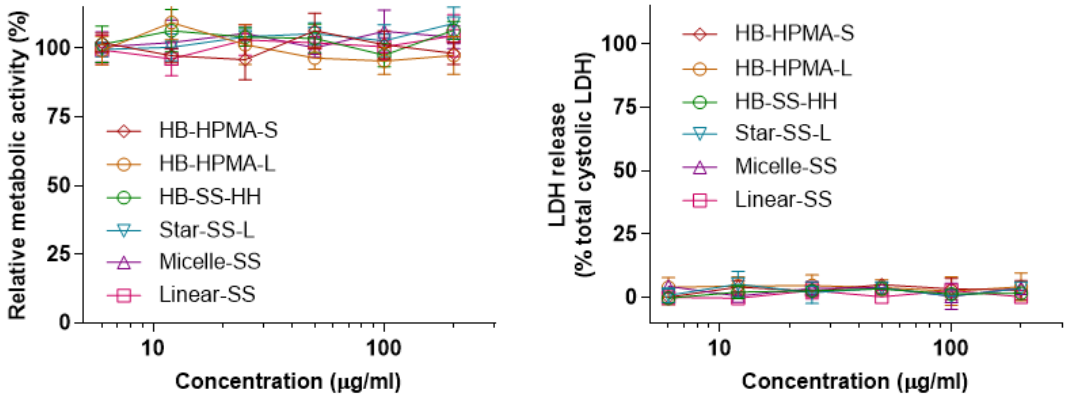

Figure S7. *In vitro* data depicting A) metabolic activity and B) LDH release of pHPMAs after 24h incubation with RAW 264.7 macrophages

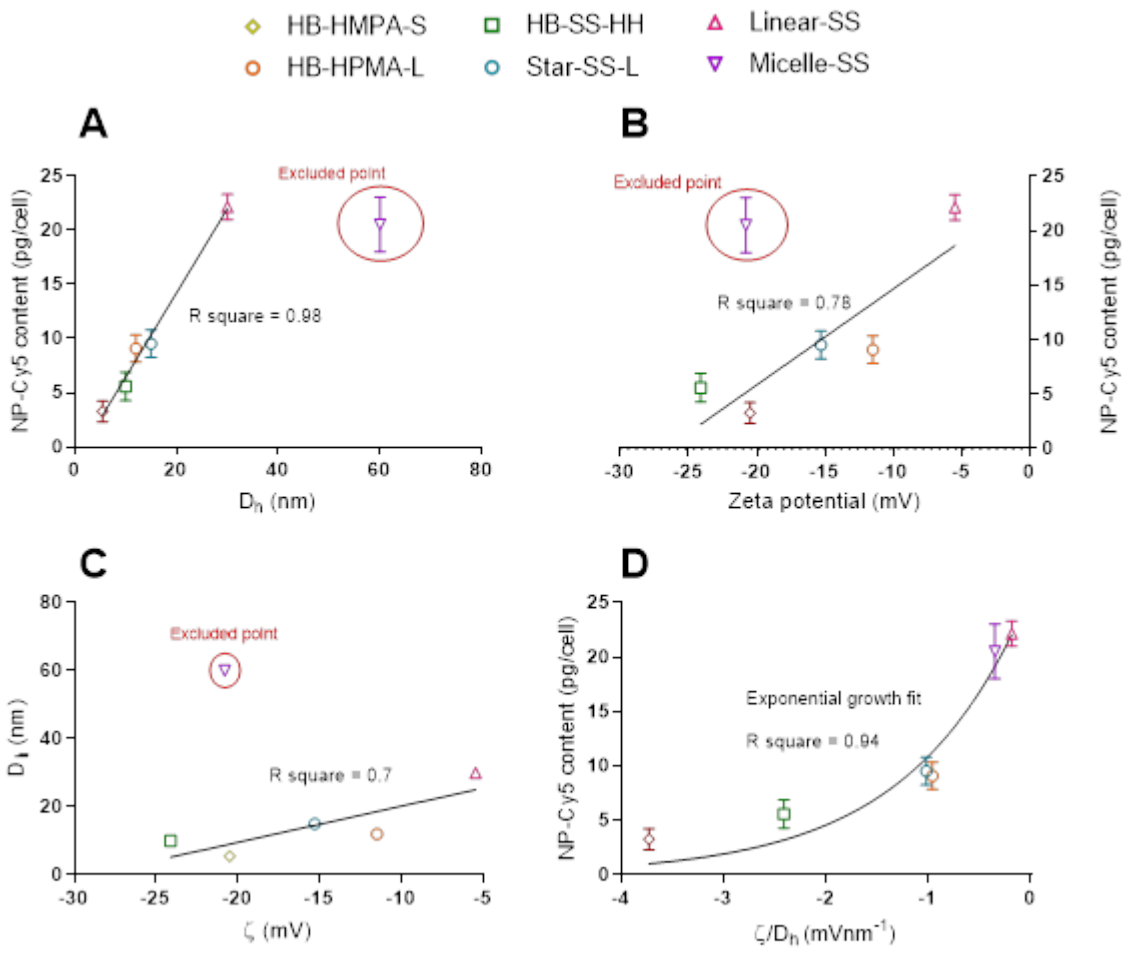

Figure S8. Macrophage uptake data showing A) influence of size on uptake (4h), B) influence of zeta-potential on uptake (4h), C) zeta-potential vs size of nanoparticles and D) ratio of zeta-potential / size vs uptake.

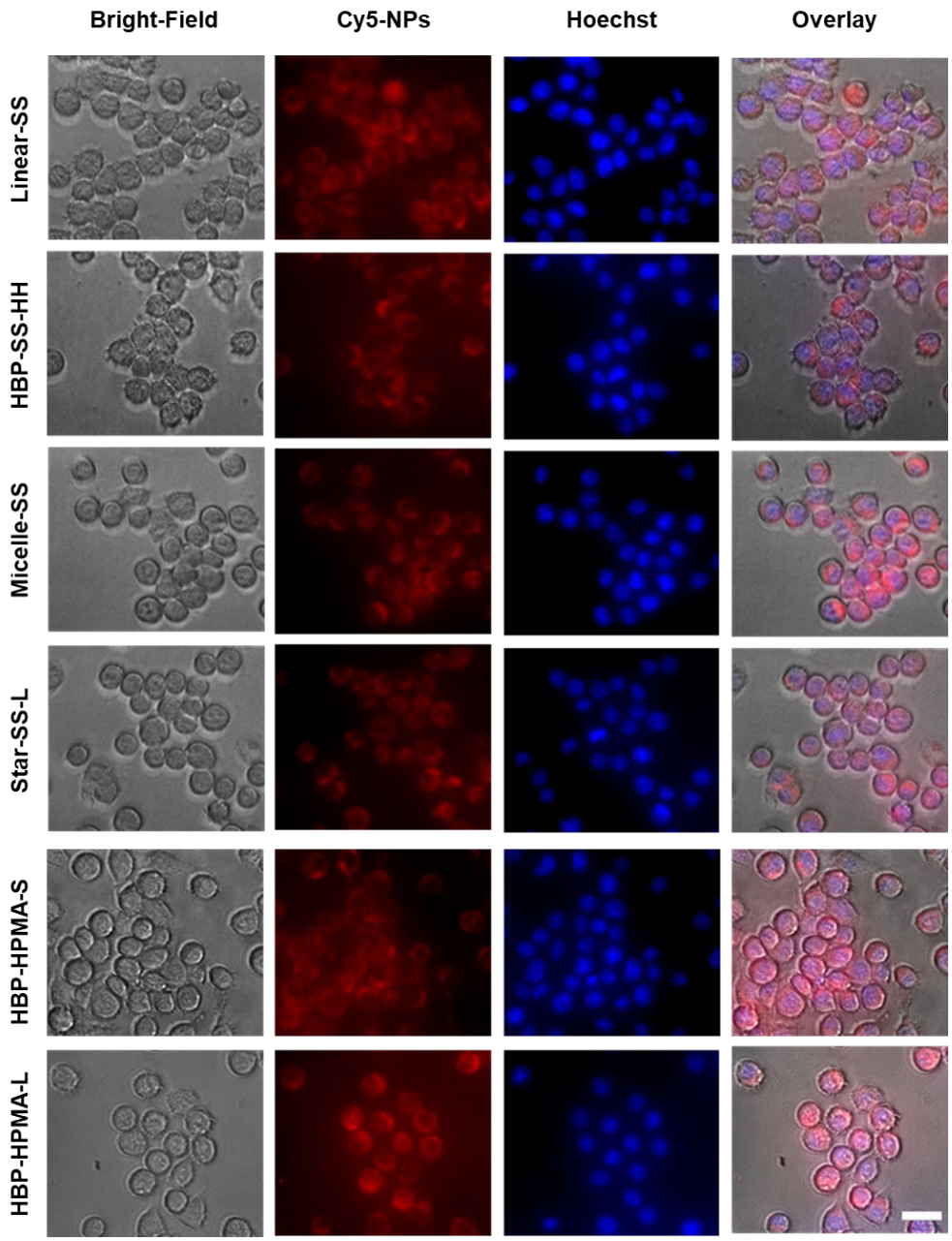

Figure S9. Fluorescence microscopy confirming intracellular localization of pHPMAs during macrophage uptake studies (scale bar: 25 μm).

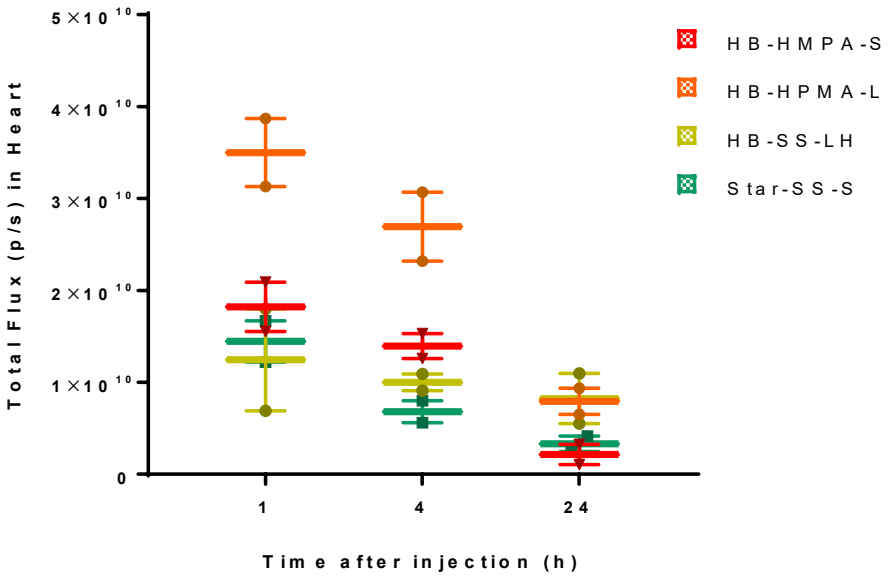

Figure S10. Quantitation of the fluorescence signal in the heart at all times, indicative of circulating nanoparticles (n = 2 ± SD).

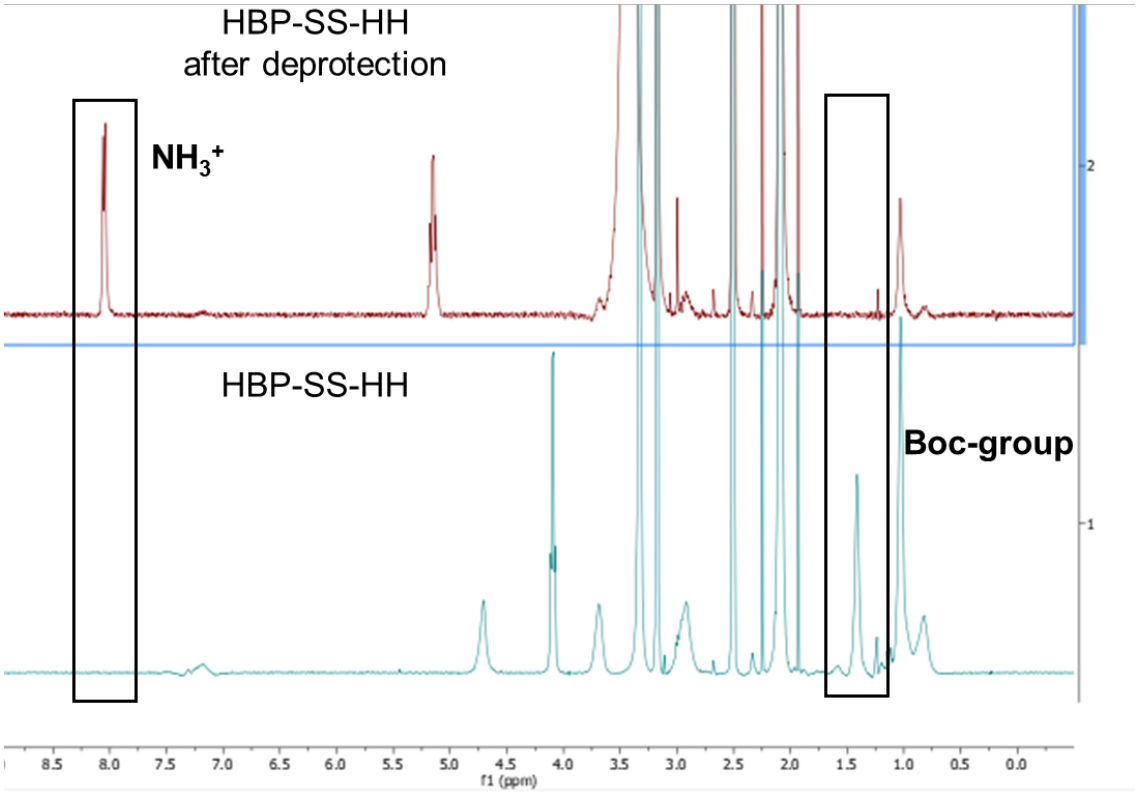

Figure S11. ^{1}H NMR spectra of a representative pHPMA before and after Boc-deprotection.

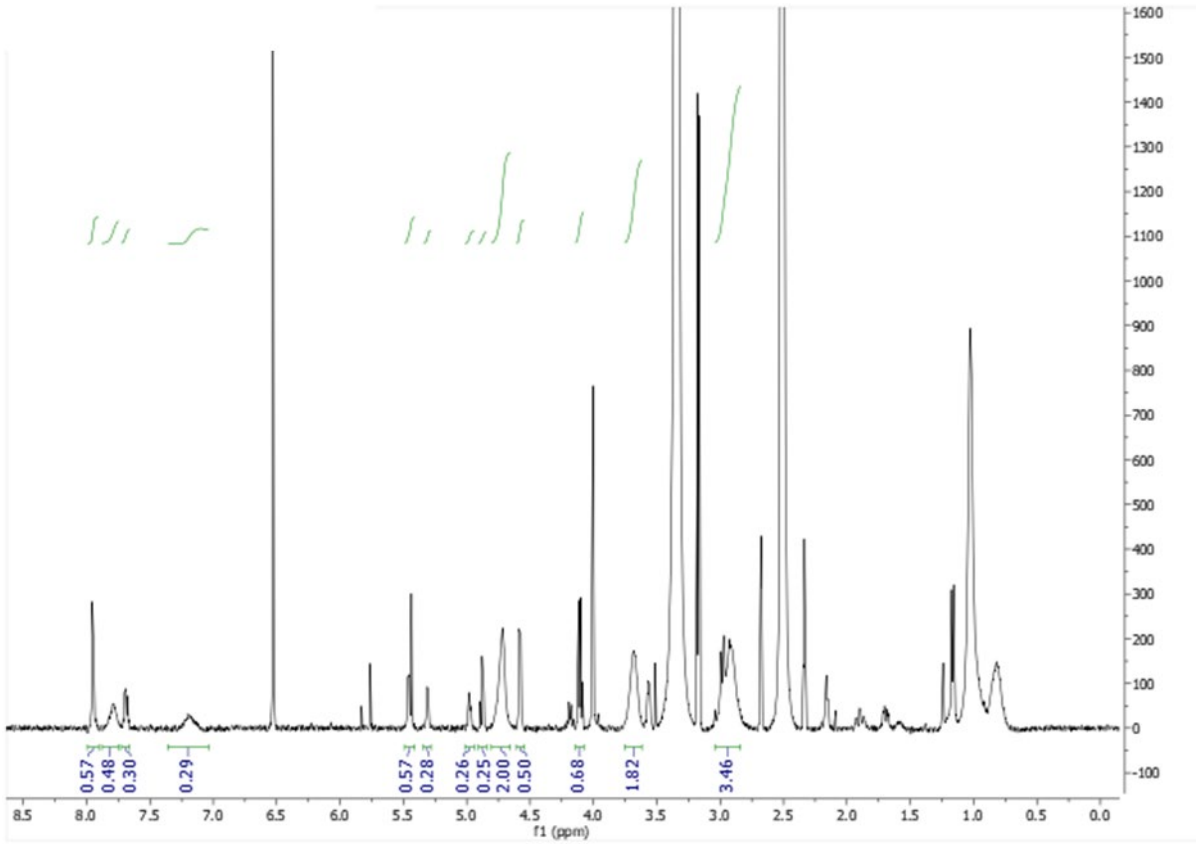

Figure S12. Assigned ^{1}H NMR spectra of HB-SS-HH-DOX following purification by precipitation and dialysis.

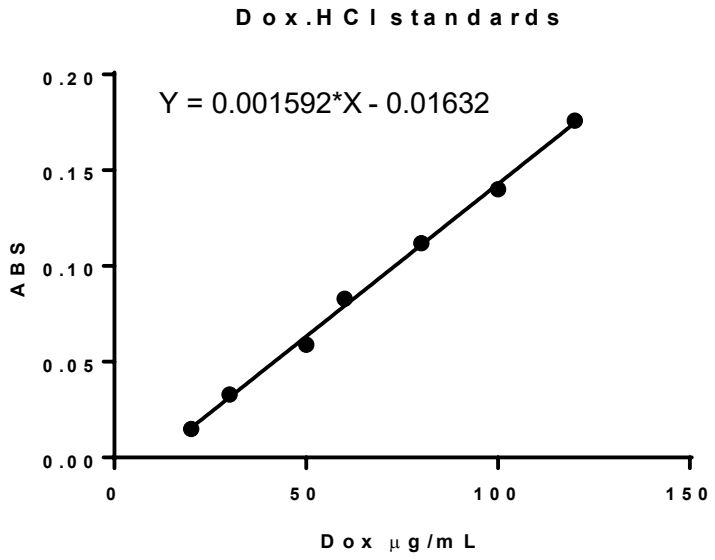

Figure S13. UV-Vis calibration curve of DOX in PBS.

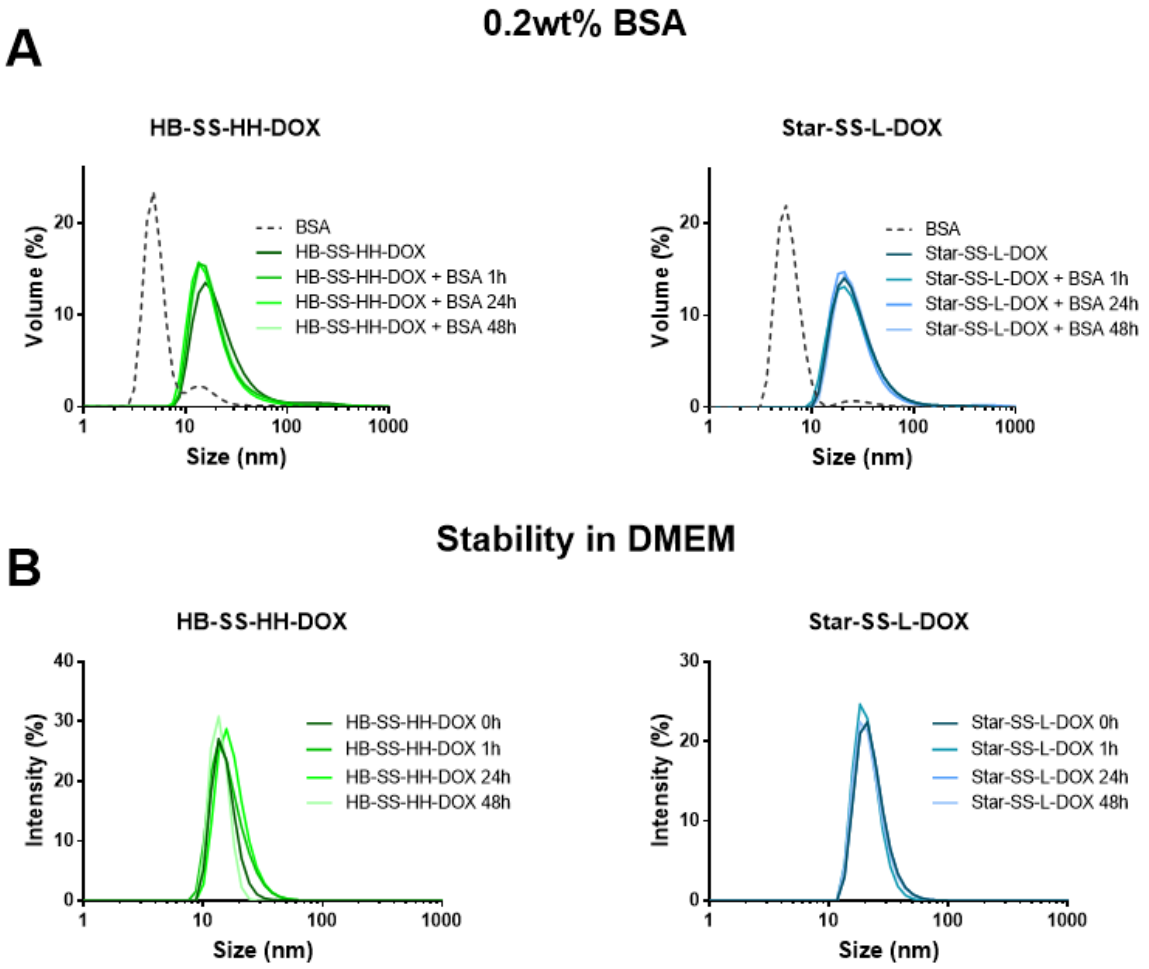

Figure S14. Stability of polymer prodrugs in 0.2wt% BSA and DMEM cell culture medium.

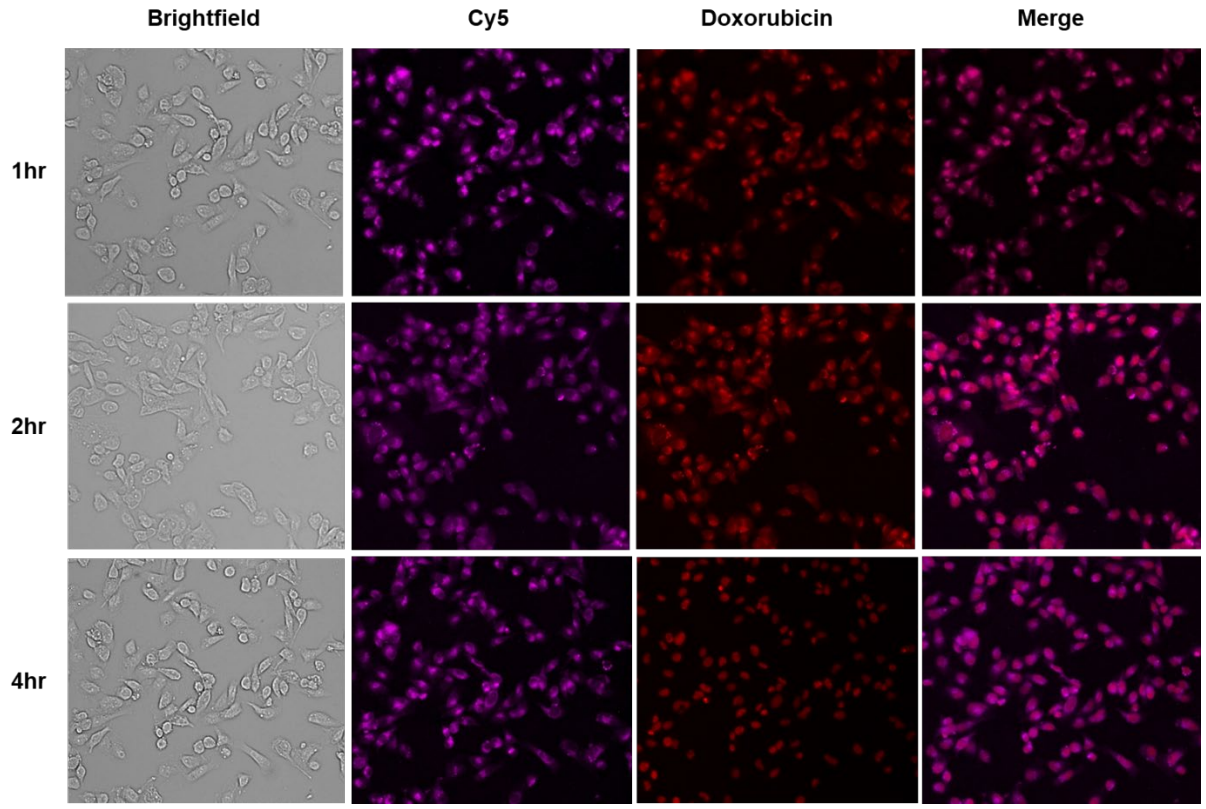

Figure S15. Fluorescence microscopy images of HB-SS-HH-DOX into MDA-MB-231 cells after 1h, 2h and 4h of incubation at 10x optical zoom.

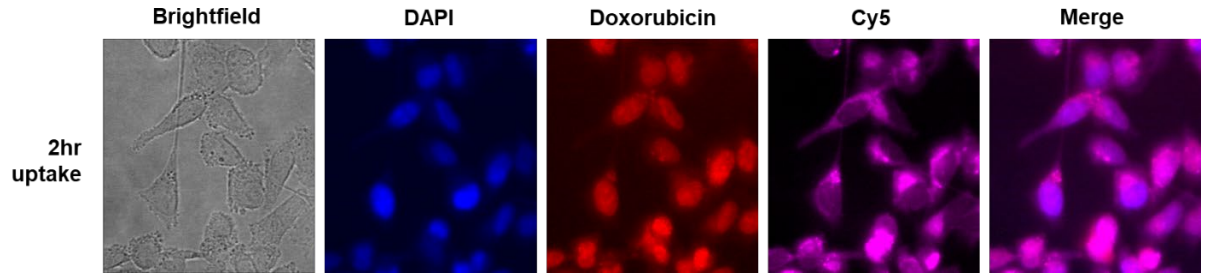

Figure S16. Fluorescence microscopy images of HB-SS-HH-DOX into MDA-MB-231 cells after 2h of incubation at 20x optical zoom.

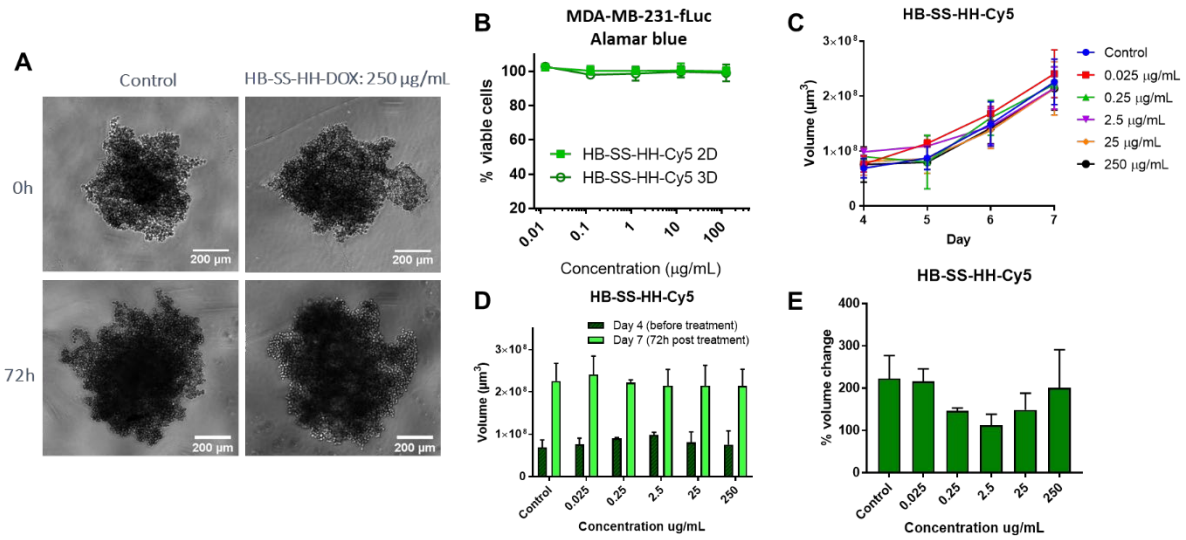

Figure S17. Spheroid cytotoxicity assay of the blank Cy5 labelled polymers to confirm no impact on spheroid growth from the blank nanoparticles. A) spheroid growth over 72h treatment, B) cytotoxicity of Cy5 polymers in 2D and 3D culture, C) growth of spheroids expressed as volume each day, D) volume comparison between day 4 and day 7 and E) % change in volume over the treatment period.

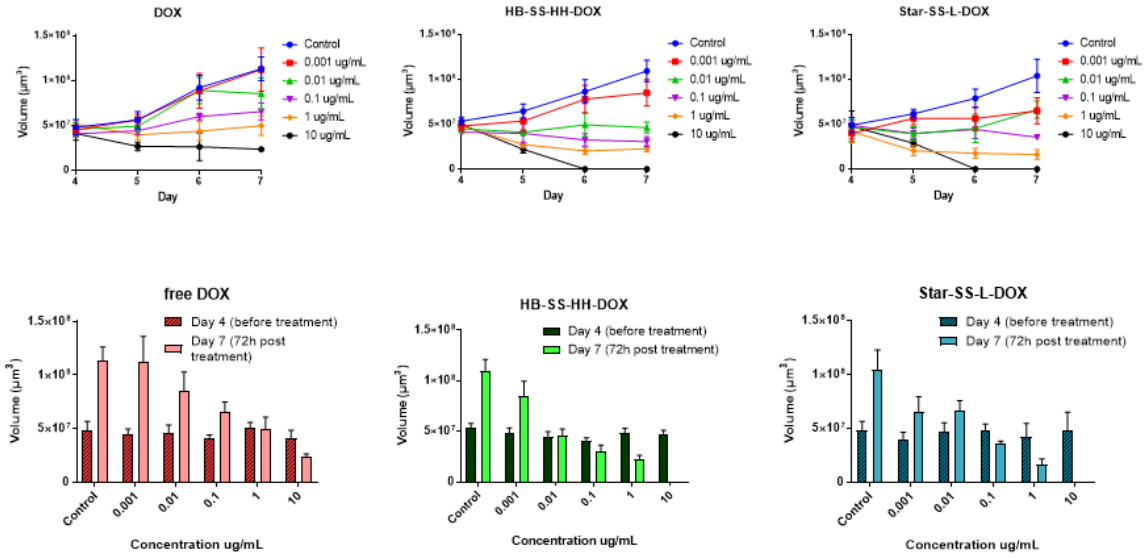

Figure S18. Growth of MDA-MB-231 spheroids over the 3-day treatment period expressed as top) volume on each day and bottom) volume comparison between day 4 and 7.

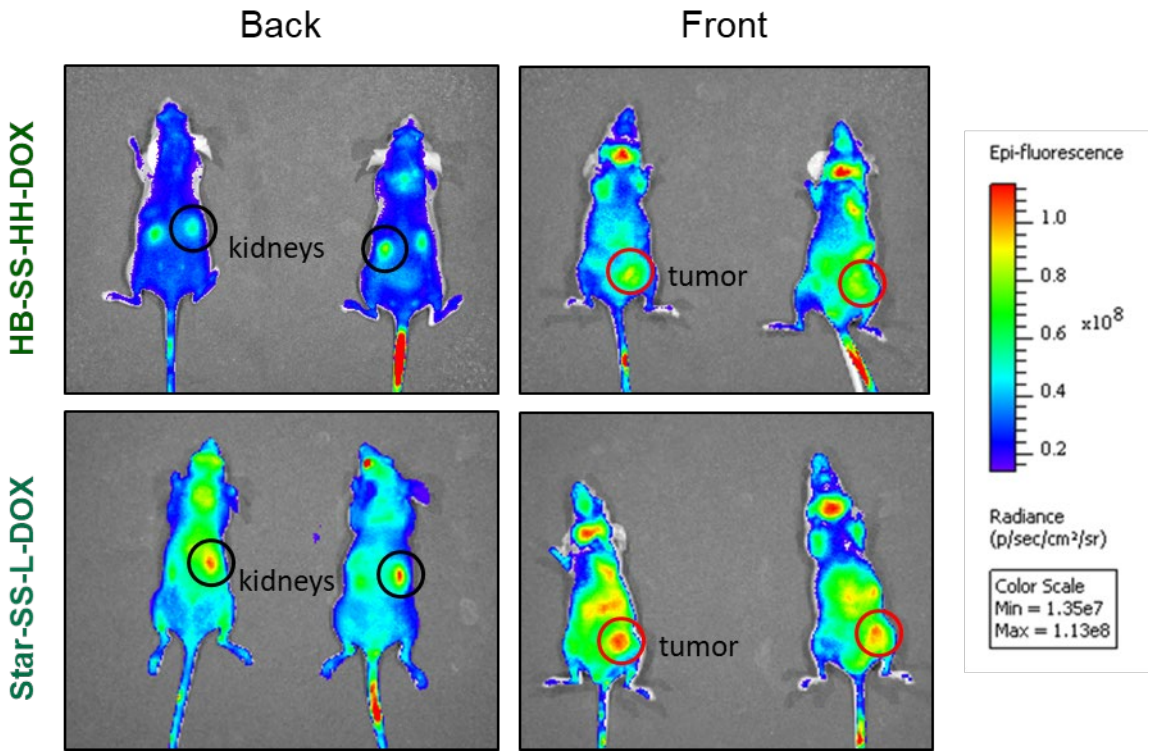

Figure S19. Fluorescence images of Cy5-labelled nanoparticles captured 24h after injection into MDA-MB-231 tumor-bearing mice. It can be seen for both nanoparticles that there is Cy5 signal from within the region of the tumor, as well as within the kidneys as observed in the non-drug-loaded nanoparticle biodistribution experiments.

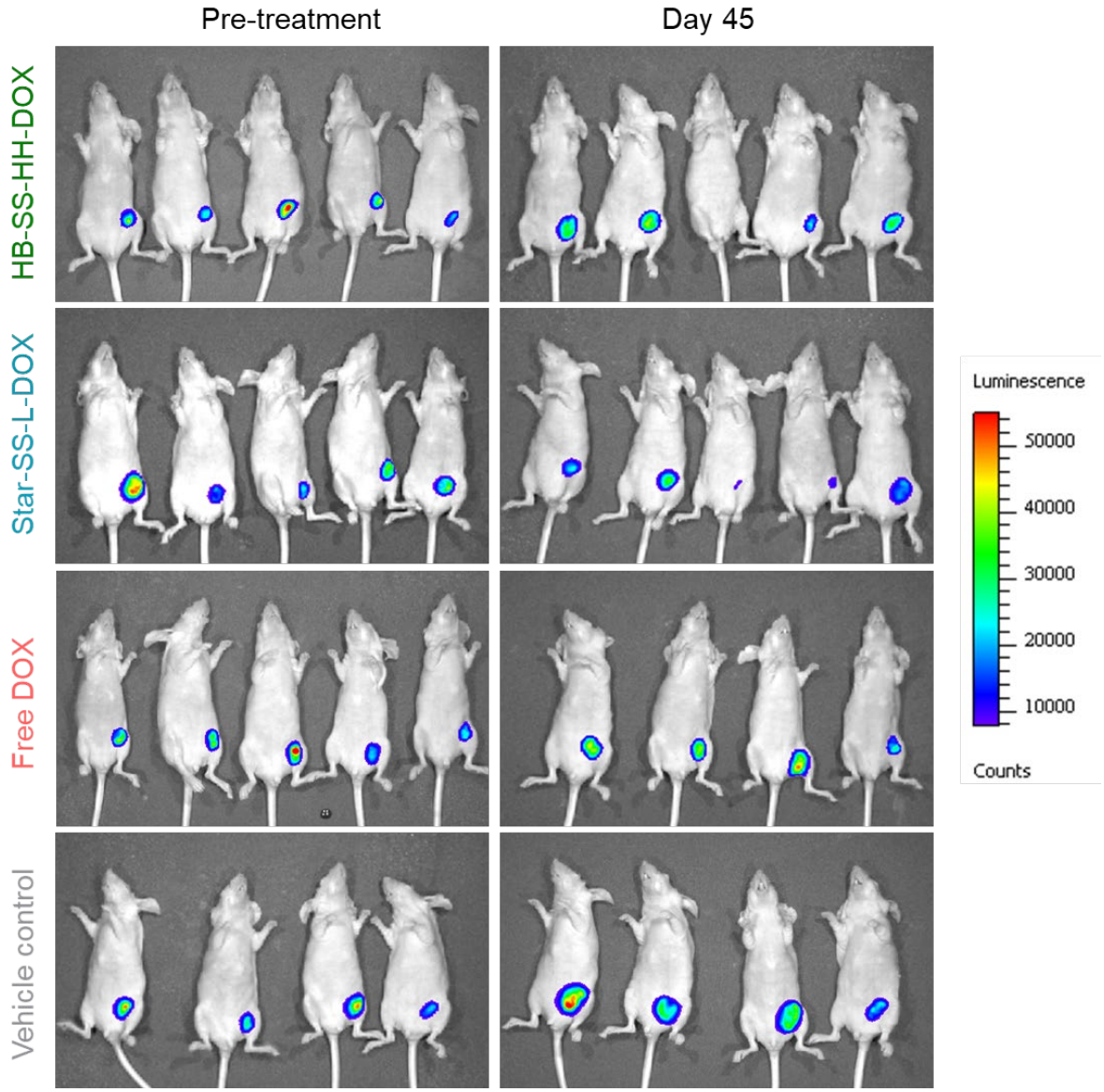

Figure S20. Luminescence images of MDA-MB-231 tumor-bearing mice at day 17 (pre-treatment) and prior to termination (Day 45) for the control and treatment groups.

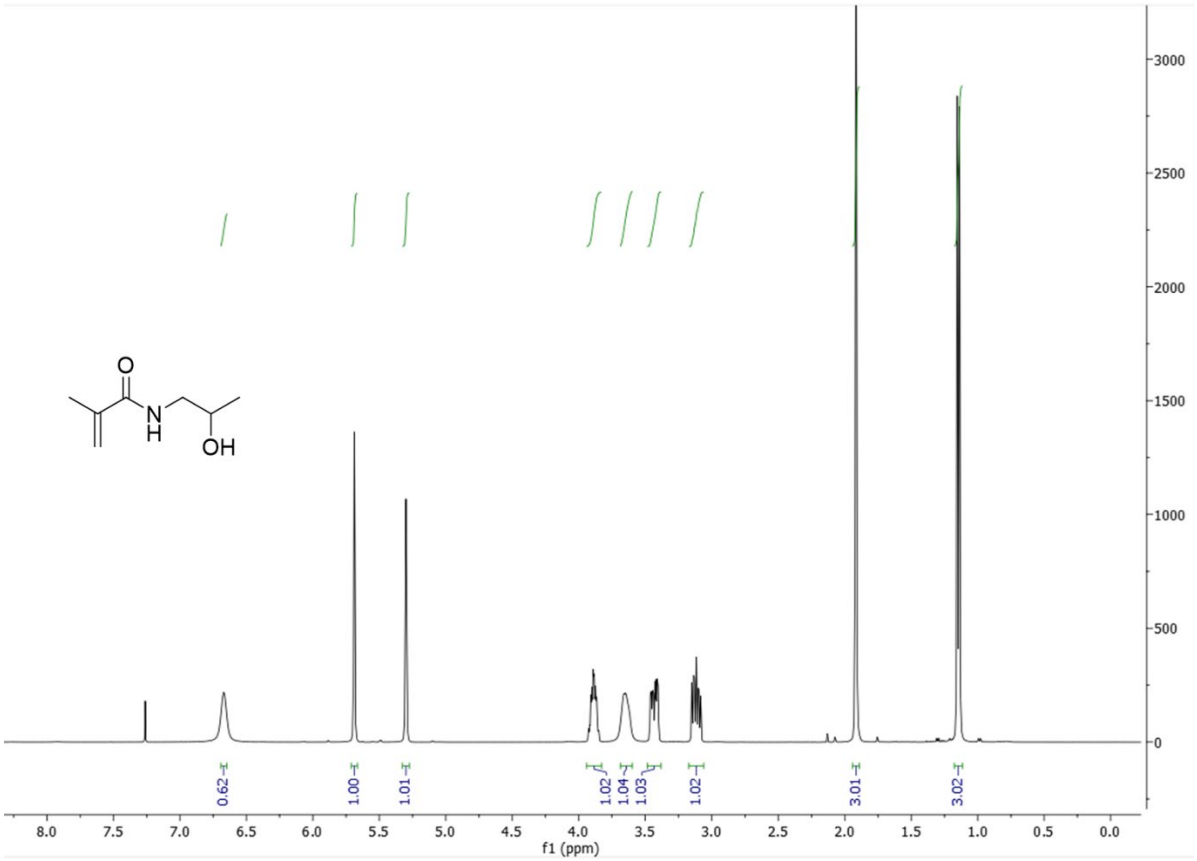

Figure S21. ^{1}H NMR spectra of HPMA

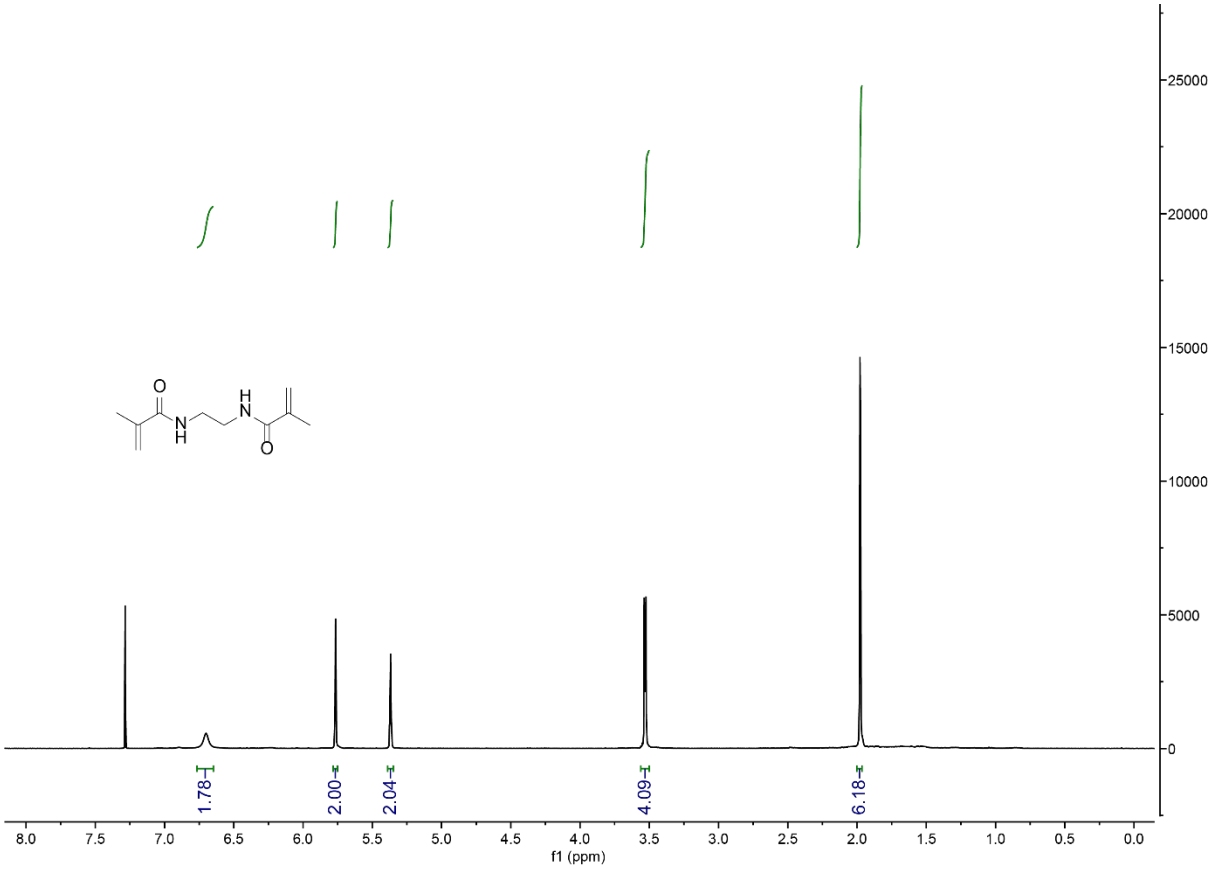

Figure S22. ^{1}H NMR spectra of EDMA

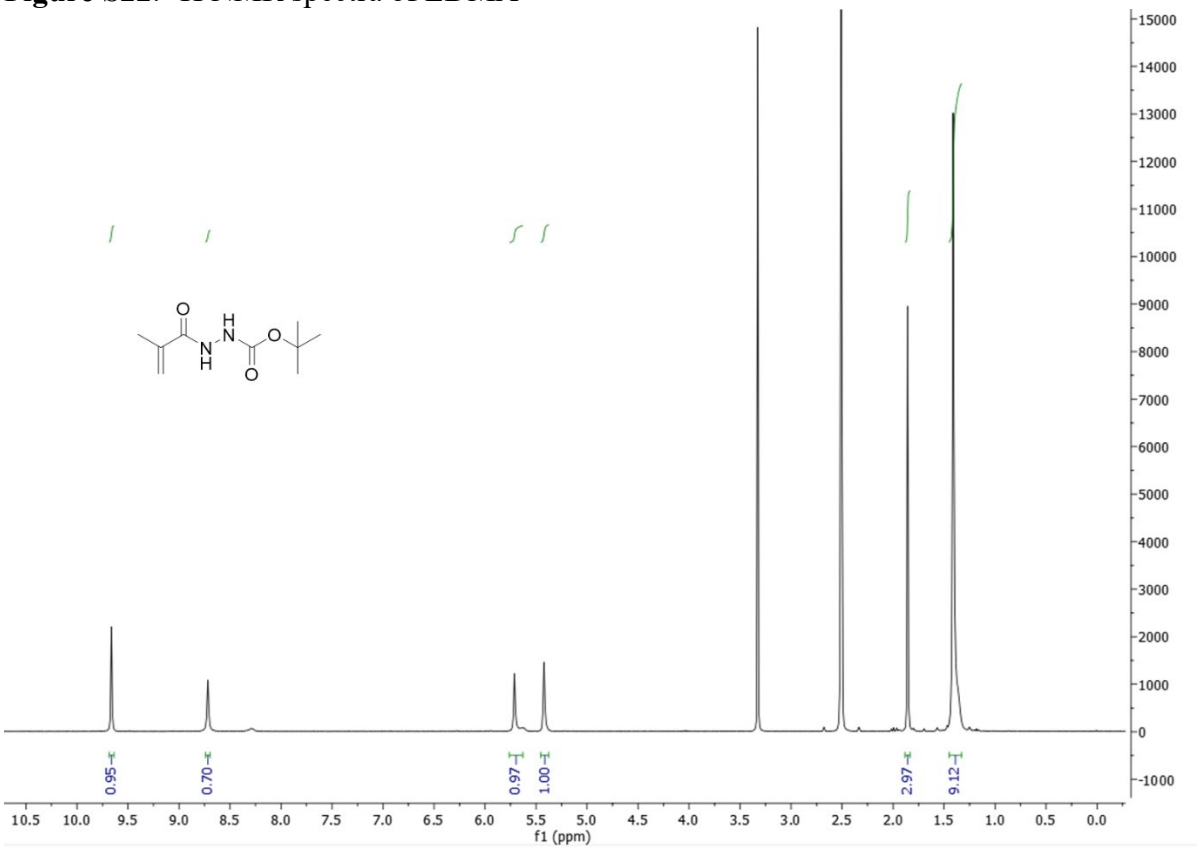

Figure S23. ^{1}H NMR spectra of tBHM

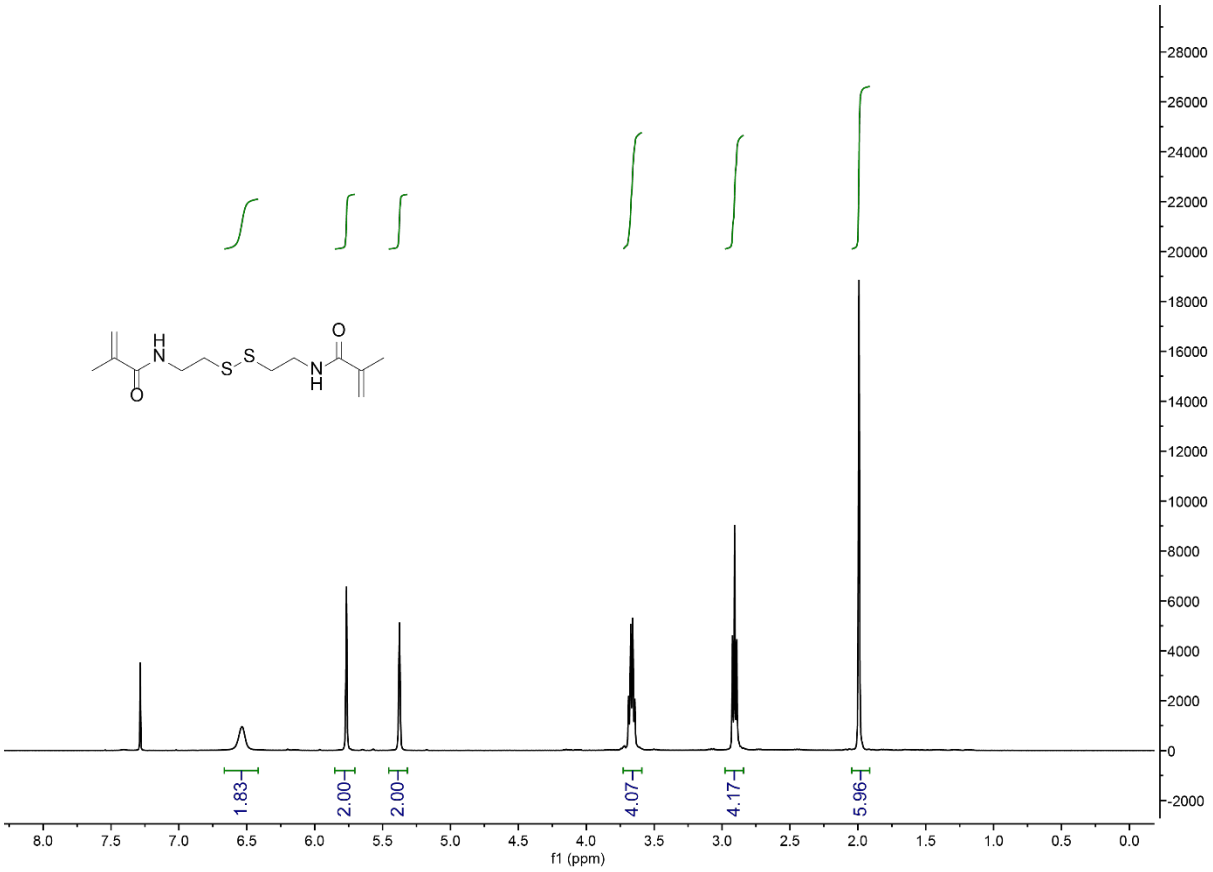

Figure S24. ^{1}H NMR spectra of CBMA

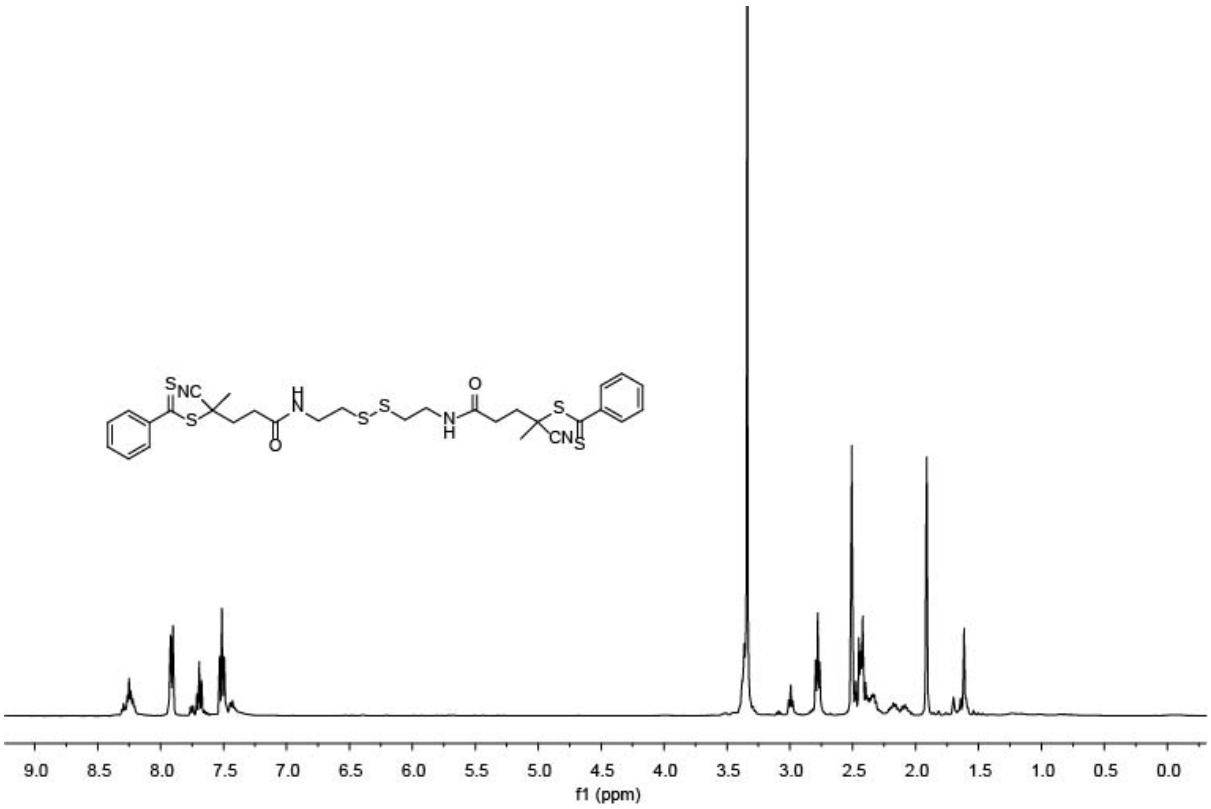

Figure S25. ^{1}H NMR of CADB-SS-CADB

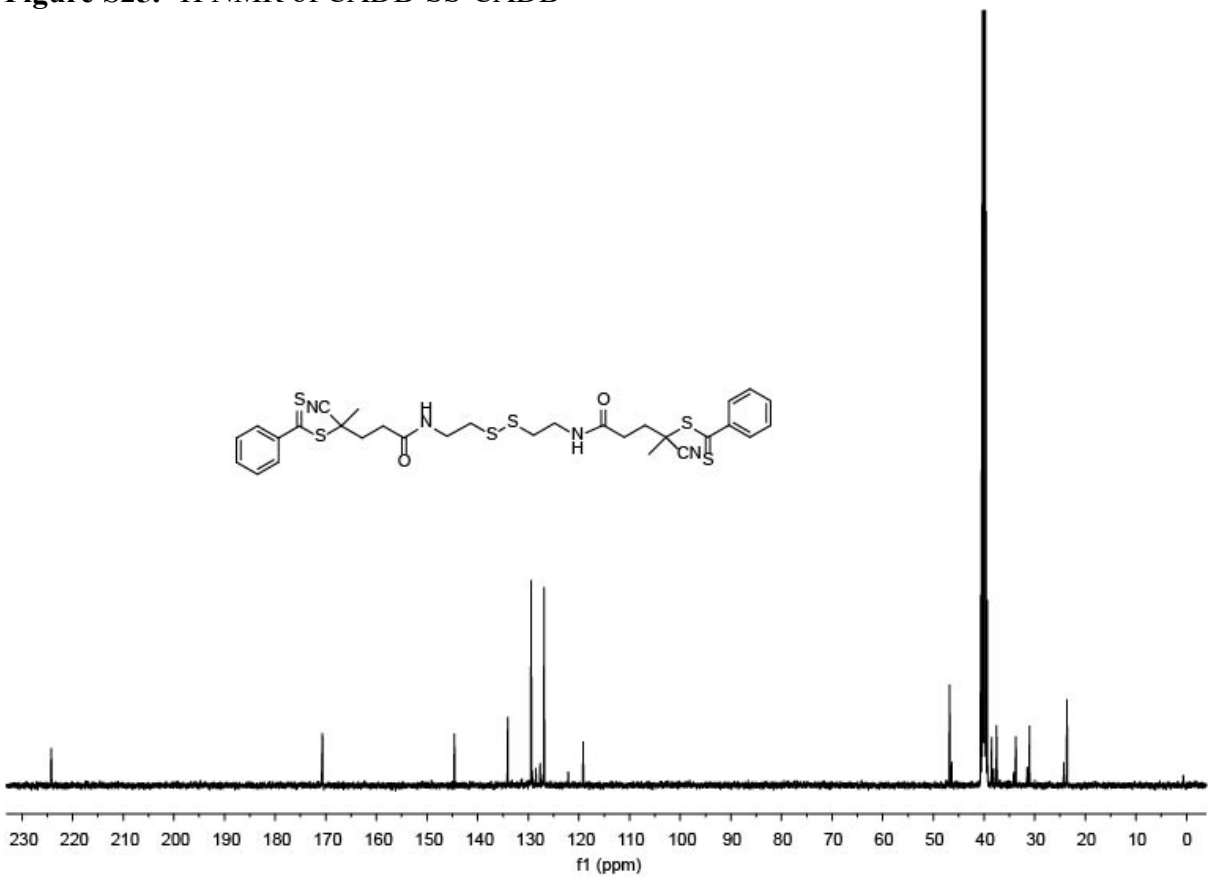

Figure S26. ^{13}C NMR spectra of CADB-SS-CADB