

Electronic Supplementary Information

Polymerization-Induced Self-Assembly (PISA) – Control Over the Morphology of Nanoparticles for Drug Delivery Applications

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EXPERIMENTAL SECTION

Materials

Styrene (ST) was deinhibited by passing through a column of basic alumina. Oligo(ethylene glycol) methacrylate (OEGMA) ($M_n = 300 \text{ g mol}^{-1}$) and 3-vinyl benzaldehyde were used as received. 2,2-Azobis(isobutyronitrile) (AIBN, Fluka, 98%) was purified by recrystallization from methanol. 4-Cyanopentanoic acid dithiobenzoate (CPADB) was prepared according to a procedure described elsewhere.¹ All the other reagents were used as purchased unless otherwise specified.

Instrumentation

¹H-NMR spectra were recorded using a Bruker 300MHz spectrometer. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonances. The molecular weight and polydispersity of the prepared polymers were measured by SEC. The eluent was DMAc (that contained 0.03% w/v LiBr and 0.05% w/v 2,6-dibutyl-4-methylphenol (BHT)) at 50 °C (flow rate of 1 mL min⁻¹) with a Shimadzu modular system comprising an SIL-10AD auto-injector, a Polymer

Laboratories 5.0 μ m bead-size guard column (50 \times 7.5mm²) followed by four linear PL (Styragel) columns (10⁵, 10⁴, 10³, and 500 Å) and an RID-10A differential refractive-index detector.

The sizes and morphologies of the block polymers were observed using a transmission electron microscopy JEOL1400 TEM at an accelerating voltage of 100 kV. The solution of the block polymer in dispersion medium was directly taken and diluted with methanol (2 mg mL⁻¹) and deposited onto copper grid (ProSciTech). Uranyl acetate staining was applied.

DLS measurements were performed using a Malvern Zetasizer Nano Series running DTS software and using a 4 mW He-Ne laser operating at a wavelength of 633 nm and an avalanche photodiode (APD) detector. The scattered light was detected at an angle of 173°.

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) measurements were performed using a Bruker IFS66\S Fourier transform spectrometer by averaging 128 scans with a resolution of 4 cm⁻¹.

Synthesis of poly(oligo (ethyleneglycol) methacrylate) (POEGMA) macro-CTA via RAFT polymerization

POEGMA was prepared by RAFT polymerization in the presence of 4-cyanopentanoic acid dithiobenzoate (CPADB) as RAFT agent. For this purpose, OEGMA (12 g, 4 \times 10⁻² mol), CPADB (0.224 g, 8 \times 10⁻⁴ mol), AIBN (1.64 \times 10⁻² g, 1 \times 10⁻⁴ mol) and 50 mL acetonitrile were mixed in a 100 mL round bottom flask and sealed with rubber septum. The reaction mixture was immersed into an ice-bath and saturated with nitrogen by continuous gas flow for 30 minutes. Polymerization reaction was carried out for 8 h at 70 °C. Then the polymerization reaction was terminated by rapid cooling and the resulting polymer was recovered by precipitation in an 50 mL diethyl ether and petroleum spirit (boiling range of 40-60°C) mixture (1:1, v/v). Dried POEGMA macro-CTA's molecular weight was determined by size exclusion chromatography (SEC). $M_{n,SEC}$ (DMAc as mobile solvent and PST standards) of 9500 g mol⁻¹, PDI= 1.09 and the theoretical number average molecular weight ($M_{n,NMR}$) was close to 11,100 g mol⁻¹ from the conversion which was calculated from ¹H-NMR by comparing the vinyl peaks (6.05 and 5.5 ppm) to that of aliphatic proton peaks (0.93 and 0.74 ppm). The number of repeating units was also calculated from ¹H-NMR of the purified polymer sample by comparing RAFT agent's aromatic protons appears between 7.9-7.4 ppm to that of methylene protons adjacent to ester linkage at 4.1 ppm and was found to be 36. Resulting POEGMA was used as macro-CTA for further block copolymer synthesis with styrene or styrene-vinyl benzaldehyde.

Polymerization kinetics study of OEGMA was done using the following procedure: OEGMA (6 g, 2 \times 10⁻² mol), CPADB (0.112 g, 4 \times 10⁻⁴ mol) and AIBN (8.2 \times 10⁻³ g, 5 \times 10⁻⁵ mol) were dissolved in 25 mL acetonitrile. The clear reaction mixture was then equally (5 mL each) divided into 6 vials, sealed with rubber septum and put into an ice-bath under continuous nitrogen flow for 30 minutes. The polymerization reaction

was started by immersing the flasks into an pre-heated oil bath at 70 °C. Then each reaction flask was taken over 2 h time intervals up to 12 h and rapidly cooled in an ice-bath to terminate the polymerization. Conversion of the monomer during the polymerization was followed by ¹H-NMR spectrum of the reaction solutions. The resulting polymers were purified by precipitating three times in 50 mL diethyl ether and petroleum spirit mixture (1:1, v/v) and dried under vacuum at room temperature for 24 h. Obtained results are collected in Table-1.

Preparation of core functional POEGMA-*b*-P(ST-*co*-VBA) amphiphilic block copolymer via RAFT polymerization

Core-functional POEGMA-*b*-P(ST-*co*-VBA) block copolymer with various morphologies were synthesized in methanol by RAFT dispersion polymerization with a molar feed ratio of ST : VBA: POEGMA : AIBN = 4750:250:1:0.5. For this purpose, POEGMA as a macro-CTA (0.222 g, 2.00×10^{-5} mol), styrene (9.89 g, 9.50×10^{-2} mol), VBA (0.661 g, 5.00×10^{-3} mol) as a functional monomer and azobisisobutyronitrile (AIBN, 1.64×10^{-3} g, 1.00×10^{-5} mol) were dissolved in 13.6 mL methanol (total monomer weight = total solvent (MeOH) weight). The reaction mixture was divided equally into eight vials to study the kinetics of the reaction. Each vial was sealed carefully and gently purged with nitrogen for 20 minutes. The reaction mixtures were then immersed in an oil bath at 70°C, and vials were taken out at specific time points, 4, 8, 12, 18, 24 and 36 hours. The polymerization was terminated by placing the reaction mixture into an ice-bath for 5 minutes and exposure to air. The polymer was purified three times by dialysis in methanol using a dialysis tube with a molecular weight cut off of 12,000 – 14,000. Morphologies and number average diameter of the block copolymer were investigated directly from the block copolymerization dispersion solution by diluted samples with methanol (2 mg/mL). Resulting 36 h sample was used for crosslinking study.

Preparation of core crosslinked POEGMA-*b*-P(ST-*co*-VBA) amphiphilic block copolymer

Core crosslinking of the block copolymer was achieved by reaction of aldehyde groups in the core with 1,3-diaminopropane in methanol at room temperature. For this purpose, 2.3×10^{-4} mol 1,3-diaminopropane was reacted to 2.3×10^{-5} mol of aldehyde group containing (36 h sample) amphiphilic polymer dispersion in methanol and the reaction mixture was stirred for 48 h. Crosslinking was tested by dissolving a few droplets in THF, resulting solution did not lose its cloudiness. And the obtained crosslinked sample was characterized by FT-IR, DLS and TEM measurements.

Doxorubicin loading

In the presence of triethylamine (50 μL), 20 mg of **POEGMA-*b*-P(ST-*co*-VBA)** and 2 mg of DOX.HCl were dissolved in DMSO (2 mL) and the mixture stirred at room temperature for 1 h. The mixture was first

dialyzed (MWCO 3500 Da) against methanol for 24 h, and then against buffer solution for 48 h to remove solvent, triethylamine, and free DOX. The solution absorbance at 485 nm was measured on a CARY 300 spectrophotometer (Bruker) to determine the concentration. The amount of encapsulated DOX in nanoparticles was quantified to be ~ 5.1 wt% using a calibration curve of DOX.HCl in DMSO. The concentration of the polymers was determined by freeze dried a known volume.

Cell culture

MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium: Nutrient Mix F-12 (DMEM) supplemented with 10% (v/v) Fetal Calf Serum (FCS) in a ventilated tissue culture flask T-75. The cells were incubated at 37° C in a 5% CO₂ humidified atmosphere and passaged every 2-3 days when monolayers at around 80% confluence were formed. Cell density was determined by counting the number of viable cells using a trypan blue dye (Sigma-Aldrich) exclusion test. For passaging and plating, cells were detached using 0.05% trypsin-EDTA (Invitrogen), stained using trypan blue dye, and loaded on the haemocytometer. All the experiments were done in triplicate.

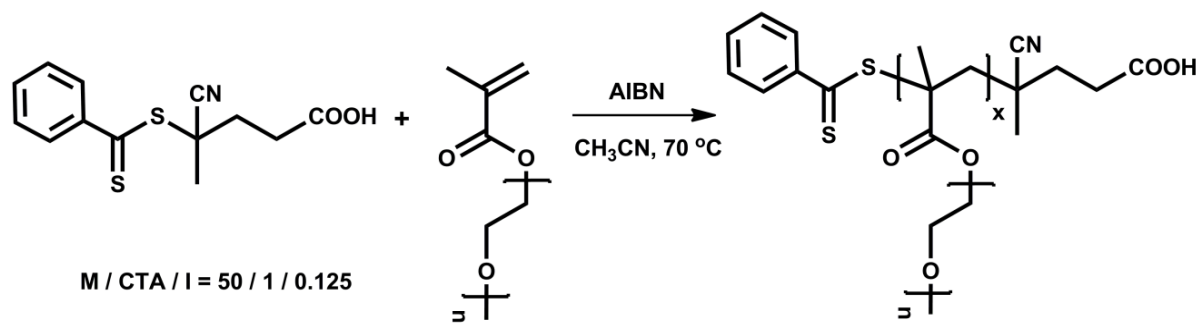
Cell viability

The cytotoxicity of free DOX, dextran nanoparticles, and DOX-loaded nanoparticles was tested *in-vitro* by a standard Alamar Blue Assay. The assay is based on the ability of living cells to convert blue redox dye (resazurin) into bright red resorufin which can be read in a spectrophotometric reader. Nonviable cells rapidly lose metabolic capacity and thus do not generate a colour signal. The intensity of the color is proportional to the cell viability. The cells were seeded at 10,000 cells/well for MCF-7 in 96 well tissue culture plates and incubated for 24 h. The medium was then replaced with fresh medium containing free DOX, dextran nanoparticles, and DOX-loaded nanoparticles over an equivalent DOX concentration range of 0.001 – 50 µM. At 72 h post drug/nanoparticle incubation, treatments were removed and fresh media was added (100 µL) followed by the addition of Alamar Blue dye (20 µL) to each well and the cells were incubated for 6 h. Cell viability was determined as a percentage of untreated control cells, and IC₅₀ values were calculated via regression analysis using Microsoft Excel.

In-vitro cell uptake by flow cytometry and confocal microscopy

To examine nanoparticle uptake, MCF7 cells were seeded at a density of 2×10^5 cells/well in 6-well tissue culture plates. The cells were left to grow for 24h in DMEM media containing 10% FBS at 37 °C in 5% CO₂ atmosphere. After 24 h, doxorubicin loaded micelles were added to the wells (concentration of 0.125µM based on DOX) and the cells were incubated for 30 min, 1 h, 6h, or 24 h. Following particle incubation, cells were rinsed twice with PBS to remove any non-uptaken nanoparticles. Cells were

harvested by trypsinization and resuspended in 500 μ l of PBS for flow cytometry analysis using the FACScanto flow cytometer (BD Biosciences). Data shown are the mean fluorescent signal for 10,000 cells. MCF-7 cells that were not treated with nanoparticle solution were used as a control.



Scheme S1. Schematic illustration of macro-CTA synthesis via RAFT polymerization.

Table S1 Conversion, molecular weight and polydispersity data for POEGMA homopolymers.

Time (h)	Conversion (%) ^a	$M_{n(\text{NMR})}$ g mol^{-1} ^b	$M_{n(\text{SEC})}$ g mol^{-1} ^c	Yield (%) ^d	PDI
2	13.94	2400	4600	8.97	1.11
4	25.96	4200	6300	22.11	1.09
6	38.87	6100	8400	37.24	1.10
8	52.63	8200	10100	50.46	1.11
10	61.85	9600	11600	60.07	1.11
12	67.43	10400	12100	64.32	1.12

^a OEGMA conversion was determined from ¹H-NMR of the reaction mixture by comparing the vinyl peaks (6.05 and 5.5 ppm) to the aliphatic proton peaks (0.93 and 0.74 ppm).

^b NMR molecular weight was calculated according to $M_n = ([M]_0 / [RAFT]_0) \times \alpha \times M_{w_{\text{monomer}}} + M_{w_{\text{RAFT}}}$, where $[M]_0$, $[RAFT]_0$, α , $M_{w_{\text{monomer}}}$, $M_{w_{\text{RAFT}}}$ are monomer RAFT agent concentration, monomer conversion molecular weights of monomer and RAFT agent, respectively.

^c The experimental M_n and PDI were determined by SEC using dimethyl acetamide as eluent solvent with polystyrene standards (the molecular weight ranging from 168 to 10^6 g mol^{-1}).

^d Polymerization yield was determined gravimetrically.

Table S2. Summary of the RAFT dispersion block copolymerization of styrene and vinyl benzaldehyde in the presence of POEGMA as a macro-CTA.

Time (h)	VBA Conv. (%) ^a	ST Conv. (%) ^b	Total Conv. (%) ^c	$M_{n(\text{NMR})}$ gmol ^{-1d}	$M_{n(\text{SEC})}$ gmol ^{-1e}	PDI
4	7.2	3.0	3.21	28300	26300	1.16
8	8.6	5.6	5.75	41700	33600	1.20
12	10.1	6.6	6.77	47200	37600	1.21
18	17.9	9.0	9.44	61500	40300	1.20
24	21.7	9.2	9.82	63800	43300	1.20
36	24.3	9.4	10.14	65600	44000	1.20

^a VBA conversion was calculated from ¹H-NMR spectrum of the reaction mixture by dividing of aldehyde protons of the poly(vinylbenzaldehyde) peak (9.75 ppm) integral with the sum of aldehyde protons of vinyl benzaldehyde (10.03 ppm) and PVBA peak integrals.

^b ST conversion was calculated from ¹H-NMR spectrum of the reaction mixture by dividing the sum of the polystyrene, poly(vinyl benzaldehyde) aromatic protons and one vinyl proton of the both monomer peaks integrals between 7.2 ppm to 6.3 ppm with the sum of ST and VBA vinyl peaks (5.8 and 5.3 ppm).

^c Total conversion was calculated using; $\text{Conv}_T = 0.95 * \text{Conv}_{(ST)} + 0.05 * \text{Conv}_{(VBA)}$

^d NMR molecular weight was calculated according to $M_n = ([M_{ST}]_o / [RAFT]_o) * x_{ST} * M_{W_{ST}} + ([M_{VBA}]_o / [RAFT]_o) * x_{VBA} * M_{W_{VBA}} + M_{W_{RAFT}}$, where $[M_{ST}]_o$, $[M_{VBA}]_o$, $[RAFT]_o$, x_{ST} , x_{VBA} , $M_{W_{ST}}$, $M_{W_{VBA}}$ and $M_{W_{RAFT}}$ are monomers and RAFT agent concentration, monomers conversion, molecular weight of monomer and RAFT agent, respectively.

^e The experimental M_n and PDI were determined by SEC using dimethyl acetamide as eluent solvent with polystyrene standards (the molecular weight ranging from 168 to 10⁶ gmol⁻¹).

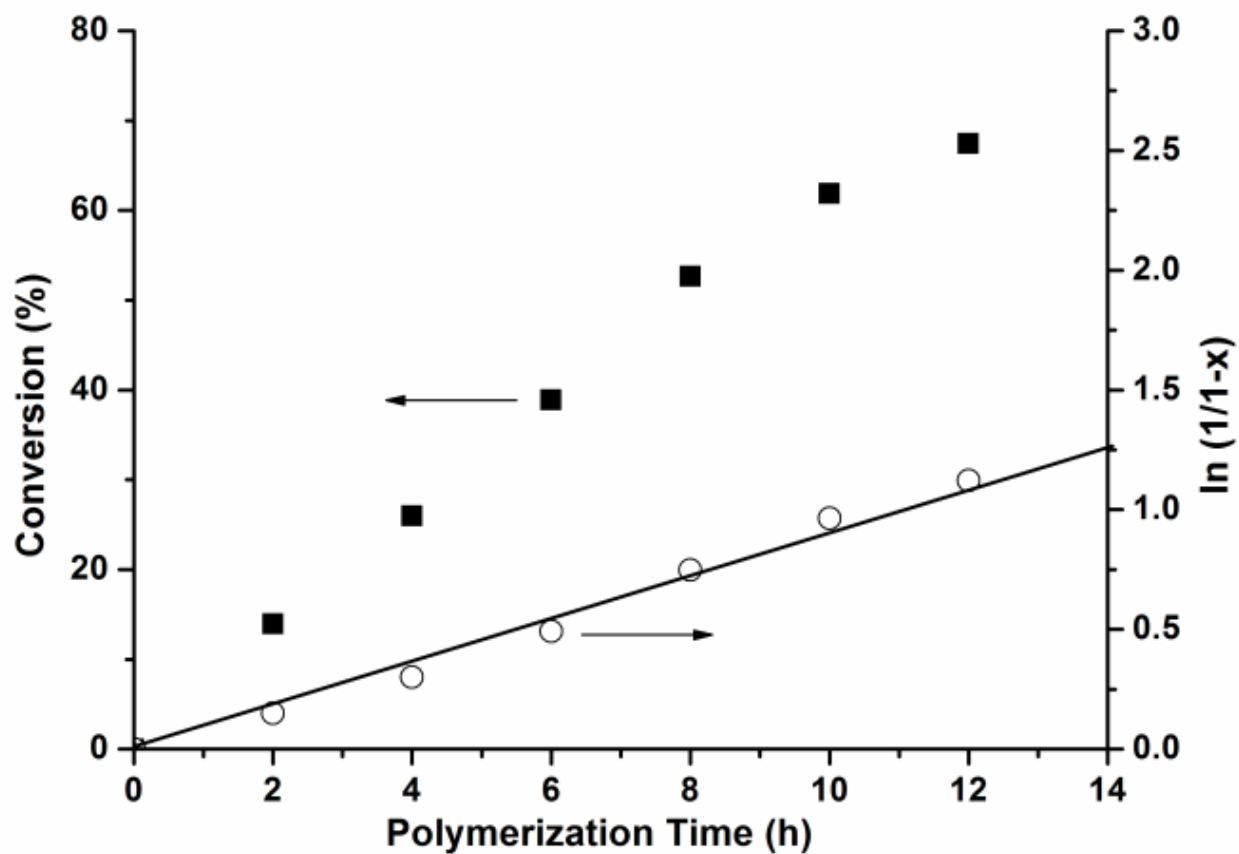


Figure S1. Time-conversion and first-order kinetic plots for the polymerization of OEGMA in the presence of CPADB (left side).

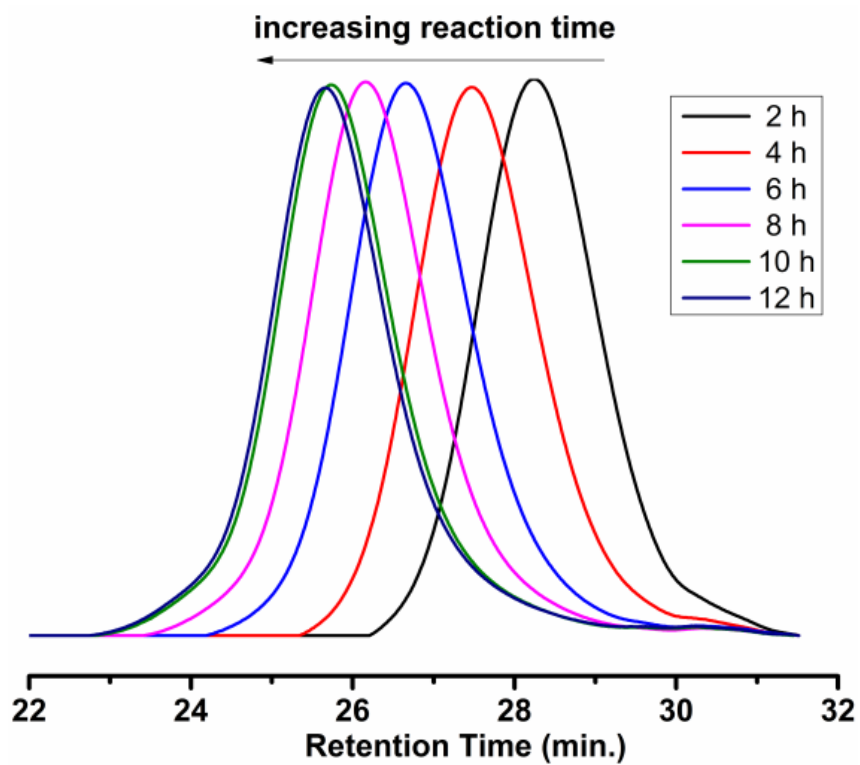


Figure S2. SEC traces of POEGMA at different polymerization times (right side).

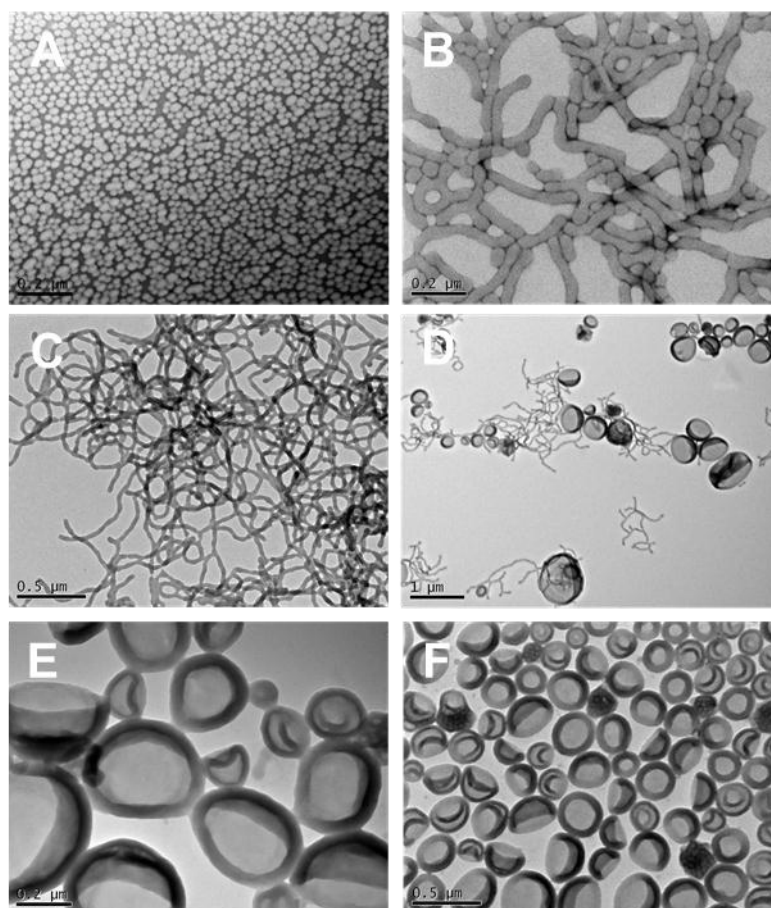


Figure S3. Additional transmission electron microscopy (TEM) pictures of different polymerization solutions after purification by dialysis against methanol (pictures from another batch). Note: A after 4 h of polymerization, B after 8 h of polymerization, C after 12 h of polymerization, D after 16 h of polymerization, E after 24 h of polymerization and F after 36 h of polymerization. Note: Uranyl acetate staining was applied for sample A, B and C.

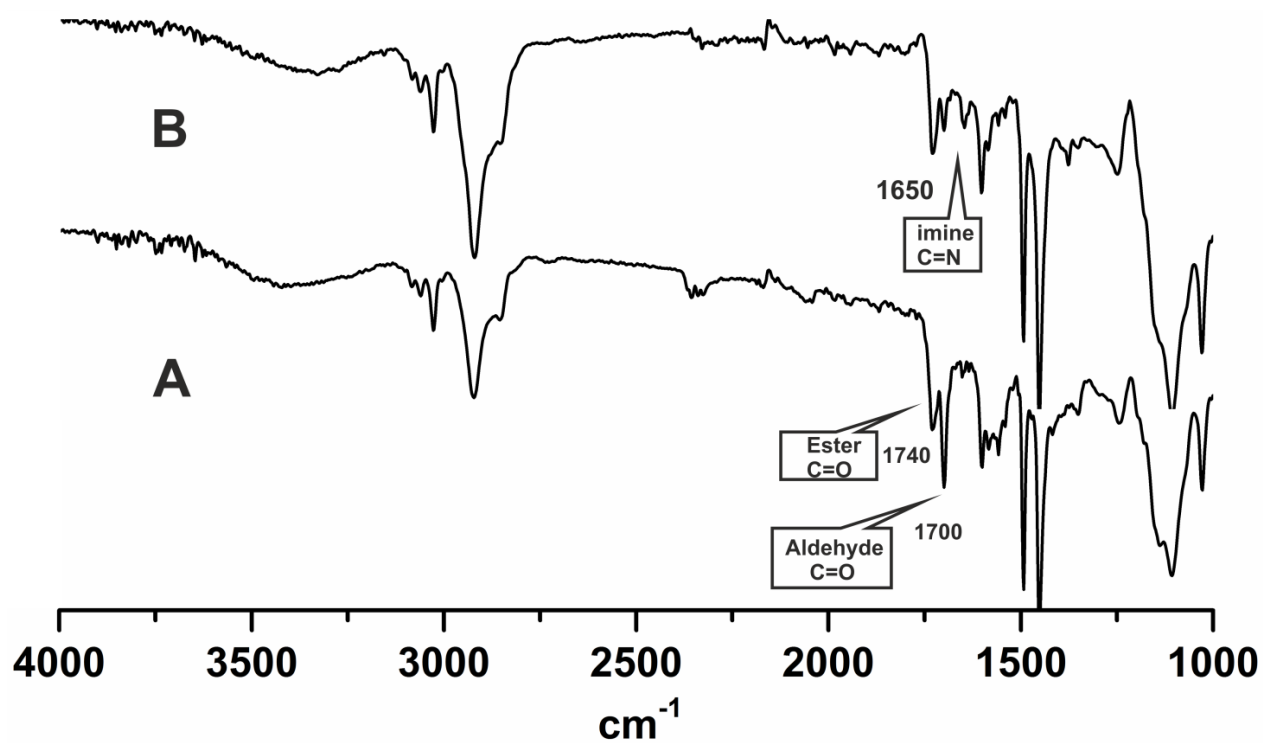


Figure S4. FT-IR spectra before (A) and after crosslinking (B) of the POEGMA-*b*-PS-*co*-PVBA block copolymer (36 h sample).

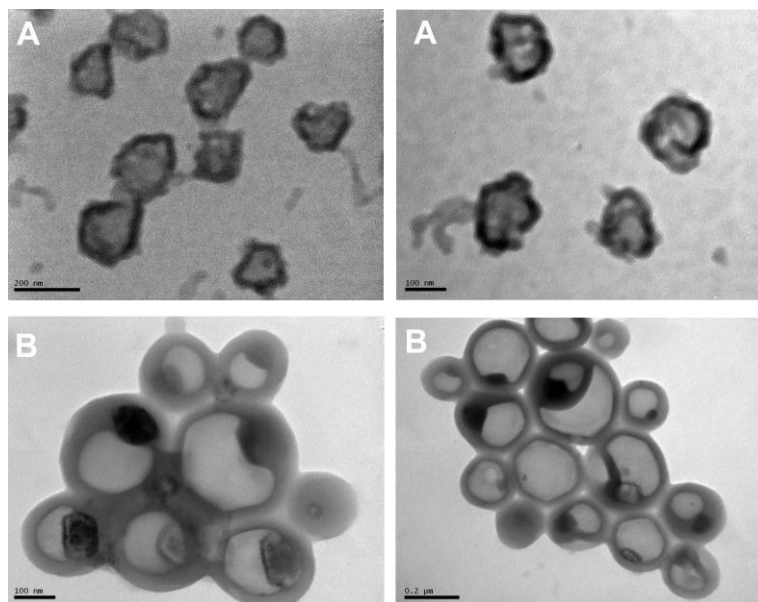


Figure S5. Transmission electron microscopy (TEM) of different cross-linked core nanoparticles dispersed in THF A- before reduction with sodium borohydrate; B- after reduction with sodium boroydrate.

Note: THF is a good solvent for both blocks. Uncrosslinked vesicles dissolve in THF.

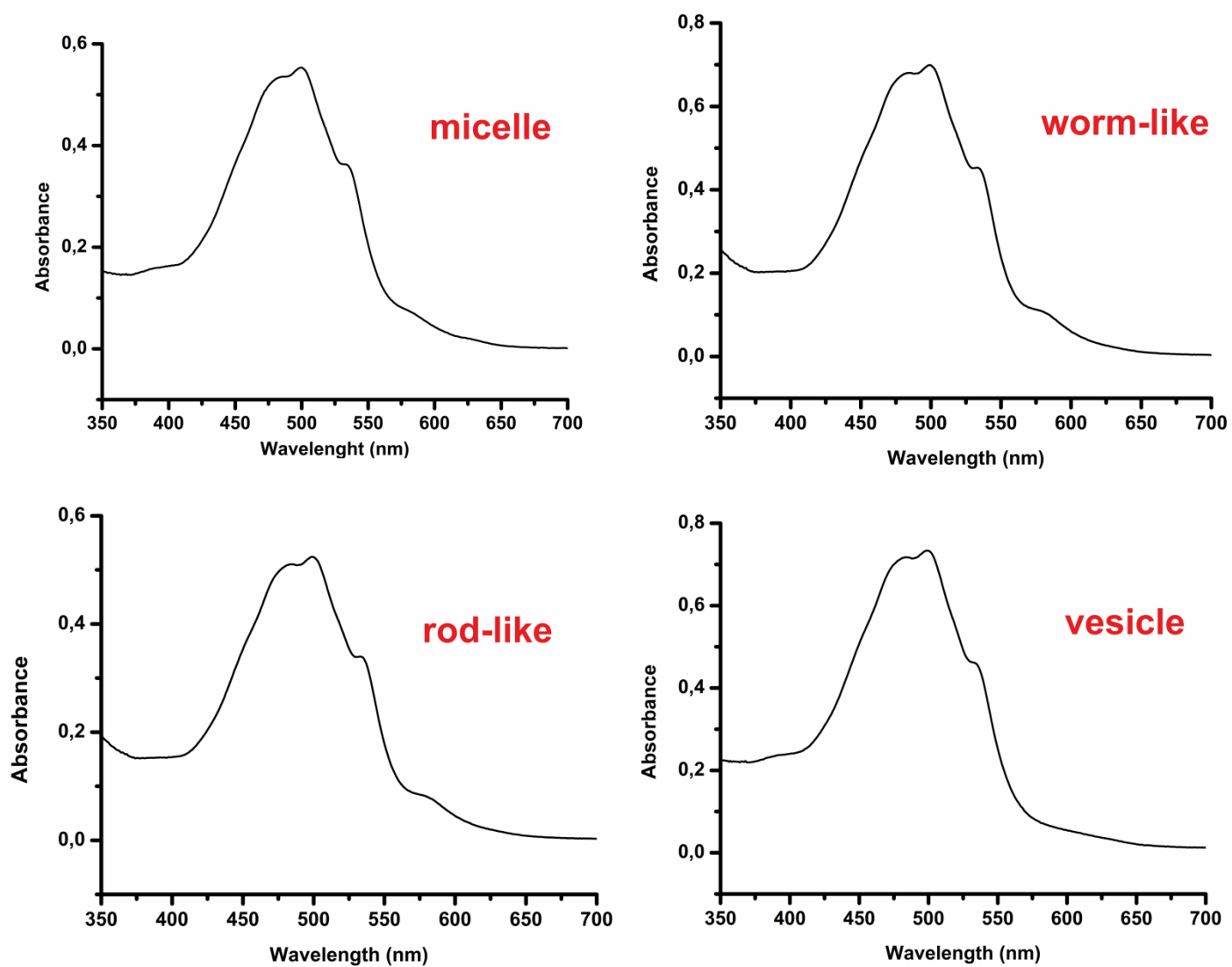


Figure S6. Examples of UV-visible absorbance spectra of DOX conjugated micelle, worm, rod-like and vesicle.

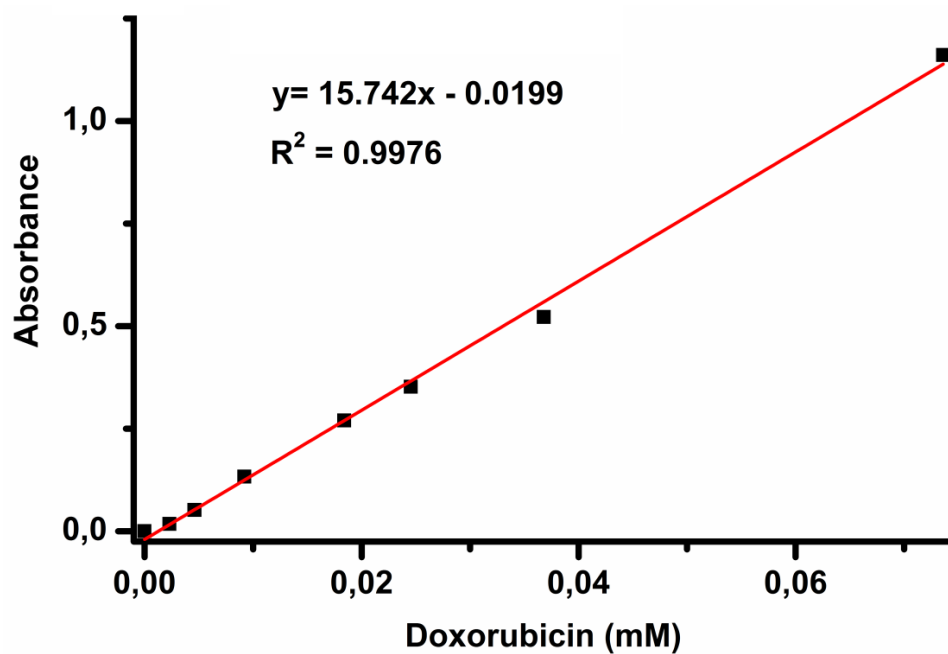


Figure S7. Calibration curve of doxorubicin using UV-visible (absorbance determined at 495 nm).

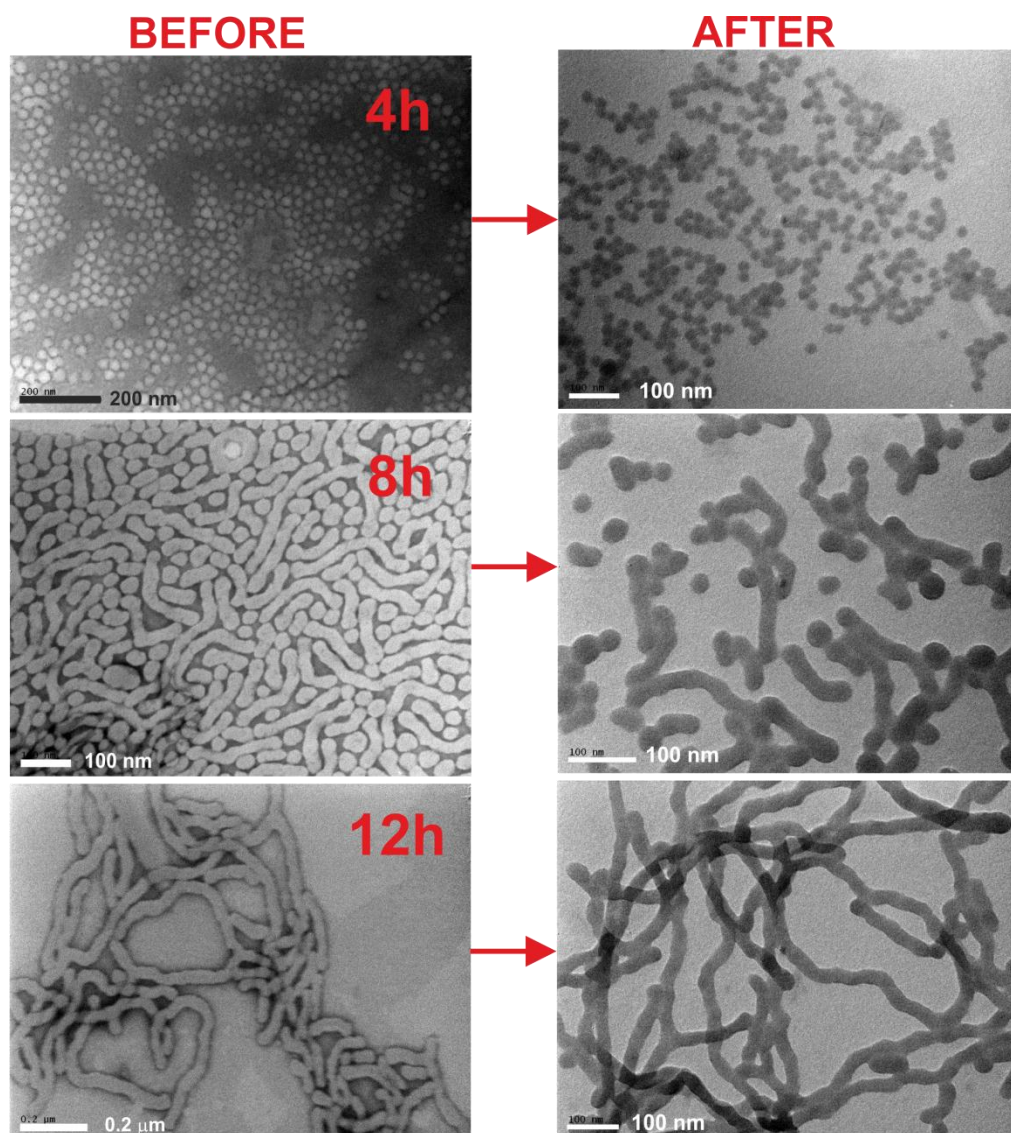


Figure S8. Transmission electron microscopy (TEM) micrographs of (left) unloaded nanoparticles and (right) different DOX loaded nanoparticles in water (after purification by dialysis against methanol, and then water).

Note: Uranyl acetate staining was applied for samples 4, 8 and 12 h without DOX to improve the contrast. In the case of the DOX loaded samples, we found that the staining was not required.

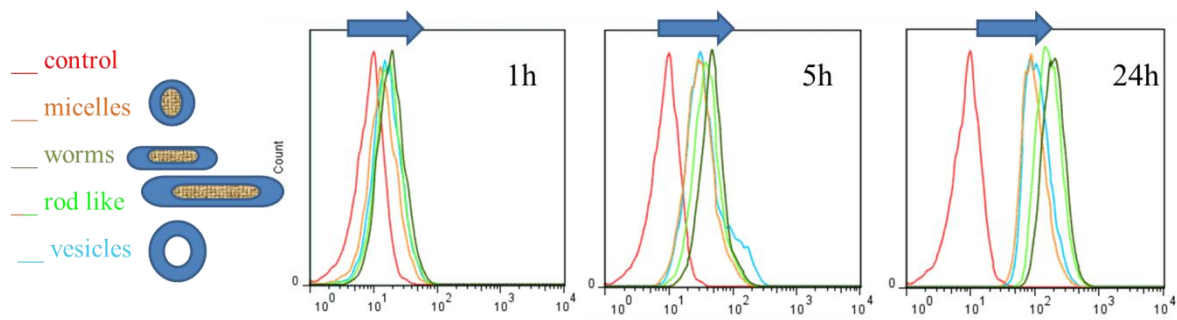


Figure S9. Cell uptake of different nanoparticle morphologies using MCF-7 breast cancer cells using flow cytometry at different time points (1 h, 5 h and 24 h).

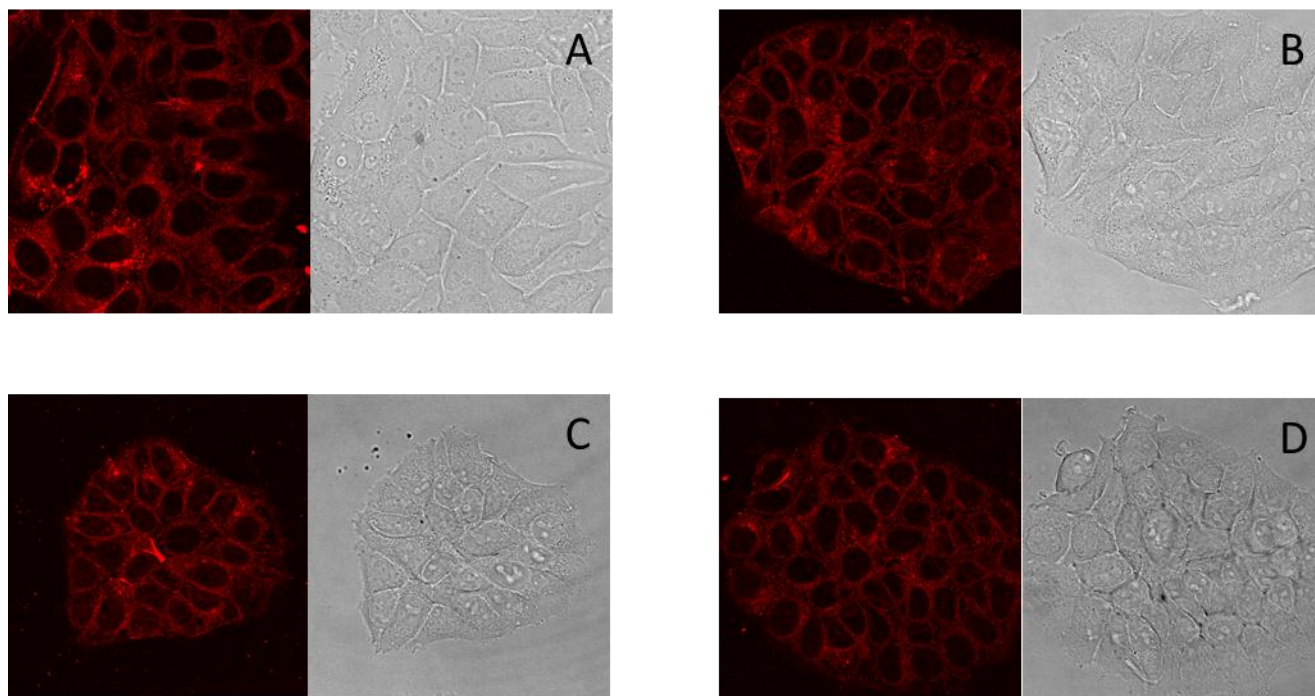


Figure S10: Confocal laser scanning microscopy showing cell uptake of different morphological nanostructures (A) micelles, (B) worm, (C) rod-like and (D) vesicle using MCF-7 cells after 3 hours of incubation.

Note 1: DOX fluorescent images were acquired at $\lambda_{excitation} = 485 \text{ nm}$ and $\lambda_{emission}$ was collected between 565 and 630 nm.

Note 2: DOX is localized in the cytoplasm in the cells.

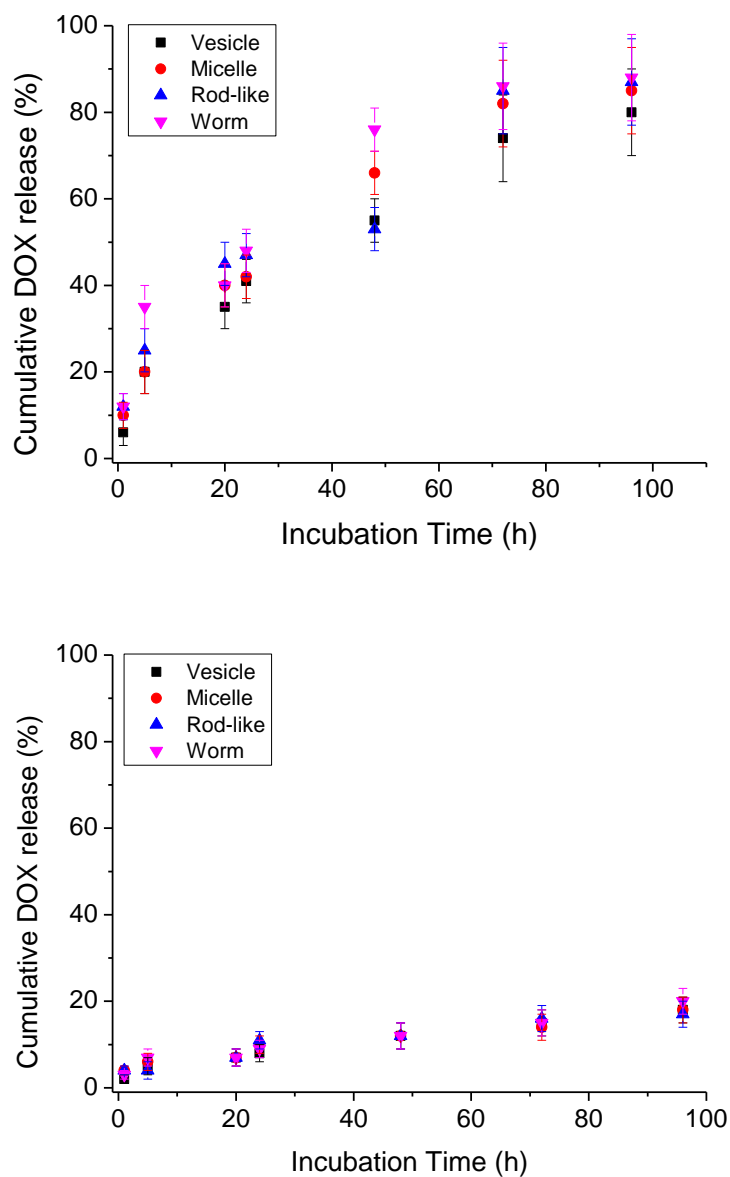


Figure S11. DOX released from different nanoparticles (up) at pH 5.0 and (bottom) at pH 7.4.

Additional References:

- 1- Y. Mitsukami, M. S. Donovan, A. B. Lowe, C. L. McCormick, *Macromolecules*, 2001, **34**, 2248