Chain-Extension in Hyperbranched Polymers Alters Tissue Distribution and Cytotoxicity Profiles in Orthotopic Models of Triple Negative Breast Cancers

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Materials and Methods

Materials

1-Amino-2-propanol (>99%), methacryloyl chloride (97%), 4,4'-azobis(4-cyanovaleric acid) (V-501, >97%), 1-Bromobenzene (99%), cysteamine dihydrochloride (>98%), pentafluorophenol (>99%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (98%), magnesium turnings (98%), 4-(dimethylamino)pyridine (98%), carbon disulphide (99%), iodine (99.99%), methacrylic anhydride (94%), pyridine (99.8%), triethylamine (TEA, >98%), doxorubicin-hydrochloride (Dox.HCl, >98%) and tert-butyl carbazate (98%) were purchased from Sigma Aldrich. Cyanine5 (Cy5) amine fluorescent dye was purchased from Lumiprobe. 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 an argon flow before use. All other chemicals were used as received unless otherwise stated.

Synthesis of Polymers

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

HPMA was synthesized as previously reported.[1] 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, $CDCl_3$) 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)

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 °C 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 colour change from clear to deep orange was observed, and the reaction mixture was allowed to reach room temperature. The crude magnesium bromo 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) were 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.

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 °C 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, $CDCl_3$) 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.

Redox-responsive hyperbranched polymer architectures – HB-SS-HH

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 characterised by ^{1}H NMR and SEC (**Figure S1**).

Redox-responsive star architecture – CE-HB-SS

Star architecture polymers were achieved through chain extension of a HB core polymer with HPMA to form a branched molecule. Briefly, HB-SS-HH (100 mg, 0.0167 mmol) was dissolved in distilled water (900 µL) along with HPMA (239 mg, 1.67 mmol, CE-HB-SS) 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 characterised by ^{1}H NMR and SEC (**Figure S2**).

Labelling of pHPMA with Cy5

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. Polymer (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

150 mg of HB-SS-HH or CE-HB-SS was dissolved in dry MeOH (2 mL). TFA (0.5 mL) was slowly added to the polymer mixture and allowed to stir for 1 hr. Polymers were purified by precipitation into cold acetone,

washed once and analyzed by ^{1}H NMR. The resultant polymers, HB-SS-HH-$NHNH_2$ or CE-HB-SS-$NHNH_2$ were stored at 4 °C until use.

Doxorubicin conjugation

HB-SS-HH-$NHNH_2$ or CE-HB-SS-$NHNH_2$ (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. Attachment of doxorubicin was confirmed by ^{1}H NMR and quantified by UV–Vis spectroscopy (ex = 480 nm).

Characterisation

^{1}H 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 analysed 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 25 °C (scattering angle 173°, laser of 633 nm) or a Viscotek 802 DLS with a laser wavelength of 830 nm at 20 °C. Polymer solutions were prepared as 1 mg/mL in PBS. Data was analysed 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.

***In Vivo* Evaluation of Polymers**

In vivo biodistribution of Cy5-labelled polymers

Experiments were performed to assess biodistribution, organ accumulation and clearance of Cy5-labelled polymers. Female 8–9 week-old immunodeficient CD-1 NuNu (Crl:CD1-*Foxn1*^{nu}) 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 °C 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 irradiated water (SLS, UK) was offered *ad libitum*. The condition of the animals was monitored throughout the study by an experienced animal technician. After a week's acclimatisation, the mice were randomly allocated by weight to the study groups of 6 mice per polymer type. 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.

In vivo efficacy and biodistribution of pHPMA-Dox conjugates

As above, 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. For the efficacy experiments, 6-7 week-old female immunodeficient CD-1 NuNu mice were purchased from Charles River UK. Mice were maintained as described above prior to beginning the study. After a week's acclimatization, the mice were initiated with tumours as below.

The cells were maintained *in vitro* in RPMI culture medium (Sigma, UK) containing 10 % (v/v) heat inactivated fetal bovine serum (Sigma, Poole, UK) and 2 mM L-glutamine (Sigma, UK) at 37 °C 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 2 x 10^6 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. Cells with viability of > 90 % were re-suspended for *in vivo* administration in growth factor reduced matrigel at 2 x 10^6 cells/ml for injection of 100 µL into the left mammary fat pad just inferior to the nipple. Tumour establishment and growth was monitored during the experiment by 2D optical imaging, carried out under anaesthesia in an IVIS Spectrum weekly, and were also measured by Vernier callipers (Camlab) twice weekly and animals weighed weekly. The tumour volumes were calculated using a standard volume formula as previously reported (tumour volume = ½ (length × width^2). Dosing commenced on day 26 when the tumours had reached a suitable size as established by calliper measurement and bioluminescent imaging, average diameters around 6 mm.

Supplementary Figures:

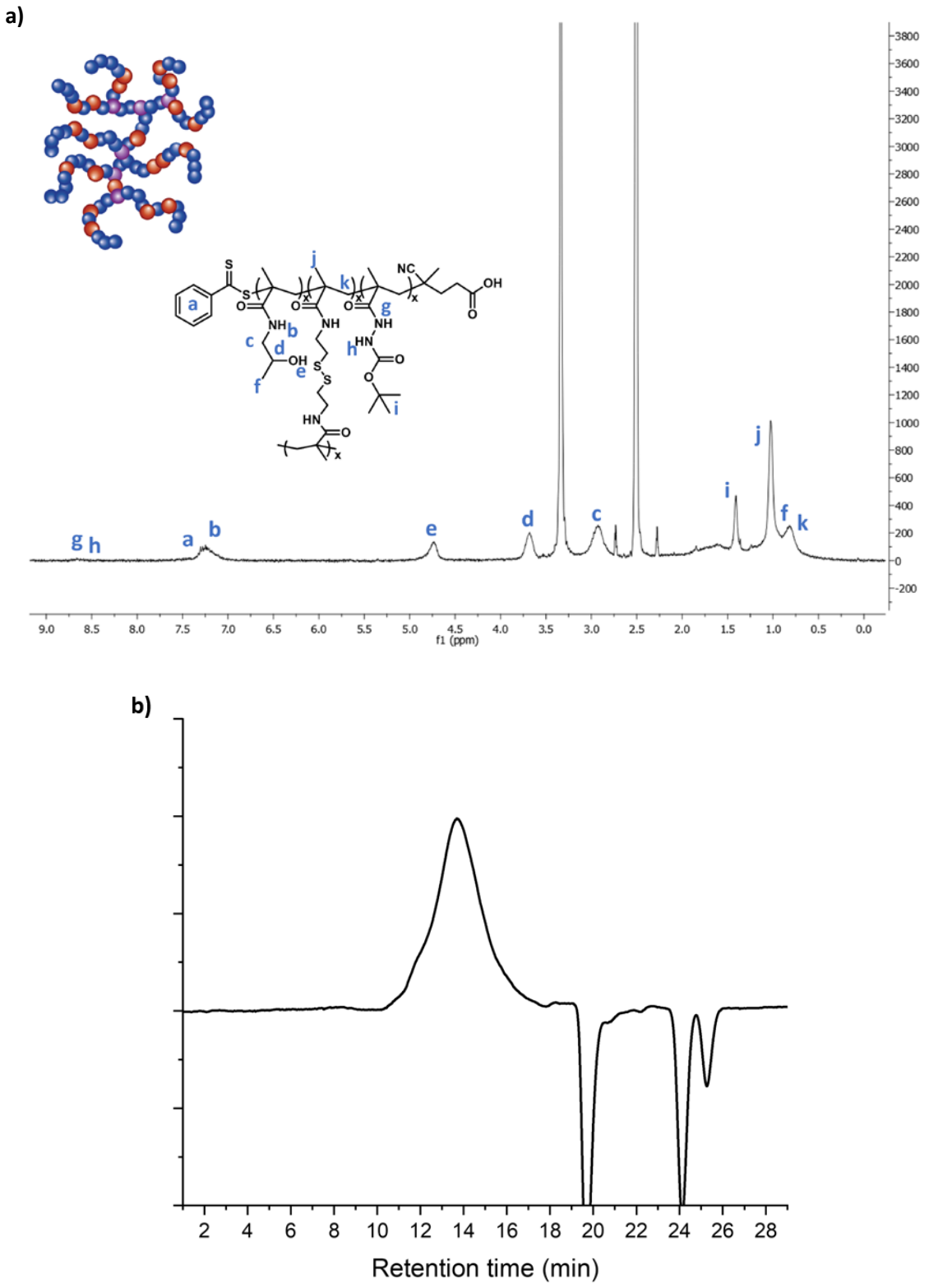

Figure S1. (a) Assigned ^{1}H NMR spectra and **(b)** GPC spectra of HB-SS-HH following purification.

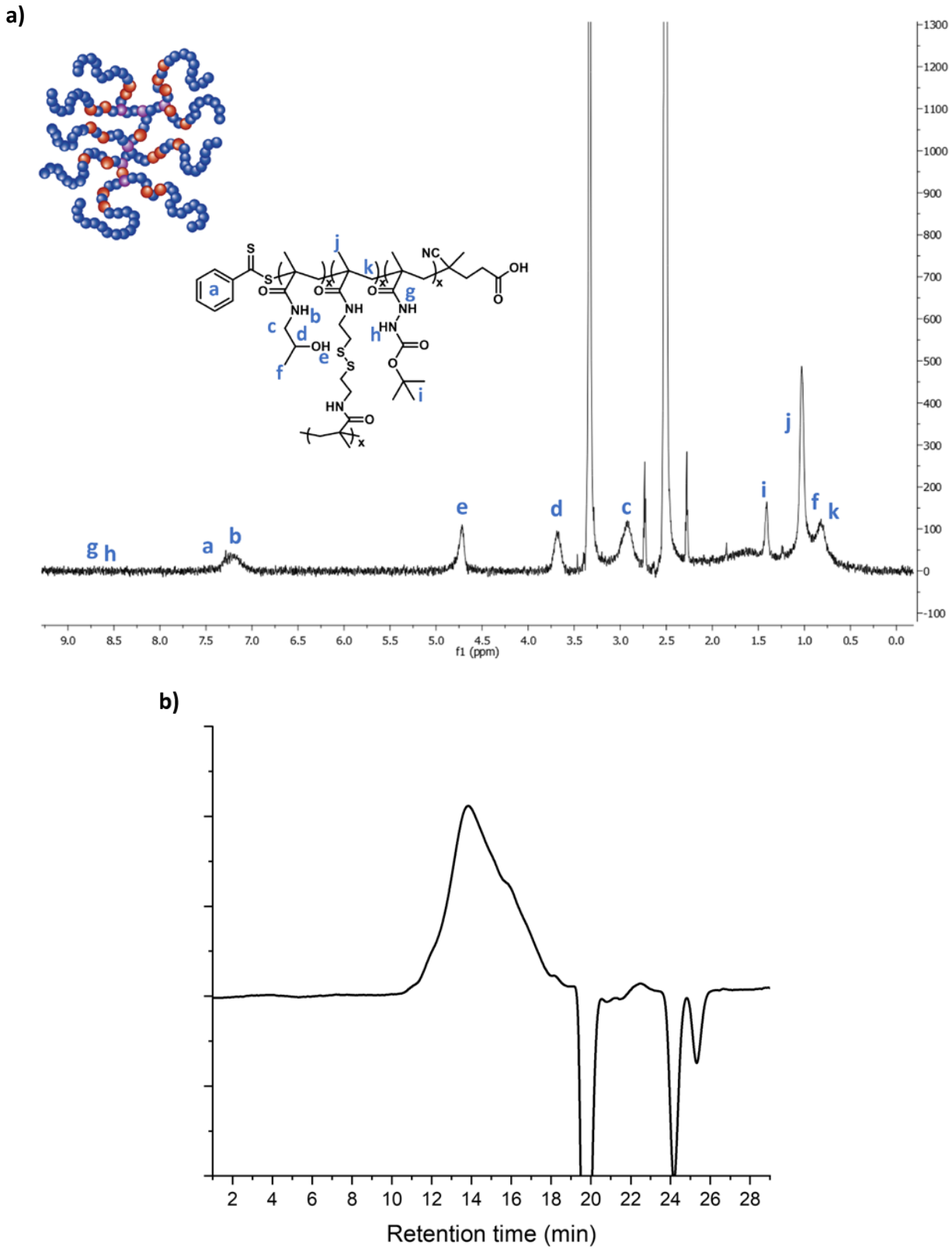

Figure S2. (a) Assigned ^{1}H NMR spectra and **(b)** GPC spectra of CE-HB-SS following purification.

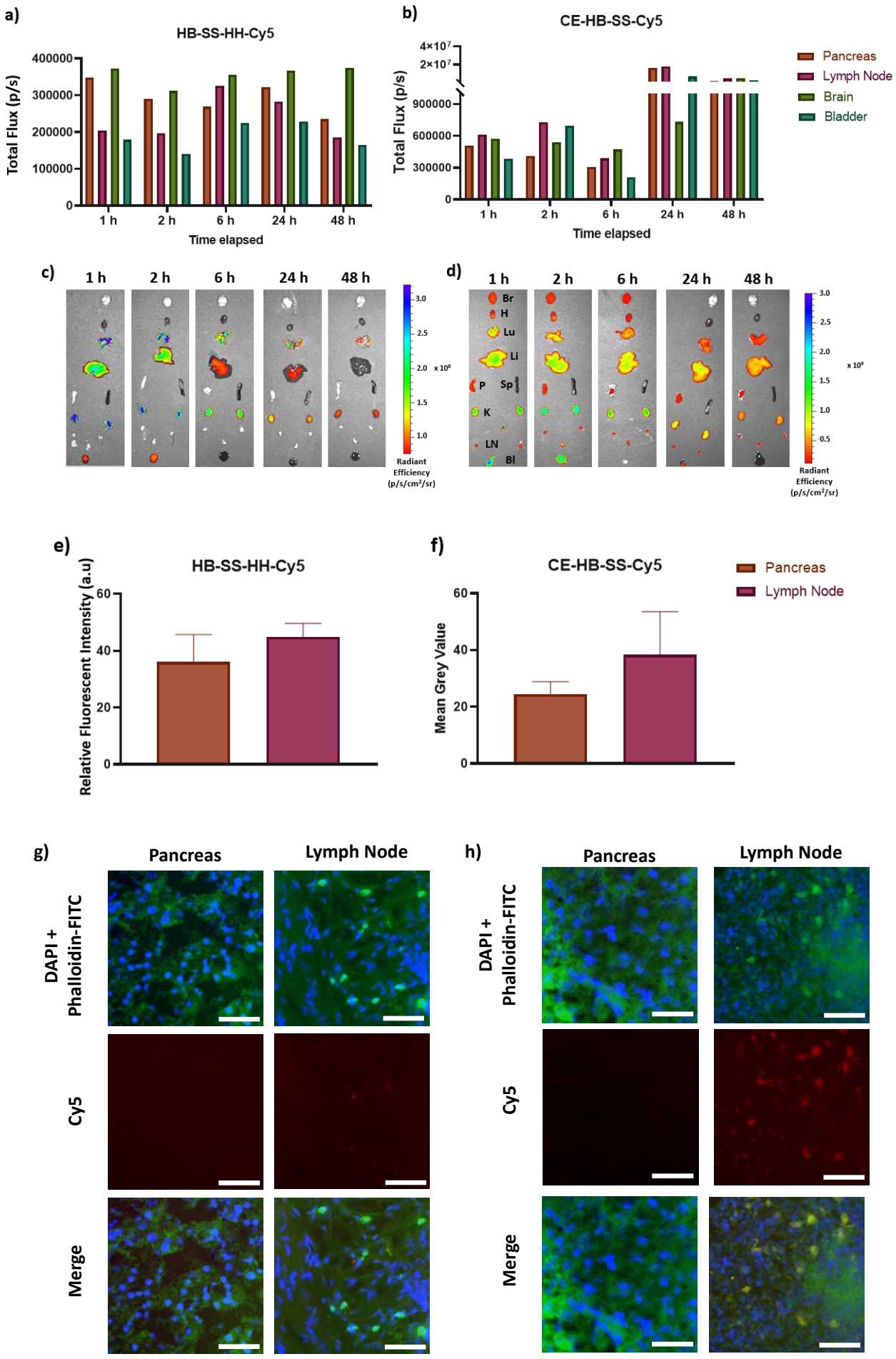
a)
HB-SS-HH-Cy5
Total Flux (p/s)
400000
300000
200000
100000
0
1 h
2 h
6 h
24 h
48 h
Time elapsed
b)
CE-HB-SS-Cy5
Total Flux (p/s)
4×10⁷
2×10⁷
900000
600000
300000
0
Pancreas
Lymph Node
Brain
Bladder
c)
d)
Br
H
Lu
Li
P
Sp
K
LN
Bl
Radiant Efficiency (p/s/cm²/sr)
e)
HB-SS-HH-Cy5
Relative Fluorescent Intensity (a.u)
f)
CE-HB-SS-Cy5
Mean Grey Value
g)
h)
Pancreas
Lymph Node
DAPI + Phalloidin-FITC
Cy5
Merge

Figure S3. (a) and **(b)** Recorded fluorescent signal measured for selected organs (pancreas, lymph node, brain, and bladder) using IVIS imaging up to 48 hours post injection of 100 µL of 2 mg/mL HB-SS-HH-Cy5 and CE-HB-SS-Cy5, respectively, in healthy mice. **(c)** and **(d)** Corresponding representative IVIS images of all excised organs at 1, 2, 6, 24 and 48 hours post-injection of HB-SS-HH-Cy5 and CE-HB-SS-Cy5, respectively (Br: brain, H: heart, Lu: lung, Li: liver, P: pancreas, Sp: spleen, K: kidney, LN: lymph node and Bl: bladder). **(e)** and **(f)** Quantified Cy5 signal in excised pancreas and lymph node 1 hour post-injection for HB-SS-HH-Cy5 and CE-HB-SS-Cy5, respectively. Following excision, organs were sectioned to 10 µm thickness and analysis was carried out in several distinct regions of the sections. **(g)** and **(h)** Representative fluorescence microscopy images of corresponding organ sections 1 hour post-injection indicating polymer deposition of HB-SS-HH-Cy5 and CE-HB-SS-Cy5, respectively (scale bar 50 µm; Blue = DAPI, Green = Phalloidin-FITC, Red = Cy5).

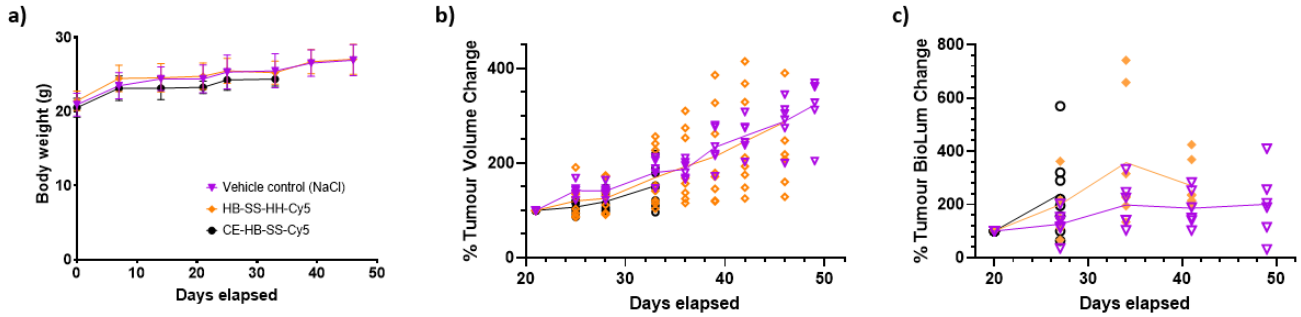

Figure S4. Recorded **(a)** body weight of subjects, **(b)** tumour volume change as a percentage of pre-treatment, and **(c)** recorded bioluminescence change for tumour as a percentage of pre-treatment for control, HB-SS-HH-Cy5 and CE-HB-SS-Cy5 treatment groups over the course of the study.

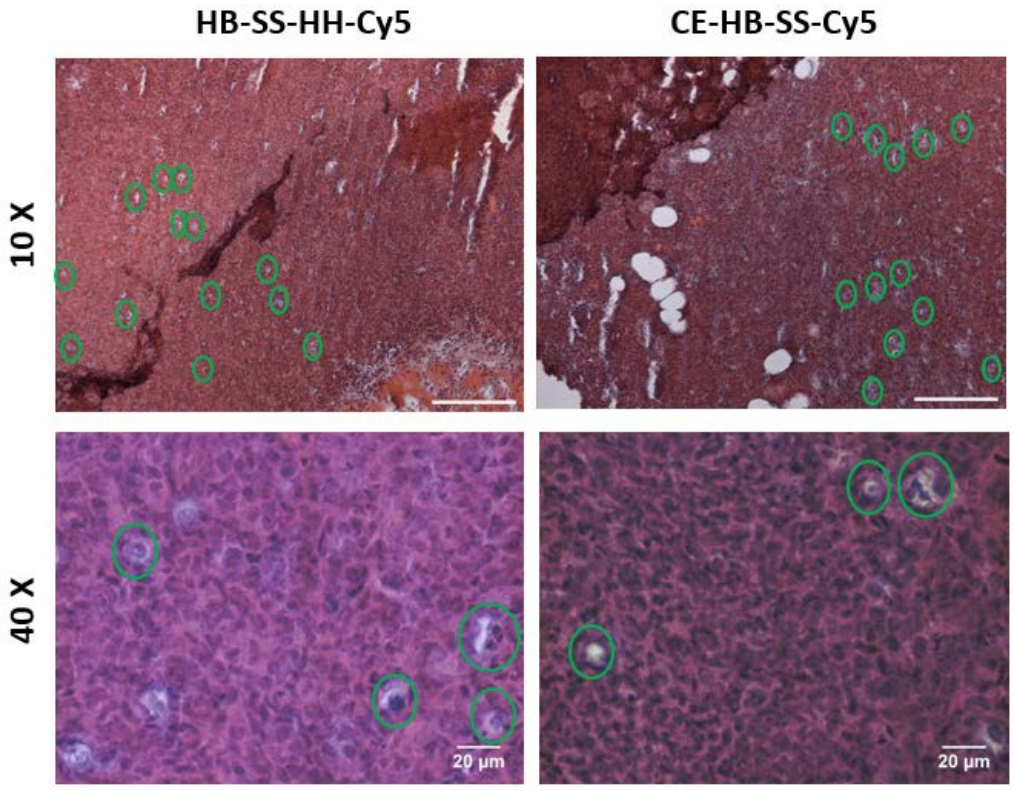

Figure S5. Representative H&E images of tumours removed at the end of the study for HB-SS-HH-Cy5 and CE-HB-SS-Cy5 treated groups taken at 10 X magnification (top row, scale bar = 200 µm) and 40 X magnification (bottom row, scale bar = 20 µm). Scattered apoptotic cells have been highlighted with green circles (○).

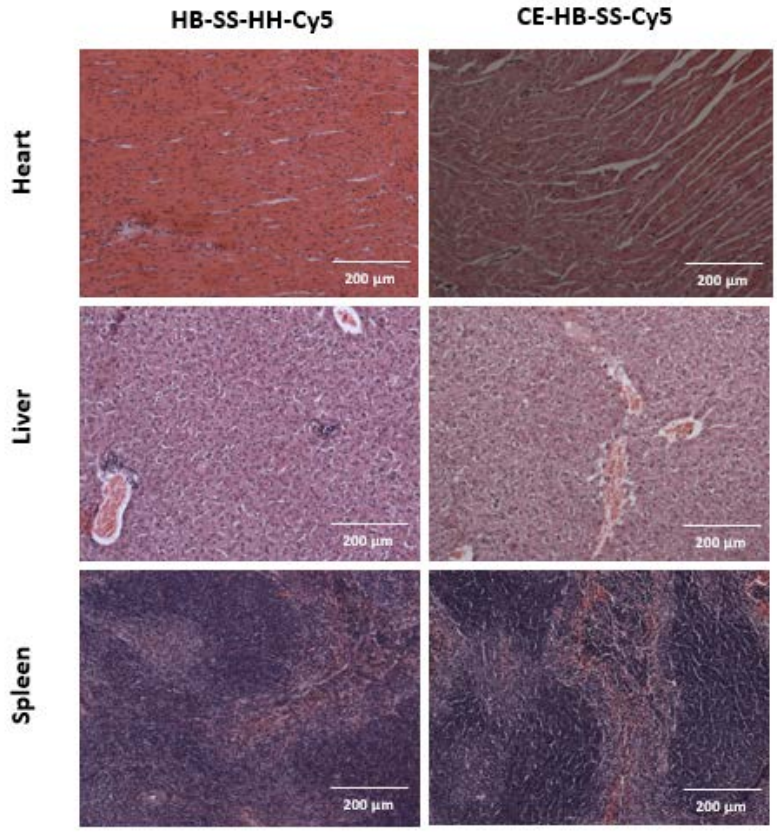

Figure S6. Representative H&E images of heart, liver and spleen removed at the end of the study for HB-SS-HH-Cy5 and CE-HB-SS-Cy5 groups taken at 10X magnification, showing retention of normal architectures following treatment with Cy5-polymers.

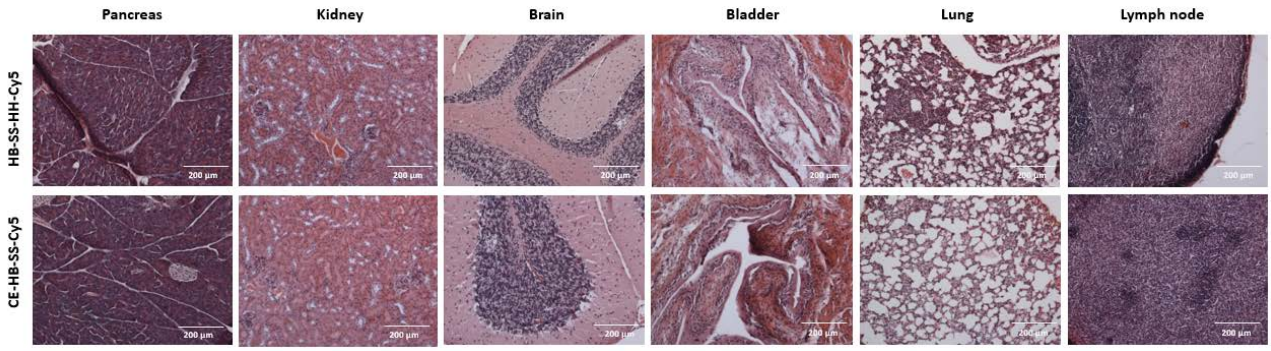

Figure S7. Representative H&E images of pancreas, kidney, brain, bladder, lung and lymph node removed at the end of the study for HB-SS-HH-Cy5 and CE-HB-SS-Cy5 groups taken at 10X magnification, showing retention of normal architectures following treatment with Cy5-polymers.

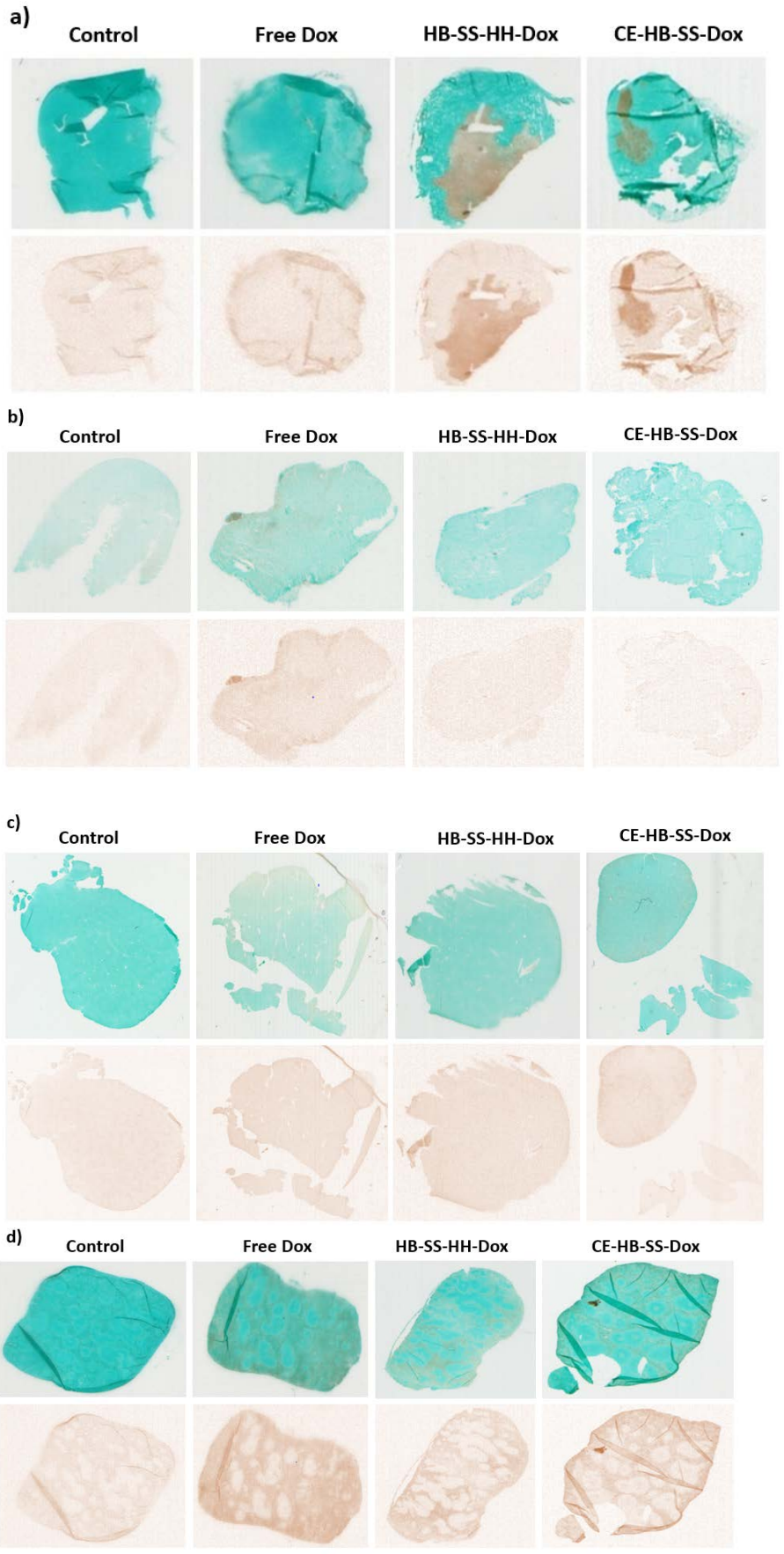

Figure S8. Representative images following TUNEL assay staining for whole sections of **(a)** tumour, **(b)** heart, **(c)** liver and **(d)** spleen, across control, free Dox, HB-SS-HH-Dox and CE-HB-SS-Dox treated groups, taken at 20X magnification. The top line of each image shows both healthy (green/blue) and apoptotic cells (brown), while the bottom line shows apoptotic cells only.

References

(1) Kopecek, J.; Bazilova, H. Poly[N-(2-hydroxypropyl)methyacrylamide] - I. Radical Polymerization and Copolymerization. *Eur. Polym. J.* **1973**, *9*, 7 - 14.