Electronic Supplementary Information

Star-Polymer Unimolecular Micelle Nanoparticle to Deliver Payload Across the Blood-Brain Barrier

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Experimental Methods

Materials: 1,4-Cyclohexanediol, potassium t-butoxide, tertiary butyl acrylate, pyridinium chlorochromate (PCC), molecular sieves (4Å), m-chloroperbenzoic acid (m-CPBA), dipentaerythritol, tin(II) 2-ethylhexanoate ($Sn(oct)_2$), caprolactone (CL), pyrene, horse-liver esterase, α-Chymotrypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DAPI, paraformaldehyde, hematoxylin, eosin, sodium azide, trizma base, EDTA, SDS and heparin were acquired from Sigma Aldrich. Doxorubicin hydrochloride was purchased from Alfa Aesar. All the salts were purchased locally (Rankem chemicals). Wild-type mouse embryonic fibroblast cells (WT-MEF) and breast cancer cells (MCF 7) were maintained in DMEM (phenol red free medium: Gibco) containing 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin–streptomycin at 37 °C under a 5 % CO_2 humidified atmosphere. Cells were trypsinised using 0.05 % trypsin (Gibco) and seeded in 96 well (Eppendorf) or 4-well live cell chamber (Labtek) (as per experiment) flat bottomed plastic plates for all assays. Lysotracker Red DND-99 (1 mM) was procured from Thermo Fisher Scientific. Acetonitrile (ACN), methanol, hexane, diethyl ether, trifluoroacetic acid (TFA) and DMSO solvents were all HPLC grade. The optimal cutting temperature (OCT) medium was purchased from Leica (14020108926). DPX mountant and p-Xylene were procured from Thermo Fisher Scientific. Thiopentone injections (Thiosol sodium) was bought from Neon laboratories (50mg/kg). The primary antibody, i.e., Chicken anti-MAP-2 Antibody (ab92434), NeuN (ABN91, Chemicon) and GFAP (Z0334, Dako) was procured from Abcam and the secondary antibody (Goat anti-chicken alexa fluor 647) was obtained from Jackson ImmunoResearch. Vectashield (Vector labs H-1000) was used as the mounting medium for the MAP 2 antibody staining protocol. The cytokine analysis kit used was purchased from BD Life sciences (BD Cytometric Bead array Mouse Th1/Th2/Th17 Cytokine kit). Poly-D-lysine used for coating the coverslips was obtained from Sigma (P0899-10MG). Hanks balanced Salt solution was obtained from HiMedia (TL1010-500ML). 0.25% Trypsin-EDTA was purchased from Invitrogen (25200056). Neurobasal medium (10888022), B27 supplement 50X (17504-044)) and L-Glutamine 200 Mm (25030-081) used for primary neuronal culture were purchased from Invitrogen. The female balb/c, C57BL/6 male mice and C57/B6J mice pups were obtained from the IISER Animal House facility, National Facility for Gene function in Health and Disease (NFGFHD).

Methods: Jeol 400 MHz spectrophotometer was used for recording NMR samples in $CDCl_3$, CD_3OD and $(CD_3)_2SO$ solvent using TMS as the standard. Gel Permeation Chromatography (GPC, also referred to as Size Exclusion Chromatography) data acquisition

was carried out by a Viscotek VE 1122 pump, Viscotek VE 3580 RI, 3210 UV-Vis and Light scattering detectors. Samples for GPC were prepared in HPLC tetrahydrofuran (THF) and executed from method files obtained after calibration using polystyrene standards. The Perkin-Elmer thermal analyzer STA 6000 model was used to determine the polymer's thermal stability at 10 °C/min heating rate under nitrogen atmosphere. Thermal properties of polymers were analyzed using a TA Q20 differential scanning calorimeter (DSC), wherein the polymers were first heated to melt in order to remove any pre-thermal history and then subsequent heating-cooling cycles were recorded at 10 °C/min under inert conditions to generate the respective thermograms. Perkin-Elmer Lambda 45 UV-Visible Spectrophotometer was used for the absorption studies. Emission spectra for determining the Critical Micelle Concentration (CMC) were recorded using a SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer. A 450 W Xe lamp serves as the source of excitation at room temperature. Pyrene was used as the fluorophore, which has excitation maxima at 337 nm. The emission spectrum gives five distinct vibronic levels, amongst which the intensity of levels I_1 and I_3 are known to be extremely sensitive of the hydrophobic environment. As a result, pyrene when encapsulated within a micelle will give different I_1 and I_3 values. The ratio of I_1/I_3 v/s logC is plotted in order to determine the critical aggregate concentration (CAC), which stays constant in a unimolecular micelle. Dynamic light scattering (DLS) was performed using Nano ZS-90 setup (Malvern instruments). DLS utilizes a 633 nm laser as the light source, and the detector collects the scattered light at 90° angle. This gives information about the correlation function $[g^2(t)]$, from which the diffusion coefficient (D) is calculated using cumulant method and further the diameter of the particle is determined using the Stokes-Einstein equation. The experiment was performed thrice with independent amphiphilic solutions to yield reproducible data. SAXS was caried out on the Nanoviewer instrument (made in Rigaku, Japan) that is calibrated with silver behenate. It comprises of a rotating anode X-ray source Micromax 007 of 40 kV and 30 mA, HyPix 3000 photon-counting detector and an X-ray collimator system (3-pin hole with an evacuated beam path). FE-SEM analysis requires the sample to be drop casted on silicon wafer and the experiment was done on a Zeiss Ultra Plus scanning electron microscope. Polymer solutions were drop casted on mica plates for carrying out atomic force microscopic (AFM) analysis using Agilent instruments. HR-TEM samples were prepared by drop-casting the dialyzed polymer solutions on copper TEM grids and the images were recorded using Jeol JEM2200FS 200 KeV systems. Again, the reproducibility of the data was confirmed using three independent polymer solutions. The cell viability determination was done by measuring the absorbance of the formazan crystals on a microplate reader (Varioskan Flash). Confocal

microscopic analysis for the cellular uptake study was done using Leica SP8 microscope. The tissue samples were embedded in the tissue freezing medium (Leica) and sectioned using a cryotome. Confocal microscopic analysis of the DAPI stained tissue sections was done using Leica SP8 microscope. H&E-stained tissue sections were imaged using the bright field microscope (Carl Zeiss) for histological analyses. Cytokine Analysis was carried out on the BD Celesta Flow Cytometer at the BD Biosciences FACS facility at IISER, Pune. Cell Lysis buffer (200 mL), used for the dissolution of the tissue samples, was prepared from 2 mL of 1 M Tris-HCl (pH = 8), 0.4 mL of 0.5 M EDTA and 2 mL of 10 % SDS in 150 mL distilled water (volume made up to 200 mL). The *in vivo* and *whole-organ* imaging was carried out using the In vivo imaging system (IVIS) Spectrum, Perkin Elmer. The experiment was performed at λ_{exc} = 745 nm and λ_{em} = 820 nm, exposure time = 1 s, binning = 4, f-stop = 2, field of view = 24.

Synthesis of S-Y Macroinitiator (MI): A specially designed melt reactor set up with an overhead stirrer and was employed in order to carry out ring opening polymerization over a multi-arm initiator (6-arm). The polymerization protocol of ε-caprolactone using dipentaerythritol as the initiator and tin (II) 2-Ethylhexanoate [$Sn(Oct)_2$] as the catalyst, which generates the active initiator *in situ*, has been explained here for the **S-60** macro initiator. The degree of polymerization was varied by controlling the [Monomer]/[Initiator] ([M]/[I]) ratio. The catalyst equivalents were calculated such that for every equivalent of the initiator, three equivalents of the catalyst were fed to the reactor. Dipentaerythritol (37.2 mg, 0.1462 mmol) and the catalyst $Sn(Oct)_2$ (177.7 mg, 0.4386 mmol) were weighed in a flame dried tailor-made schlenk tube followed by addition of the caprolactone monomer (1.0 g, 8.7719 mmol). The schlenk tube was evacuated under high vacuum (0.01 mbar) prior to the start of the polymerization for 45 mins. The polymerization reaction was continued at 110 °C under neat conditions for 8 h with constant overhead stirring set at 130 rpm. The polymer was dissolved in acetonitrile (HPLC) and precipitated in cold methanol. The precipitation was repeated thrice in order to obtain highly pure polymer. Yield = 0.85 g (85 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.05 (t, 2.00 H), 3.64 (t, 0.19 H), 3.38 (s, 0.06 H), 2.30 (t, 1.99 H), 1.64 (m, 4.04 H), 1.37 (m, 2.03 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.6, 64.2, 62.6, 34.1, 32.3, 28.3, 25.5, 24.6. FT-IR (cm^{-1}): 2953.57, 2869.63, 1728.41, 1644.79, 1459.08, 1418.07, 1392.87, 1366.46, 1277.23, 1235.36, 1187.44, 1162.94, 1065.67, 1037.24, 964.50, 927.11, 846.51, 743.08, 564.83, 520.43.

S-110 macro-initiator: Dipentaerythritol (18.6 mg, 0.0731 mmol), $Sn(Oct)_2$ (88.8 mg, 0.2193 mmol), caprolactone (1.0 g, 8.7719 mmol). The polymerization was carried out for 8 h at 110 °C. Yield = 0.75 g (75 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.05 (t, 2.00 H), 3.63 (t, 0.11 H), 3.38 (s, 0.04 H), 2.29 (t, 2.00 H), 1.64 (m, 4.00 H), 1.37 (m, 2.00 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.6, 64.2, 62.7, 34.2, 32.4, 28.4, 25.6, 24.6. FT-IR (cm^{-1}): 2952.19, 2868.82, 1728.69, 1459.78, 1418.77, 1391.53, 1363.48, 1275.02, 1234.41, 1162.74, 1066.93, 1039.11, 965.35, 929.70, 847.71, 740.46, 561.76, 514.61.

S-35 macro-initiator: Dipentaerythritol (74.4 mg, 0.292 mmol), $Sn(Oct)_2$ (355.4 mg, 0.8772 mmol), caprolactone (1.0 g, 8.7719 mmol). The polymerization was carried out for 6 h at 110 °C. Yield = 0.7 g (70 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.05 (t, 2.00 H), 3.63 (t, 0.34 H), 3.36 (s, 0.11 H), 2.30 (t, 1.98 H), 1.64 (m, 3.99 H), 1.37 (m, 2.00 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.7, 64.2, 62.7, 34.2, 32.4, 28.4, 25.6, 24.6. FT-IR (cm^{-1}): 2952.36, 2868.90, 1728.53, 1459.75, 1419.32, 1363.71, 1233.85, 1161.63, 1066.32, 1039.20, 965.21, 929.68, 847.76, 740.19, 564.54, 514.34.

L-60 macro-initiator: Triethyleneglycol monomethylether (23 mg, 0.14 mmol), $Sn(Oct)_2$ (28.4 mg, 0.0701 mmol), caprolactone (1.12 g, 9.8245 mmol). The polymerization was carried out for 8 h at 110 °C. Yield = 0.80 g (71 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.05 (t, 2.00 H), 3.65 (t, 0.20 H), 3.37 (s, 0.05 H), 2.30 (t, 2.00 H), 1.64 (m, 4.08 H), 1.37 (m, 2.03 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.6, 64.2, 62.7, 34.2, 28.4, 25.6, 24.6. FT-IR (cm^{-1}): 2952.99, 2869.31, 1728.64, 1459.65, 1419.16, 1364.04, 1275.63, 1234.61, 1162.68, 1066.55, 1038.73, 964.93, 929.28, 847.53, 741.08, 561.14, 516.07.

Synthesis of L-10 macro-initiator: ε-caprolactone (800 mg, 7 mmol), 1-Nonanol (101 mg, 0.7 mmol), $Sn(Oct)_2$ (142 mg, 0.35 mmol). Polymerization was done for 1 hour at 110^0C and precipitated in cold methanol (60mL). Yield = 0.85 g (95%) white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.05 (t, 22H), 3.64 (t,2H), 2.30 (t, 23H), 1.68-1.58 (m, 45H), 1.41-1.33 (m, 22H), 1.31-1.26 (m, 16H), 0.87 (t, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.71, 64.28, 62.75, 34.35, 32.44, 31.97, 29.60, 29.36, 28.47, 25.65, 24.80, 22.78, 14.22

Synthesis of star-shaped block copolymers SB-X: The typical synthetic protocol for the block copolymer has been outlined here for **SB-60**. The purified **S-60** polymer served as the macro-initiator for polymerization of tertiary butyl ester substituted caprolactone monomer (*t*-BECL). The melt reactor was charged with the S-60 MI (307.5 mg, 0.0419 mmol) and $Sn(Oct)_2$ (51.03 mg, 0.126 mmol) in a 1:3 ratio followed by the addition of the *t*-BECL monomer (650 mg,

2.5194 mmol). The schlenk tube was evacuated under high vacuum (0.01 mbar) prior to the start of the polymerization for 45 mins and the reaction was continued for 12 h at 130 °C with constant overhead stirring set at 130 rpm. The polymer was dissolved in minimum amount of acetonitrile and precipitated in cold 20 % hexane/diethyl ether. This precipitation was repeated thrice in order to obtain polymer in high purity. Yield = 0.6 g (63 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.12 (m, 2.00 H), 4.05 (t, 2.00 H), 3.64 (m, 2.19 H), 3.43 (m, 0.97 H), 3.38 (s, 0.06 H), 2.43 (t, 2.00 H), 2.36 (t, 2.01 H), 2.29 (t, 2.01 H), 1.84 (m, 1.98 H), 1.77 (m, 1.98 H), 1.64 (m, 4.00 H), 1.44 (s, 8.45 H), 1.37 (m, 2.03 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.6, 173.5, 170.9, 80.6, 75.5, 64.8, 64.2, 61.2, 36.5, 36.3, 34.1, 32.9, 29.7, 28.9, 28.3, 28.1, 25.5, 24.6. FT-IR (cm^{-1}): 2954.68, 1727.50, 1457.89, 1418.25, 1392.41, 1366.87, 1254.04, 1158.22, 1102.28, 1064.60, 964.60, 928.40, 845.68, 752.09, 559.84, 512.86.

SB-100 block copolymer: S-110 MI (309.9 mg, 0.0242 mmol), $Sn(Oct)_2$ (29.4 mg, 0.0726 mmol), *t*-BECL monomer (750 mg, 2.9069 mmol). The polymerization was carried out for 12 h at 130 °C. Yield = 0.7 g (66 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.12 (m, 1.86 H), 4.05 (t, 2.00 H), 3.64 (m, 1.96 H), 3.43 (m, 0.93 H), 3.38 (s, 0.04 H), 2.43 (t, 1.84 H), 2.36 (t, 1.91 H), 2.30 (t, 2.00 H), 1.85 (m, 1.90 H), 1.77 (m, 1.90 H), 1.64 (m, 4.00 H), 1.44 (s, 8.75 H), 1.37 (m, 2.02 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.7, 173.6, 170.9, 80.7, 75.5, 64.9, 64.2, 61.3, 36.5, 36.4, 34.2, 33.0, 29.8, 29.0, 28.4, 28.2, 25.6, 24.6. FT-IR (cm^{-1}): 2955.32, 1726.52, 1457.53, 1366.52, 1253.48, 1159.15, 1101.68, 1064.58, 964.63, 929.05, 845.47, 752.03, 554.74, 515.39.

SB-35 block copolymer: S-35 MI (281.9 mg, 0.0664 mmol), $Sn(Oct)_2$ (80.8 mg, 0.1993 mmol), *t*-BECL monomer (600 mg, 2.3256 mmol). The polymerization was carried out for 12 h at 130 °C. Yield = 0.55 g (62 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.13 (m, 2.01 H), 4.05 (t, 2.00 H), 3.65 (m, 2.34 H), 3.43 (m, 0.99 H), 3.37 (s, 0.11 H), 2.43 (t, 2.02 H), 2.37 (t, 1.99 H), 2.30 (t, 1.98 H), 1.85 (m, 2.06 H), 1.78 (m, 2.00 H), 1.64 (m, 4.06 H), 1.44 (s, 9.02 H), 1.37 (m, 2.04 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.6, 173.5, 170.9, 80.7, 75.5, 64.9, 64.2, 61.3, 36.5, 36.4, 34.2, 33.0, 29.8, 29.0, 28.4, 28.2, 25.6, 24.6. FT-IR (cm^{-1}): 2955.84, 2926.20, 1727.04, 1458.18, 1366.74, 1253.88, 1159.58, 1101.96, 1064.45, 963.83, 928.45, 845.80, 751.71, 565.11, 517.31.

LB-60 block copolymer: L-60 MI (294.1 mg, 0.0419 mmol), $Sn(Oct)_2$ (8.5 mg, 0.0209 mmol), *t*-BECL monomer (650 mg, 2.5194 mmol). The polymerization was carried out for 12 h at 130 °C. Yield = 0.65 g (69 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.13 (m, 1.67 H), 4.05 (t, 2.00 H), 3.65 (m, 1.87 H), 3.44 (m, 0.80 H), 3.38 (s, 0.05 H), 2.43 (t, 1.67 H), 2.37 (t, 1.64 H), 2.30

(t, 2.06 H), 1.85 (m, 1.75 H), 1.78 (m, 1.75 H), 1.64 (m, 4.06 H), 1.44 (s, 7.49 H), 1.37 (m, 2.02 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.6, 173.5, 170.9, 80.6, 75.5, 64.8, 64.2, 61.2, 36.5, 34.1, 32.9, 29.7, 28.9, 28.4, 28.1, 25.5, 24.6. FT-IR (cm^{-1}): 2955.55, 2923.18, 1727.32, 1644.59, 1457.86, 1418.50, 1367.34, 1280.78, 1254.76, 1161.16, 1065.58, 965.05, 928.19, 845.62, 749.67, 563.10.

Synthesis of LB-10 block copolymer: t-BECL monomer (400 mg, 1.55 mmol), L-PCL-10 macroinitiator (201 mg, 0.13 mmol), $Sn(Oct)_2$ (26 mg, 0.06 mmol). Polymerization was done for 1 hour at 130^0C and precipitated in cold mixture of hexane in diethyl ether (60mL). Yield = 0.5 g (80%) viscous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.18-4.10 (m, 22H), 4.05 (t, 22H), 3.68-3.60 (m, 24H), 3.46-3.42 (m, 13H), 2.43 (t, 23H), 2.36 (t, 22H), 2.30 (t, 23H), 1.88-1.73 (m, 46H), 1.68-1.60 (m, 49H), 1.44 (s, 100H), 1.39-1.35 (m, 22H), 1.27-1.20 (m, 14H), 0.87 (t, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 173.71, 173.62, 171.00, 80.71, 75.58, 64.92, 64.29, 61.36, 36.59, 34.24, 33.05, 29.82, 29.02, 28.22, 25.65, 24.70, 171.18, 155.32, 80.34, 70.68, 62.95, 46.95, 46.76, 44.32, 33.98, 33.42, 27.12. 14.07.

Synthesis of star-shaped random copolymer SR-60: The random copolymer synthesis outlined here is for **SR-60** polymer, wherein the feed for both the monomers CL and *t*-BECL was kept as 60. Herein, the 6-arm dipentaerythritol initiator (11.15 mg, 0.0439 mmol) was added to the schlenk tube along with the catalyst $Sn(Oct)_2$ (53.3 mg, 0.1315 mmol) maintaining their molar ratio as 1:3 followed by addition of the two monomers CL (300 mg, 2.6315 mmol) and *t*-BECL (679 mg, 2.6315 mmol) simultaneously. The tube was evacuated under high vacuum (0.01 mbar) before the start of the polymerization for 45 mins. The polymerization was continued at 130 °C for 12 h with constant overhead stirring set at 130 rpm. The polymer was dissolved in acetonitrile (2.5 mL) and precipitated in cold 20 % hexane/diethyl ether. This was repeated thrice to result in high purity polymer. Yield = 0.65 g (66 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.13 (m, 2.01 H), 4.05 (t, 2.00 H), 3.64 (m, 2.20 H), 3.43 (m, 1.09 H), 2.43 (t, 2.01 H), 2.37 (t, 2.01 H), 2.30 (t, 2.01 H), 1.85 (m, 2.03 H), 1.78 (m, 2.04 H), 1.64 (m, 4.26 H), 1.44 (s, 9.02 H), 1.38 (m, 2.00 H).

Synthesis of the carboxylic acid substituted star-block copolymers SB-X: Deprotection of the tertiary butyl group in the polymer SB-60 (214 mg) was carried out using trifluoroacetic acid (TFA) at 0 °C for 30 min. TFA (2.0 mL) was added slowly along the sides of the round bottom flask containing the polymer solution (in DCM) and the reaction was continued at 0 °C for 30 min with constant stirring. TFA was removed by repeated washings with DCM and then

the polymer was dissolved in DCM followed by precipitation in cold 20 % hexane/diethyl ether. The solution was vortexed at 1000 rpm and then the supernatant solution was decanted. This procedure was repeated thrice in order to ensure the complete removal of TFA. Yield = 150 mg (82 %). ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ ppm: 4.03 (m, 2.01 H), 3.98 (t, 2.00 H), 3.55 (m, 2.18 H), 2.39 (t, 2.01 H), 2.31 (t, 1.97 H), 2.27 (t, 2.01 H), 1.70 (m, 3.99 H), 1.54 (m, 4.06 H), 1.29 (m, 2.02 H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ ppm: 173.3, 173.1, 75.3, 64.7, 63.9, 61.2, 35.4, 33.8, 32.8, 29.7, 29.1, 28.3, 25.3, 24.5. FT-IR (cm^{-1}): 2253.15, 2126.47, 1727.73, 1657.83, 1550.29, 1226.44, 1048.82, 1023.57, 1000.01, 822.81, 760.38, 617.53, 512.85.

SB-100 block copolymer: SB-100 butyl ester substituted polymer (150 mg), TFA (1.5 mL). Yield = 100 mg (78 %). ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 4.15 (m, 1.86 H), 4.06 (t, 2.00 H), 3.69 (m, 1.96 H), 3.49 (m, 0.92 H), 2.50 (t, 1.87 H), 2.40 (t, 1.83 H), 2.32 (t, 2.02 H), 1.86 (m, 1.83 H), 1.79 (m, 1.84 H), 1.63 (m, 4.02 H), 1.38 (m, 2.03 H). ^{13}C NMR (100 MHz, CD_3OD) δ ppm: 173.6, 173.5, 75.5, 64.9, 64.2, 61.3, 35.4, 33.9, 33.0, 29.7, 29.0, 28.3, 25.6, 24.6. FT-IR (cm^{-1}): 2251.29, 2124.31, 1726.87, 1656.91, 1514.46, 1050.43, 1024.08, 1002.39, 821.67, 758.95, 617.52.

SB-35 block copolymer: SB-100 butyl ester substituted polymer (105 mg), TFA (1.0 mL). Yield = 76 mg (85 %). ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ ppm: 4.04 (m, 2.00 H), 3.98 (t, 2.00 H), 3.56 (m, 2.36 H), 2.39 (t, 2.06 H), 2.32 (t, 2.07 H), 2.27 (t, 2.04 H), 1.69 (m, 4.01 H), 1.54 (m, 4.01 H), 1.29 (m, 1.98 H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ ppm: 173.4, 173.2, 75.5, 64.7, 64.0, 61.1, 35.4, 33.8, 32.8, 29.7, 29.1, 28.3, 25.3, 24.5. FT-IR (cm^{-1}): 2251.24, 2124.63, 1725.97, 1657.18, 1514.26, 1201.78, 1050.55, 1023.98, 1002.54, 821.61, 758.84, 618.29.

LB-60 block copolymer: LB-60 butyl ester substituted polymer (220 mg), TFA (2.0 mL). Yield = 130 mg (70 %). ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ ppm: 4.04 (m, 1.68 H), 3.98 (t, 2.00 H), 3.56 (m, 1.87 H), 2.39 (t, 1.74 H), 2.32 (t, 1.65 H), 2.26 (t, 2.03 H), 1.69 (m, 3.38 H), 1.52 (m, 3.99 H), 1.28 (m, 2.02 H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ ppm: 173.4, 173.2, 75.3, 64.7, 63.9, 61.2, 35.4, 33.8, 32.8, 29.7, 29.1, 28.3, 25.3, 24.5. FT-IR (cm^{-1}): 2253.01, 2126.38, 1656.71, 1515.18, 1049.08, 1023.53, 1000.23, 822.67, 760.13, 616.11, 520.82.

Synthesis of LB-10: L-PCL10-*b*-CPCL (150 mg), TFA (1.5 mL), DCM (2 mL). After deprotection polymer was precipitated in cold mixture of hexane in diethyl ether (30mL). Yield = 85 mg, viscous solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 4.05-40.03 (m, 22H), 3.98 (t, 23H), 3.57-3.54 (m, 24H), 3.37-3.3.36 (m, 14H), 2.39 (t, 23H), 2.32 (t, 22H), 2.25 (t, 23H), 1.71-1.61 (m, 46H), 1.56-1.48 (m, 49H), 1.32-1.27 (m, 22H), 1.27-1.21 (m, 14H), 0.85 (t, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 173.0, 172.74, 158.49, 64.31, 63.59, 35.00, 33.45, 32.65, 29.367, 27.86, 24.96, 24.16.

The star homopolymer SH-60 and star random copolymer SR-60 were deprotected using trifluoroacetic acid employing the same protocol as mentioned before and characterized using ^{1}H NMR to ascertain the disappearance of the t-butyl peak at 1.44 ppm.

Self-assembly of copolymers: The polymer **SB-60** (0.5 mg) was dissolved in 0.8 mL DMSO and this solution was then added dropwise to 4.2 mL milli-Q water with constant stirring for 4 h at 25 °C. The solution was then transferred to a semi-permeable membrane of MWCO = 3.5 kD and dialyzed against milli-Q water for 24 h. The water was replenished at regular intervals in order to substantiate the removal of DMSO. Similar protocol for self-assembly was adapted for all the carboxylic acid substituted star and linear block copolymers.

Photophysical studies: The self-assembled star and linear block copolymer micelles were subjected to the critical micelle concentration (CMC) study using pyrene as the probe employing fluorescence spectroscopy. Herein, a pyrene stock was prepared in acetone (HPLC) and 1 mL of this stock was added to each sample vial such that the final pyrene concentration turned out to 0.6 μM (for 3 mL of the sample). The solvent was evaporated completely followed by addition of different concentrations of polymer dialyzed solutions ranging from 10^{-6} mg/mL to 0.5 mg/mL maintaining the overall volume to 3ml. These sample vials were then sonicated for 3 h followed by equilibration at room temperature for 12 h. Prior to acquiring the samples, all the vials were purged with nitrogen in order to eliminate all the dissolved oxygen. The fluorescence measurements were carried out by exciting the pyrene molecule at 337 nm and the emission was collected at 347-500 nm. The I_1 and I_3 vibronic levels were monitored, and these values helped determine the CMC for all the dialyzed solutions.

pH dependent self-assembly studies: Block copolymers were designed constituting peripheral carboxylic acid groups on each repeating unit. Based on pH, the carboxylic acid groups can be protonated or deprotonated which will have direct influence on the self-assembly aspects such as surface charge and sizes of the nanoparticles. To carry out pH dependent studies, solutions of self-assembled SB-60 nanoparticles (0.5 mg/mL) were prepared in 10 mM Britton-Robinson saline buffers (pH 2 to 14) and they were subjected for zeta potential and size measurements. The resulting size and zeta potentials were plotted against the corresponding pH values using origin software. All measurements were done at least thrice and obtained results are reported as the average with standard error of mean.

Drug Encapsulation in the Copolymer Scaffolds: Typical encapsulation procedure is given for the star-block polymer. **SB-60** (0.5 mg) and DOX (0.2 mg, DOX.HCl pre-treated with triethylamine) were dissolved in 0.8 mL DMSO followed by dropwise addition of the cocktail to 4.2 mL water with continuous stirring for 4 h. The dialysis was carried out in a dialysis tube of MWCO = 3.5 kD against large amount of milli-Q water for 48 h. At regular intervals the water was changed in order to facilitate the removal of un-encapsulated DOX and DMSO.

The drug loading efficiency (DLE) and drug loading content (DLC) were determined by UV-Visible spectroscopy using the following equations:

DLE (%) = {weight of drug in micelles/weight of drug in feed} X 100%

DLC (%) = {weight of drug in micelles/weight of drug-loaded nanoparticles} X 100%

***In vitro* DOX Release kinetics:** The star-block copolymers comprise of a complete polyester backbone that is known to cleave in the presence of enzymes in the lysosomal compartment rendering these nano-assemblies enzyme-responsive. This investigation was carried out by incubating SB-60+DOX nano-assemblies with two lysosomal enzymes, i.e., horse-liver esterase (10U) and α-Chymotrypsin (8U). The enzyme containing DOX solutions were taken in a dialysis tube of MWCO = 1 kD and this tube was then immersed in 4 ml of 1X PBS (pH = 7.4) maintained at 37 °C with constant stirring. In another control setup, the dialyzed solution was taken alone in the tube and exposed to pH = 7.4 (1X PBS) at 37 °C. The nanoparticle degradation was monitored in terms of the amount of DOX released from the tube into the outside 1X PBS solution and these values were determined using absorbance spectroscopy at various time points up to 48 h. Comparing these values with the amount of DOX inside the dialysis tube at time t = 0 h, resulted in the cumulative drug release values.

$$\text{Cumulative release (\%)} = C_n \times V_0/m \times 100$$

where C_n represents the amount of DOX in the n^{th} sample, V_0 is the total volume of 1X PBS in the container, and m is the total amount of DOX loaded in nanoparticles taken inside the dialysis tube.

Cell Viability Assay (MTT Assay): The biocompatibility of the star block copolymers along with their DOX loaded nano-formulations was investigated via the cell viability assay in wild type mouse embryonic fibroblast cell lines (WT-MEF) and neuroblastoma cells (SH-SY5Y) employing the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). In a typical experiment, 10^3 or (2 X10^3 for SH-SY5Y) cells were seeded in each well

of a 96-well plate (Eppendorf) in 100 µL of complete DMEM (or F-12) containing 1 % penicillin streptomycin and 10 % FBS (fetal bovine serum). The cells were incubated at 37 °C in a 5 % CO_2 humidified atmosphere for 16 h (72h in case of SH-SY5Y) allowing the cells to adhere following which media aspiration was carried out from each well. The desired concentrations of the nascent polymers, free DOX and the DOX loaded nano-scaffolds were then added to each of the wells for the cell treatment and all of these were done in triplicates. For each of these experiments, there was a control without any compound containing only the complete DMEM and the cells were then incubated at conditions mentioned before for 72 h. At the end of the incubation, the compound containing media was replaced with a freshly prepared MTT solution (0.5 mg/mL) in complete DMEM followed by 4 h incubation at 37 °C, 5% CO_2. The mitochondrial dehydrogenase enzyme present in the viable cells reduces the tetrazolium salt into formazan crystals, whose purple colour gives the readout for number of viable cells. At the end of 4 h, the MTT containing media was aspirated and the purple formazan crystals were dissolved in 100 µL DMSO; the plate was then subjected to absorbance measurements at 570 nm using the Varioskan flash microplate reader. The absorbance values from the sample treated wells were compared with the control wells considering the control value to be 100 % and thus, the cell viability % was determined for all the samples as mean ± SEM.

The cellular uptake of SB-60+DOX was investigated in neuroblastoma (SH-SY5Y) cells. SH-SY5Y cells (10^5) seeded onto each cover slip in 1.2 mL of complete F-12 DMEM containing 10 % FBS and 1 % antibiotic in the 6-well plate followed by incubation at 5 % CO_2 at a temperature of 37 °C for 4 days. Media was aspirated and replaced with fresh media having SB-60+DOX nanoparticles (DOX concentration = 5 µg/ml) were added to separate wells and then incubated for three time points, i.e., 2, 4 and 8h. At the end of the time point, compounds containing media was aspirated and cells were washed with PBS and subsequently fixed with 3.5% PFA. Nuclei were stained with DAPI, and coverslip were mounted on sterilized glass slide and imaged with confocal microscope (Zeiss Anisotropy). Laser having $\lambda = 405$ and $\lambda = 488$ nm excitation were used for DAPI and DOX detection. The imaging analysis was done using Fiji software

Live cell imaging: The cellular uptake of free DOX and SB-60+DOX was monitored with the aim of understanding the uptake mechanism and kinetics. To this effect, live cell experiment was devised with Lysotracker staining (stains the lysosomal compartment) in a time-dependent manner in WT-MEF cells. The 4-well live cell chamber plates were seeded with 25,000 cells per well in 1 mL of complete DMEM containing 10 % FBS and 1 % antibiotic followed by

incubation at 5 % CO_2 at a temperature of 37 °C for 16 h. The requisite concentrations of free DOX and DOX loaded polymeric micelles (DOX concentration = 5 μg/ml) were added to separate wells and then incubated for three time points, i.e., 60, 90 and 180 min. At the end of the time point, the compound containing media was removed followed by addition of 50 nM lysotracker red DND-99 solution in DMEM and these were then imaged within 10 min using Leica SP8 while being incubated in a chamber maintained at 37 °C and 5 % CO_2 humidified atmosphere. The confocal imaging was done on a Leica SP8 system with a sequential scan setting using the lasers λ = 488 nm for DOX excitation and λ = 561 nm for excitation of the lysotracker red dye. The imaging analysis was done using Fiji software.

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Mice handling and injection of nano-vehicles:

All the animal procedures were carried out at National Facility for Gene Function in Health and Diseases (NFGFHD, Indian Institute of Science Education and Research (IISER) Pune, India) with compliance of Institutional Animal Ethics Committee India (IAEC) guidelines. Animal ethics committee approval number is IISER/IAEC/2018-02/07.

Female balb/c mice of 8-12 weeks old (~ 25g in weight) were chosen for the *in vivo* experiments and these were divided into 3 groups of n = 3 each, namely free DOX, SB-60+DOX, and SB-60. The aim behind the *in vivo* study was to unravel the effect of polymer topology on the biodistribution and pharmacokinetics parameters whilst comparing the same

against the small molecule DOX, hence the two groups constituted were free DOX and SB-60+DOX. The other group corresponded to nascent polymer scaffold as control to its DOX-loaded counterpart to help evaluate the immunogenicity and cytotoxic effects, if any, of polymer alone. One mouse was injected with PBS only without any compound; the organs and plasma procured from this mouse served as the control. For all the experiments, the PBS, free DOX and all the nano-formulations were delivered using intraperitoneal injections. The DOX dosage for the biodistribution study was fixed at 2 mg/kg mice, which in this case translated to 50 μg DOX corresponding to 25 g mice and the concentration of DOX-loaded nano-formulations were determined using the drug loading content. Mice from group 1 were injected with 175 μL of DOX alone and group 2 mice were injected with 184 μL of unimolecular SB-60+DOX. The aforementioned calculation helped us to determine the polymer amount required to inject 50 μg DOX and the same polymer concentration was injected into the mice in the nascent polymer group. Collection of blood sample was done from retro-orbital sinus, alternating between the sinuses during the sequential collection. Blood was collected at 2, 4, 8 and 24 h after the injection. Absorbable hemostatic gelatin sponge (AbGel) was used if the bleeding did not stop on its own. Animals were left undisturbed, maintained *ad libitum* in between the time points of blood collection. Mice were sacrificed at the end of 24 h post injection by transcardial perfusion and organs were collected to be further utilized either for biodistribution or biochemical analysis. All experimental mice were kept in individually ventilated cages in a temperature- and humidity- controlled animal facility and a 12-hours light/dark cycle was maintained in these rooms. All animal care and procedures were in accordance with the Institutional Animal Ethics Committee (IAEC) at IISER Pune and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Biochemical analysis of organs and blood samples: Blood sample was collected from each animal (all 3 groups) in heparin coated vials. For each time point, approximately 15 μL of blood was withdrawn from one of the retro-orbital sinuses. The blood was then centrifuged immediately for 20 minutes at 10000 rpm at 4°C to separate plasma from RBCs. The plasma in the supernatant was collected separately in new heparin coated vials and were stored at -80°C until the cytokine assay was carried out. Brain, Heart, Liver, Kidneys and Spleen were obtained at 24-hour time point after the injection for the DOX containing 2 groups. Mice received Thiopentone injections intra-peritoneally (Thiosol sodium, Neon laboratories, 50mg/kg) to achieve deep anesthesia state in order to carry out perfusion. Transcardial perfusion was done

using 1X Phosphate buffered saline (PBS). Organs were dissected out and were put in separate 15 mL falcons filled with 4 % Paraformaldehyde (PFA). Organs were kept immersed in 4 % PFA at 4 °C for 24 h. They were then transferred to falcons filled with 0.01 % Sodium Azide in PBS (1X) and kept at 4 °C until required for the assays.

Histological analyses of organs: To carry out Hematoxylin & Eosin (H&E) staining, 10 µm organ sections were taken on gelatin-formaldehyde coated glass slides in a cryotome. Before cryotomy, organs were further transferred to falcons filled with 30 % sucrose for 24-48 hours for cryo-preservation. Upon obtaining the sections, the slides were air-dried at 37 °C for 30 minutes before further processing was done. The solutions required for H&E staining were stored in Couplin jars kept at room temperature. Slides were moved through a sequential series of couplin jars containing Hematoxylin, Scott's tap water, Eosin, grades of alcohol (95%, 100%, 50% Xylene-50% alcohol) and 100% Xylene. Sections on slides were then mounted using DPX mountant (Thermo Fisher scientific) and stored at 4 °C before carrying out microscopy. The sections were imaged using bright field microscope (Carl Zeiss) to carry out qualitative analysis of any alterations in the tissue sections obtained from the organs of different groups of mice.

Quantification of biodistribution of Doxorubicin in organs using confocal microscopy: Organs were separately embedded in optimal cutting temperature (OCT) medium (Leica,14020108926) and sectioned at 50 µm thickness in a cryotome. Freely floating sections were collected at 300-350 µm intervals in 24 well/6-well plates, as per the requirement, filled with 1X PBS and stored at 4 °C. DAPI (Sigma, 1:500 in 1X PBS) staining was carried out for a total of 5-6 sections per organ (approx. 300-350 µm apart). Sections were mounted on glass slides using Mowiol based aqueous mounting medium and stored at 4 °C until imaged. Confocal microscopy to image the amount of DOX in several field of views (FOVs) was carried out for 5 sections. Biodistribution quantification was done by calculating the mean gray value of the DOX intensity in the three groups of mice using Fiji Image J. The FOVs that were chosen for calculation were devoid of blood clots (checked using the DIC mode).

MAP 2, NeuN and GFAP Antibody staining: The 50 µm thick brain tissue sections of groups free DOX and SB-60+DOX obtained via cryotome were subjected to MAP 2 antibody staining. For permeabilization and blocking, 2/3 large sections of the brain were added to a single well of a 24-well plate followed by addition of 250 µL of blocking solution (5 % Normal goat serum (NGS) and 1 % triton-X in 1X PBS) in each well and incubation at room temperature for 2 h. After the 2 h incubation, removed the blocking solution from each well. Added 1:1000 dilution

of MAP 2 antibody (1 µL in 1 mL) in the blocking solution (1 % NGS and 0.2 % triton-X in 1X PBS), mixed well and then added 250 µL of this solution to each well. The plate was then incubated on the shaker at 25 rpm for 20 h at 4 °C, followed by three 1X PBS washes, wherein for each wash the plate was kept on the shaker at 25 rpm, room temperature for 15 min. After the third wash, the 1X PBS was inoculated from each well. The secondary antibody was added to the blocking solution (1 % NGS in 1X PBS) in a 1:500 dilution (2 µL in 1 mL) and was thoroughly mixed before adding 250 µL in each well followed by incubation at room temperature for 2 h on the shaker at 25 rpm in the dark. After the incubation, wells were washed with 1X PBS thrice as mentioned before. This was followed by DAPI staining at1:500 dilution (2 µL in 1 mL) in the blocking solution (1 % NGS in 1X PBS), 250 µL of this solution was added to each well, covered the plate with foil and kept on the shaker at 25 rpm for 10-12 mins at room temperature. The sections were mounted using the Vectashield mounting medium and stored at 4 °C until imaged. Similar protocol was adopted for the NeuN and GFAP antibody staining of the 50 µm thick brain tissue sections of the SB-60+DOX group.

Immunogenicity Analysis: The BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine kit was employed to quantify the expression of cytokines, i.e., Interleukin (IL)-2, IL-4, IL-17A, Interferon-gamma (IFN-ɣ) and Tumor necrosis factor alpha (TNF-α) in the plasma samples corresponding to the 24 h time point for all the mice (n = 6) across the 3 groups and the one control mice injected with PBS. The plasma samples were thawed at 4 °C for 2-3 h prior to the experiment. The lyophilized Mouse Th1/Th2/Th17 standard spheres were reconstituted with 2 mL of Assay diluent, referred to as the "Top standard", equilibrated for ~ 15 min at room temperature followed by gentle mixing of the spheres (reconstituted protein) by pipetting. Serial dilutions were performed of the Top standard with the Assay Diluent in the ratios 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and one negative control was prepared containing only the Assay diluent. The next step was mixing of the five capture beads IL-2, IL-4, IL-17A, IFN-ɣ and TNF-α, wherein 10 µL of each capture bead for each assay tube was taken together and vortexed thoroughly. The plasma samples were diluted by the desired dilution factor (1:2, 1:10, 1:50 or 1:100) using the Assay diluent and then mixed thoroughly for the assay. The mixed capture beads were vortexed before adding 50 µL of the stock to each of the assay tubes (10 cytokine standards and plasma samples). To the control tubes, 50 µL of the cytokine standard dilutions were added such that the cytokine concentrations varied from 20 pg/mL (1:256 dilution) to 5000 pg/mL (Top standard). Further 50 µL of the plasma samples (diluted as mentioned above) were added to assay tubes containing the mixed capture beads. To all the assay tubes, 50 µL of the PE detection reagent was added followed by 2 h incubation

at room temperature. Then 1 mL of wash buffer was added to all the assay tubes and centrifuged at 200 g for 5 min. The supernatant from each assay tube was decanted followed by addition of 300 µL wash buffer to each of the assay tube in order to resuspend the pellet. The samples were acquired on the BD Celesta (3 laser, 12 color) flow cytometer and analyzed using the FCAP Array software, wherein using the cytokine standard values the amount of each of the 5 cytokines was determined in all the plasma samples.

Hemolysis Assay: For the assay, blood sample (0.4 ml) was collected from C57B1/6 male mice (~ 9 weeks old) via the retro-orbital plexus puncture in heparin coated eppendorfs. The sample was centrifuged at 8000 rpm, room temperature for 10 min to separate the plasma from the red blood cells (RBCs). The supernatant plasma was discarded carefully leaving behind the RBCs and these were then washed with freshly prepared 1X PBS buffer thrice. Discarded the supernatant and diluted the RBCs using 1X PBS (5 % v/v). 100 µL of this diluted RBCs stock was taken in a 96-well plate followed by addition of 100 µL of various polymer concentrations ranging from 10 µg/mL to 1000 µg/mL. PBS was chosen to be the negative control and Triton-X was the positive control required for the determination of % hemolysis. All the samples were recorded in triplicates. The 96-well plate was incubated at 37 °C for an hour followed by centrifugation at 1400 rpm for 10 min. 100 µL of the supernatant was then transferred to another 96-well plate and the erythrocyte rupture was determined as a function of the amount of hemoglobin released by taking the absorbance read-out at 576 nm. Hemolysis (%) was determined using the formula:

Hemolysis (%) = [($O.D._{576nm}$ in polymer sample - $O.D._{576nm}$ in PBS)/($O.D._{576nm}$ in Triton-X - $O.D._{576nm}$ in PBS)] X 100

IVIS mediated biodistribution analysis and *whole-organ* imaging: 12 weeks old female balb/c mice (~ 25g in weight) were chosen for the IVIS biodistribution study and these were divided into 2 groups of n = 3 each, namely free IR780 and SB-60+IR780 along with a control mouse (injected with 1X PBS). The PBS, free IR780 and the nano-formulation were injected intra-peritoneally wherein the IR780 dosage was fixed at 0.5 mg/kg, that translated to 10 µg IR780 per mouse according to the body weight of ~ 25g. The dye loading content of SB-60+IR780 was used to determine the exact concentration of the nano-formulation to be injected. Following the injections, blood samples were collected via retro-orbital plexus puncture at the time points 8h, 24 h, 48 h and 72 h. Absorbable hemostatic gelatin sponge (AbGel) was used in case the bleeding didn't stop. The biodistribution of the free IR780 dye and the SB-60+IR780 UMM nano-formulation was monitored in real time by employing IVIS. Herein, the mice were

imaged at time points 24 h, 48 h and 72 h while under anesthesia capturing both the dorsal and ventral view using the excitation and emission filter of λ_{exc} = 745 nm and λ_{em} = 820 nm. Mice were then sacrificed at the 72 h time point by cervical dislocation and the brain was procured in 1X PBS for *whole-organ* imaging analysis employing the same excitation and emission filters. Using the blood samples, plasma was isolated following the aforementioned procedure. The plasma samples obtained from n = 3 mice of the groups free IR780 and SB-60+IR780 along with the control mouse (IX PBS) were inoculated in a 96-well plate at a 1:10 dilution (5 µL plasma sample+45 µL cell lysis buffer). These samples were then subjected to the IVIS instrument analysis and the amount of the dye IR780 was determined by measuring the radiant efficiency at λ_{exc} = 745 nm.

Primary neuronal culture and immunocytochemistry: The olfactory bulb and cortex were dissected from postnatal day 1 C57/B6J mice pups and the tissues were minced in hanks balanced salt solution. The tissues were further digested using 0.25 % Trypsin EDTA(1X) for 15 min at 37 ºC and neutralized with media containing serum. After multiple rounds of washes with wash buffer (HBSS containing 1% Pen Strep and 0.0033 M HEPES), the pellet was dissolved in Neurobasal medium supplemented with 10 % fetal bovine serum, 1X B27 supplement, 0.25 % L-Glutamine and 0.5 % of Penicillin-streptomycin. The cells were seeded on a coverslip coated with poly-D-lysine. After 24 h, the culture was made serum free and the media was changed every three days. The SB-60+DOX nanoparticle was dissolved in Neurobasal medium and the cells were incubated with the solution for 1 h and 4 h. The cells were fixed using 4 % paraformaldehyde for 10 minutes and immunocytochemistry was performed. The cells were stained for MAP2(Microtubule associated protein2) protein using Anti-chicken MAP2 antibody with 1:1500 dilution. Alexa Fluor chicken 647 antibody (1:500) was used as the secondary antibody and DAPI (1:500) was used for nuclear staining. The cells were then imaged using Leica SP8 and processed via the Fiji Image J software.

Blood-brain barrier (BBB) integrity studies using Evan blue dye extravasation method: Integrity of Blood-brain barrier was studied using the widely used Evans blue (EB) dye extravasation method *in vivo*. Normally EB cannot cross BBB. Any alteration in BBB permeability in BBB can be confirmed by presence of EB in brain tissue specifying that BBB integrity has been compromised. Ideally, any polymeric nanoparticle having ability to cross BBB must not affect the integrity of BBB. EB dye extravasation studies was used to evaluate BBB integrity and permeability qualitatively as well as quantitatively by measuring EB content in the brain. Adult Female balb/c mice of age 8-10 weeks weighing 20-22 g were used to carry

out these studies. Total 6 mice were randomly divided into two groups (n=3). Each mice of group one was injected with 100 µL of PBS saline buffer (1X) intraperitoneally and this group was considered as control group because saline buffer will not have any effect on BBB integrity. Group two were injected with SB-60 nanoparticles (10 mg/kg) in 100 µL of PBS saline buffer (1X) intraperitoneally. After the injection mice were kept in regular habitat of animal house for 48 hours to ensure complete circulation of nanoparticles throughout body. After 48 hours every mouse was intravenously injected with 100 µL of EB dye solution (2% w/v) in normal saline via lateral tail vein. Animals started to turn blue within 10-20 seconds of injection ensuring the successful intravenous administration of EB dye. Further, animals were kept in cage for an hour and monitored. After 1 hour the animals were immediately anesthetized using isoflurane and their thoracic cavities were opened and hearts were accessed using surgical tools to avoid excretion of EB dye. Further animals were pericardially perfused using normal saline (40-50 mL of 0.9%) and brains were harvested and photographed before further assay. Weights of all brain tissues were recorded using an analytical balance. Each brain sample was manually homogenized containing 500 µL trichloroacetic acid (50% w/v in distill water) in tissue grinder. Homogenates were centrifuged at 10,000 g for 20 min at room temperature and supernatants were collected in fresh tubes. Supernatants were diluted using ethanol (1:4) and 100 µL (in triplicates) of each sample were taken in 96 well plate and subjected for absorbance measurement in microplate reader using wavelength at 632 nm. The concentration of EB in each brain was determined using standard curve of EB in similar solvent generated in same microplate. Standard curve was generated by measuring absorbance of 100 µL of EB solutions at various concentration (1000, 750, 500, 250 and 100 ng/mL of EB) in same solvent. Calculated amount of EB in brain samples are expressed in ng EB dye /mg of brain tissue.

Blood-brain barrier (BBB) integrity studies using BBB impermeable dye IR780 iodide: IR 780 iodide dye is impermeable to BBB and thus can be utilized to evaluate any alteration caused in BBB permeability by detecting its fluorescence signal in brain tissue. The SB-60+IR780 nanoparticle can permeate the BBB because of the ability of SB-60 nanoparticle to cross BBB. Therefore, a total of 9 female balb/c mice of 8 to 12 weeks old were divided into three groups (n=3), wherein one group was injected with free IR780 dye (IR780 dose 0.5 mg/kg, 10µg in 100 µL PBS buffer) which was considered as control group and group two was injected with SB-60+IR780 (IR780 dose 0.5 mg/kg, 10µg in 100 µL PBS buffer). Group three was injected with SB-60 nascent nanoparticles dissolved in 100 µL PBS buffer (SB-60 nanoparticles dose was 10 mg/kg). All injections were done intraperitoneally and all three group were subjected for IVIS whole body fluorescence imaging using excitation filter 745 nm and

emission filter 800 nm. Imaging was done at 1 h, 2 h, 4 h, 6 h and 24 h timepoints. After 24 hours the group one and two mice were euthanized for further organ extraction. Group three which was already injected with SB-60 nascent nanoparticles were kept for 24 hours to ensure complete circulation of nanoparticles throughout body and organs. Then group three was again intraperitoneally injected with free IR 780 (IR780 dose 0.5 mg/kg, 10μg in 100 μL PBS buffer) and subjected for IVIS whole body imaging for similar timepoint as other groups. After 24h group three animals were euthanized for organ extraction. Organs were extracted by anesthetizing animals using Isoflurane and their thoracic cavities were opened to access heart with the help of surgical tool. Once heart was accessed and small cut was made on right atrium and needle was injected in left ventricle which was pumped with PBS saline buffer to remove all blood from animal (pericardial perfusion). After perfusion all the organs of all animals were harvested, washed with saline and subjected for IVIS fluorescence imaging. All the imaging data were analyzed using PerkinElmer IVIS software and plotted using Graph pad prism 8.0 software. Statistical tests were performed using same software.

Notes Animal ethics committee approval number is IISER/IAEC/2018-02/07.

Statistics: All the analyses were done using Graphpad Prism 8.0 (Graphpad Software Inc, USA). Ordinary One-way ANOVA Turkey test and unpaired t-test were performed, and the values are represented as mean ± SEM.

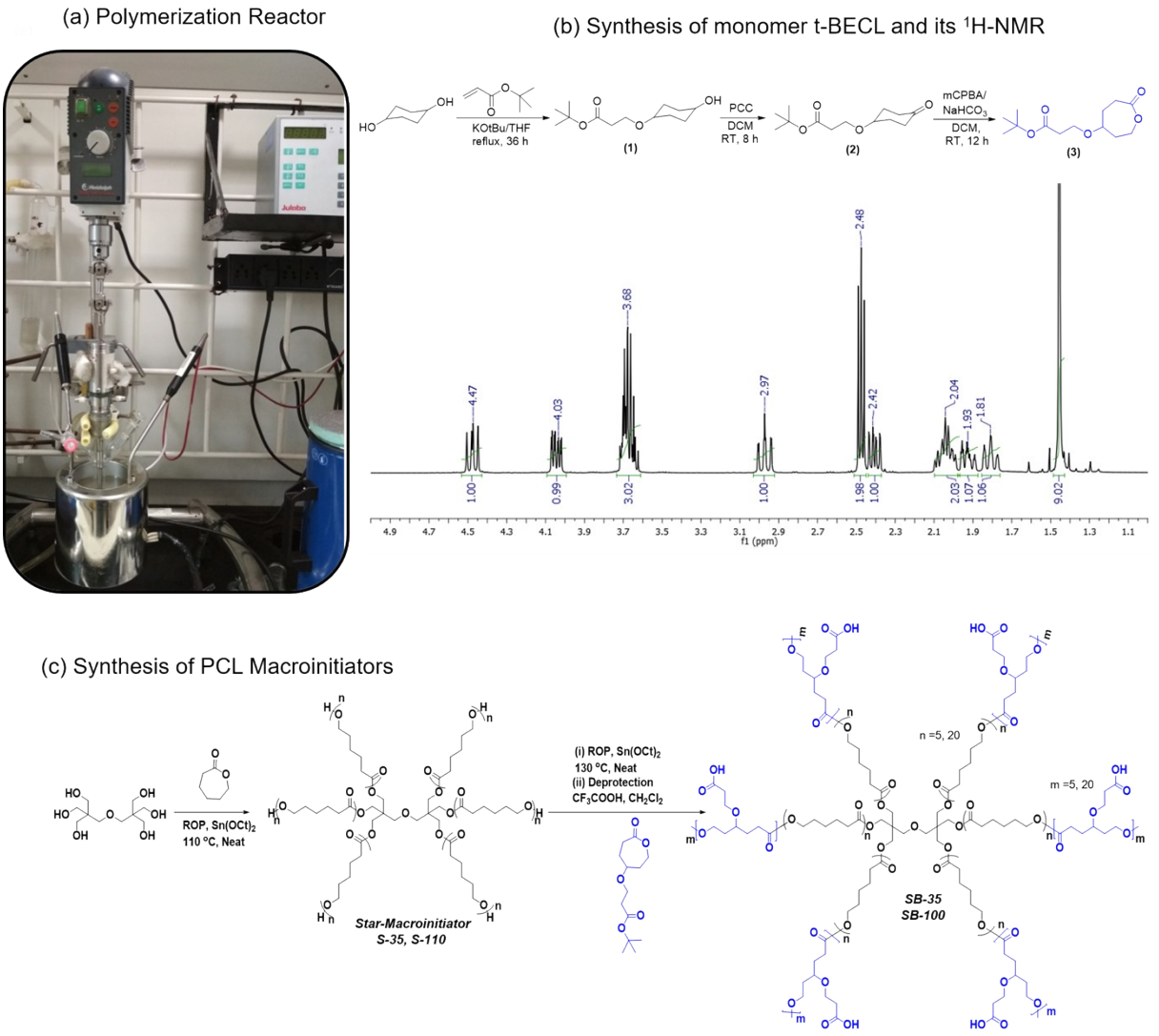


Figure S1: (a) In-house specially designed melt reactor set-up with an overhead stirrer for ring opening polymerization methodology. (b) Multi-step synthetic scheme of the tertiary butyl ester substituted caprolactone monomer (*t*-BECL) and its ^{1}H-NMR in $CDCl_3$. (c) Ring opening polymerization of the star macroinitiators S-35, S-110 and block copolymers SB-35, SB-100.

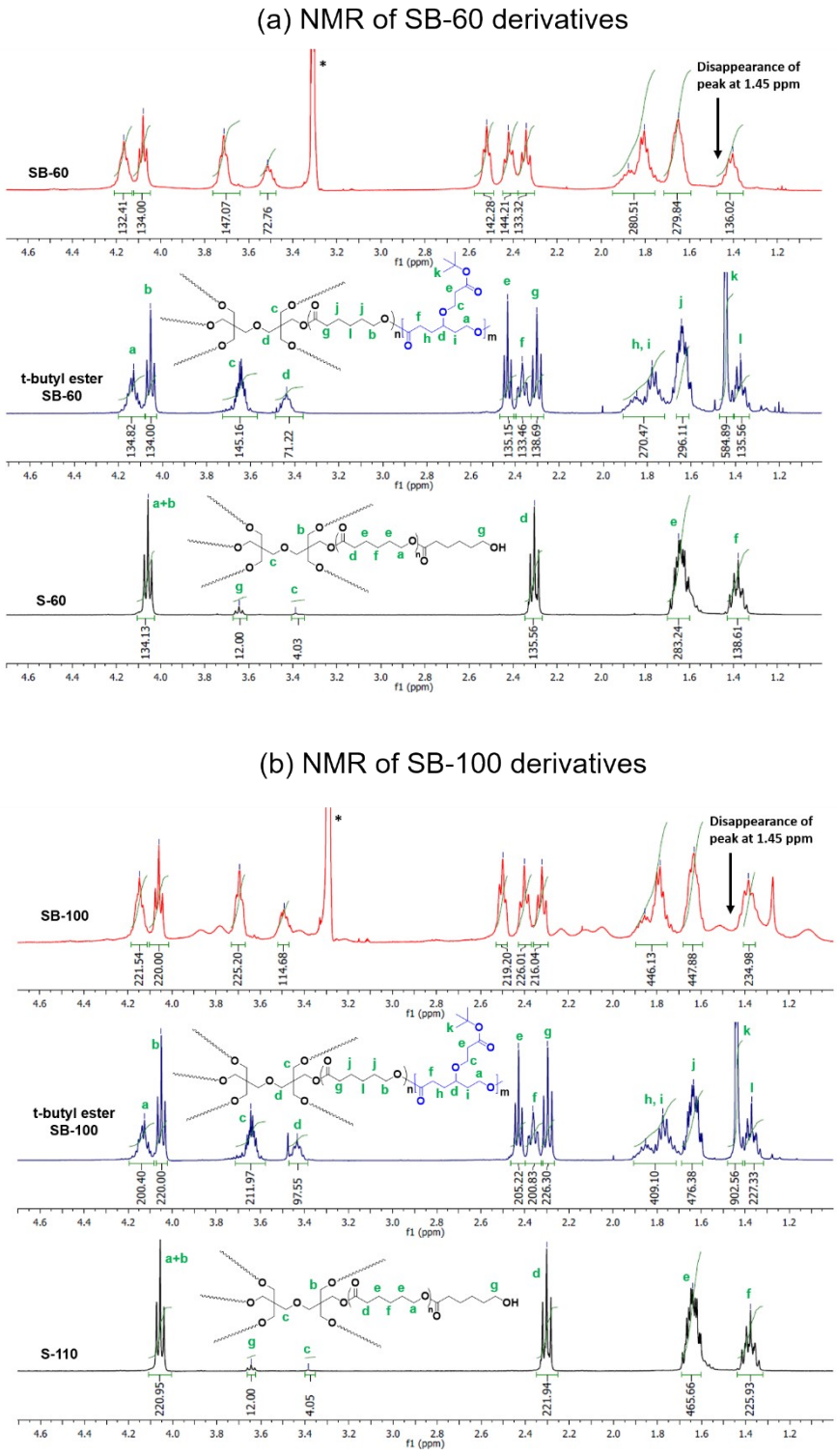


Figure S2: (a) ^{1}H NMR stack plot of S-60, SB-60 and carboxylic substituted SB-60 in $CDCl_3$ and $(CD_3)_2SO$. (b) ^{1}H NMR stack plot of S-110, SB-100 and carboxylic substituted SB-100 in $CDCl_3$ and CD_3OD. The spectra are plotted up to 4.70 ppm for simplicity.

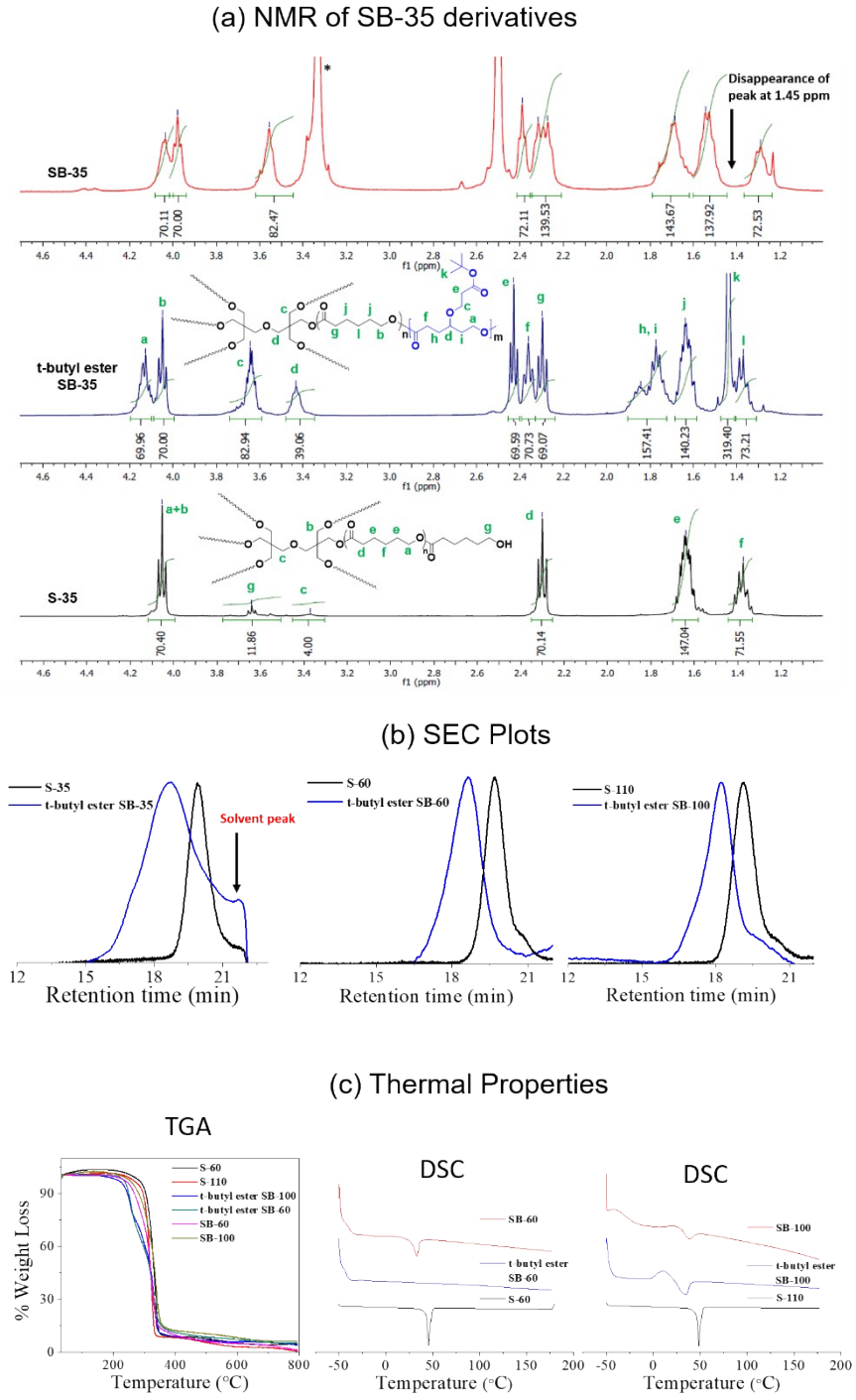


Figure S3: ^{1}H NMR stack plot of S-35, SB-35 and carboxylic substituted SB-35 in $CDCl_3$ and $(CD_3)_2SO$. The spectra are plotted up to 4.70 ppm for simplicity. (b) Gel permeation chromatogram of t-butyl ester substituted star block copolymers alongwith their respective macroinitiators. (c) Thermogravimetric Analysis (TGA) plot and Differential Scanning Calorimetry (DSC) thermograms of star macroinitiators and block copolymers (heating cycle).

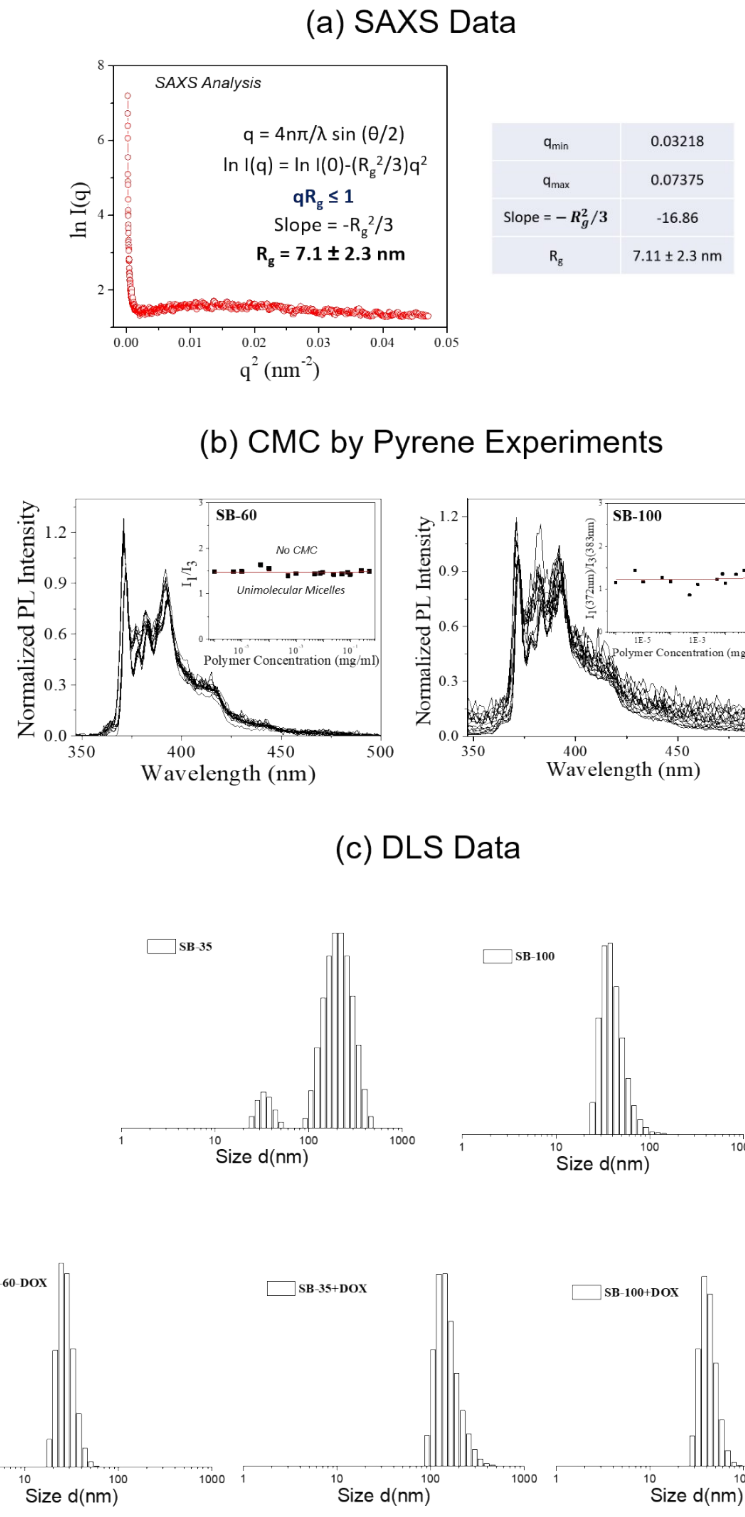


Figure S4: (a) Small Angle X-ray Scattering (SAXS) analysis and determination of radius of gyration (R_g) for the star block copolymer SB-60. (b) Critical Micelle Conccentration (CMC) plots of SB-60 and SB-100 polymers. (c) DLS histograms of star block copolymer nascent and DOX-loaded nano-scaffolds.

Important points for Figures S1 to S4 Analysis

Note: ^{1}H NMR stack plot of SB-60 star-block copolymer and the macroinitiator S-60 is shown here. In order to determine the degree of polymerization (X_n), the integration of the peak intensities from the initiator were compared to those of the polymer backbone in ^{1}H-NMR. As can be seen in lowest panel corresponding to the S-60 macroinitiator, the chain end terminal protons appeared at 3.64 ppm corresponding to the 12 protons adjacent to the hydroxyl group. The peak at 3.39 ppm represents the 4 ether protons of the dipentaerythritol initiator. The newly formed ester peaks in the polycaprolactone part were found to appear at 4.06 ppm and 2.30 ppm. The dipentaerythritol initiator 12 protons next to the ester also appear at 4.06 ppm together with the polycaprolactone peak. The terminal protons at 3.64 ppm were chosen as the reference peak and given an integration of 12, corresponding to which the integration of the PCL part at 4.06 ppm was determined and hence, comparing the intensities of the aforementioned peaks gave the incorporated degree of polymerization (X_n). For feed [M]/[I] = 60 in the star macroinitiator: the actual incorporation as determined from the ^{1}H-NMR was 64 ± 3 repeating units confirming the statistical distribution of 10-11 units per arm. To estimate the degree of polymerization in star-block copolymer; the appearance of new peak corresponding to t-butyl ester PCL was compared with macroinitiator PCL units. In case of the polymer SB-60 (second panel), additional peaks corresponding to the BECL part were seen when compared to the S-60 NMR, wherein the new ester peaks appeared at 4.13 ppm and 2.37 ppm reiterating the formation of the star-block copolymer. Since the S-60 macroinitiator was employed to yield the star-block copolymer, hence comparing the intensities of the two ester peaks at 4.06 ppm (PCL part) and 4.13 ppm (BECL part) gave us the number of repeating units for the BECL unit. The number average degree of polymerization (X_n) for the t-butyl ester PCL block was estimated to be 64 ± 3 (for feed [M]/[MI] = 60) indicating the formation of average 10-11 substituted PCL units per arm. The deprotection of the t-butyl group into carboxylic acid was confirmed via the disappearance of the 1.45 ppm peak, as can be seen in the top panel corresponding to carboxylic substituted SB-60.

Note: In all the following figures, the carboxylic acid substituted star block copolymers will be referred to as **SB-35**, **SB-60** and **SB-100**.

Note: The carboxylic acid substituted star block polymers were characterized using ^{1}H NMR. The integartion of all the respective peaks was maintained followed by deprotection using TFA and thus, the structural integrity of the polymer backcone was unperturbed. These polymers are only soluble in Methanol and DMSO, hence, their GPC chromatograms could not be recorded due to the unavailability of a GPC system with the aforementioned mobile phases.

Note: Differential scanning calorimetry (DSC) thermograms exhibited both S-60 and S-110 macroinitiators as semi-crystalline having melting transitions at 45.6 °C and 48.6 °C, respectively. The star-block copolymer SB-60 was found to be sluggish to crystallize and the polymer displayed only glass transition (T_g) at -42.5 °C. On the other hand, the SB-100 polymer exhibited a cold crystallization peak at 10.5 °C followed by a melting transition at 34.4 °C. The carboxylic acid substituted star-block copolymers SB-60 and SB-100 exhibited semi-crystalline behaviour wherein both the glass transition as well as the melting transitions were observed.

Note: The scattering intensity, I is determined as a function of the scattering vector "q" and according to the Guinier approximation, the value of I depends on the Radius of gyration R_g as per the equation given above. The Guinier approximation focusses on the lower q values of the SAXS scattering curve and the plot of ln I(q) vs q^2 is what is referred to as the Guinier plot, whose slope gives us the value of R_g (under the condition that $qR_g \leq 1$)

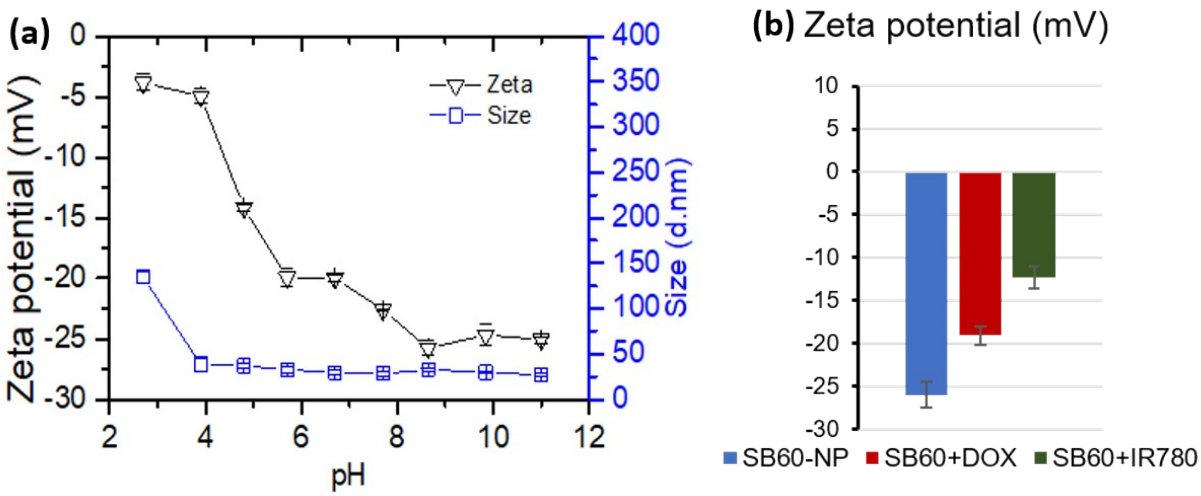
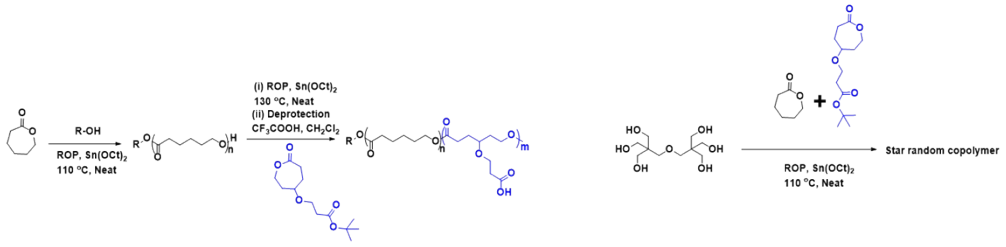
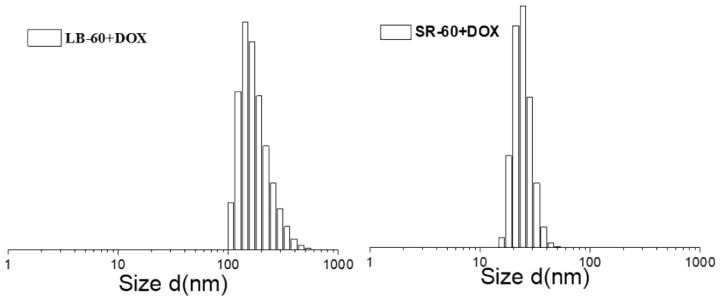


Figure S5: (a) Variation in zeta potential and size of SB-60 UMM nanoparticle upon changing the pH from 3.0 to 11.0. (b) Zeta potential values (mV) for the nascent SB-60, SB60+DOX and SB60+IR780 nanoparticles.

(a) Polymer Topology Control



(b) Size, Zeta potential (ξ) of Nanoparticles



Architecture	Sample	D_h (nm)	ξ (mV)	PDI	Sample	D_h (nm)	ξ (mV)	PDI
		Nascent Nanoparticles				DOX Loaded Nanoparticles		
Linear Di-block	LB-60	170 ± 10	-10.9	0.6	LB-60+DOX	143±10	-23.9	0.2
Random Star	SR-60	30 ± 5	-3.1	0.4	SR-60+DOX	25±3	-21.5	0.4

(c) Self-assembly of Linear Block Copolymer

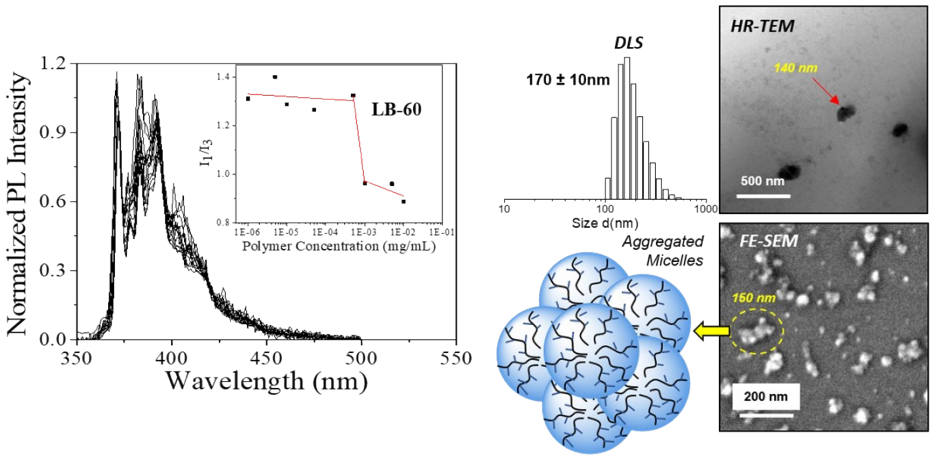


Figure S6: (a) Polymer topology control in the PCL block copolymers. Synthetic scheme of the linear di-block LB-60 and star random copolymer SR-60. All the polymers have identical molecular weights with 60 PCL units and 60 carboxylic PCL units. (b) Size, Zeta potential and other details of the nascent and DOX loaded liner di-block LB-60 and star random copolymer SR-60. (c) CMC deterimination of LB-60, DLS, FE-SEM and HR-TEM images of linera di-block exhibiting aggregated micelles.

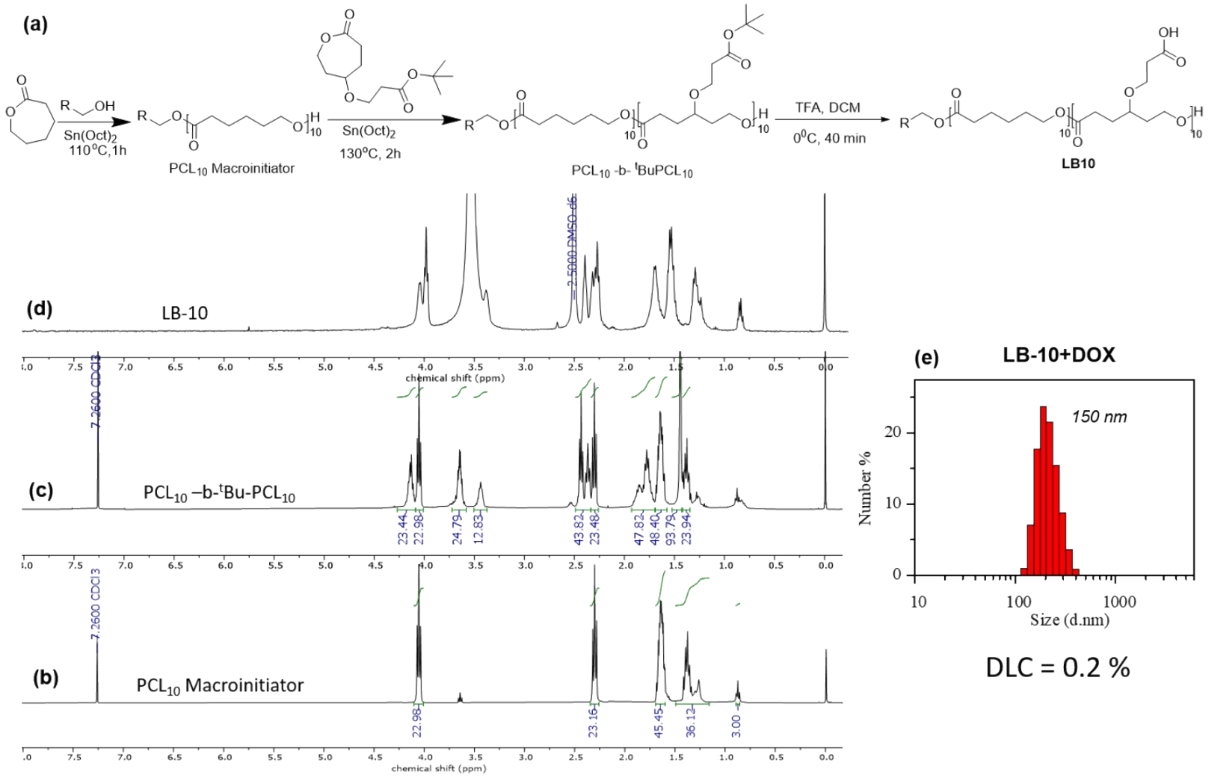


Figure S7: (a) Synthetic scheme of linear block copolymer LB-10. (b) ^{1}H NMR stack plot of PCL_{10} MI, PCL_{10}-b-^{t}BuPCL_{10} (c) and carboxylic substituted LB-10 (d) in $CDCl_3$ and $(CD_3)_2SO$. The spectra are plotted up to 8.00 ppm for simplicity. (e) Size and DLC of LB-10+DOX nanoparticle.

Sample	DLC (%)	DLE (%)
SB-35+DOX	0.95	4.75
SB-60+DOX	14.20	35.20
SB-100+DOX	13.10	32.75
LB-60+DOX	2.0	10.00
LB-10+DOX	0.11	1.10
SR-60+DOX	3.00	15.00
SB+60+IR780	5.00	50.00

Figure S8: Table summerizing the drug loading content (% DLC) and drug loading efficiency (% DLE) for all the drug and dyes loaded samples.

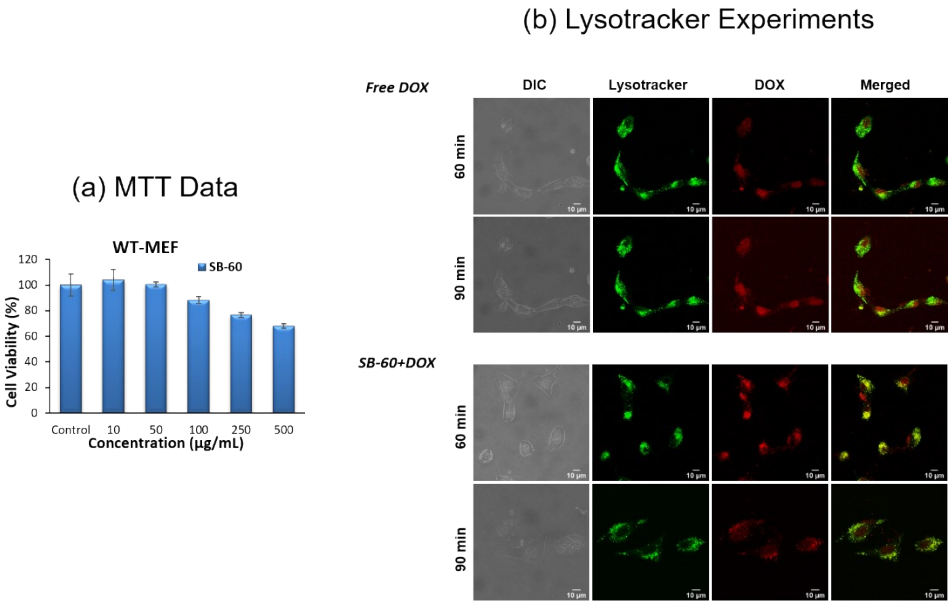


Figure S9: (a) MTT plot depicting the cytocompatibility of the star block copolymer SB-60 upto a concentration of 500 µg/mL. (b) Representative CLSM images depicting live cell uptake of free DOX and SB-60+DOX at different time points 60 and 90 min in WT-MEF cells.

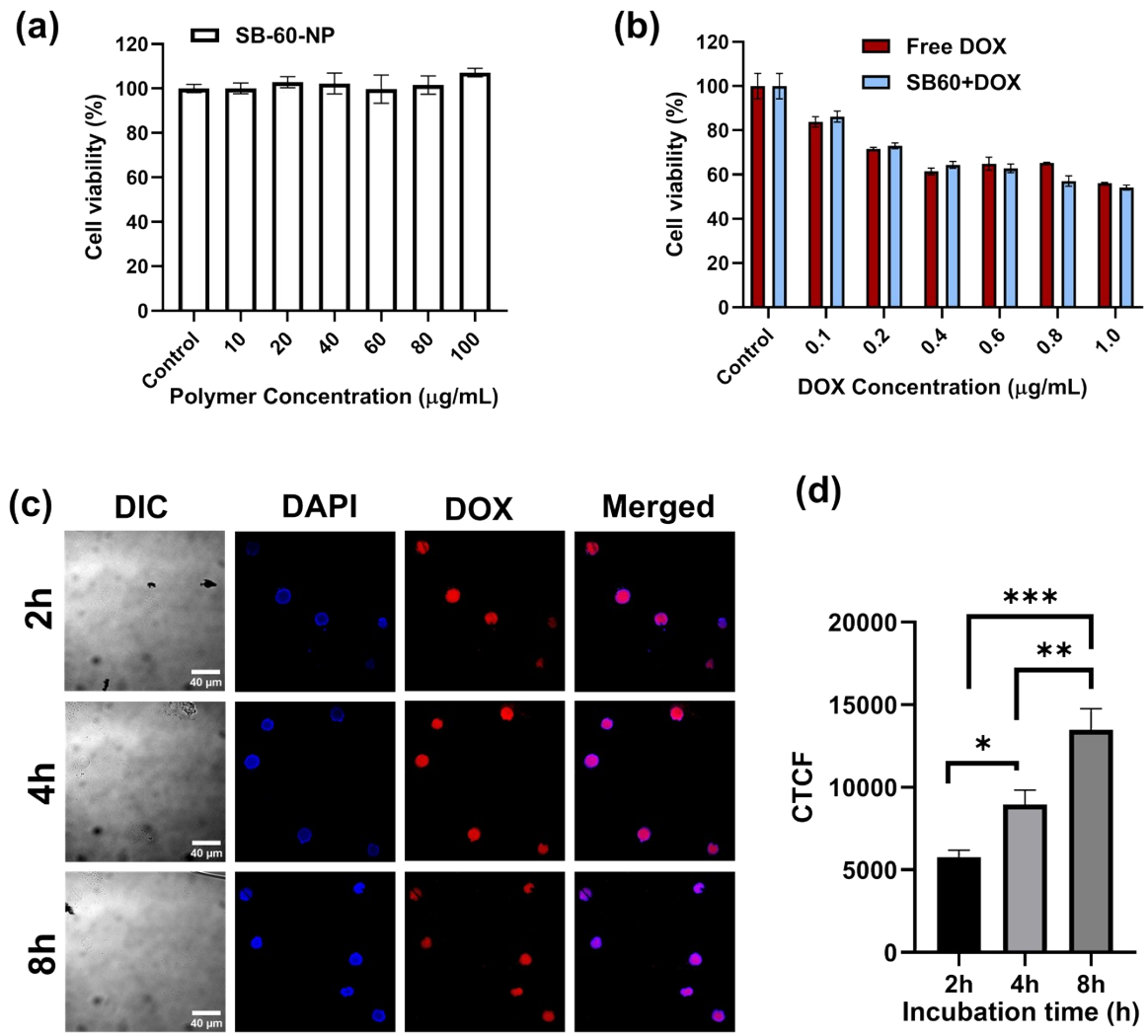


Figure S10: (a) MTT plot depicting the cytocompatibility of the star block copolymer SB-60 upto a concentration of 100 μg/mL in SH-SY5Y neuroblastoma cells after 72 h incubation. (b) MTT plot depicting the toxocity of the nascent DOX and SB-60+DOX nanoparticles upto a concentration of 1.0 μg/mL in SH-SY5Y neuroblastoma cells after 72 h incubation. (c) Representative CLSM images depicting cellular uptake of SB-60+DOX at different time point in SH-SY5Y cells. (d) Plot of CTCF for cellular uptake of SB-60+DOX in SH-SY5Y cells (Data represented as mean ± S.D., *n=3*, *p*<0.05, p**=0.0024, p***=0.0001, ANOVA test*).

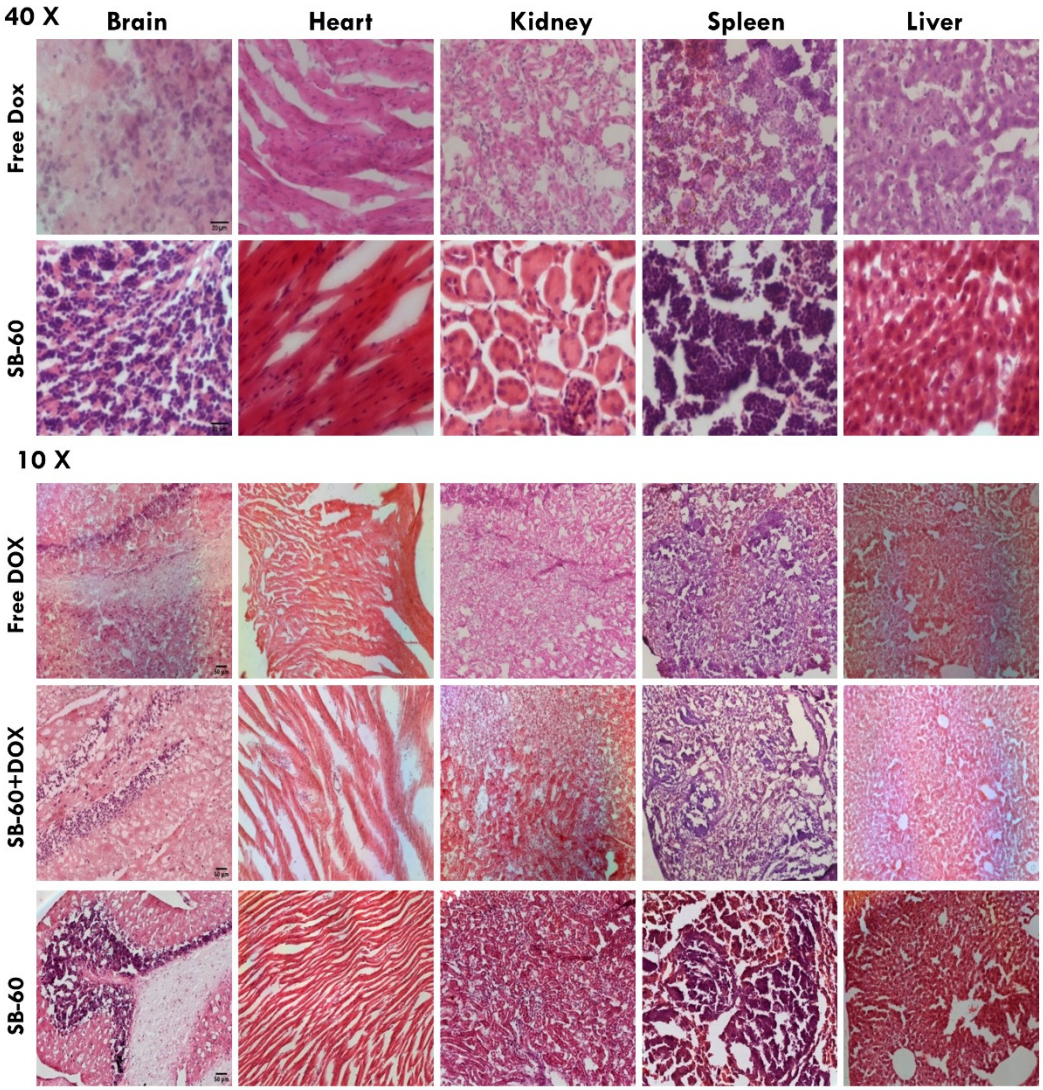


Figure S11: Representative images (40X and 10X) of H&E-stained tissue samples. Scale bar = 20 µm and 50 µm, respectively

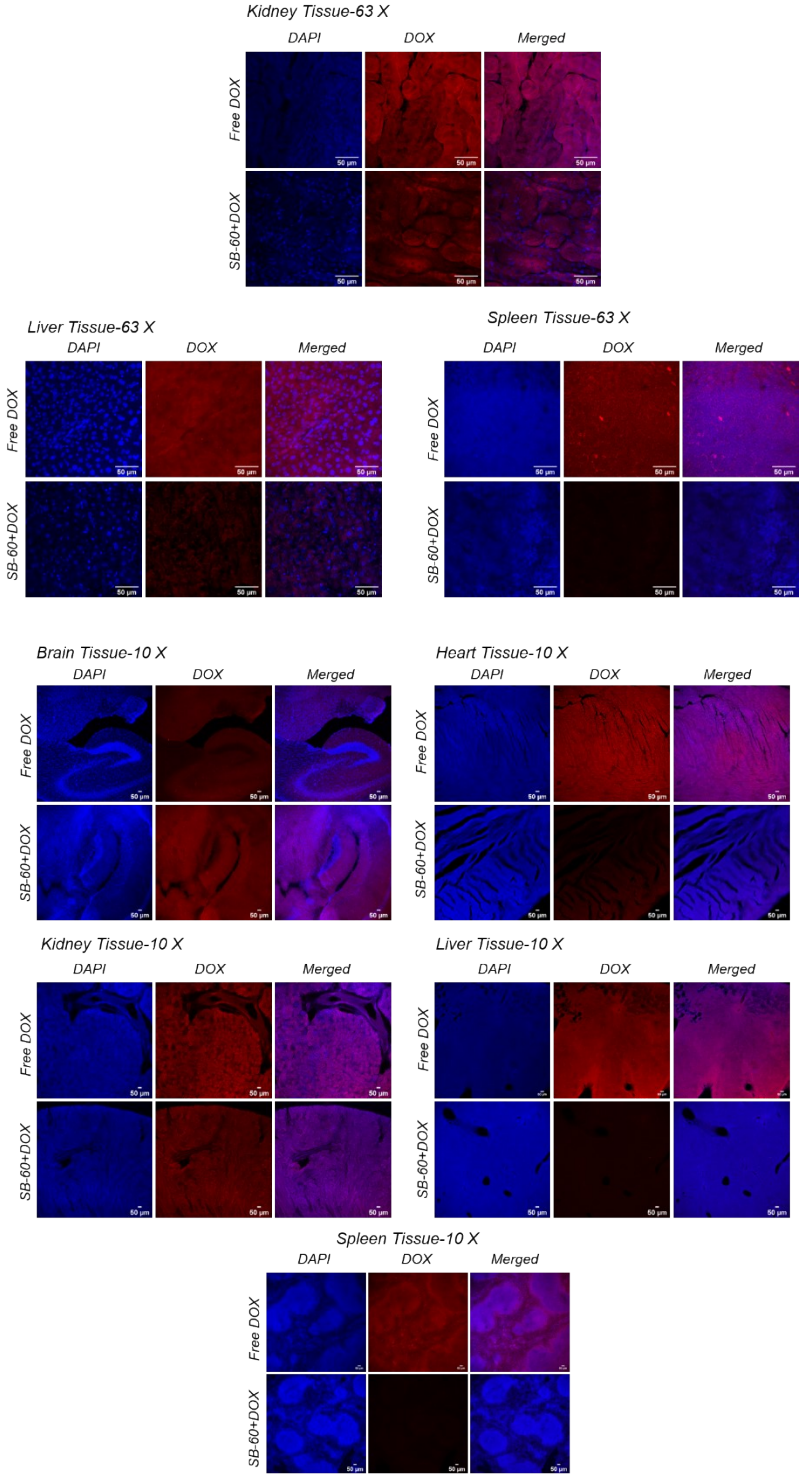


Figure S12: Representative CLSM images (63 X and 10X) depicting the uptake of free DOX and SB-60+DOX for the brain, heart, kidney, liver, and spleen tissue.

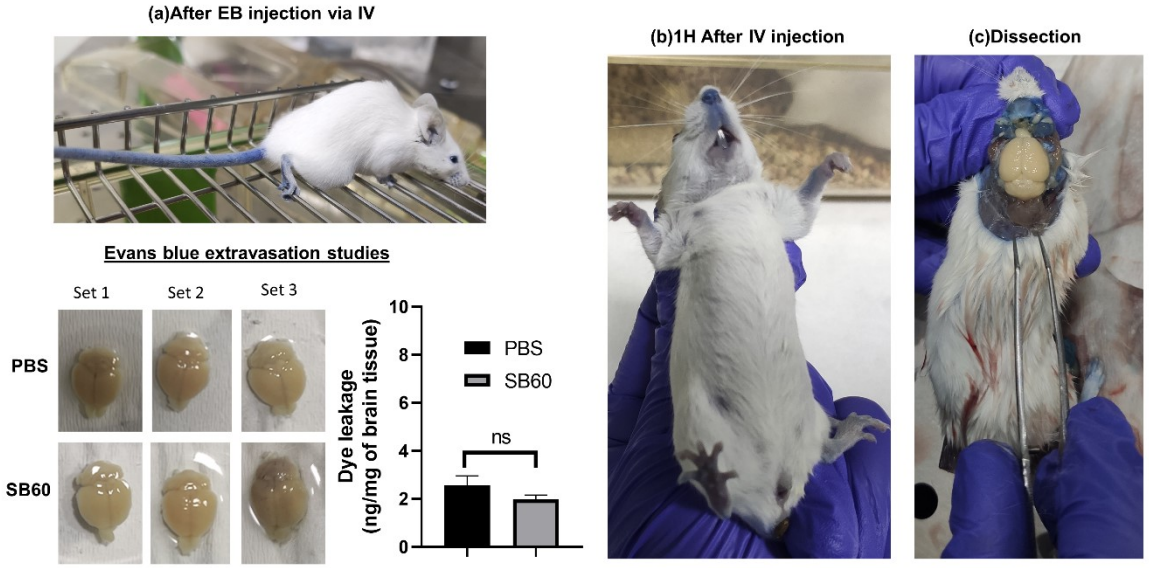


Figure S13: Evans blue extravasation studies. (a) Mice photographed after EB injection via IV route. Pictures of brain tissue harvested from mice injected with PBS and SB60, followed by EB and the dye leakage quantified for both the groups. (b) Anesthetized mice 1 h after EB injection. (c) Dissection to harvest the brain tissue after pericardial perfusion.