Supporting Information

Uniform Block Copolymer Nanofibers for the Delivery of Paclitaxel in 2D and 3D Glioblastoma Tumor Models.

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1. Supplementary Materials and Methods

General considerations

The synthesis and characterization of PFTMC$_{26}$-*b*-PDMAEMA$_{424}$ have been previously reported by Street et al. and Parkin et al.[S1,S2] The PFTMC homopolymer degree of polymerization was determined by matrix-assisted laser desorption/ionization mass spectrometry and the coronal-block degree of polymerization was determined by integrations of coronal block peaks in ^{1}H NMR relative to peaks of the PFTMC block. RAFT-CTA was dried via vacuum desiccation over phosphorus pentoxide prior to use. Paclitaxel was purchased from Sigma-Aldrich (Canada). All other reagents and solvents were purchased from Sigma-Aldrich (Canada), Combi-Blocks (USA), VWR (Canada), or Fisher Scientific (Canada) and

used without further purification. Solvents for self-assembly were HPLC grade and were filtered through PTFE, nylon, or cellulose filters with a pore size of 200 nm before use.

Instrumentation

Gel permeation chromatography (GPC)

Gel permeation chromatograms were acquired on a Malvern OMNISEC triple-detector (refractive index, UV-Vis photodiode detector, light scattering detector and viscometer) chromatograph. Prepared samples were of 1 mg/mL concentration in HPLC grade THF, and were filtered through a PTFE filter with a 200 nm pore size prior to measurement. The eluent used was *n*-Bu_4NBr/THF (1 % *v/v*) at a flow rate of 1 mL/min. Two columns were employed, the first of grade T3000 and the second of grade T5000, operated at 35 °C. Universal calibration was constructed using a polystyrene standard.

Preparatory Gel permeation chromatography (Prep GPC)

Preparatory gel permeation chromatography was performed on a Shimadzu Prep GPC equipped with a CBM-20A communications bus module, LC-20AP solvent delivery unit, SIL-10AP autosampler, CTO-40C column oven, SPD-40 UV-Vis detector, RID-20A refractive index detector, and FRC-10A fraction collector. An initial injection of polymer in THF (1 mL, 10 mg/mL) at a flow rate of 3 mL/min using HPLC grade THF as eluent was used to gather the retention times of the species in solution. Using this data, the fraction collector was calibrated to separate the desired peaks into separate vials. Subsequent injections (3 mL, 10 mg/mL, 3 mL/min) were repeated until the desired volume was collected. The resulting solutions were concentrated in vacuo to yield the final polymer.

Ultrasonication

Micelle sonication was carried out using a Fisherbrand 112xx series advanced ultrasonic cleaner (FB-11203), operated in sweep mode at 80 % power and 37 MHz at 10 °C.

Transmission electron microscopy (TEM)

TEM images were obtained on a JEOL 1011 microscope equipped with an 11 Megapixel CCD camera, operated at 80 kV. Nanoparticle solutions (1.5 μL, 1 mg/mL) were drop-casted onto a carbon-coated copper grid. Uranyl acetate solution (8 μL) in EtOH (3 wt %) was subsequently drop-casted on top, and the grids were left to dry overnight. Copper grids (400 mesh) were

purchased from Ted Pella. Carbon films (ca. 6 nm) were prepared by carbon sputtering mica sheets with a Leica ACE 600 carbon coater. The carbon films were deposited onto copper grids via floatation on water and the grids were allowed air dry.

For micelle length analysis, a minimum of 200 nanofibers were traced manually using the FIJI software package. The number average micelle length (L_n) or width (W_n) and weight average micelle length (L_w) were calculated using eq. S1-2 from the individual contour lengths (L_i) of the micelles. Here, N_i is the number of micelles of length L_i, and n is the number of micelles examined in each sample. The distribution of micelle lengths is characterized by $Đ = L_w/L_n$.

$$L_n = \frac{\sum_{i=1}^{n} N_i L_i}{\sum_{i=1}^{n} Ni} \qquad L_w = \frac{\sum_{i=1}^{n} N_i L_i^2}{\sum_{i=1}^{n} NiLi} \qquad \text{(eq. S1-2)}$$

Dynamic Light Scattering (DLS) Measurements and ζ-Potential Measurements

Dynamic light scattering (DLS) and ζ-potential experiments were carried out using a Malvern Zetasizer Pro. Aqueous samples for DLS were prepared at 50 μL of 1 mg/mL. The correlation function was acquired in real-time and analysed by Cumulant analysis. This process allowed the diffusion coefficients for the nanoparticles to be determined. These were expressed as the effective hydrodynamic radius (R_H) using the Stokes-Einstein relationship for coated spheres in water (Refractive Index = 1.33, Dispersant Viscosity = 0.887, Dispersant Dielectric Constant = 78.5) with core properties of polystyrene latex (Refractive Index = 1.590, Absorption = 0.010). For ζ-potential measurements, the Smoluchowski approximation was used. A minimum of five measurements per sample were taken, consisting of between 10 and 100 cycles per run. The average ζ-potential was calculated from the individual measurements taken.

2. Self-Assembly Procedures

The composition of all solvent mixtures is given as *v:v*. All nanofibers were ultimately transferred into Milli-Q® water *via* a modified procedure of the preformed-nanoparticle solvent-switch loading (PNSL) method as reported by Garcia-Hernandez et al.[S3] Modifications include slow drop-wise addition of water from a micropipette, followed by organic solvent evaporation over the course of 2 days. No filtration through a syringe filter was performed.

General self-nucleation procedure. A solution of diBCP dissolved in THF (unimer) (20 mg/mL – 200 mg/mL) was diluted with an appropriate amount of THF. Subsequently, unimer solution was slowly added via a micropipette into a selective solvent (EtOH) to yield solutions which had final diBCP concentrations between 1 mg/mL – 10 mg/mL with 20:80 THF:EtOH solvent ratios. Each solution was manually shaken for ~10 s, agitated using a vortex mixer for ~10 s, and heated to 70 °C for 3 h. The resulting length-disperse nanofibers were analyzed via TEM.

General preparation of seed nanofibers. Length disperse nanofibers (1 mg/mL – 10 mg/mL, 20:80 THF:EtOH) were sonicated for at least 3 h using a Fisherbrand 112xx series advanced ultrasonic cleaner (FB-11203) for 3 h at 10 °C in sweep mode at 37 MHz and 80% power. The resulting seed nanofibers were analyzed by TEM.

3. Cell culture assays

All cell work was performed in a biological safety cabinet (BSC) using sterile technique. All materials were autoclaved and disinfected with 70 % ethanol prior to being placed in the BSC. U87 MG human GBM cells (CRL-1573, ATCC) were cultured in Dulbecco's Modified Eagle Medium, high glucose (DMEM, Thermo Fisher), supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C and 5% CO_2. Media changes were performed every two days. Cells were passaged at ~80% confluency using 0.25% Trypsin-EDTA (15400054, Thermo Fisher) diluted in PBS. Cell counting was performed using the dye exclusion method with Trypan blue on an automated cell counter (DeNovix CellDrop FL). Reseeding of cells was performed at a density of 1.5×10^4 cells/cm^2. Cryopreservation was performed at 1×10^6 cells/mL density in CryoStor® CS10 cell freezing medium (STEMCELL Technologies). Vials were first placed in a Freezing container (Nalgene) and stored at -80 °C overnight before being transferred to liquid nitrogen.

2D cell culture. Solutions of PTX-loaded nanofibers were made up at 10 – 40% w/w (PTX/nanofibers). U87 MG cells were seeded in a black-walled 96-well plate in culture medium containing 10% FBS at a density of 10,000 cells/well and left to adhere for 24 h. All media was then aspirated and replaced with 100 µl of sample or control solutions in FBS-free DMEM. Non-loaded nanofibers or PTX-loaded nanofibers were used at concentrations ranging from 0.1 – 25 µg/ml. Free PTX or albumin-NP bound PTX (Abraxane®, Celgene) were used as positive controls. After 24, 48, and 72 h, media were aspirated and replaced with 10% alamarBlue™ in DMEM. Plates were incubated for 2 h, after which fluorescence readings were

taken directly from the plate using a microwell plate reader (λ_{ex} = 560/9 nm; λ_{em} = 590/20 nm). The absolute half maximal inhibitory concentration (IC_{50}) values were calculated for nanofibers and PTX content in the samples using GraphPad Prism 10 software by non-linear regression *via* fitting of a variable slope four-parameter function.

3D cell culture. Patient-derived GBM cells were generously provided by Dr. David Nathanson (UCLA, GS025), which have been modified with an amplified epidermal growth factor receptor.[S4] Single cells were expanded in suspension culture in DMEM/F12 media supplemented with 200 nM GlutaMAX™, 10 U/ml Penicillin/Streptomycin, B27 supplement w/o vitamin A (all Thermo Fisher) and 5 µg/ml heparin, 20 ng/ml FGFb and 20 ng/ml EGF (all Gibco). Cells were passaged every 7 days using 1x TrypLE (Life Technologies). Following passaging, single cells were seeded into 24-well AggreWell™ plates (STEMCELL Technologies), seeded at 900k cells/well, equivalent to 3000 cells/microwell, and a volume of 2 mL. Cells were distributed by gentle agitation, placed in an incubator at 37 °C, and left undisturbed for 72 h to induce spheroid formation. Exposure to PTX-loaded nanofibers, free PTX, or ABX was then performed by aspirating 1 mL from each well and gently adding 1 ml of sample solution at 2x concentration to achieve the desired final sample concentration. Samples analyzed 3 days after application.

Live/Dead staining of spheroids. GBM spheroids treated with nanofibers, PTX, or ABX were stained for live and dead cells using a 2 µM Calcein-AM and 3 µM Ethidium homodimer-1 (both Invitrogen) staining solution in PBS. 1 mL of cell culture medium was gently aspirated and replaced with the staining solution to achieve the final concentration.

Cellcyte or Cytation 5 Imaging Procedure. Brightfield and fluorescence images were taken either on a Cellcyte X™ or Biotek Cytation 5 multimode plate reader, 30 minutes post-addition of live/dead stain. The images were taken of the microwell plate directly, with the lid on. Imaging on the Cytation was performed at 4 x and 20 x magnification on a minimum of 12 separate spheroids at 4 x and 3 separate spheroids at 20 x. The green fluorescence protein filter (λ_{ex} = 469/35 nm; λ_{em} = 525/39 nm) was employed for measuring fluorescence intensity from calcein-AM, and the Texas Red fluorescence filter (λ_{ex} = 586/15 nm, λ_{em} = 647/57 nm) was utilized for measuring fluorescence from Ethidium Homodimer-1. All settings were kept constant between samples. Imaging on the Cellcyte was performed at 10 x magnification, and a minimum of 3 spheroids were imaged continuously over the course of 3 h. Green fluorescence was measured at an excitation of 473/91 nm and emission of 502/61 nm, and red fluorescence

was measured at an excitation of 580/98 nm and an emission of 612/80 nm. The first timepoint was selected for calculation of the corrected total fluorescence. All settings were kept constant between samples.

Calculation of corrected total cell fluorescence (CTCF). Spheroid images were opened in FIJI software. The red and green fluorescence of 3 separate spheroids was measured, along with 3 separate areas of the background. To calculate the corrected total cell fluorescence, eq. S3 was utilized. From the integrated density (given by FIJI), the multiplication product of the cell area and the mean of the background readings was subtracted. The sum of the green and red fluorescence was calculated for each spheroid, and the percentage of each relative to the total fluorescence was computed. The standard deviation was then calculated for the three separate percentages, giving an average red:green ratio, which gave a value for percent alive, versus percent dead cells.

$$CTCF = Integrated\ density - (area\ of\ cell \times mean\ background\ fluorescence)$$

(eq. S3)

4. Supplementary Tables

Table S1. Summary of molar mass data for PFTMC$_{26}$-*b*-PDMAEMA$_{424}$ polymer.[S1,S2] Data is reproduced with permission from the Royal Society of Chemistry, and American Chemical Society.[S3]

Polymer	M_n (g/mol) GPC	M_w (g/mol) GPC	$Đ_M$ GPC	DP_n NMR	M_n (g/mol) NMR	DP_n MALDI-TOF
PFTMC$_m$-*b*-PDMAEMA$_n$	87,900	102,100	1.16	m = 26 n = 424	73,500	m = 26

Table S2. Summary of self-assembly data for PFTMC$_{26}$-*b*-PDMAEMA$_{424}$ nanofibers.[S1,S2] Data is reproduced with permission from the Royal Society of Chemistry, and American Chemical Society.

Morphology	Length in THF/EtOH (nm) via TEM	Length in Water (nm) via TEM	L_w/L_n in water via TEM
Nanofiber	25 ± 8	26 ± 6	1.05

Table S3. ζ-potential of free PTX and PTX-loaded nanofibers, recorded in water.

PTX (wt %)	ζ-potential (mV) Free PTX	ζ-potential (mV) PTX + Seed Nanofibers
0	-	+ 27
1	+ 9	+ 23
10	– 21	+ 20
20	– 8	+ 27
30	– 5	+ 33
80	-	+ 38

Table S4. IC_{50} values for nanofiber samples loaded with given amounts of PTX, over the course of 72 h, with measured relative PTX IC_{50} values for each respective sample. Nanofiber IC50 refers to the IC50 as a function of nanofiber concentration [μg/ml], while PTX IC50 refers to the IC50 as a function of the nominal final PTX dosing [μM] of each sample. Displayed are mean values ± standard deviation.

	Nanofiber IC_{50} [μg/mL]			PTX IC_{50} [μM]		
Sample (wt % PTX)	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
0	9.52 ± 0.23	10.22 ± 0.25	11.37 ± 0.26	-	-	-
1	4.56 ± 0.21	4.12 ± 0.07	3.12 ± 0.06	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
10	2.72 ± 0.18	1.72 ± 0.08	0.57 ± 0.02	0.32 ± 0.02	0.20 ± 0.01	0.07 ± 0.00
20	2.06 ± 0.28	1.13 ± 0.13	0.48 ± 0.02	0.48 ± 0.07	0.26 ± 0.03	0.11 ± 0.01
30	2.70 ± 0.08	2.05 ± 0.26	0.69 ± 0.13	0.95 ± 0.03	0.72 ± 0.09	0.24 ± 0.05
40	2.92 ± 0.11	2.10 ± 0.27	0.78 ± 0.14	1.37 ± 0.05	0.98 ± 0.12	0.37 ± 0.07
60	2.69 ± 0.39	1.06 ± 0.22	0.86 ± 0.04	1.89 ± 0.28	0.74 ± 0.15	0.60 ± 0.03
80	2.96 ± 0.12	1.85 ± 0.15	0.76 ± 0.09	2.77 ± 0.11	1.73 ± 0.14	0.71 ± 0.08
100	3.86 ± 0.21	2.06 ± 0.18	0.60 ± 0.18	4.52 ± 0.02	2.41 ± 0.02	0.70 ± 0.02
Free PTX	-	-	-	13.21 ± 5.27	0.26 ± 0.02	0.15 ± 0.07
Abraxane	-	1.78 ± 0.31	0.46 ± 0.03	undefined	0.23 ± 0.04	0.06 ± 0.00

5. Supplementary Figures

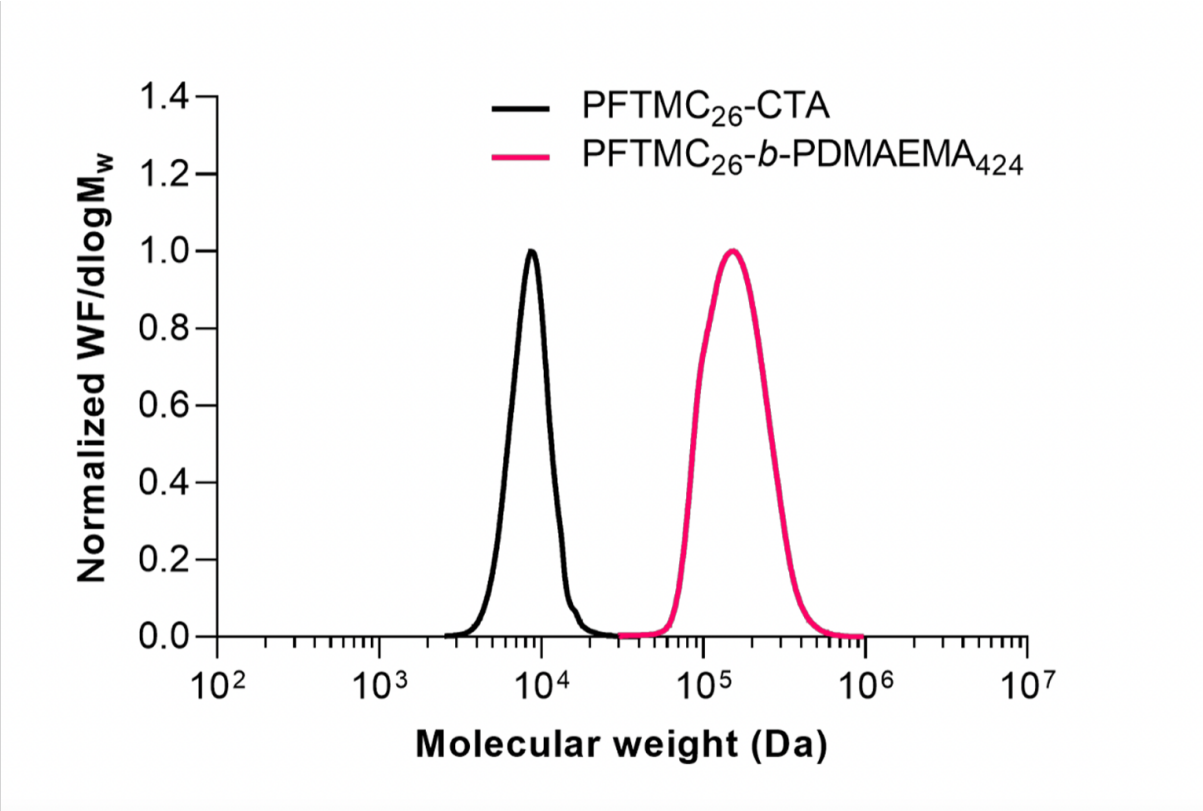

Figure S1. GPC chromatograms (refractive index detector) of $PFTMC_{26}$ homopolymer (black trace) and $PFTMC_{26}$-based block copolymer (pink trace), measured in *n*-Bu_4NBr/THF. Figure reproduced with permission from the Royal Society of Chemistry.[S2]

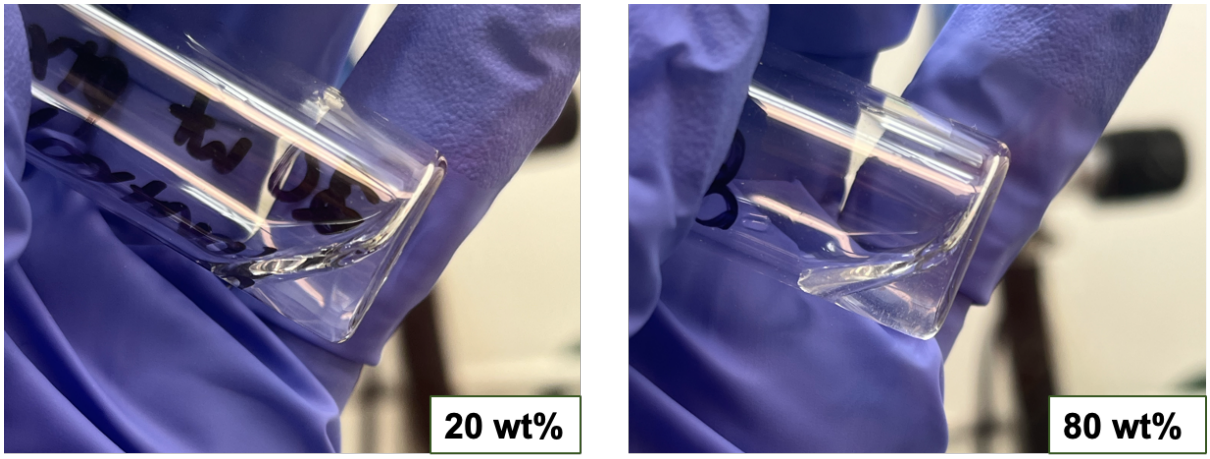

Figure S2. Representative photographic images of PTX-loaded nanofibers at 20 wt% (left) and 80 wt% (right). Macroscopically observable nanofiber precipitates are clearly visible at 80 wt%, but not at 20 wt%.

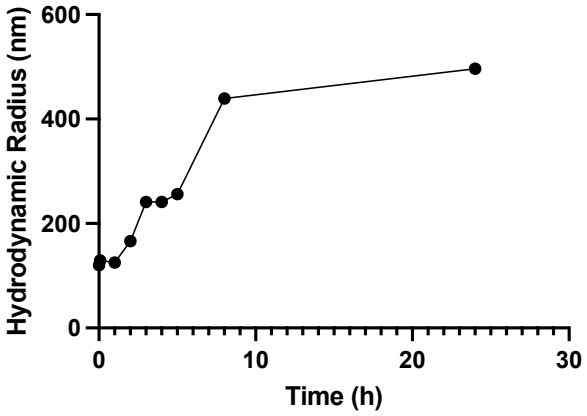

Figure S3. Kinetic measurements over 24 h of average hydrodynamic radius of 20 wt% PTX-loaded nanofibers at 37 °C. PTX-loaded nanofibers display an increase in hydrodynamic radius over time, reaching a plateau at 24 h.

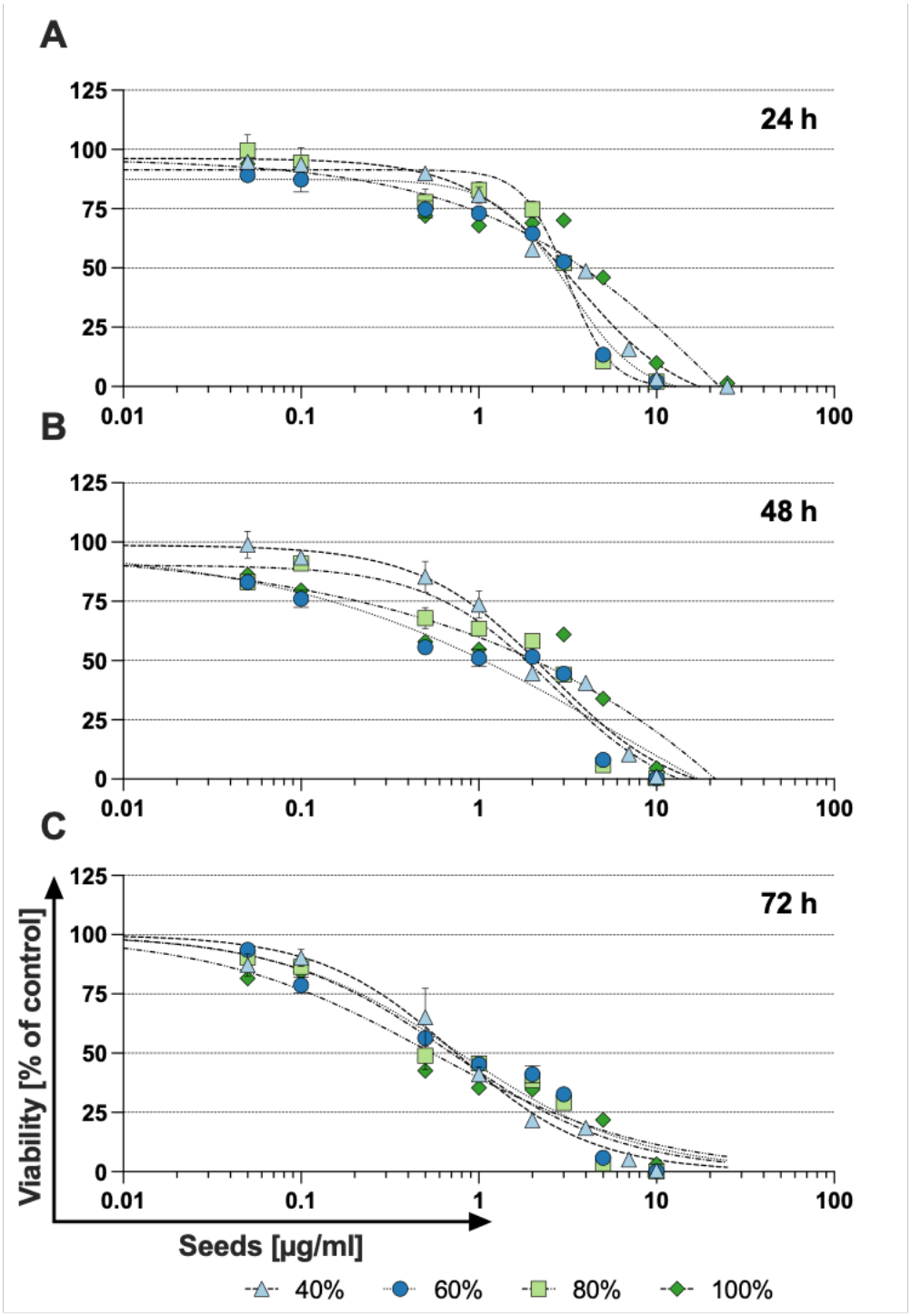

Figure S4. Cell viability curves utilized to calculate IC_{50} values for seeds loaded with ≥ 40 wt % PTX over (A) 24 h, (B), 48 h, and (C) 72 h.

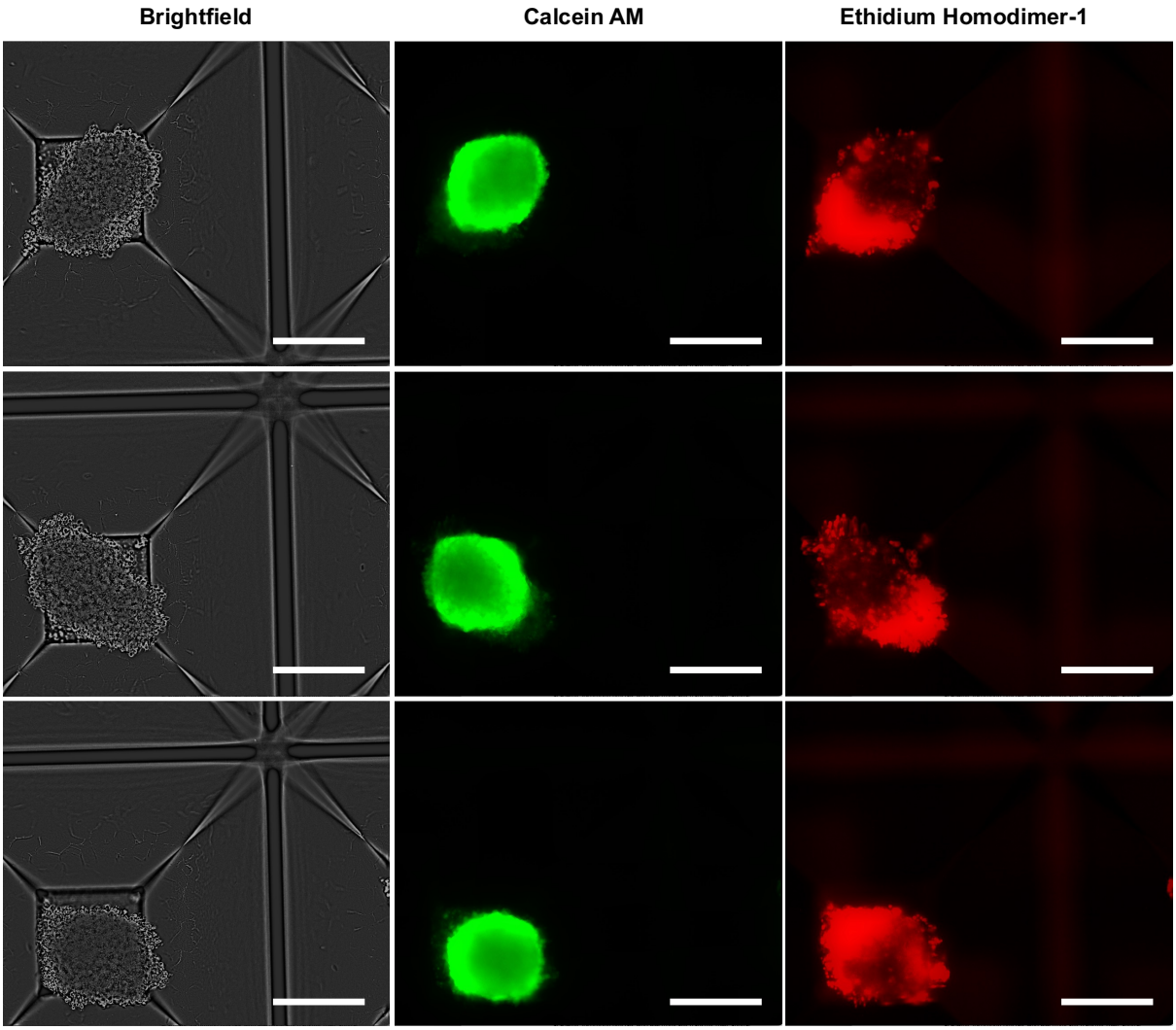

Figure S5. Three distinct GBM tumor spheroids in DMEM/F12 cell culture media, stained with calcein-AM and ethidium homodimer-1. All scale bars represent 200 μm.

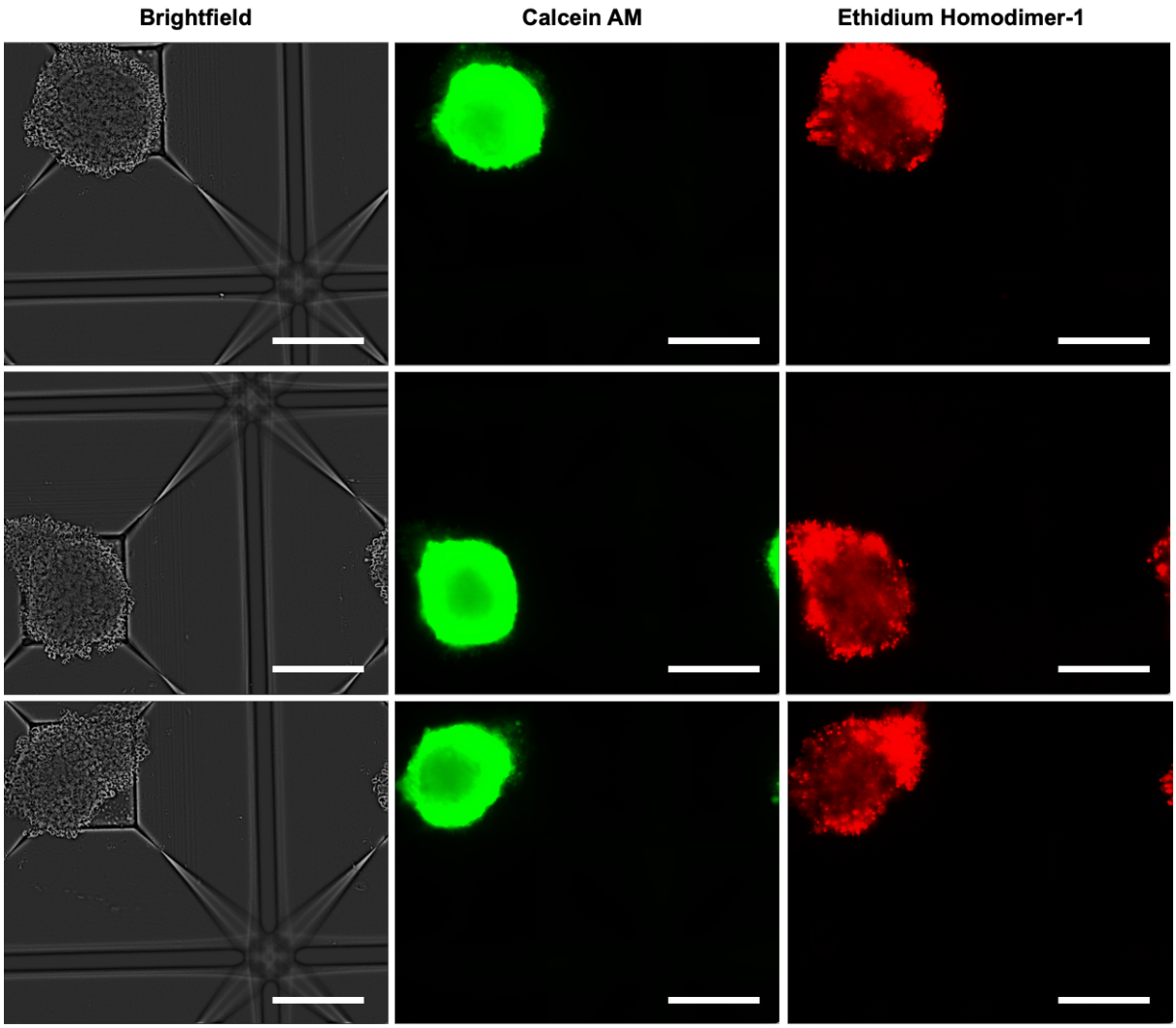

Figure S6. Tumor spheroids upon incubation with seed nanofibers (0 wt % PTX) and stained with calcein-AM and ethidium homodimer-1. All scale bars represent 200 μm

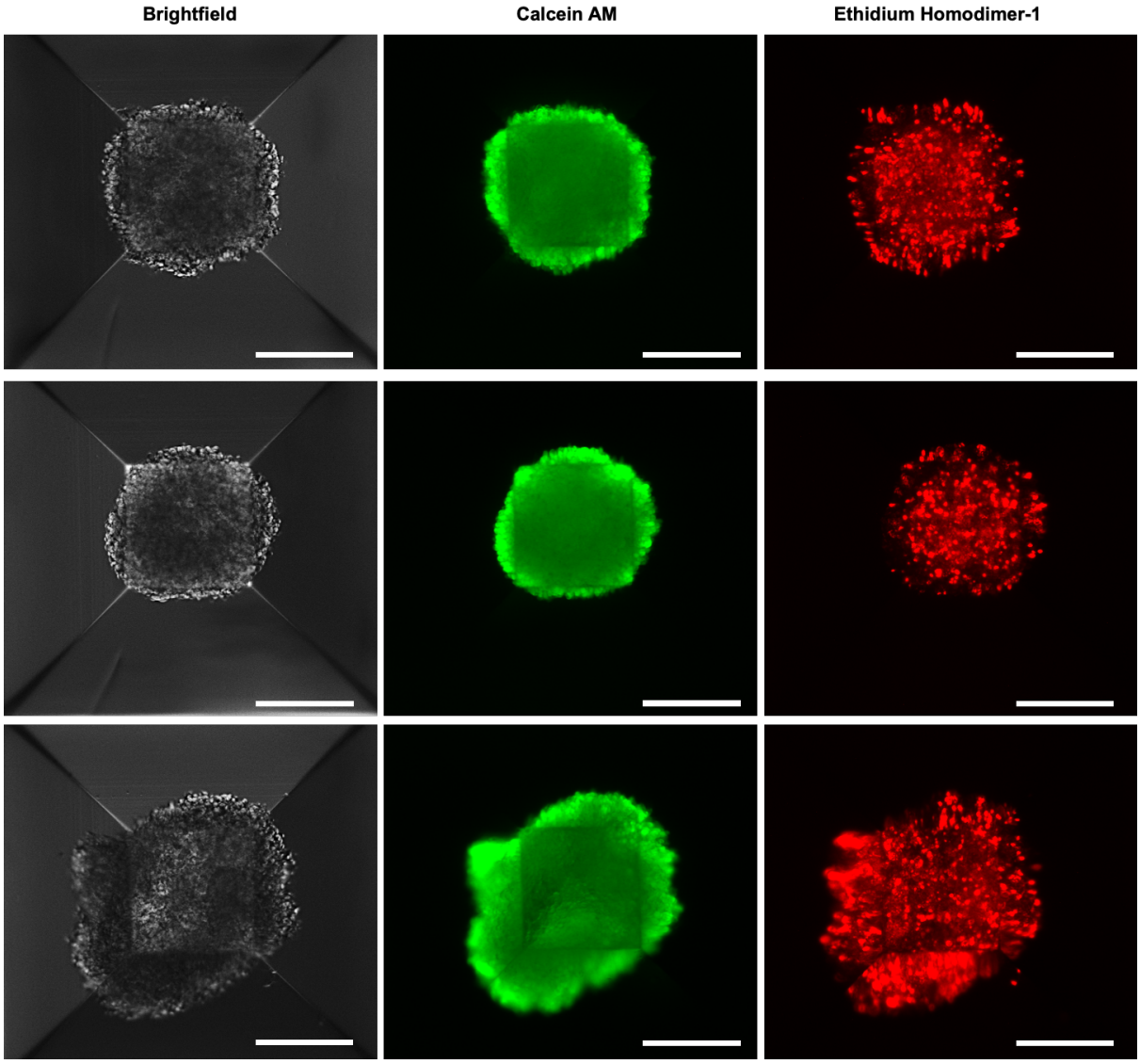

Figure S7. GBM tumor spheroids upon incubation with free PTX solubilized in H_2O/DMSO (5 %). Images were taken using a Cytation 5 plate reader at 4x magnification. Calcein-AM fluorescence was detected with a green fluorescence protein fluorescence filter, and ethidium homodimer-1 fluorescence was detected with a Texas Red fluorescence filter. All scale bars represent 200 μm.

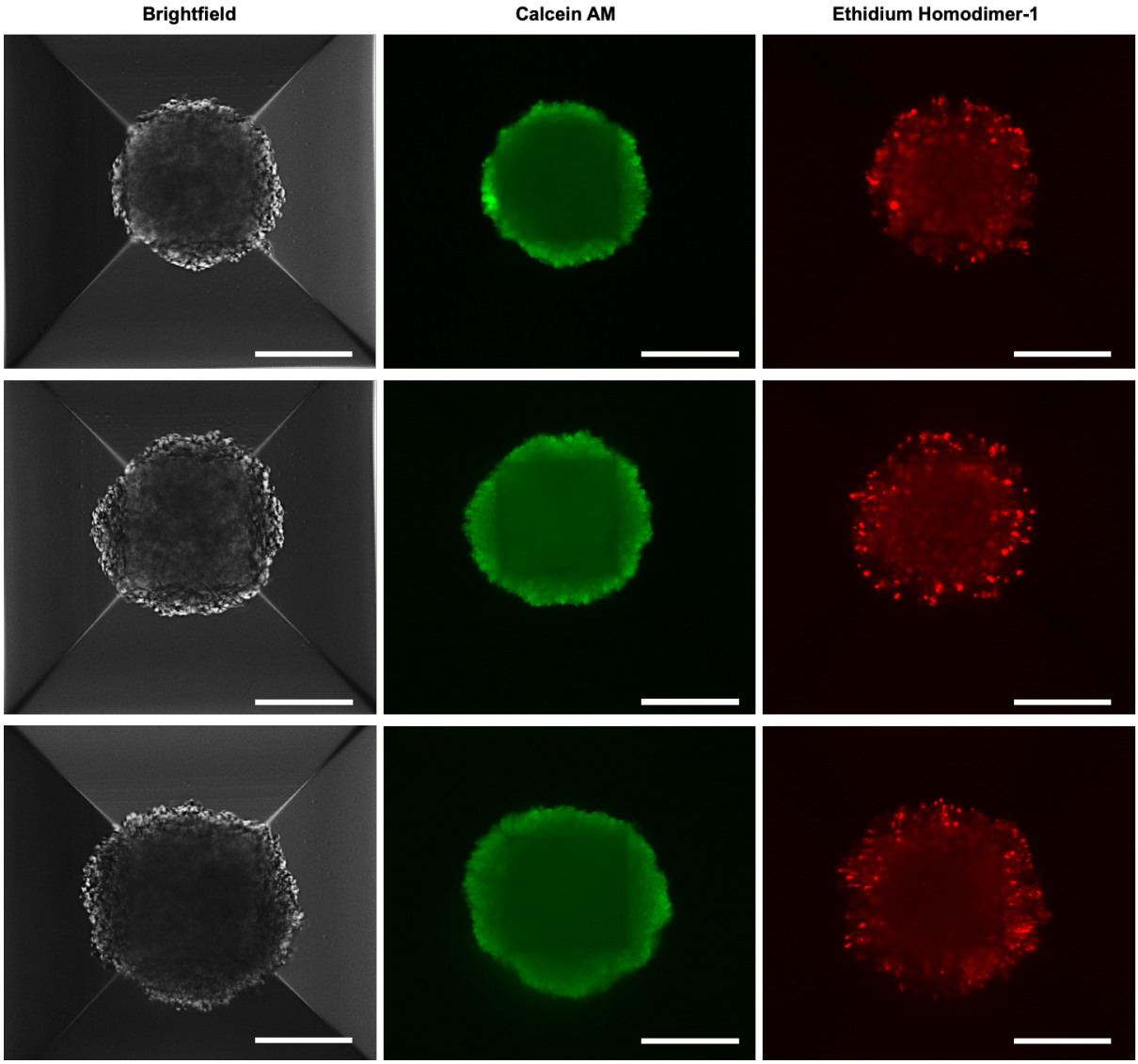

Figure S8. GBM tumor spheroids upon incubation with ABX. Images were taken using a Cytation 5 plate reader at 4x magnification. Calcein-AM fluorescence was detected with a green fluorescence protein fluorescence filter, and ethidium homodimer-1 fluorescence was detected with a Texas Red fluorescence filter. All scale bars represent 200 μm.

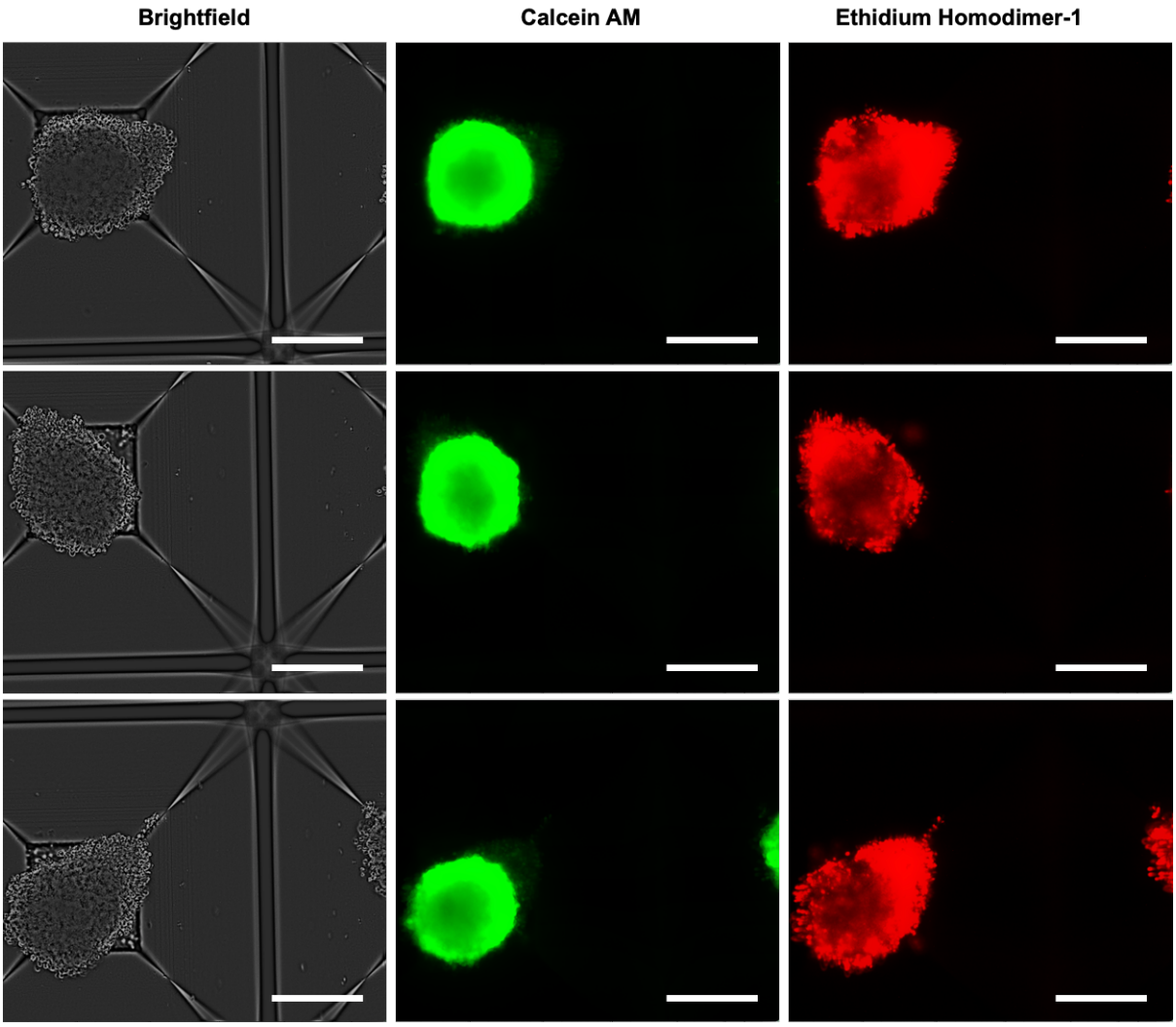

Figure S9. Tumor spheroids post-incubation with seed nanofibers loaded with 20 wt % PTX. Green fluorescence from calcein-AM measures live cells, and red fluorescence from ethidium homodimer-1 measures dead cells. All scale bars represent 200 µm.

6. References

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S3. Garcia-Hernandez, J. D.; Street, S. T.; Kang, Y.; Zhang, Y.; Manners, I. Cargo Encapsulation in Uniform, Length-Tunable Aqueous Nanofibers with a Coaxial Crystalline and Amorphous Core. *Macromolecules* **2021**, *54* (12), 5784–5796. DOI:10.1021/acs.macromol.1c00672.

S4. E. Tsang, L. M. Urner, G. Kim, K. Chow, L. Baufeld, K. Faull, T. F. Cloughesy, P. M. Clark, M. E. Jung and D. A. Nathanson, *ACS Medicinal Chemistry Letters*, 2020, **11**, 1799–1809.