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Supplementary Information

Drug Governs Morphology of Polyalkylated Block Copolymer Aggregates.

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Materials & Methods

Copolymers

Polyalkylated copolymers have been prepared as described previously by anionic polymerization and a thiolene coupling reaction.[1] C_6, C_{12} and C_{18} copolymers were found to have molecular weights (M_n) of 11200, 13300 and 15400, respectively, with polydispersity ≤ 1.05. The critical micelle concentrations of the copolymers were found to be 0.17 ≤ CMC (μM) ≤ 0.02.

Copolymer Aggregates and Formulations (F-DOX-C_n)

The copolymer aggregates and F-DOX-C_n were prepared using the dialysis method.[2] Briefly, 45 mg of copolymer was dissolved in 2 mL of THF. A 12 mg sample of DOX was dispersed in 1 mL of acetonitrile and 1 mL of THF and 9 μL of trimethylamine were then added. After a period of two hours under stirring (in the dark), the copolymer and DOX solutions were combined, mixed and added dropwise to 8 mL of saline 0.9% (NaCl) with stirring. The solution was then transferred into a dialysis bag (6-8 KDa cut off) and dialyzed against saline (1L) for 24hr.The formulation was centrifuged (5000 rpm / 15 min) and the supernatant was purified by ultrafiltration (100 KDa) (3 volumes).

TEM and Cryo-TEM

For analysis of each copolymer solution a five microliter sample was pipetted onto the surface of a glow discharged Quantifoil grid (Quantifoil GMb) that had 2 micron holes in 2 micron spacing. Excess fluid was wicked off with filter paper and the grid was then plunged into liquid ethane at -190 deg C. The grid was loaded into a Gatan Cryo-Holder at liquid nitrogen temperature. The holder was loaded into the FEI Tecnai G2 F20 TEM. Imaging was done at 200 kV at spot size 6 with the Gatan 4K bottom mount CCD camera and the Gatan Digital Micrograph software.

AFM

Two microliters of each sample was deposited onto freshly cleaved mica and imaged after drying in air. All images were acquired in tapping mode using a Digital Instruments Nanoscope IIIa Multimode 2 AFM equipped with a "J" scanner (maximum lateral scan area 125 x 125 μm) using TESP-V2 tips (nominal resonant frequency: 320 kHz; nominal spring constant: 42 N/m; nominal length: 125 μm; nominal width: 40 μm) (Bruker AFM probes, Camarillo,

CA). Images were collected at a resolution of 512 x 512 pixels at 1 Hz scan rate. At least three areas were acquired to accurately assess the morphology of each sample. Image analysis was performed using Nanoscope software version 5.12r3 (Digital Instruments).

SANS Analysis

The scattered intensity $I(q)$ of samples were measured using the Bio-SANS instrument at the High Flux Isotope Reactor (HFIR, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA). Samples were prepared in D_2O and filled into quartz cuvettes (Hellma Analytics, Müllheim, Germany). Data reduction and background subtraction were performed employing software provided by the beam line facility. The data $I(q)$ vs. momentum transfer q was fitted using a form factor for wormlike micelles as described in Vogtt et al., *Langmuir* **31**:8228. This approach envisions wormlike chains as a chain of cylindrical subunits and separates the scattered intensity accordingly. The first contribution indicated with the index 1 stems from the cylindrical subunits described via their length L and radius R. The second part with the index 2 comprises the contribution of the assembly of the cylindrical subunits in terms of the radius of gyration R_g and the fractal dimension d_f. The cylindrical subunits are assumed to perform a self-avoiding walk by setting the minimum dimension d_{min} = 1.67, where the connectivity dimension c is given by the ratio d_f / d_{min}. Fitting was done with a self-programmed algorithm employing the software Igor Pro™.

The block copolymer aggregates in the presence of DOX were modelled as prolate ellipsoids. Here, the equatorial semi-axes R were likewise described by a log-normal distribution with a median value $\langle R \rangle$ and a distribution width parameter s_R. The length of the semi-principal axis is given by mR. Fitting was performed using the software SASfit (https://kur.web.psi.ch/sans1/SANSSoft/sasfit.html).

Table 1 depicts the values obtained from the fitting procedures. Here $f_v(Dr)^2$ denotes the product of volume fraction times the square of the scattering length density difference between particle and solvent. $\langle R_1 \rangle$ and s_R are the median and the width parameters for the radius of the cylindrical subunits modelled as a log-normal distribution. L_1 is the corresponding length. G_2, $R_{g,2}$ and $d_{f,2}$ are the scattered intensity at zero angle, radius of gyration and fractal dimension of the overall wormlike assembly, respectively.

Table 1: Results of fitting procedure modelling the block copolymer aggregates as wormlike micelles (A) and as ellipsoids in the presence of doxorubicin (DOX) (B).

A	$\phi_V (\Delta\rho)^2 /(10^{20}$ cm^{-4})	$\langle R_1 \rangle$ / Å	σ_R / Å	L_1 / Å	G_2 / cm^{-1}	$R_{g,2}$ / Å	$d_{f,2}$
C_6	0.142 +/- 0.002	50 +/- 1	0.144 +/- 0.003	228 +/- 3	151 +/- 9	2600 +/- 100	1.67
C_{12}	0.1741 +/- 0.0001	62.62 +/- 0.07	0.166 +/- 0.001	229.3 +/- 0.3	1300 +/- 30	2610 +/- 30	1.67
C_{18}	-	-	-	-	-	-	-

B	$\phi_V (\Delta\rho)^2 /(10^{20}$ cm^{-4})	$\langle R \rangle$ / Å	σ_R / Å	μ
C_6 + DOX	0.160 +/- 0.003	54.6 +/- 0.1	0.147 +/- 0.002	2.57 +/- 0.01
C_{12} + DOX	0.201 +/- 0.003	66.86 +/- 0.07	0.103 +/- 0.002	2.222 +/- 0.006
C_{18} + DOX	0.174 +/- 0.003	59.63 +/- 0.08	0.123 +/- 0.002	2.659 +/- 0.009

Cell Culture

Human ovarian carcinoma cells (OVCAR8) were obtained from the Biological Testing Branch of the National Cancer Institute (NCI; Frederick, MD, USA). Cells were cultured in RPMI-1640 supplemented with 10 % FBS and 1 % penicillin-streptomycin as monolayers at 37 °C in 5 % CO_2 and 90 % relative humidity.

Monolayer Cytotoxicity

Cells were plated in 96-well plates at a density of 4000 cells/well, and treated with F-DOX-C_6, F-DOX-C_{12}, F-DOX-C_{18} or free DOX for 48 hours. Following treatment, cells were washed with PBS, and then 100 µL of freshly prepared reaction buffer (2 mg/mL *p*-nitrophenyl phosphate in 0.1 M sodium acetate buffer (pH 5.5) containing 0.1 % Triton X-100) was added to each well. Following two hours of incubation at 37 °C, 10 µL of 1 M sodium hydroxide was added to each well, and the UV absorbance at 405 nm was measured using a plate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA).

MCTS Growth Inhibition

MCTS of OVCAR8 cells were grown using the liquid overlay technique as previously reported by our group [3]. Briefly, sub-confluent cells were trypsinized and seeded at a density of 1000 cells/well onto non-adherent 96-well round-bottom Sumilon PrimeSurface™ spheroid plates (MS-9096U; Sumitomo Bakelite, Tokyo, Japan). Cells were incubated in complete growth media at 37 °C in 5 % CO_2 and 90 % relative humidity for seven days until they reached a diameter of approximately 500 µm. During the growth period, 50 µL of media was carefully removed without disturbing the MCTS, and replaced with 50 µL of fresh media every other day. Prior to initiation of treatment, MCTS were imaged using a light microscope with a 10x objective lens (VWR VistaVision). MCTS were treated with media (control) or 0.75 µM of F-DOX-C6, F-DOX-C12, F-DOX-C18 or free DOX for 48 hours. Following treatment, MCTS were washed five times with fresh media by carefully removing 50 µL of media containing treatment, and replacing with 50 µL of fresh media. Every other day, media was changed as described above, and the images of MCTS were captured for volume analysis as described previously [3]. Data is expressed as the mean percentage growth of 3 - 6 MCTS ± SD.

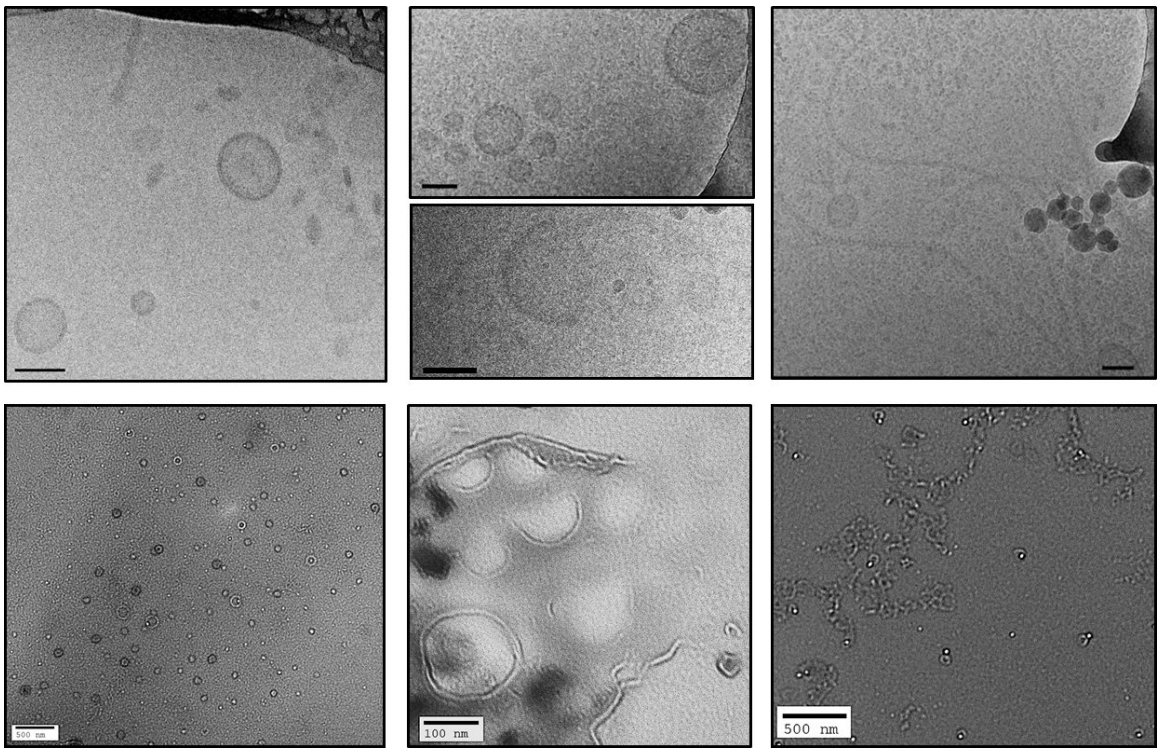

Figure S-1. Representative cryo-TEM (upper) and TEM (lower) images of mPEG-*b*-(AGE-C_{12})$_{25}$ aggregates (0.5 wt% copolymer, 0.3% U.A. stain). Scale bars in upper cryo-TEM images represent 100 nm and scale bars for lower images (TEM) represent 100 or 500 nm as shown.

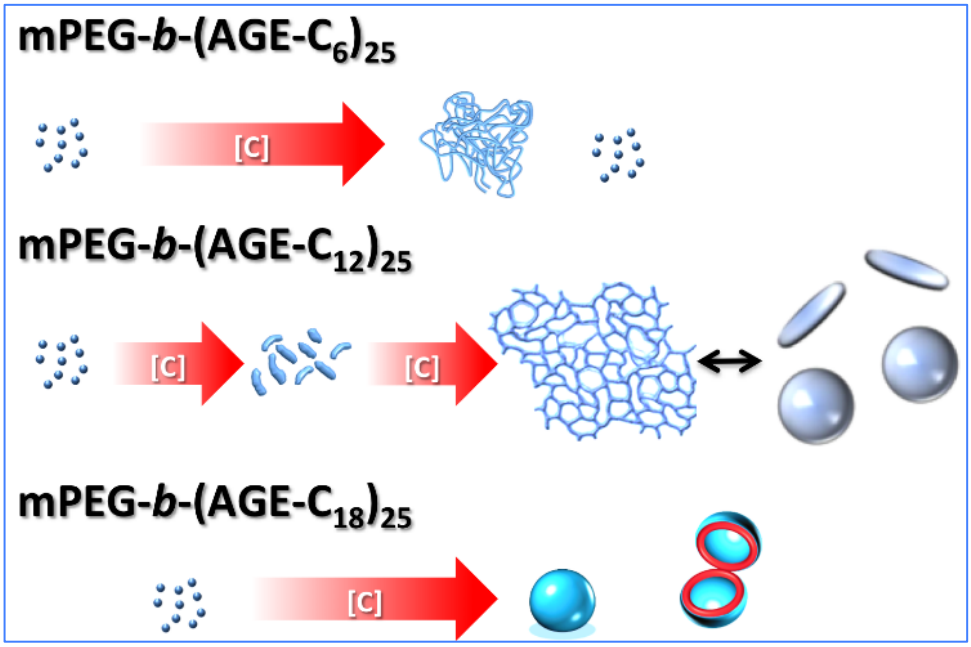

Figure S-2. Postulated evolution of morphologies of the amphiphilic PCAs with an increase in copolymer concentration.

Overall, the copolymer concentration significantly influenced the PCA morphology in the case of the amorphous C_{12} PCAs (coexistence of morphologies), whereas PCAs bearing shorter alkyl chains (i.e. C_6 PCAs, spheres + filomicelles) or semi crystalline chains (i.e. C_{18} PCAs, bilayers) reached their final morphology at low copolymer concentration and did not evolve further at 0.5 wt % copolymer (Fig.S-2).

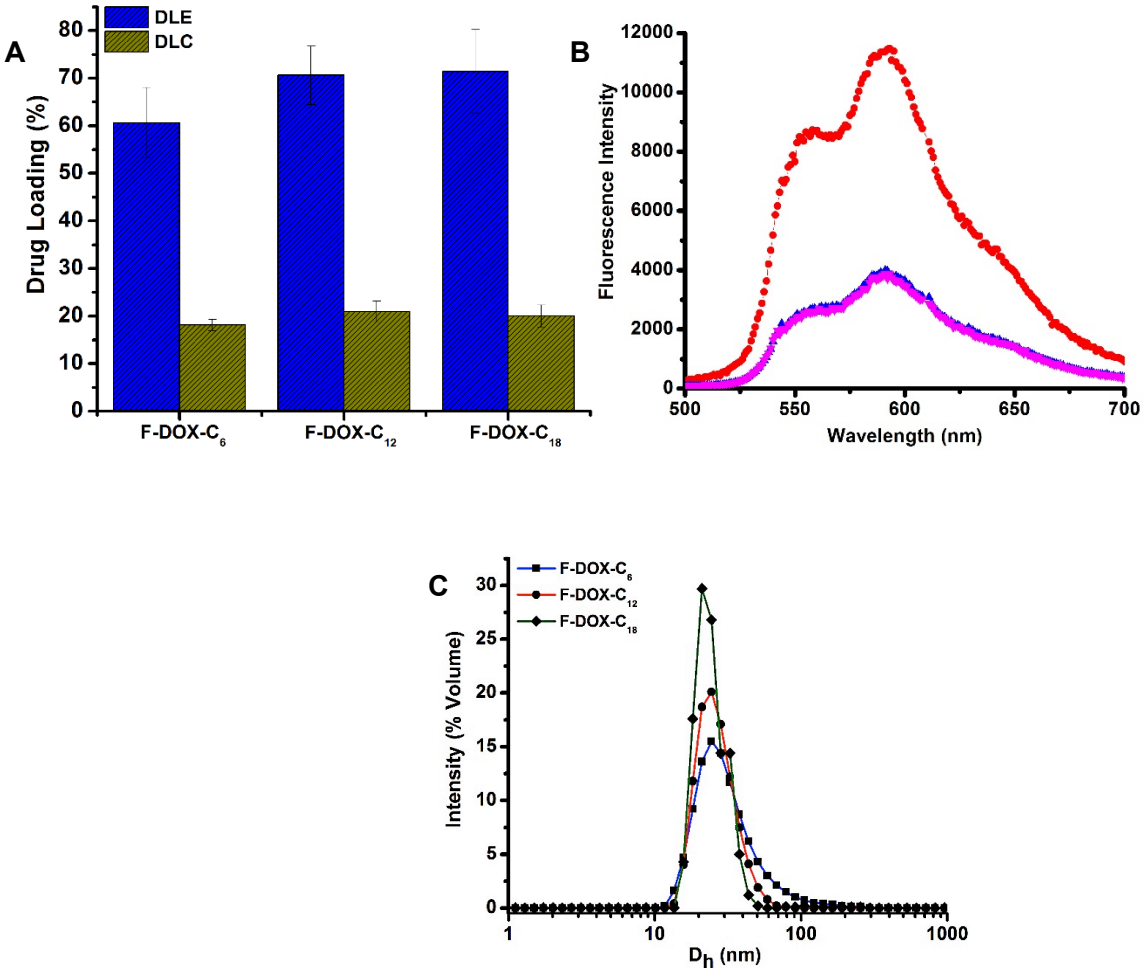

Figure S-3. A) Drug loading efficiency (DLE) and capacity (DLC) in w/w % for the three formulations F-DOX-C_n. **B)** Fluorescence spectra of free doxorubicin (red) and F-DOX-C_n formulations (in blue and pink) at a DOX concentration of 10 µg/mL in PBS pH = 7.4.

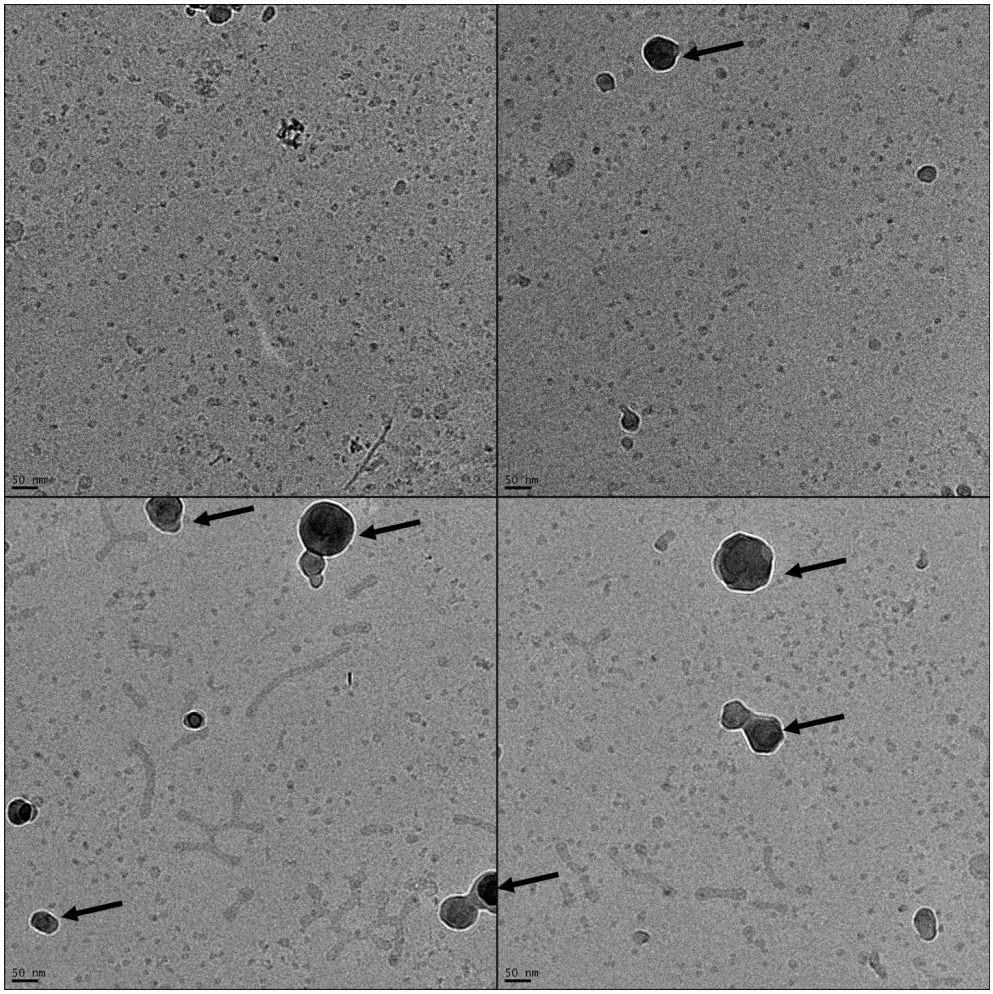

Figure S-4. Representative cryo-TEM images of vitrified solutions of F-DOX-C_6. Scale bars represent 50 nm. Arrows on the vitrified samples identify ice contamination.

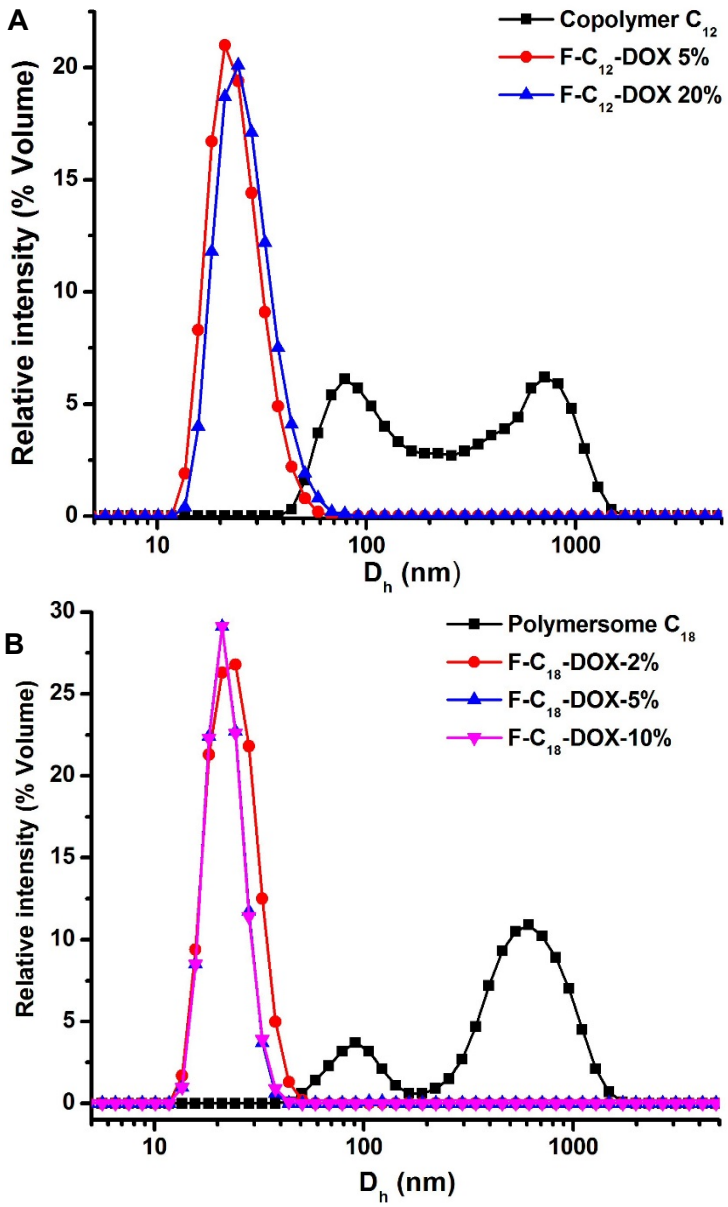

Figure S-5. Impact of drug incorporation, at different DOX loading levels (w/w %), as investigated by dynamic light scattering A) copolymer C_{12} and B) copolymer C_{18}.

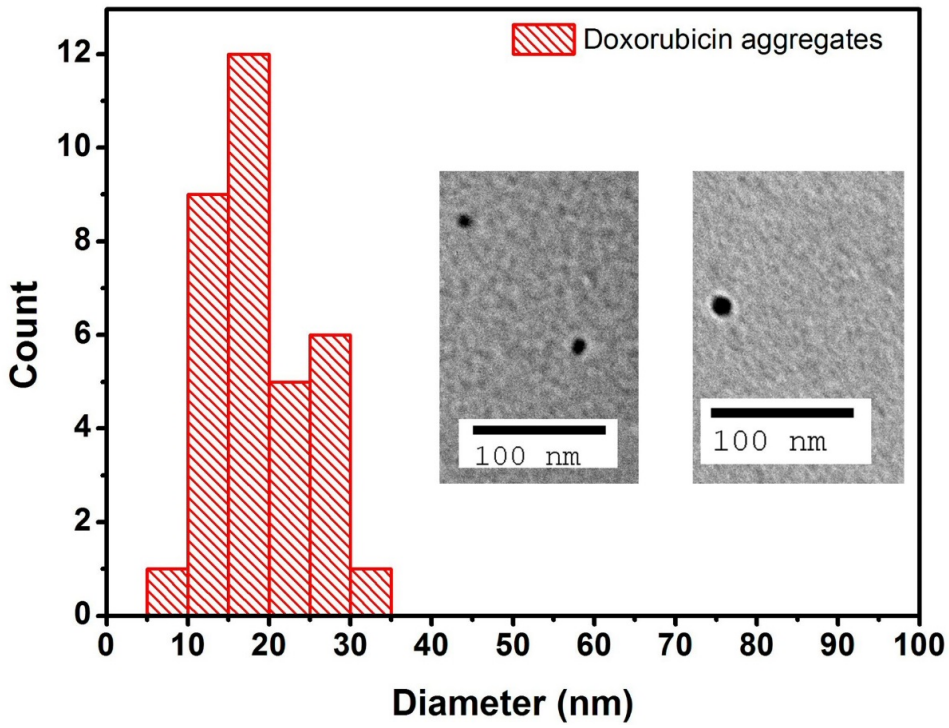

Figure S-6. Size distribution of aggregates formed from free drug (count ~ 50 particles) as observed by TEM without staining. Free DOX was treated as described for preparation of the copolymer formulations. After dialysis in saline, DOX was diluted in distilled water prior to deposition on copper grid (no staining) and analysed by TEM.

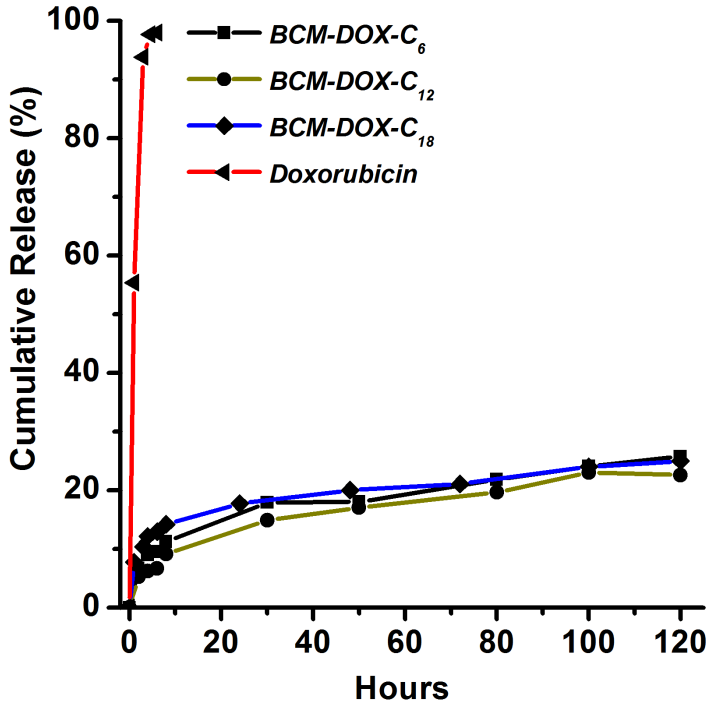

Figure S-7. Release profiles of DOX from the F-DOX-C_n formulations in PBS 0.1M pH = 7.4 (n=3 individual experiments). For more clarity, S.D (≤ 5%) values have been removed from the traces.

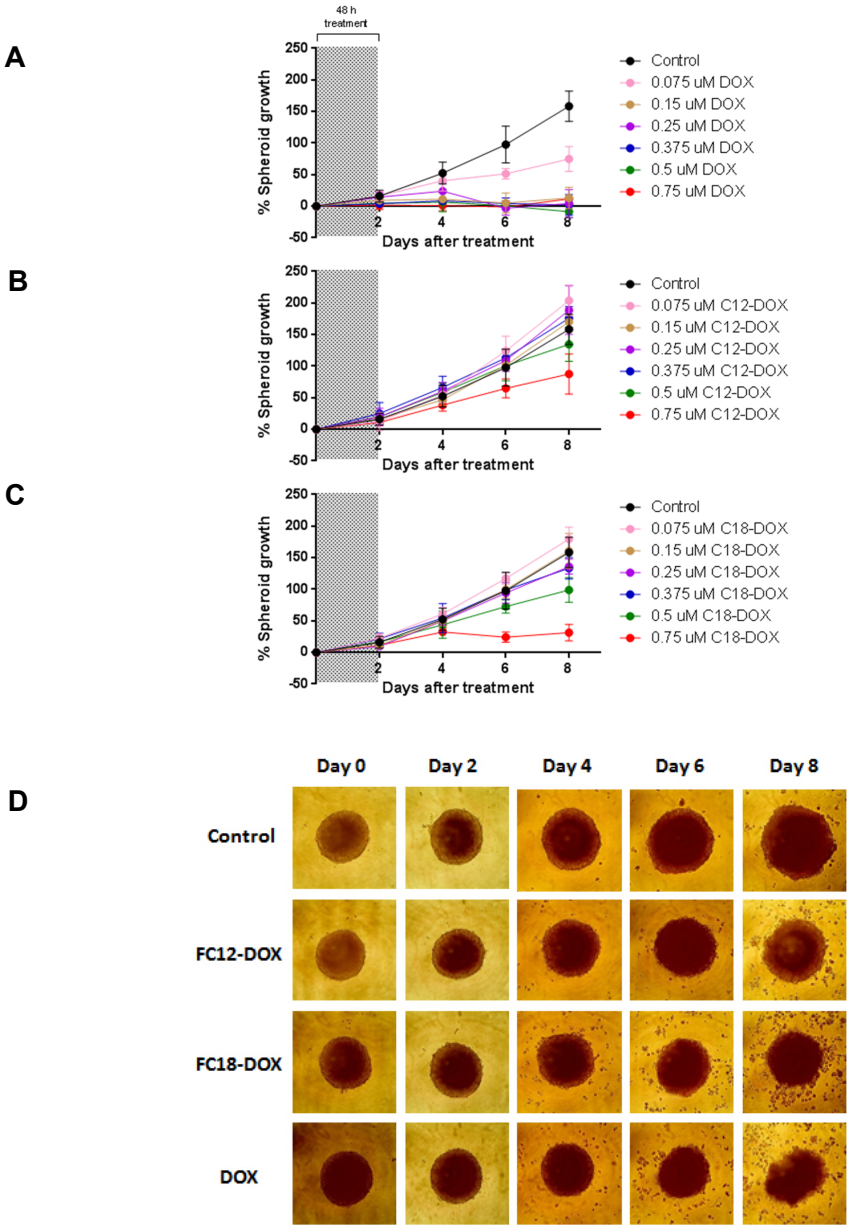

Figure S-8. Growth inhibition of OVCAR8 MCTS by free DOX (A), F-DOX-C_{12} (B) and F-DOX-C_{18} (C) at different concentrations of drug. Data represent means of percent spheroid growth (n = 3 – 6 MCTS) ± SD.

1. Le Devedec, F., et al., *Postalkylation of a Common mPEG-b-PAGE Precursor to Produce Tunable Morphologies of Spheres, Filomicelles, Disks, and Polymersomes.* ACS Macro Letters, 2016. **5**(1): p. 128-133.
2. Le Devedec, F., L. Houdaihed, and C. Allen, *Anionic Polymerization of an Amphiphilic Copolymer for Preparation of Block Copolymer Micelles Stabilized by π-π Stacking Interactions.* 2016(116): p. e54422.
3. Mikhail, A.S., S. Eetezadi, and C. Allen, *Multicellular Tumor Spheroids for Evaluation of Cytotoxicity and Tumor Growth Inhibitory Effects of Nanomedicines In Vitro: A Comparison of Docetaxel-Loaded Block Copolymer Micelles and Taxotere (R).* Plos One, 2013. **8**(4).