Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

Aspect-ratio-dependent interaction of molecular polymer brushes and multicellular tumour spheroids

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S1 Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMA, average M_n=300 g·mol^{-1}), glycidyl methacrylate (GMA, 97%), N,N,N',N',N",N"'-hexamethyltriethylenetetramine (HMTETA, 97%), and anisole were obtained from Sigma-Aldrich. Sodium azide (99%) was received from AJAX. Ammonium chloride (NH_4Cl, 98%) was received from Scharlau. Copper(I) chloride (CuCl, 98%) was received from Merck. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. Alkyne-derivatives of Atto 488 and Atto 647 was purchased from Sigma Aldrich (Australia).

High purity (Milli-Q, MQ) water 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.

S2 Characterisation methods

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded in deuterated solvents using a 300 MHz Bruker Avance system at 25 °C. Size exclusion chromatography (SEC) measurements were performed on an UFLC Shimadzu Prominence GPC system running using DMAc/LiBr and a flow rate of 1 mL min^{-1} at 50 ^{o}C. Samples (<1 g L^{-1}) were dissolved and pressed through a 450 nm PTFE filter prior to injection. Fluorescence spectra of aqueous MPB solutions (1 g·L^{-1}) were recorded on a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Cryogenic transmission electron microscopy (cryo-TEM) was performed on a FEI Tecnai 12 Bio-Twin microscope at 120 kV in bright field mode. Images were acquired using a CCD camera (Gatan). Vitrified samples were prepared using a FEI Vitrobot Mark IV (in 100 % humidity). 3 µL MPB solution was pipetted onto a plasma-treated holey carbon copper grid, blotted with filter paper and plunged into a -170 °C ethane/propane mixture. Vitrified samples were cryo-transferred using a Gatan cryo-holder with LN_2 cooling. The specimen temperature was maintained at -184 °C. Atomic force microscopy (AFM) imaging was performed in air using a Bruker Multimode-8 with tapping-mode cantilevers (48 N·m^{-1}, Tap190Al-G, Budget Sensors, Bulgaria). Prior to AFM measurement, freshly cleaved mica was drop-cast with an aqueous solution of MPBs (0.25 g·L^{-1}) and blown dry under a stream of nitrogen. Confocal light/laser scanning spectroscopy (CLSM) on spheroids was performed on a Leica SP5 confocal microscope with HC PL FLUOTAR 10.0x0.30 DRY (for spheroids) and HCX PL APO

CS 63.0x1.20 WATER (for monolayers) objective lens. Spheroid image acquisitions were performed with λ_{ex} = 891 nm (MP), and λ_{em} = 495 nm – 600 nm. Z-stacks were obtained with a slice thickness of 25 µm. Images were analysed using ImageJ.

S3 Synthesis of MPBs

MPBs 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 earlier.[1] The detailed description of the grafting procedure from a poly(2-(2-bromoisobutyryloxy)ethyl methacrylate) (PBIEM) polyinitiator backbone can be found below. Table S1 gives an overview of the synthesised MPBs used in this study.

Synthesis of polyinitiator backbones. The 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.

Synthesis of molecular 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 4.1 mg HMTETA were added and the mixture was degassed by three freeze-pump-thaw cycles (FPT) using nitrogen (N_2). Then, 1.8 mg CuCl was added to the Schlenk flask under a gentle N_2 stream and immediately evacuated to high vacuum after addition. The ratios of [PBIEM]:[PEGMA]:[GMA]:[HMTETA]:[CuCl] were 1:425:75:1:1. The Schlenk flask was let to stir at 68 ^{o}C. The polymerisation was stopped at appropriate time points (typically between 90 and 120 min), cooled to room temperature and exposed to air. The brush composition was calculated from the crude reaction mixture using the monomer conversions and previously reported grafting efficiencies of ~50 % for PEGMA from PBIEM.[2] To purify the brushes, the reaction mixture was pushed through a short silica gel column, precipitated directly into hexane

and dissolved immediately in acetone. Dialysis in acetone was used to remove remaining monomers. Note, dried MPBs were not able to be re-dissolved.

Post-modification of MPBs. 50 mg of a GMA-containing MPB solution in 4 mL DMF. Subsequently, 5 mg sodium azide and 5 mg ammonium chloride were added. The reaction mixture stirred for 18h at 55 °C. For purification, the reaction mixture was dialysed into acetone/methanol mixture and finally stored in acetone (at 4 °C).

MPB labeling via click chemistry. 30 mg of azido-MPBs (in acetone) were concentrated under a gentle N_2 stream, before re-dissolved in MeOH/MQ (1:1; v/v). Then, 10 µL Atto-alkyne was added and the reaction was stirred overnight at room temperature. For purification, the reaction mixture was dialysed into MeOH and then MQ (subsequent solvent changes over 3 days), and finally concentrated through solvent evaporation. All MPB solutions were stored under refrigeration at 4 °C.

Table S1. *Overview of synthesised MPBs*

MPB name	MPB composition[a]	Aspect ratio[b]	Molecular weight[a] [t·mol^{-1}]	Dyes per MPB[c]
MPB-sphere	PBIEM$_{56}$-*co*-PBIEM$_{56}$-*graft*-[PEGMA$_{128}$-*co*-GMA$_{19}$]	~1	2.33	~1.3
MPB-rod	PBIEM$_{1350}$-*co*-PBIEM$_{1350}$-*graft*-[PEGMA$_{90}$-*co*-GMA$_{12}$]	~4-5	39.5	~12
MPB-filament	PBIEM$_{3750}$-*co*-PBIEM$_{3750}$-*graft*-[PEGMA$_{95}$-*co*-GMA$_{12}$]	~25	115	~33

[a]Composition and MW were calculated from ^{1}H NMR using reported grafting efficiencies (~50%) for PEGMA from PBIEM backbones. [b]Estimated from AFM images. [c]Estimated from the initial ratio of Atto dye and MPB in each click reaction.

S4 Cell and spheroids culture

DLD-1 cells (colorectal adenocarcinoma cell line, obtained from ATCC) were grown in Advanced DMEM (ThermoFisher Scientific, Australia) supplemented with 2% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 (v/v) and 37 °C. The cells were routinely passaged until 80−90% confluence.

For DLD-1 multicellular spheroid preparation, DLD-1 cells were trypsinised and seeded at a density of 5,000 or 10,000 cells/well into a round-bottom 96-well plate (Costar 7007, Corning). Spheroids formed over a 72-h incubation period (total DMEM volume: 100 µL). PBIEM$_{56}$-*co*-[PEGMA$_{128}$-*co*-GMA$_{19}$]$_{56}$, PBIEM$_{1350}$-*co*-[PEGMA$_{90}$-*co*-GMA$_{12}$]$_{1350}$ or PBIEM$_{3750}$-*co*-[PEGMA$_{95}$-*co*-GMA$_{12}$]$_{3750}$ MPBs in MQ water were dispersed into DMEM media to various concentrations. MPB-DMEM suspension were dosed to individual wells (containing one spheroid each) to give different concentrations (0.25, 0.5 and 1.0 mg·mL^{-1}) and incubated for 22 hours in a total volume of 200 µL. The volume factions of MQ water to DMEM media was below 12%.

‘Control spheroids’ were treated with equal volumes of MQ water. Individual spheroids were harvested using a trimmed P200 pipette tip, transferred to a 1.5 mL Eppendorff tube and washed with PBS three times. The spheroids were gently transferred to Nunc 4-well chamber slides (ThermoFisher Scientific) containing 1 mL of PBS for CLSM imaging.

S5 Cryo-TEM

Cryo-TEM of the MPBs in water highlighted the transition from spherical/ellipsoidal to rod- and filament-like MPBs.

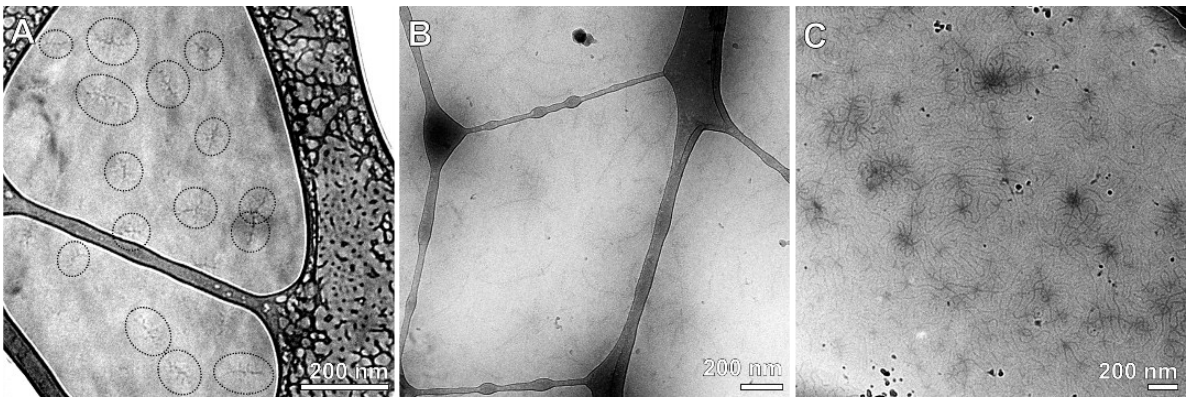

Fig. S1. Cryo-TEM images of A) sphere-like MPBs, B) rod-like MPBs and C) filament-like MPBs in MilliQ water. Scale bars are 200 nm.

S6 ^{1}H NMR

^{1}H NMR spectra were recorded in deuterated solvents by precipitating MPBs from acetone into cold hexane and swiftly re-dissolving them in $CDCl_3$. The solution was then evaporated under a gentle N_2 stream and re-dissolved in $CDCl_3$. Despite the presence of some solvents in the spectra, a qualitative assessment of the polymer was still possible. A representative NMR spectrum is shown in Fig. S1 below.

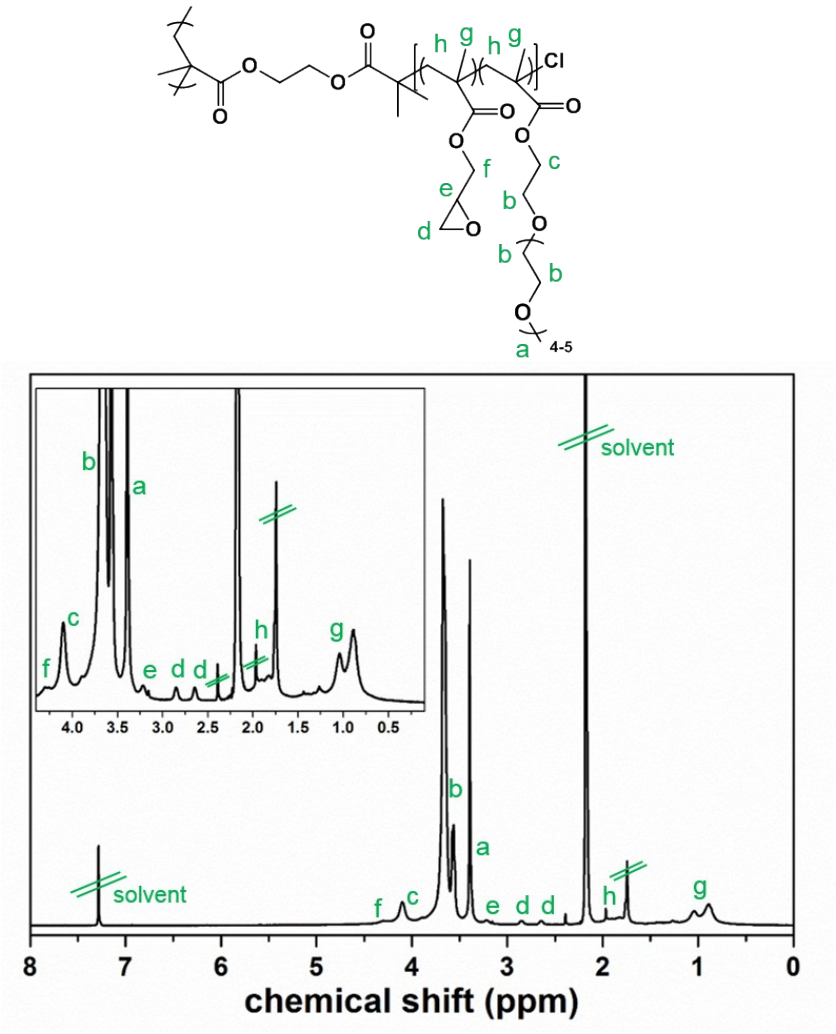

Fig. S2. HNMR of PBIEM$_{56}$-*co*-PBIEM$_{56}$-*graft*-[PEGMA$_{128}$-*co*-GMA$_{19}$] in $CDCl_3$.

S7 SEC

SEC did not indicate any brush-to-brush intermolecular coupling during polymerisation. SEC traces of all MPBs showed narrow molecular weight distributions. A small shoulder towards higher elution volumes was noticed. Using a linear PEGMA-*co*-GMA copolymer of comparable MW to the brush side chains revealed that the shoulder does not stem from free polymer side chains. The peak maximum of a $PEGMA_{95}$-*co*-GMA_9 was at 18.7 mL. A possible explanation for the shoulder in the MPB traces might be that some brushes have fragmented when pushed through the filter (450 nm pores) during SEC sample preparation.

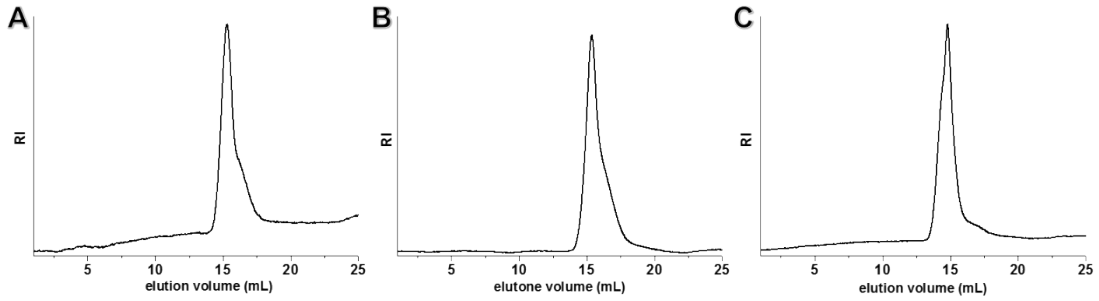

Fig. S3. SEC traces of azido-functionalised A) $PBIEM_{56}$-*co*-$PBIEM_{56}$-*graft*-[$PEGMA_{128}$-*co*-GMA_{19}], B) $PBIEM_{1350}$-*co*-$PBIEM_{1350}$-*graft*-[$PEGMA_{90}$-*co*-GMA_{12}] and C) $PBIEM_{3750}$-*co*-$PBIEM_{3750}$-*graft*-[$PEGMA_{95}$-*co*-GMA_{12}] in DMAc/LiBr.

S8 Fluorescence

Fluorescence spectroscopy underlined the effectiveness of click chemistry to functionalise MPBs with comparable amounts of fluorophores (Atto 488).

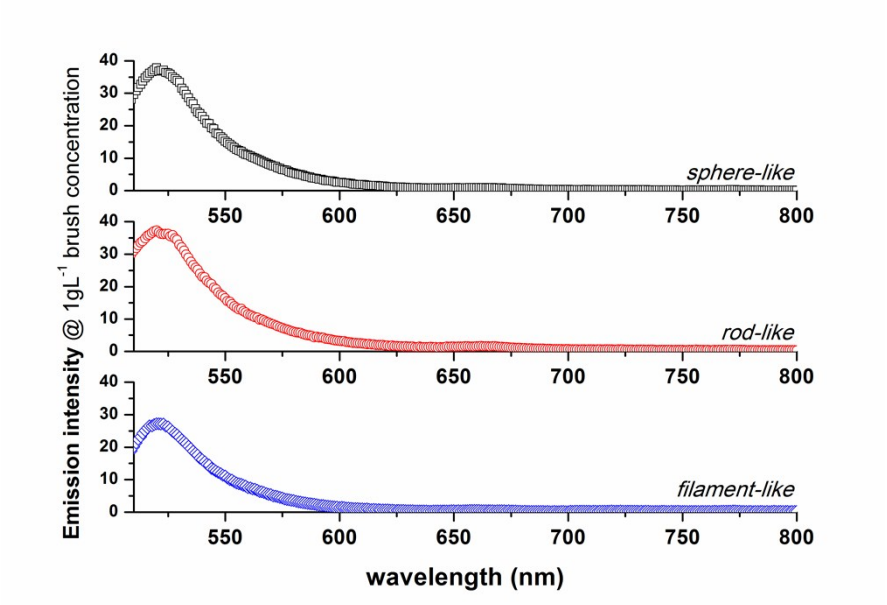

Fig. S4. Emission spectra of the three aqueous solutions of Atto 488 labelled MPBs. Solution concentration were 1 g·L^{-1}. Excitation wavelength was 488 nm.

S9 AFM cross-sections

Samples were deposited on mica from aqueous MPB solutions (concentration ~0.25 $g \cdot L^{-1}$). The cross-sectional heights of MPBs decreased due to drying and flattening on the mica substrate. The overall thicknesses of MPBs were comparable - when considering possible tip-sample convolution phenomena.

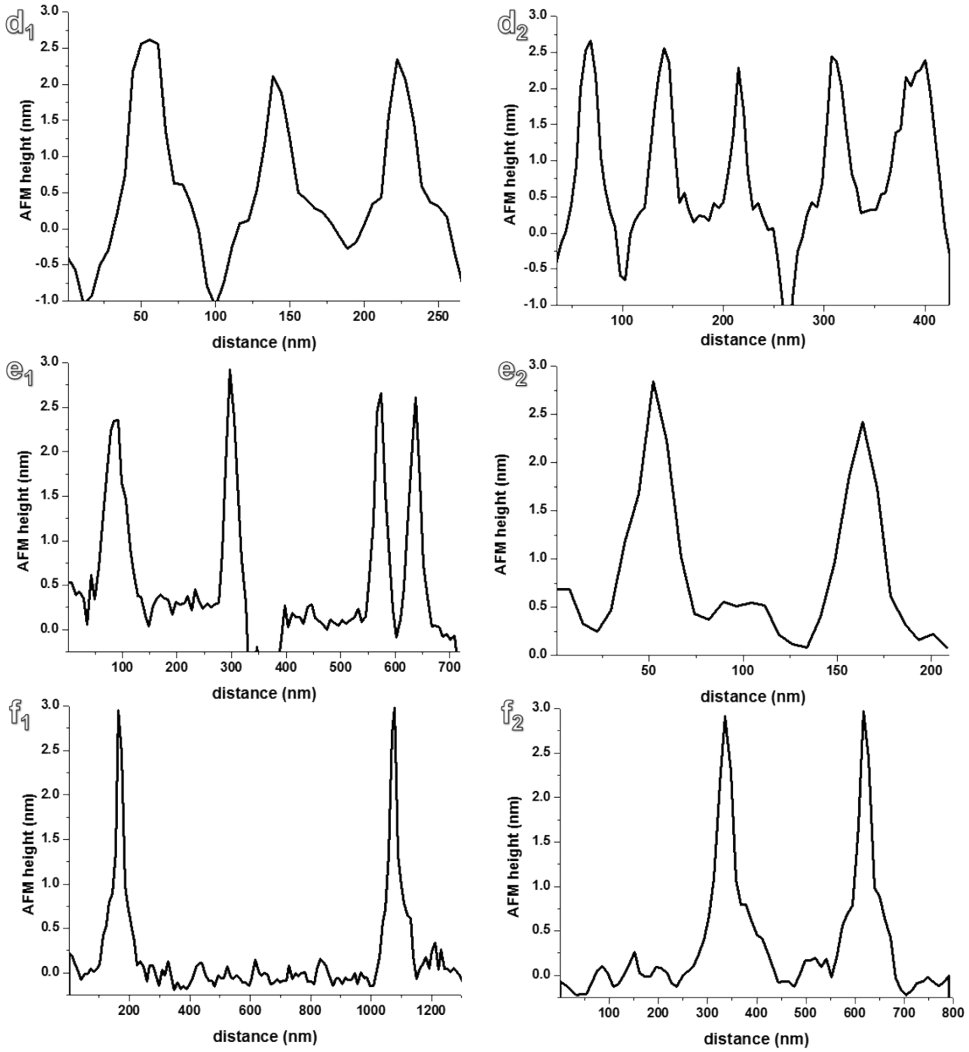

Fig. S5. AFM cross-sections of d) $PBIEM_{56}$-*co*-$PBIEM_{56}$-*graft*-[$PEGMA_{128}$-*co*-GMA_{19}], e) $PBIEM_{1350}$-*co*-$PBIEM_{1350}$-*graft*-[$PEGMA_{90}$-*co*-GMA_{12}] and f) $PBIEM_{3750}$-*co*-$PBIEM_{3750}$-*graft*-[$PEGMA_{95}$-*co*-GMA_{12}] on freshly cleaved mica (deposited from MQ water).

S10 Fluorescence intensity profiles

We used ImageJ to measure the fluorescence intensity across the indicated areas in the Figure 3G-I.

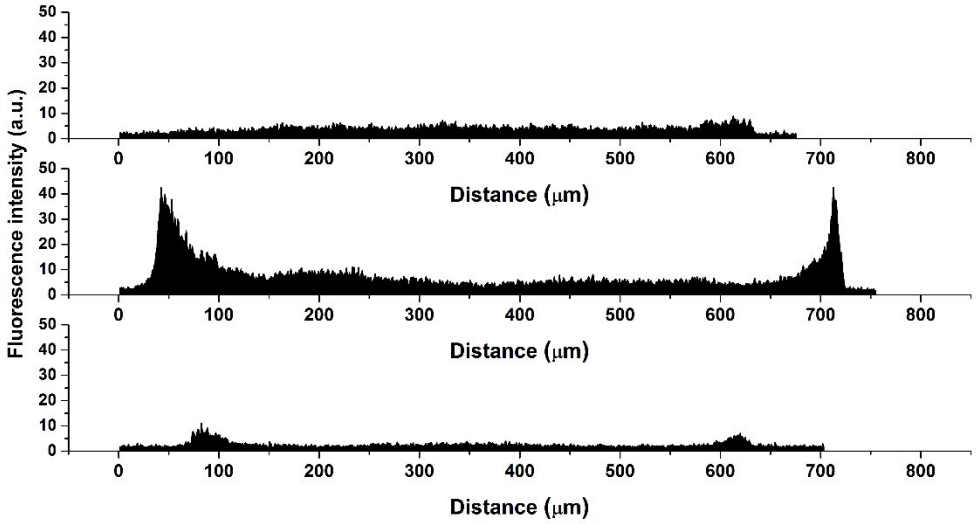

Fig. S6. Fluorescence intensity plots of the spheroid mid-plane cross-sections indicated in Figure 3G (top), 3H (middle) and 3I (bottom).

S11 Additional cell images

- 5K DLD-1 MCS

Additional analysis using smaller MCS (formed from 5×10^3 DLD-1 cells) have confirmed the trend observed for larger spheroids, where MPB rod outperformed spheres and filaments.

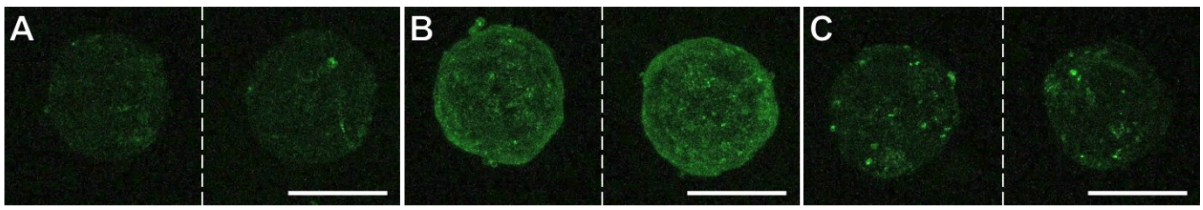

Fig. S7. CSLM images of 5K DLD-1 MCS. The individual panels show z-stack images of MCS incubated for 22h at a concentration of 1.0 $g\cdot L^{-1}$ of A) MPB-spheres, B) MPB-rods and C) MPB-filaments. Scale bars are 750 microns.

- DLD-1 monolayers

We used DLD-1 monolayers to assess the association of MPBs in 2D cell culture. To avoid effects of autofluorescence and interference with the NucBlue stain during imaging, we used MPBs that had been labelled with Atto647 (red fluorescence).

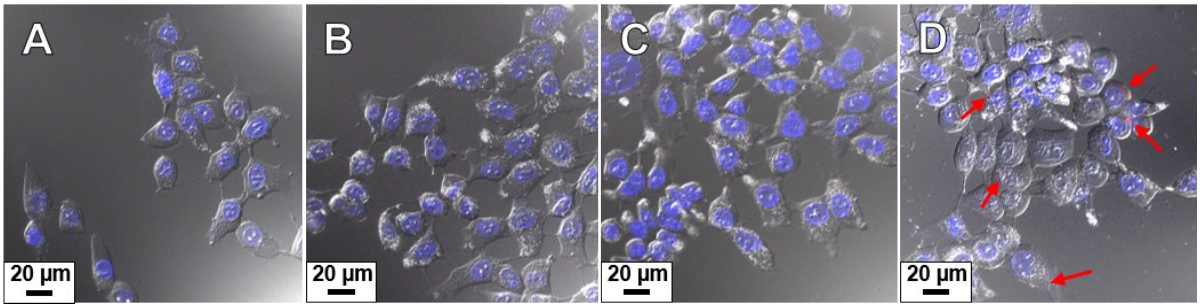

Fig. S8. DLD-1 monolayer images. The individual images show overlay images (DIC image, NucBlue, and Atto647 channels) of DLD-1 cells incubated with MPBs for 24h at a concentration of 0.5 $g \cdot L^{-1}$: A) 24h control (no brushes), B) MPB-spheres, C) MPB-rods and D) MPB-filaments. Scale bars are 20 microns. Red arrows indicate fluorescence (red channel) of associated MPBs.

- Association and penetration studies of $PBIEM_{1350}$-*co*-$PBIEM_{1350}$-*graft*-[$PEGMA_{90}$-*co*-GMA_{12}] (MPB-rods)

We studied the concentration dependence of the MPB-rod–spheroid interaction.

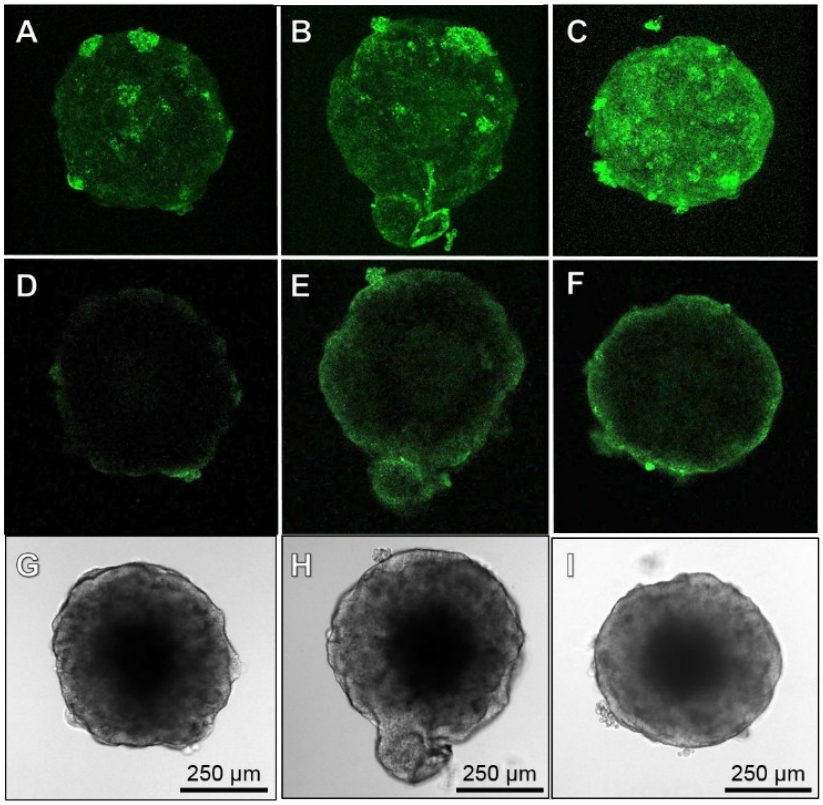

Fig. S9. Association and penetration studies using fluorescently labelled MPB-nanorods (green colour) $PBIEM_{1350}$-*co*-$PBIEM_{1350}$-*graft*-[$PEGMA_{90}$-co-GMA_{12}] at different concentrations: (A/D) 0.25 gL^{-1}, (B/E) 0.5 gL^{-1}, (C/F) 1.0 gL^{-1}. CSLM z-stack (A-C), cross-section (D-F) and DIC (G-I) images of DLD-1 MCS. Details: MCS formed from 5,000 cells over 3 days; incubation time ~ 24 h.

References

1. a) M. Müllner, T. Lunkenbein, J. Breu, F. Caruso and A. H. E. Müller, *Chem. Mater.*, 2012, **24**, 1802-1810; b) M. Zhang, T. Breiner, H. Mori and A. H. E. Müller, *Polymer*, 2003, **44**, 1449-1458.
2. a) Z. Zheng, M. Müllner, J. Ling and A. H. E. Müller, *ACS Nano*, 2013, **7**, 2284-2291; b) Y. Xu, S. Bolisetty, M. Drechsler, B. Fang, J. Yuan, M. Ballauff and A. H. E. Müller, *Polymer*, 2008, **49**, 3957-3964.