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Supplementary Information

Passive Tumour Targeting and Extravasation of Cylindrical Polymer Brushes in Mouse Xenografts

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S1. Materials and characterisation methods

Materials and reagents.

Poly(ethylene glycol) methyl ether methacrylate (PEGMA, average M_n=300 g·mol^{-1}), glycidyl methacrylate (GMA, 97%), *N,N,N′,N″,N‴*-pentamethyldiethylenetriamine (PMDETA, 99%), copper(I) bromide (CuBr, 98%), propargylamine (98%), dry dimethyl sulfoxide (DMSO) and anisole were obtained from Sigma-Aldrich. Triethylamine (TEA, 99%) was purchased from Merck. Alexa Fluor 488 *N*-hydroxysuccinimide (AF488-NHS) was purchased from Invitrogen (Australia). 4-Azidosalicylic acid (ring-5-3H) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). Deuterated chloroform ($CDCl_3$) and dimethyl sulfoxide (d-DMSO) were purchased from Cambridge Isotope Laboratories, Inc. HT1080 human adenocarcinoma cells were purchased from American Type Culture Collection (ATCC) (Salisbury, U.K.). MEM media was purchased from Sigma Aldrich (NSW, Australia). All other cell culture reagents were from Gibco® and purchased from Life Technologies (VIC, Australia) including 75 cm^2 flasks, non-essential amino acids (NEAA), Dulbecco's Phosphate Buffered Saline (DPBS) without $CaCl_2$ or $MgCl_2$, Glutamax, fetal bovine serum (FBS), penicillin-streptomycin and 0.25% trypsin-EDTA. Isoflurane inhalation anaesthetic was supplied by Provet Pty Ltd. (VIC, Australia). Heparin sodium (10000 U/mL) was purchased from Hospira Australia Pty. Ltd. (VIC, Australia) and sterile saline 0.9% in polyethylene bags from (Baxter Healthcare Pty Ltd., NSW, Australia). Tissue analytical reagents included Soluene-350 tissue solubiliser (Perkin Elmer, WA, Australia), isopropyl alcohol (Burdick and Jackson, Muskegon, MI) and 30% hydrogen peroxide (Ajax Finechem Pty Ltd. NSW, Australia). Irga Safe Plus liquid scintillation cocktail was purchased from Perkin Elmer (WA, Australia). High purity (Milli-Q) water with a resistivity of >18.2 MΩ·cm was obtained from an inline Millipore RiOs/Origin water purification system. All chemicals were used as received. All monomers were passed through a short silica gel column prior to polymerisation to remove the inhibitor.

Characterisation Methods.

Nuclear magnetic resonance (^{1}H NMR) spectra were recorded in deuterated solvents using a 300 MHz Bruker system at 25 °C. ***Gel permeation chromatography*** (GPC) measurements were performed on an UFLC Shimadzu Prominence GPC system using DMAc/LiBr and a flow rate of 1 mL min^{-1} at 50°C. Samples (8 g·L^{-1}) were dissolved and forced through a 450 nm PTFE filter prior to injection. ***FT-IR*** measurements were performed on a Perkin Elmer Spectrum Two spectrometer. ***Fluorescence spectroscopy*** was performed on a Perkin Elmer LS55 Fluorescence spectrometer (excitation wavelength: 470 nm). Non-contact mode ***atomic force microscopy*** (AFM) imaging was performed in air using a Cypher S (Asylum Research) with tapping-mode cantilevers (48 N·m^{-1}, Tap190Al-G, Budget Sensors, Bulgaria). Prior to AFM measurement, freshly cleaved mica was dipped into a solution of CPBs (0.25 g·L^{-1}) and blown dry under a stream of nitrogen. ***Dynamic light scattering*** was performed on aqueous CPB suspensions (0.2 g·L^{-1}) using a Malvern Zetasizer Nano ZS. ***Confocal microscopy*** images were captured on a Leica TCS SP8 Confocal running version 1.9 of LAS X using a 63x PL APO CS2 NA1.4 objective.

S2 Synthesis of cylindrical polymer brushes (CPBs)

CPBs were synthesised through the grafting-from approach using atom transfer radical polymerisation (ATRP). The polymer backbones used in this study have been synthesised and reported before.[1,2] The detailed description of the grafting procedure from a polyinitiator backbone can be found below. Figure S2 gives a summary of the synthesis route. Table S2 gives an overview of the synthesised CPBs used in this study.

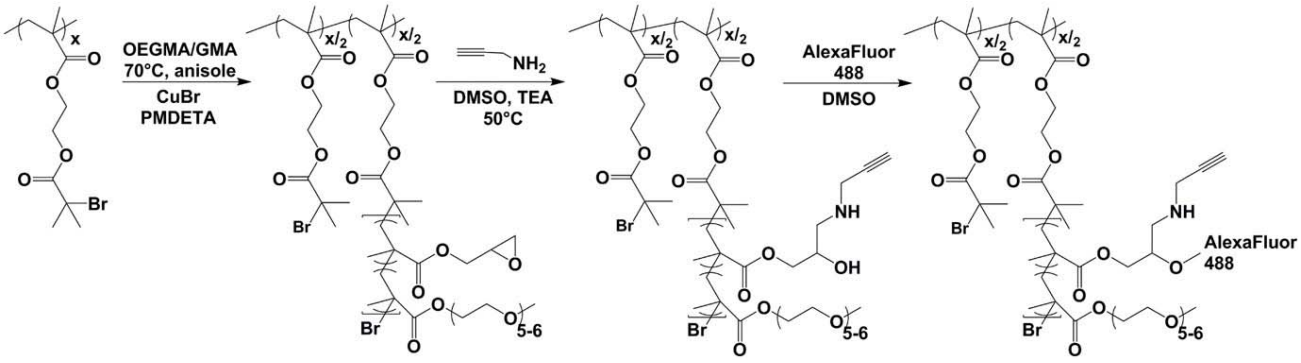

Fig. S2 Overview of the grafting process and post-polymerisation modifications.

Synthesis of polyinitiator backbones. The poly(2-(2-bromoisobutyryloxy)ethyl methacrylate) (PBIEM) polyinitiator backbones have been synthesised according to published protocols. Briefly, polymer backbones with different lengths were produced by either ATRP (to yield a backbone with a low degree of polymerisation (DP)) or anionic polymerisation (to achieve backbones with a high DP). Polymer backbones made from poly(2-(trimethylsiloxy)ethyl methacrylate) (PTMS-HEMA) can be transformed into polyinitiator backbones after a deprotection and esterification step. We have previously published the synthesis and characterisation of the long polymer backbones (DP= 2700 and 7500) as well as the short backbone (DP = 112) used in this study.[1, 2]

Synthesis of cylindrical polymer brushes. 5 mg of $PBIEM_x$ (x = 112, 2700 or 7500) was dissolved in 8.5 mL anisole. Subsequently, 2.5 mL of a mixture of PEGMA and GMA and 3.1 mg PMDETA were added and the mixture was deoxygenated by bubbling nitrogen (N_2) for 60 min. Meanwhile, 2.8 mg CuBr and a stir bar were added into a Schlenk flask and evacuated under high vacuum. The ratios of [PBIEM]:[PEGMA]:[GMA]:[PMDETA]:[CuBr] were 1:425:75:1:1. After bubbling, the mixture was transferred into the N_2-flushed Schlenk flask via syringe and let to stir at 70 °C. The polymerisation was stopped after 120 min, cooled to room temperature and exposed to air. The solution was then pushed through a short silica gel column. CPBs were purified through dialysis in acetone after precipitation into cold hexane. CPBs had to be immediately re-dissolved in acetone after precipitation. Dried CPBs were not able to be re-dissolved. To calculate the brush composition we used the monomer conversion at 120 min and previously reported grafting efficiencies of ~50 % for methacrylates (such as PEGMA, DMAEMA, etc.) from PBIEM.[3, 4]

Alkyne modification. 30 mg of a GMA-containing CPBs were dissolved in 5 mL dry DMSO. Subsequently, 10 µL propargylamine and 100 µL TEA were added. The reaction mixture was allowed to stir for 2 days at 50 °C. For purification, the reaction mixture was dialysed into acetone and subsequently into ethanol (95%).

AF488 labeling. 30 mg of alkyne-modified CPBs (in acetone) were precipitated into cold hexane and immediately re-dissolved in 5 mL dry DMSO. Then, 10 µL AF488-NHS was added and the reaction was stirred overnight. For purification, the reaction mixture was dialysed into acetone, concentrated under reduced pressure and finally dialysed into Milli-Q water. All solutions were stored under refrigeration.

Table S2. Overview of synthesised CPBs, including their molecular weight (MW), dispersity (Đ) and lengths

CPB name	CPB composition	MW (t/mol)	Đ	Length (nm)
CPB-AR1	$PBIEM_{56}$-*co*-$[PEGMA_{133}$-*co*-$GMA_{19}]_{56}$	2.4	1.18	56 ± 12
CPB-AR4	$PBIEM_{1350}$-*co*-$[PEGMA_{94}$-*co*-$GMA_{12}]_{1350}$	40.7	1.15	200 ± 50
CPB-AR19	$PBIEM_{3750}$-*co*-$[PEGMA_{102}$-*co*-$GMA_{14}]_{3750}$	123.3	1.16	1050 ± 150

Composition and MW were calculated from ^{1}H NMR using reported grafting efficiencies (~50%) for methacrylic monomers from PBIEM backbones. Đ was obtained from GPC measurements in DMAc. Length were determined from AFM images and are presented in averages ± average absolute deviation.

Radiolabelling. 4-Azidosalicylic acid [ring-5-3H] (10 µL as purchased, nominally 10 µCi based on quoted concentration of stock [1 Ci·L^{-1} in ethanol]) was added while stirring to CPBs suspended in Milli-Q water (5 mL). CPB concentrations were in the range of 5 g·L^{-1}. Copper sulphate (700 µg) and sodium ascorbate (1 mg), both as solutions in Milli-Q water, were added and stirring was continued overnight. Unreacted 4-azidosalicylic acid and copper catalyst were removed from the reaction mixture by dialysis against ethanol followed by concentration (by evaporation) and dialysis into Milli-Q water. All solutions were stored under refrigeration. Prior to use, samples were diluted to a desired concentration with Milli-Q water. Sodium chloride was added to yield an isotonic solution.

S3 Animal details, and *in vivo* studies and characterisation

Animals. Nude Balb/c mice (female, 5-6 weeks of age) were purchased from Animal Resource Centre (WA, Australia). Animals were housed on a 12 h light/dark cycle and were provided food and water at all times. All experiments were approved by the Monash Animal Ethics Committee, Monash University (Melbourne, Victoria).

Cell culture. HT1080 cells (human adenocarcinoma) were purchased from ECACC (Salisbury, U.K.). The cells were grown in 75 cm^2 flasks in MEM supplemented with 2 mM Glutamax, 1% NEAA, 10% FBS and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 and 37 °C. Cells were passaged 1:5 using 0.25% trypsin-EDTA 3 times per week. Cell lines tested negative for mycoplasma contamination.

Biodistribution of CPBs in nude mice. HT1080 cells were used between passage 5 and 10. For induction of solid tumours into nude mice, cells were passaged using trypsin-EDTA, washed once in dPBS and resuspended to 5×10^7 cells/mL in dPBS. 2.5 million cells (in a final volume of 50 µL) were injected in to the left flank of mice (n= 6-7 mice for each compound) using a 25G needle. Tumour growth was monitored with a pair of callipers every 2 days (**eq. 1**). When tumours reached approximately 100 mm^3, mice were injected with 0.2 mg of either ^{3}H-labelled CPB-AR1, CPB-AR4 or CPB-AR19 intravenously via the tail vein using a 0.5 mL insulin syringe fitted to a $29^{1/2}$ gauge needle. After 2 days, prior to euthanasia by cervical dislocation, mice were anaesthetised and blood sample was collected by cardiac puncture. A 0.05 mL volume of heparinised whole blood was weighed and transferred to a 20 mL scintillation vial for further processing. Tumour, liver, spleen, kidneys, heart, lungs, muscle (from the right thigh) and fat (from the right dorsal region) were excised after death, weighed and collected in 20 mL scintillation vials (the weight of whole liver was noted and only one lobe of liver weighing ~ 0.1 g was taken for further processing). Similarly the blood and organs from non-dosed animal were also collected and processed to provide background counts. All tissues and blood samples were solubilised in 4 mL of 1:1 v/v soluene:isopropyl alcohol by overnight heating at 60°C as described previously.[5] Tissue samples were then bleached with 0.4 mL of 30% v/v hydrogen peroxide followed by addition of 10 mL scintillation cocktail, vortexed and kept at 4°C for 96 h until counted for [^{3}H] activity using Packard Tri-Carb 2000CA liquid scintillation counter (LSC) (Meriden, CT).

$$\text{Volume } (mm^3) = \frac{4}{3}\pi(a \times b^2) \qquad (1)$$

Extravasation and tumour penetration of CPBs by confocal microscopy. HT1080 tumours were induced in 10 nude mice (n=3 for each CPB and 1 blank) as described in the above section. Tumour growth was monitored with a pair of callipers every 2 days (**eq. 1**). When tumours reached approximately 100 mm^3, mice were injected with 0.2 mg of either AlexaFluor 488 labelled CPB-AR1, CPB-AR4 or CPB-AR19 intravenously via the tail vein. After 2 days, mice were euthanized by cervical dislocation and tumours excised carefully for further processing. Whole tumour was fixed in 3% v/v paraformaldehyde-PBS solution for 24 h, immersed in a cyropreservative solution (30% w/v sucrose in PBS) overnight (or until tumours have sunk to the bottom of the vessel) at 4 °C, implanted in Tissue Tek O.C.T. (Sakura Finetek, Torrance, CA) and stored at -80 °C until sectioning. The O.C.T. embedded tumour sample was then cut into 10 µm coronal sections with a cryostat, thaw-mounted onto super Frost plus® slides (Menzel-Glaser, Braunschweig, Germany) and desiccated at room temperature. Tumour sections were immunostained for blood vessels using a CD 31 antibody. Briefly, tumour slices were blocked with blocking buffer (5% v/v normal donkey serum in 0.1M PBS containing 0.1% v/v triton-X 100) in a humidified chamber for 1 h. Sections were then incubated with rat anti-mouse CD 31 (1:100, BD Biosciences, NSW, Australia) in blocking buffer at 4°C overnight. After incubation with the primary antibody,

sections were washed three times with 0.1M PBS and incubated with donkey anti-rat Alexa Fluor® 647 or donkey anti-rat Alexa Fluor® 594 secondary antibody (1:500) in 0.1M PBS for 1 h at room temperature. After further washing, sections were stained with DAPI (1:1000) for nuclear staining and mounted with fluorescence mounting media (Dako Australia Pvt Ltd, NSW, Australia), 'coverslipped', sealed and dried for 1-2 h at room temperature before imaged using a confocal microscope.

Image Capture. Images were captured on a Leica TCS SP8 Confocal microscope running version 1.9 of LAS X using a 63x PL APO CS2 NA1.4 objective. Excitation was as follows – 405nm for DAPI, 488nm for Green, 561 for AlexaFluor 594, 633 for AlexaFluor 647. Emission was captured in the following bands – 410-480nm for DAPI, 495-550 for Green, 570-620 for AlexaFluor 594 and 630-750 for AlexaFluor 647.

S4. NMR measurements

The NMR spectra were recorded in deuterated solvents by either dissolving freshly freeze-dried CPBs in $CDCl_3$ (this only worked for the shortest CPB, *CPB-AR1*) or by precipitating CPBs from acetone into cold hexane and swiftly re-dissolving them in d-DMSO. The DMSO samples were subsequently exposed to reduced pressure to remove most solvent impurities prior to measurement. This slightly complicated route was chosen as freeze-dried CPBs with longer backbones were not able to re-dissolve in NMR solvents. However, despite the presence of some solvents in the spectra, a qualitative assessment of the polymer was still possible.

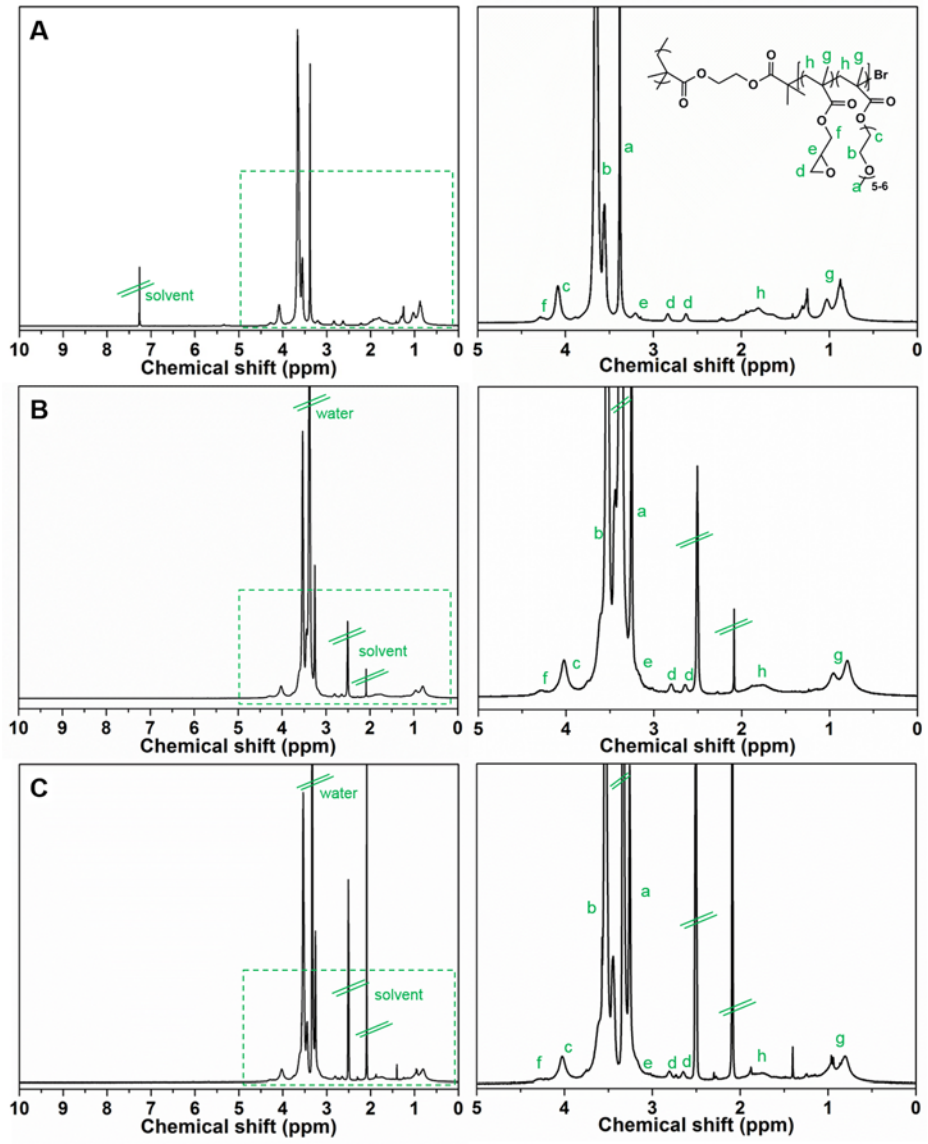

Fig. S4-1 NMR traces of all three CPBs: (A) CPB-AR1 in $CDCl_3$, (B) CPB-AR4 in *d*-DMSO and (C) CPB-AR19 in *d*-DMSO. The inset in (A) depicts the chemical composition of a polymer side chain and its assignment to the respective signals in the NMR spectra. Crossed out signals are assigned to solvent impurities.

After reaction with propargylamine, CPBs were purified and precipitated from acetone into cold hexane, and swiftly re-dissolved in d-DMSO. The DMSO samples were subsequently exposed to reduced pressure to remove most solvent impurities prior to measurement. Similar to above, this route was chosen as freeze-dried CPBs with longer backbones were not able to re-dissolve in NMR solvents. However, the loss of characteristic GMA peaks (yellow boxes) indicates a successful modification of the CPBs.

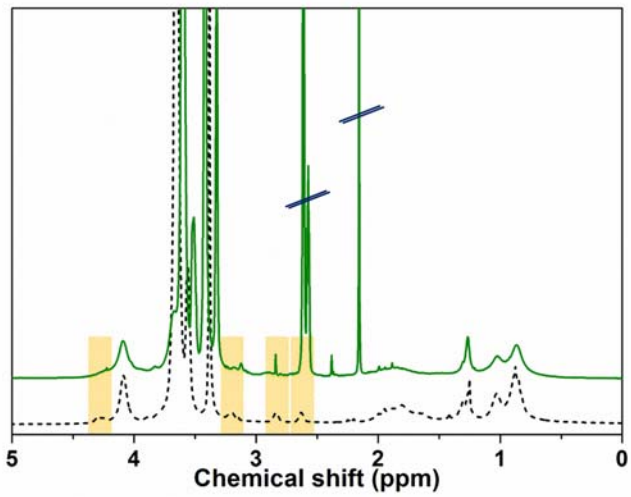

Fig. S4-2 NMR traces of CPB-AR1 in *d*-DMSO before (dashed black line) and after (solid green line) propargylamine modification. The yellow boxes highlight changes to the spectra. Crossed out signals are assigned to solvent impurities.

S5 GPC

GPC traces of all CPBs showed very narrow molecular weight distributions (Đ). Apparent molecular weights ($MW_{GPC,app}$) have been recorded as well, but have to be considered with caution, as they are derived from a polystyrene calibration curve.

Moreover, the polymer architecture and in particular the extremely high MW of these CPBs, complicate the sample preparation (e.g. filtering through 450 nm pores). Hence, we chose not to show the $MW_{GPC,app}$ values. However, the general trend of increasing molecular weight can still be seen from the decreasing elution time.

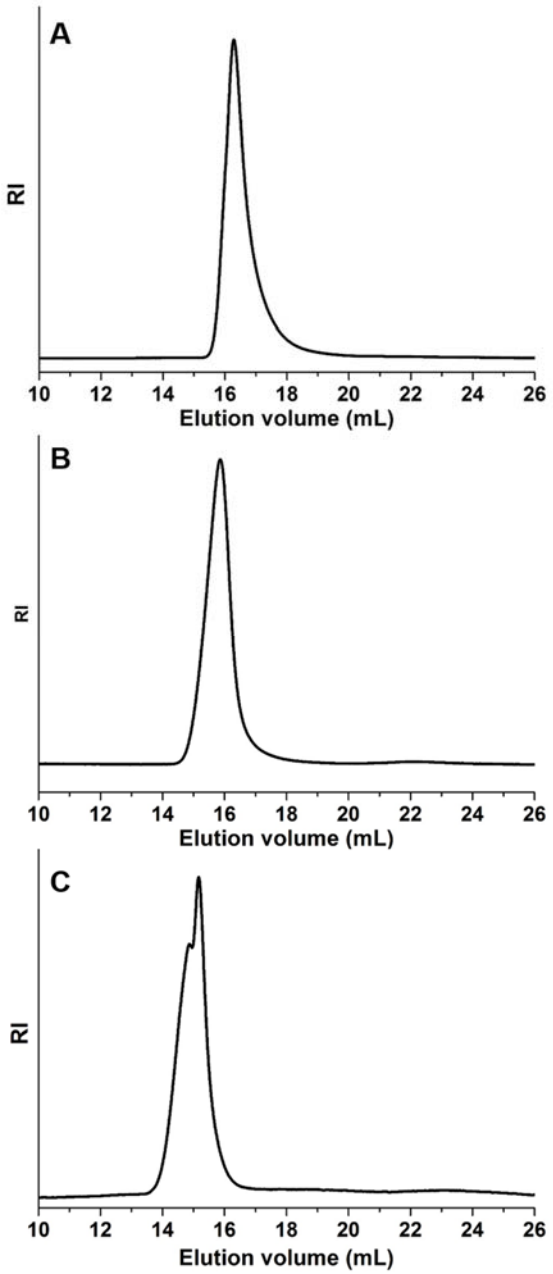

Fig. S5 GPC traces of all three CPBs in DMAc: (A) CPB-AR1, (B) CPB-AR4 and (C) CPB-AR19, respectively. Elution time decreases with increasing molecular weights from A→B→C.

S6 FT-IR

FT-IR spectra of freeze-dried CPBs before and after modification with propargylamine were recorded. Changes in the spectra around 3260 were attributed to the introduction of C-H stretch of carbon-carbon triple bonds after propargylamine modification. However, the carbon-carbon triple bond stretch (~2260-2100) was not detected. It is known that this stretch is rather weak and hence hard to detect in our polymer architectures. The attachment of radiolabels and AF488-dyes (attached to the ring-opened epoxy ring) are indication that alkyne functionality has been successfully introduced. This is in agreement with our previous study.[1]

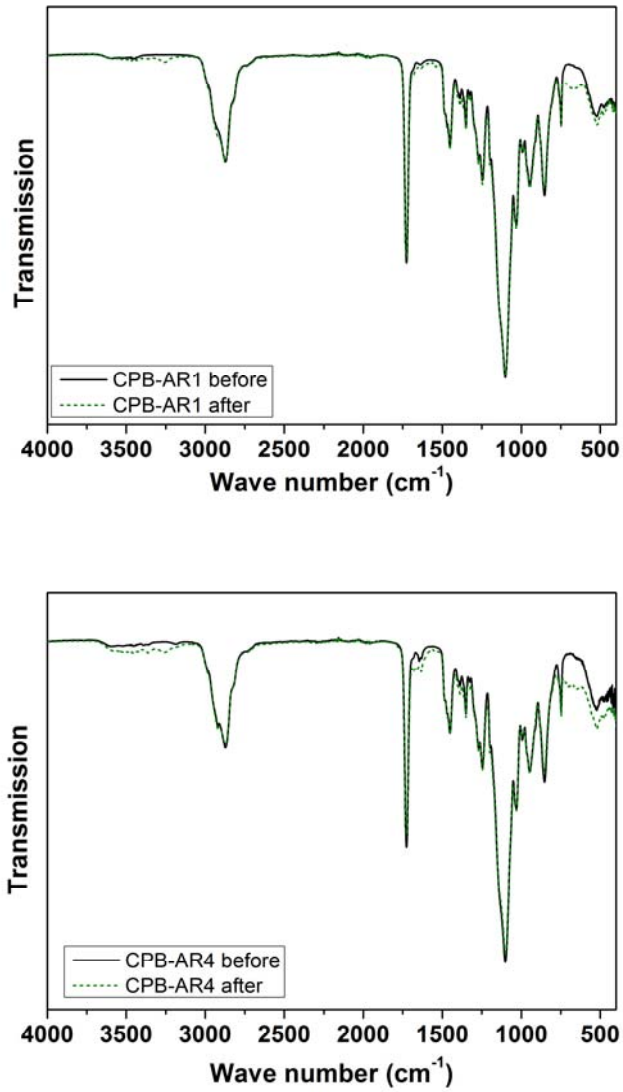

Fig. S6 Representative FT-IR spectra of CPB-AR1 (top) and CPB-AR4 (bottom) before (black solid line) and after (green dashed line) modification with propargylamine.

S7 Fluorescence spectroscopy

Fluorescence spectra highlight the successful attachment of AF488-NHS dyes to the hydroxyl groups of the ring-opened GMA units. While the pristine brush showed no fluorescence emission when excited at 470 nm, the dye-modified CPB solution showed increased fluorescence. The CPB solutions were dialysed (purified) in Milli-Q water prior to measurement.

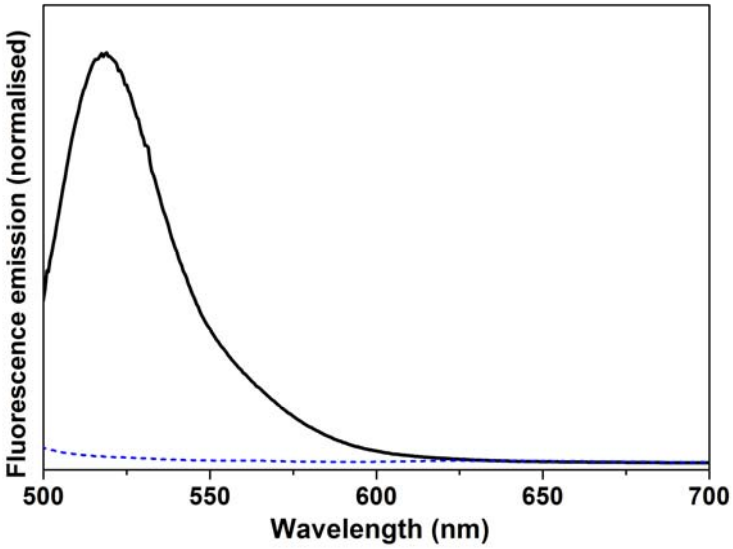

Fig. S7 Representative fluorescence spectra of CPB-AR4 before (blue dashed line) and after (black solid line) modification with AF488-NHS.

S8 AFM images and cross-sections

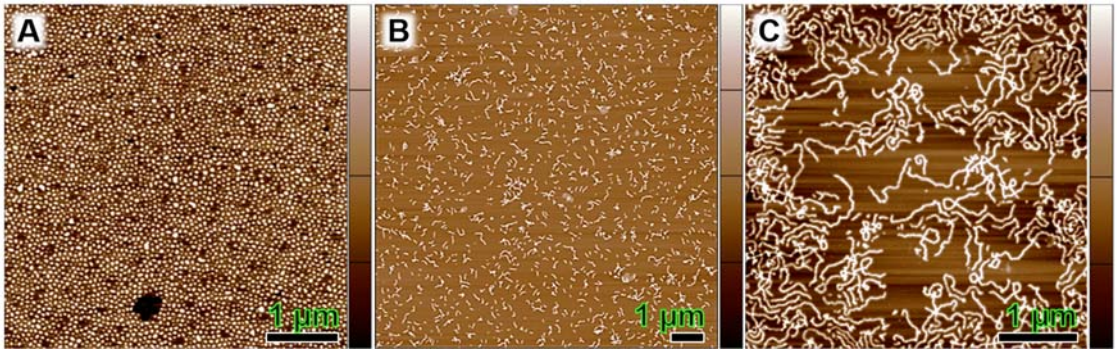

Fig. S8-1 AFM height images of CPBs deposited from chloroform on mica: (A) CPB-AR1, (B) CPB-AR4 and (C) CPB-AR19.

Samples were deposited on mica from CPB stock solutions (0.25 g L^{-1} in chloroform). The cross-sectional heights of all CPBs decrease due to drying and flattening on the substrate. However, the overall thicknesses of CPBs are comparable (55 ± 5 nm) - in particular when considering possible tip-sample convolution phenomena.

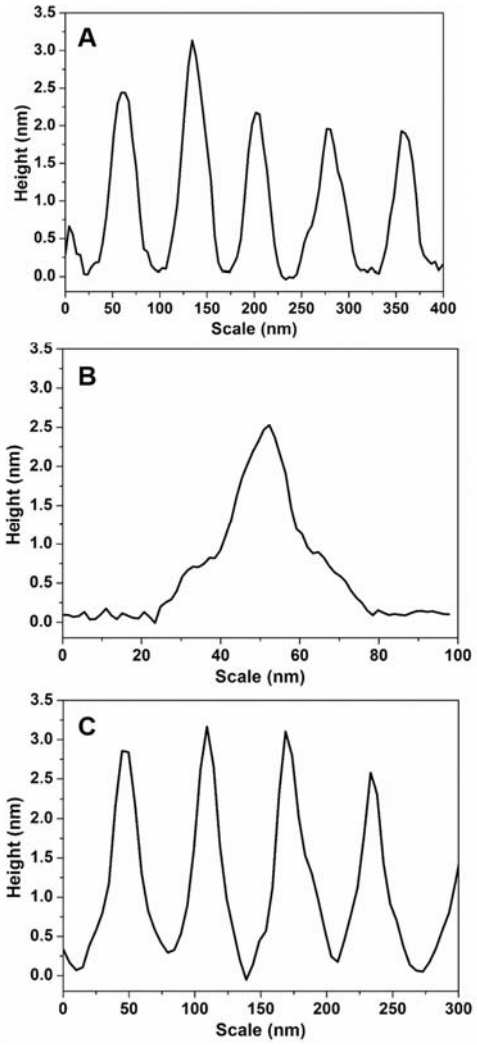

Fig. S8-2 Cross-sections of dried CPBs deposited from chloroform on mica: (A) CPB-AR1, (B) CPB-AR4 and (C) CPB-AR19, respectively.

S9 DLS

Dynamic light scattering investigation revealed that the PEGMA-based CPBs did not aggregate in aqueous suspension at concentration of 0.2 g·L^{-1}. Note that this method only presents apparent hydrodynamic diameters for shape-anisotropic nanoparticles.

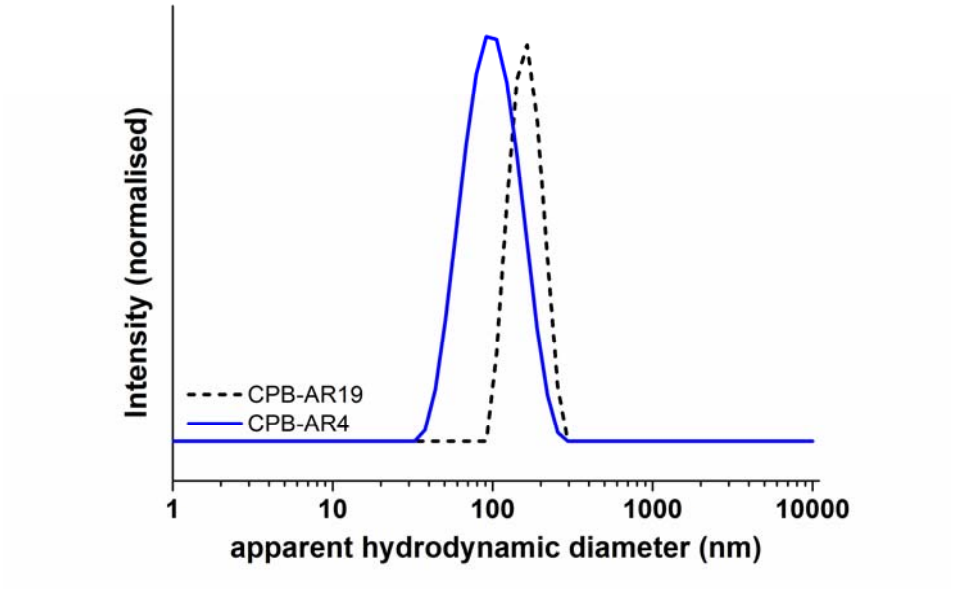

Fig. S9 DLS plots of aqueous CPB suspensions (0.2 g·L^{-1}): CPB-AR4 (blue solid line) and CPB-AR19 (black dotted line).

S10 Biodistribution

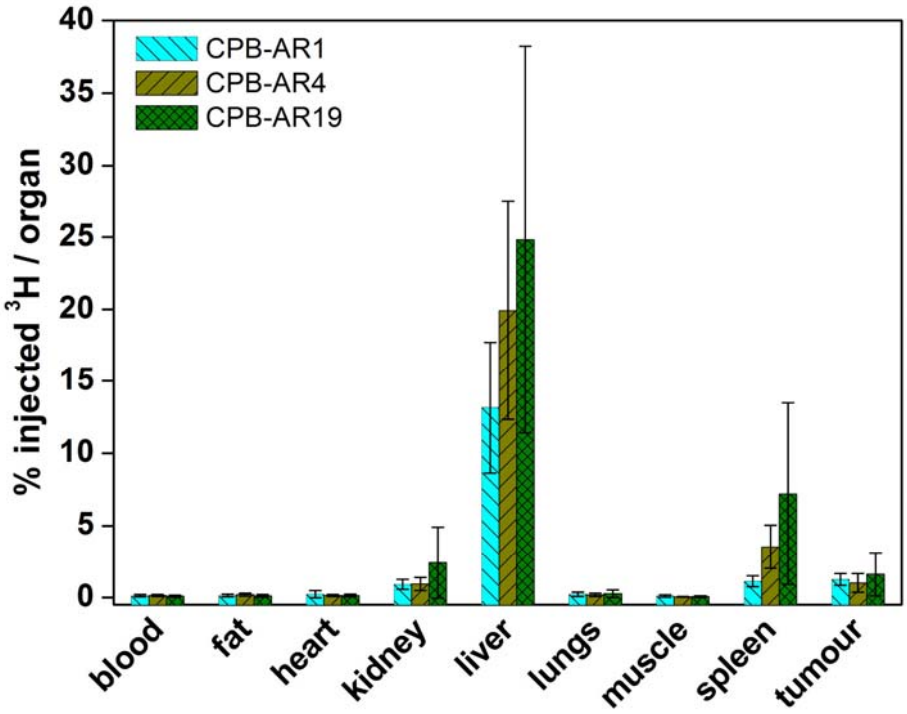

Fig. S10 Percentage of ^{3}H in organs after sacrifice at 48 h. Values are mean ± SD (n =6-7 mice).

S11 Extravasation

Image Analysis. The average distance of particles from blood vessels was assessed using the Fiji distribution of ImageJ as follows.[6] Channels were separated. A Gaussian blur (sigma=1) was applied to the particle channel to remove noise. A manual threshold was applied and converted to a binary mask. A Gaussian blur (sigma=2) was applied to the vessel channel to remove noise. The positive vessel signal was manually set to a threshold and converted to a binary mask. The mask was then used to generate a Euclidean distance map. The average intensity of each particle was measured in the Euclidean map and its intensity converted to distance (360 nm per intensity unit). The average distance of all particles from vessels was logged and saved.

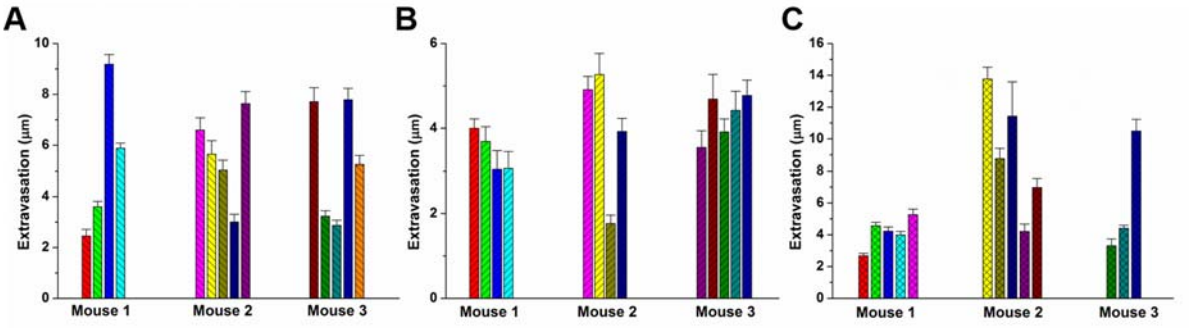

Fig. S11 Individual analyses of the extravasation of the CPBs: (A) CPB-AR1, (B) CPB-AR4 and (C) CPB-AR19. Each bar represents the analysis of a different field of interest on a tumour section. Values are mean ± SD (n = 3 mice).

References

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