Supporting Information for

Synthesis of a hyperbranched polymer multimodal molecular imaging probe

Materials

Unless otherwise stated, all reagents and solvents were purchased from Sigma Aldrich at the highest available purity and used as received. All monomers were passed through a short plug of basic alumina, immediately prior to use, to remove free radical inhibitors. Tetrahydrofuran and dimethylformamide were purified by a MB-SPS800 solvent purification system, prior to use. NOTA-Bn-NCS (Macrocyclics Ltd), IRDye® 750-NHS (NIR-750, LICOR Biosciences) and Alexa Fluor-647-NHS (Life Technologies) were used as received. Puratronic copper wire (0.1 mm dia) for AAS standards was obtained from Alpha Aesar at 99.9999% purity. Dimethyl sulfoxide was distilled over 4 Å molecular sieves, under reduced pressure and stored under argon gas. The alkyne trithiocarbonate RAFT was synthesised as per published methods.[33, 34] Distilled water with a resistivity of 18.2 MΩ.cm was obtained from an Elga ultra pure water system.

Polymer characterisation techniques

^{1}H NMR spectra of 5 w/w% polymer samples in either $CDCl_3$ or DMSO-d_6 were collected on a Bruker AVANCE 500MHz NMR spectrometer with a TXi-5z probe, using a 45° pulse, 4 sec acquisition time and using the residual solvent peak as an internal standard.

Size exclusion chromatography (SEC) was performed on a system consisting of a 1515 Isocratic pump (Waters), a 717 auto sampler (Waters), Styragel HT 6E and Styragel HT 3 columns (Waters), a 2414 differential refractive index detector (Waters) and a Dawn 8+ MALLS detector (Wyatt). THF was used as the mobile phase at a flow rate of 1 mL/min. Empower 2 (Waters) and Astra (Wyatt) software was used for data collection and processing. For the determination of molar mass, light scattering data was used with a measured dn/dc value of 0.0600 mL/g.

UV-Vis spectroscopy was performed on a Cary 4000 spectrophotometer (Varian) and analysed using the Cary WinUV software (Varian). For quantification of trithiocarbonate groups, all samples were prepared in methanol, at a concentration that gave a maximum absorbance at 307 nm less than 1.0. For quantification of dye attachment, samples were prepared in 10 mM PBS at a concentration that gave a maximum absorbance at 277 nm less than 1.0.

Hyperbranched polymer particle sizes were measured in pure water using a Malvern Zetasizer. The diameters recorded (Table 1) are number averages of 5 experiments conducted at 25 °C. Samples were filtered through 450 micron PTFE filters prior to measurement.

Atomic absorption spectroscopy (AAS) was performed using a SpectraAA220FS instrument (Varian). Analysis was performed using an air-acetylene flame and a hollow cathode lamp monochromated to 324.8 nm. [Cu^{2+}] standards were prepared by dissolving Puratronic copper wire in 30% nitric acid solution to produce a 1000 ppm solution and then diluting with distilled water to give a concentration range of 0-20 ppm.

Synthesis of hyperbranched polymers by RAFT polymerisation

Two hyperbranched polymer cores were synthesised in this work. The experimental conditions for **HBP 1A** are described in detail (Scheme 1). Conditions for synthesis of **HBP 2A** were the same, unless otherwise stated (Scheme S1, ESI). The relevant physical and chemical properties of all materials used in this work are summarised in Table 1.

Poly(ethylene glycol) methyl ether methacrylate (MW 475, 2.00 g, 4.21×10^{-3} mol), trifluoroethyl acrylate (0.162 g, 2.34×10^{-3} mol), ethylene glycol dimethacrylate (0.135 g, 5.2×10^{-5} mol), ethynyl 2-(((dodecylthio)carbonothioyl)thio)-2-methylpropanoate (alkyne RAFT, 0.069 g, 2.63×10^{-4} mol) and azobisisobutyronitrile (AIBN) (0.009 g, 5.68×10^{-5} mol) were added to a 20 mL scintillation vial and dissolved in THF. The solution was purged for 15 minutes with argon gas for a final volume of 2 mL of THF. The vessel was heated to an internal temperature of 70 °C and allowed to polymerise for 24 h. The resulting polymer was precipitated three times in hexane to yield the final hyperbranched polymer cores **HBP 1.** ^{1}H NMR (500 MHz, $CHCl_3$) δ: 0.84-2.00 ppm (m, CH_2, CH_3), 3.36-3.76 ppm (m, CH_2CH_2O), 4.06 ppm (s, sh, $COOCH_2$ PEGMA, $COOCH_2$ EGDMA), 4.31-4.44 ppm (d, $COOCH_2$ TFEA), 4.62 ppm (s, $CH_2C{\equiv}C$)

Aminolysis of trithiocarbonate groups

Aminolysis of both **HBP 1** and **HBP 2** was performed to reduce the trithiocarbonate end-groups to a thiol. In a typical reaction, 500 mg **HBP 1** was dissolved in 5 mL THF. 25 μL of hydrazine was added to the solution and allowed to react at 25 °C for 16 h. Polymers were precipitated from hexane and used without further purification to give **HBP 1-1**. Removal of trithiocarbonate end-groups was monitored by the disappearance of the characteristic trithiocarbonate absorbance at 307 nm using UV-Vis spectrophotometry. **HBP 1-1**: 80% removal trithiocarbonate groups. **HBP 2-1**: 94% removal trithiocarbonate

groups.

Attachment of Azido-PEG-amine Spacer to Hyperbranched Polymer 2

One equivalent of **HBP 1-1** (0.30 g, 9.6×10^{-6} mol HBP, 4.8×10^{-5} mol alkyne) was reacted with four equivalents (to alkyne concentration) of 11-azido-3,6,9-trioxaundecan-1-amine (55 µL, 2.7×10^{-4} mol) in 2 mL of DMF at 130 °C for 48 h. The reaction was diluted with 20 mL of distilled water and dialysed for two days in a snakeskin dialysis tubing (MWCO 3,500) against 50% methanol, exchanging to 100% ultrapure water. The sample was then lyophilised to recover **HBP 1-2**. ^{1}H NMR estimates >90 % functionalisation of the alkyne group. ^{1}H NMR (500 MHz, CHCl3) δ: 0.84-2.00 ppm (m, backbone CH_2, CH_3), 1.39 ppm, (s, NH-(C=O)O-C-(CH_3)$_3$) 3.36-3.76 ppm (m, CH_2CH_2O), 3.86 ppm (s, OCH_2CH_2(N-N=N-CH=C)), 4.06 ppm (s, sh, $COOCH_2$ PEGMA, $COOCH_2$ EGDMA), 4.31-4.44 ppm (d, $COOCH_2$ TFEA), 4.58-4.62 ppm (d, OCH_2CH_2(N-N=NCH=C)), 5.14-5.19 ppm (d, CH_2(C=CH-N-N=N)), 7.6 ppm (b s, N-N=NC*H*=C)), 8.1 ppm (b s, N-N=N-C=C*H*))

A similar procedure was used to synthesise **HBP 2-2**, replacing the DMF with 1 mL of toluene at 100 °C and reacted for 48 hours. The sample was then dialysed and lyophilised to recover **HBP 2-2**.

Attachment of NOTA-Bn-NCS to hyperbranched polymers

One equivalent of **HBP 1-2** (100 mg, 3.0×10^{-6} mol) was dissolved in 1.0 mL sodium bicarbonate buffer (0.1 M, pH 9.4) with 1.5 equivalents of NOTA-Bn-NCS (2.5 mg, 4.5×10^{-6} mol) and allowed to react at 25 °C for 16 h. The solution was diluted to 0.5 mL with ultrapure water and purified using a Zeba Spin column (7 kDa MWCO) and lyophilised to yield **HBP 1-3**. Attachment of NOTA was confirmed by ^{1}H NMR. These procedures were repeated with **HBP 2-2** to yield **HBP 2-3**.

Attachment of near infrared optical dyes to hyperbranched polymers

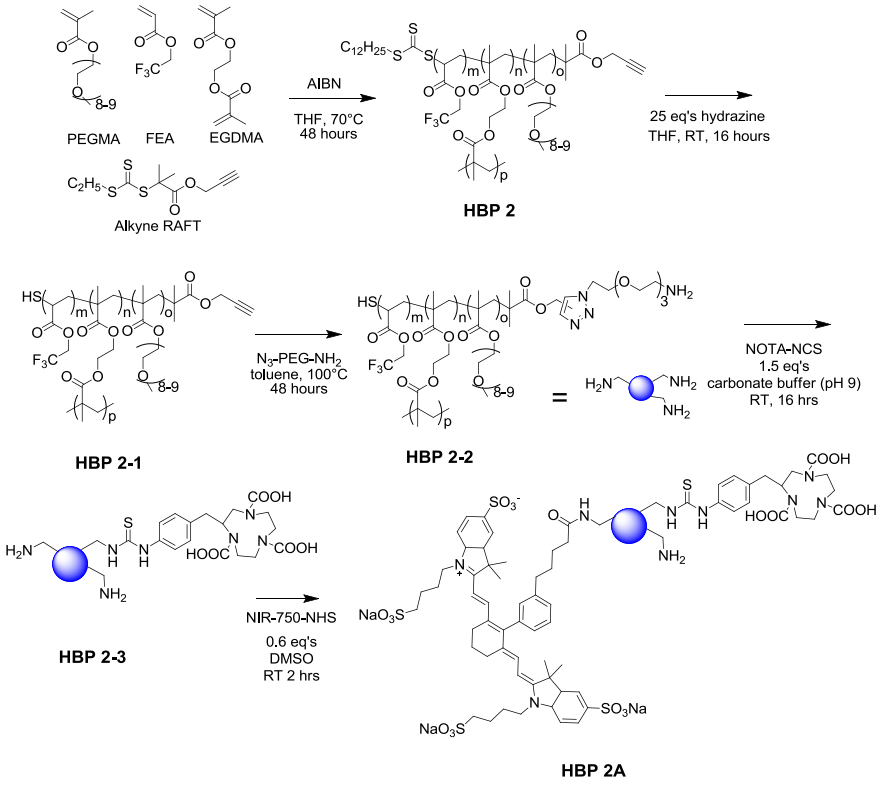
17.4 mg of **HBP 1-3** (5.6×10^{-7} mol) was dissolved with 0.5 mg Alexa Fluor-647-NHS (8.4×10^{-7} mol) in 0.2 mL of DMF and allowed to react for 4 h, protected from light. The solution was diluted with ultrapure water and dialysed for 4 d to yield **HBP 1A**. Attachment of Alexa Fluor-647 was characterised by UV-Vis analysis, using the extinction coefficient from the supplier of 239000 $M^{-1}cm^{-1}$ at 650 nm.

45 mg of **HBP 2-3** (8.5×10^{-7} mol) was dissolved with 0.55 mg NIR-750-NHS (0.5 equivalents, 4.6×10^{-7} mol) in 500 µL of anhydrous DMSO and allowed to react for two hours at room temperature, protected from light. The solution was purified using Zeba spin columns, to yield **HBP 2A**. Attachment of NIR-750 was characterised by UV-Vis analysis, using the extinction coefficient from the supplier of 260000 $M^{-1}cm^{-1}$ at 756 nm.

Validation of copper purification methodology with ^{63}Cu.

Five 20 mL scintillation vials were prepared with **HBP 1A** (50 mg, 1.4×10^{-6} mol) and dissolved in 9 mL of PBS (pH 7.2). 2mM $CuSO_4$ solution was added (1 mL) and stirred for 1.5 hours at 25°C. One vial was kept as a positive control and two vials were treated with EDTA solution (150 µL, 0.02 M). One pair of samples, consisting of one that had been treated with EDTA and one that hadn't, were purified by dialysis against distilled water (3,500 MWCO). The other pair were treated using Zeba size exclusion chromatography (SEC) spin columns, as per the manufacturer's protocol.

All samples were lyophilised and the dried product (~10 mg, mass recorded on analytical balance to three significant figures), was dissolved in 30% nitric acid solution (1 mL) and reacted at 90 °C for 2 h. Each solution was diluted to 10 mL with distilled water in a volumetric flask. Cu^{2+} concentration was measured using AAS.



Scheme 1. Synthesis of **HBP 2A** series of hyperbranched polymer multimodal molecular imaging agents.

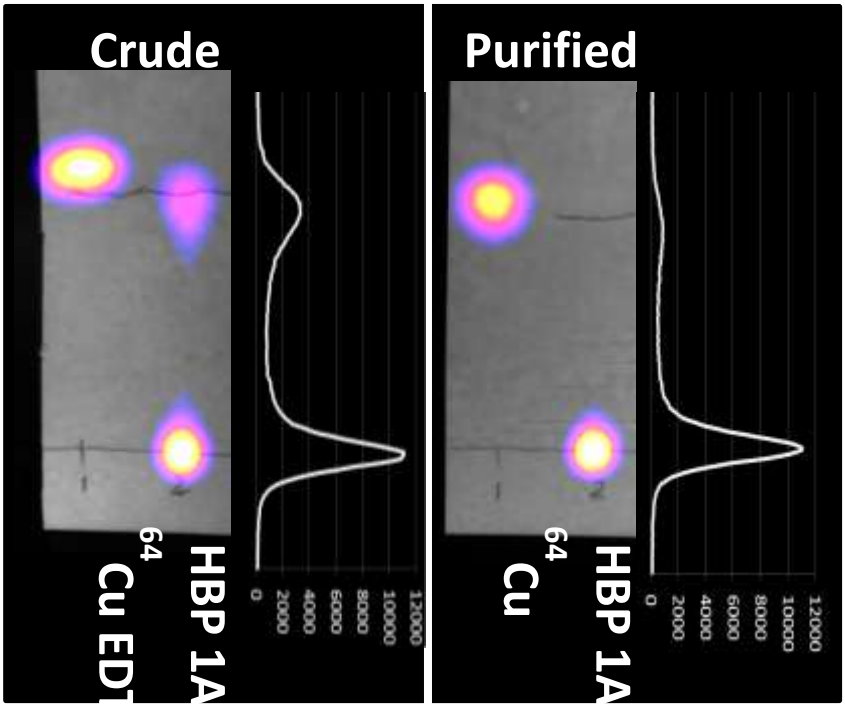


Figure 1. iTLC analysis of ^{64}Cu labelled HBPs, before and after purification with spin SEC columns with lane profile plot of **HBP 1A** (arbitrary fluorescence units). Radioisotopic images were collected on a Bruker In Vivo MS FX Pro.

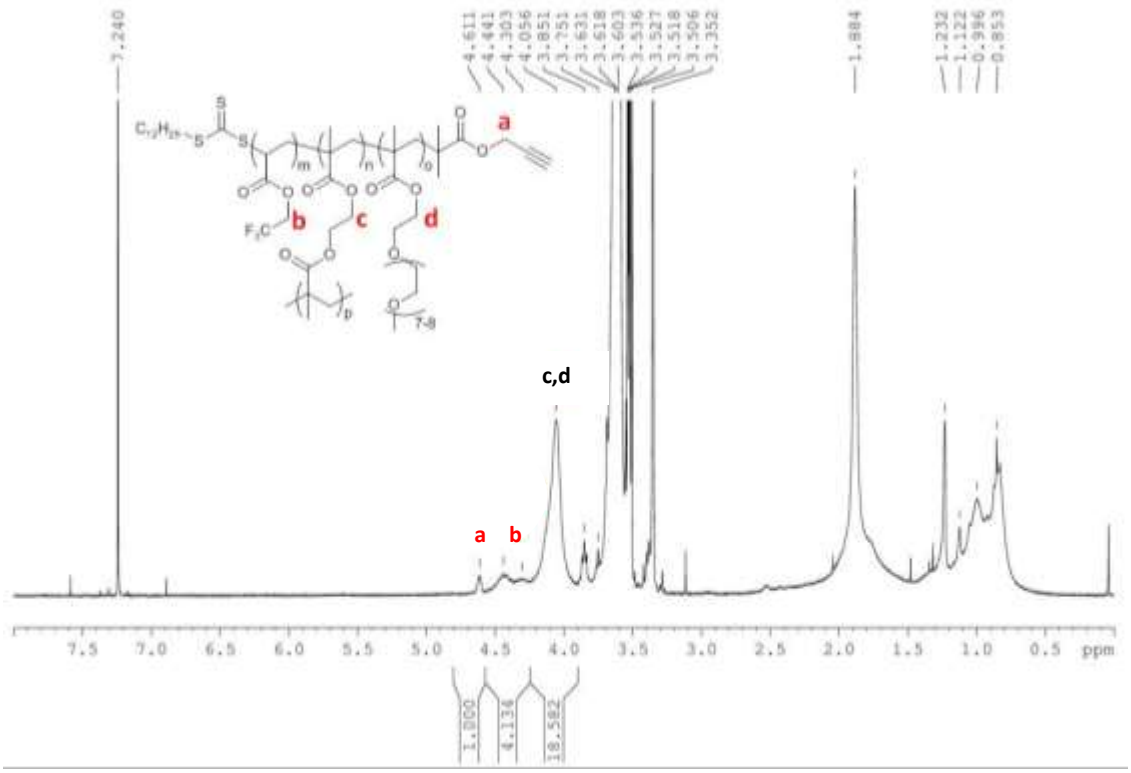


Figure 2. [1]H NMR of the base hyperbranched polymer (**HBP 1**) used for synthesis of multimodal molecular imaging agents. Characteristic monomer and end group peaks that are used for the characterisation of molar mass and monomer incorporation are labelled.

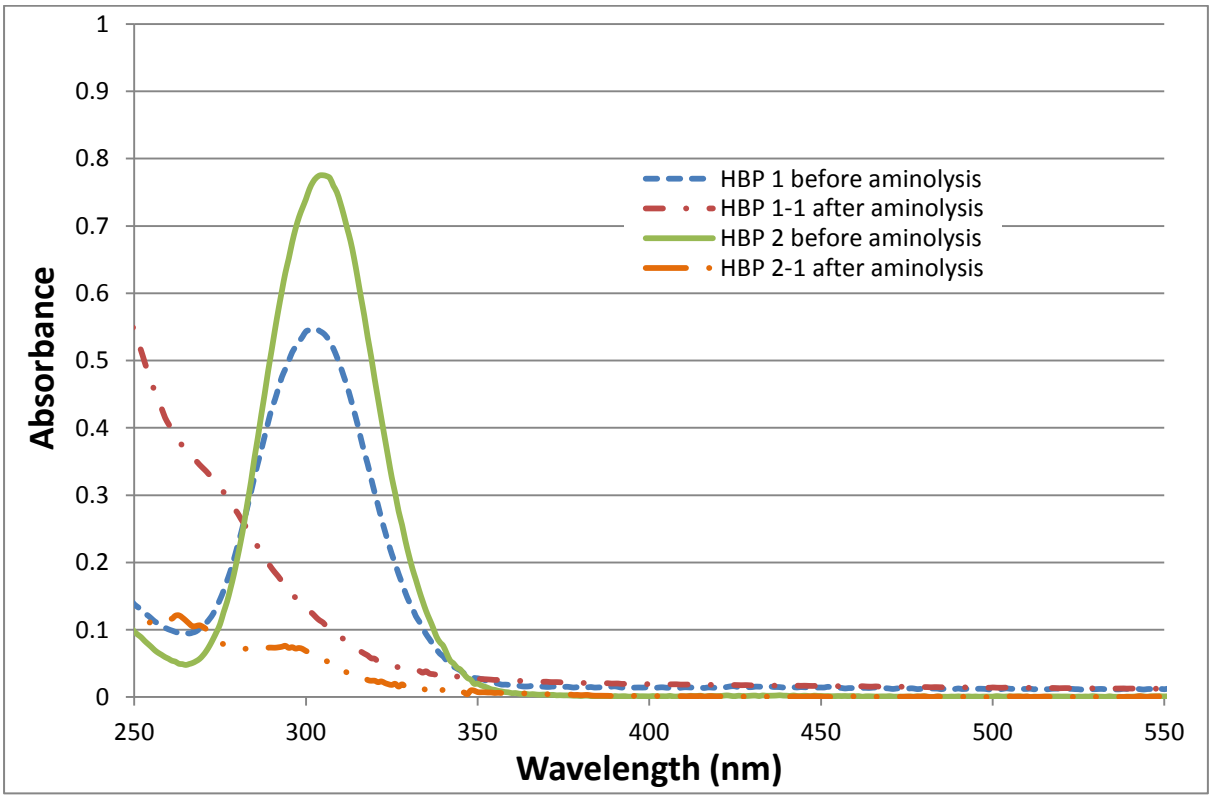


Figure 3. UV-Vis spectra of HBPs before and after aminolysis of trithiocarbonate groups with hydrazine.

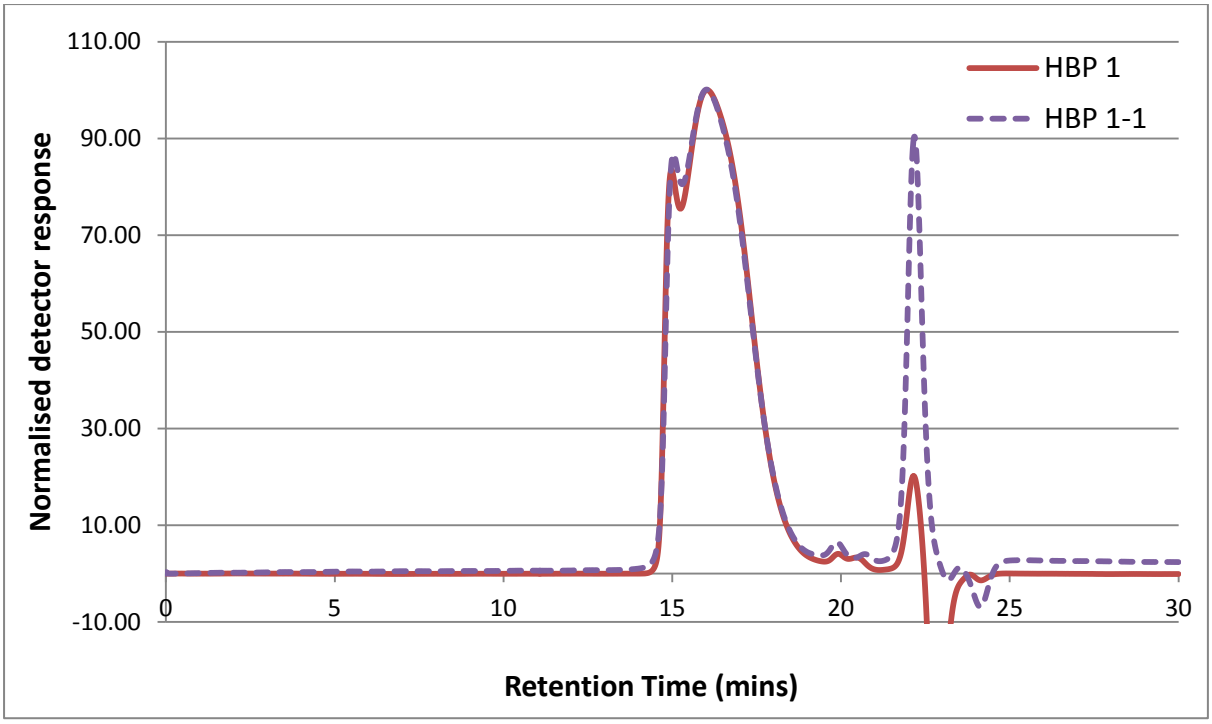


Figure 4. SEC spectra of HBPs before (**HBP 1**) and after aminolysis (**HBP 1-1**). No evidence can be seen for the formation of high molecular species due to intermolecular disulfide coupling.

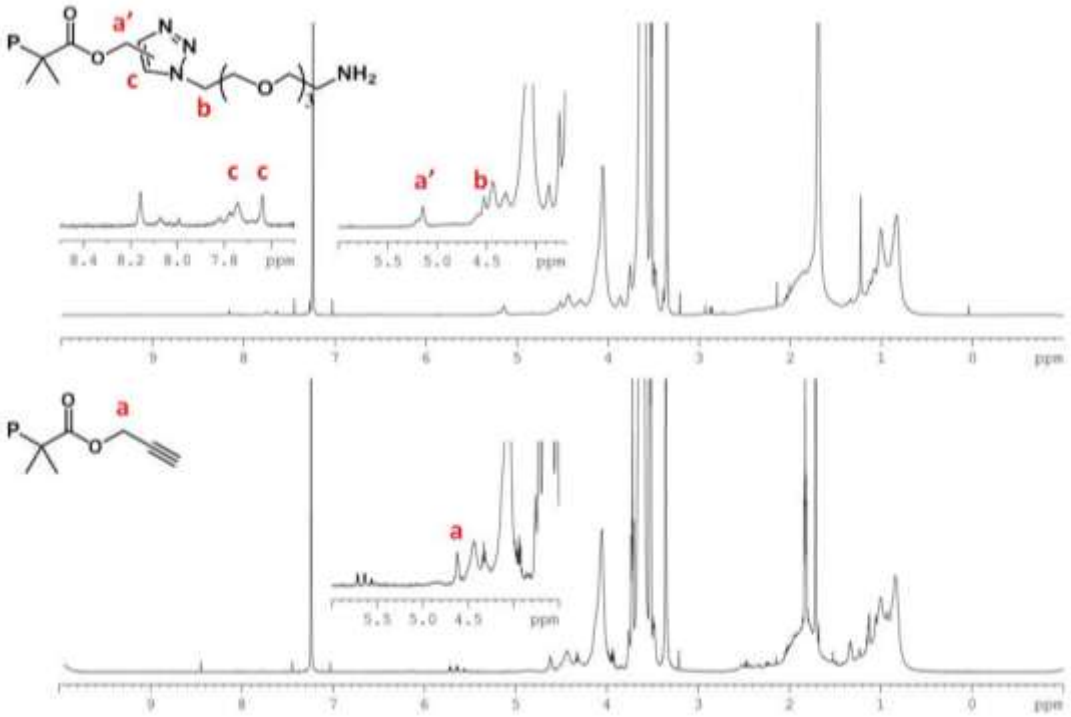


Figure 5. ^{1}H NMR of **HBP 1-1** overlaid with **HBP 1-2**, demonstrating attachment of azido-PEG-amine spacer. Characteristic peaks in **HBP 1-2** appear as multiplets, due to the presence of both regioisomers of the triazole ring being synthesised.

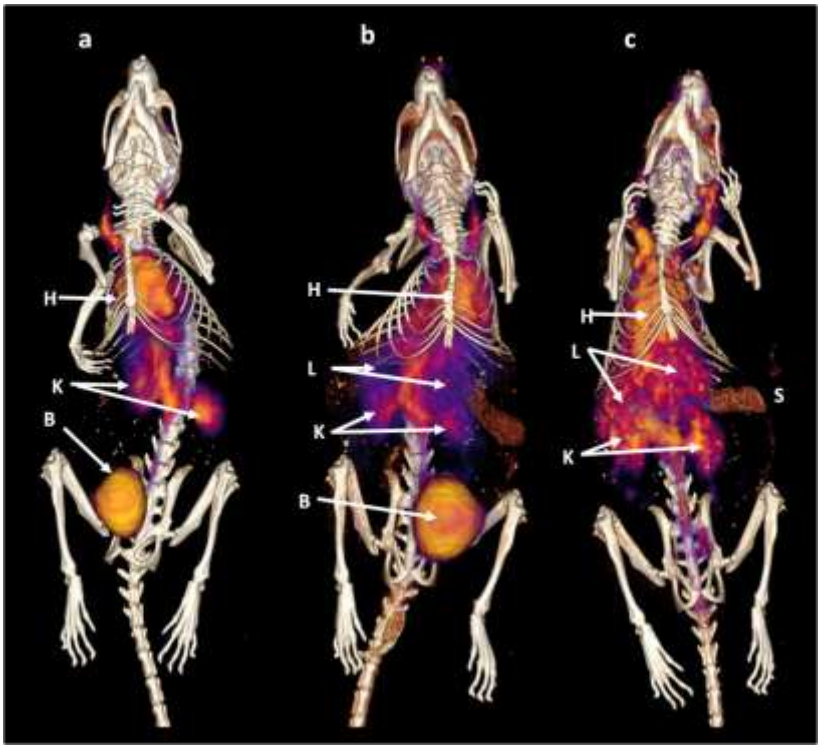


Figure 6. PET/CT images of healthy C57 BL/6 injected with **HBP 1** showing polymer biodistribution a) immediately PI, b) 3 hrs PI and c) 18 hrs PI.

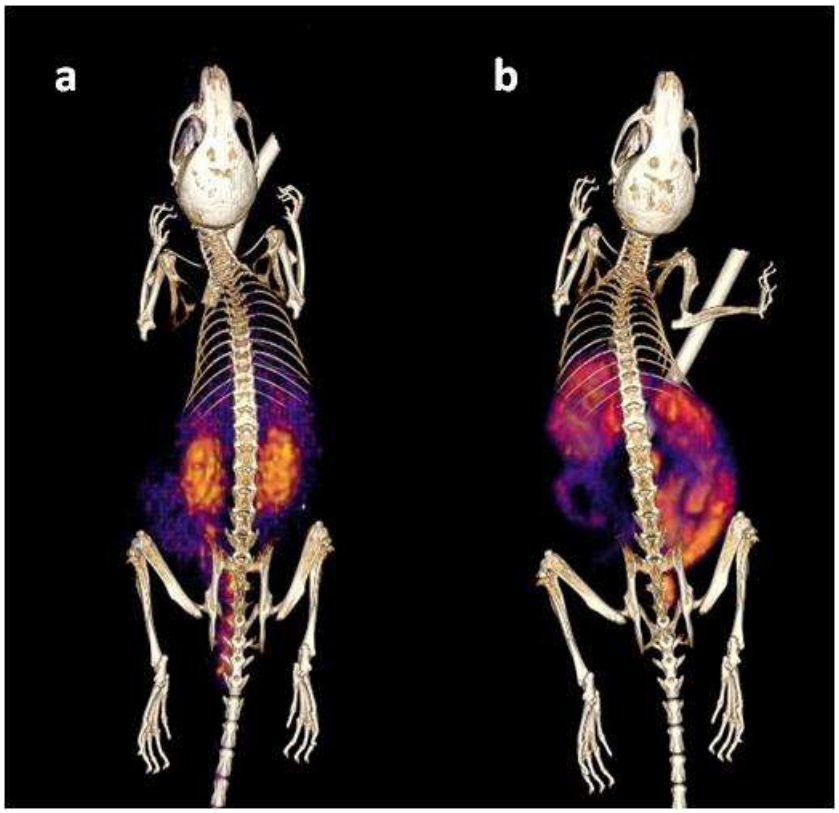


Figure 7. PET/CT images of B16 melanoma bearing C57 BL/6 mice 24 hours post injection with a) ^{64}Cu-NOTA-NCS and b) ^{64}Cu-acetate.