Decreased core crystallinity facilitated drug loading in polymeric micelles without affecting their biological performances

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Polymer synthesis and characterization

Homopolymer (PCL) and copolymer (mPEG-b-PCL) were synthesized according to literature[1] using n-butanol or $mPEG_{5k}$-OH as initiators, respectively, and $Sn(Oct)_2$ as the catalyst. All polymerizations were conducted in bulk under 120 ˚C for 24h. Synthesis of mPEG-b-PCL with different PCL lengths (10k Da, 13k Da and 16k Da) was realized by controlling the monomer/initiator ratio. The synthesized polymers were characterized by ^{1}H-NMR and gel permeation chromatography (GPC). The ^{1}H-NMR spectra of all samples were obtained using a Bruker Avance III HD NMR spectrometer at 400MHz. The solvent used was $CDCl_3$. GPC was performed on a Water 1515 GPC using three Styragel columns (Waters Corp: HT3, HT4 and HT5) in tandem and a 2414 differential refractive index detector. DMF was used as eluent at a flow rate of 1.0 mL/min with column temperature set at 35 ˚C and sample concentration was approximately 2 mg/mL. The molecular weight was calibrated using polystyrene standards.

Sample preparation

DSC: PCL/MCT blends were prepared as follows: a stock solution of MCT in dichloromethane (DCM) at a concentration of 10 mg/mL was added to approximately 10 mg of PCL at volumes of 50μL, 100μL, 200 μL, 400 μL, 600 μL and 800 μL, and then the total volume of DCM was adjusted to 1 mL. After complete mixing, the solutions prepared were added dropwise to clean glass slides at room temperature in a fume hood to allow evaporation of DCM. Then the white solid remaining on the glass slides was harvested and subjected to DSC analysis.

AFM: Sample solution (PCL and 40% MCT) was prepared similarly to DSC measurements except that the amount of PCL used was 40 mg. In a fume hood, the solution was added dropwise to a clean glass slide heated at 60 ˚C. After addition, the melted polymer was kept at 60 ˚C for another

5 min to ensure complete vaporization of THF. Then, the melted polymer was allowed to cool to room temperature. The prepared sample was wax-like in apprearance and approximately 1 mm thick. Then sample was carefully removed from the glass slide and the side in contact with the glass side was subjected to AFM measurement.

MCT-free micelles and OCMs were prepared by dialysis using $mPEG_{5k}$-b-PCL_{10k} and diluted to 0.04 mg/mL. Aliquots of the samples were pipetted on silicon substrates, after 30 min the excess liquid was removed by filter paper. AFM was conducted on an Asylum Research Cypher AFM, using Asylum Research SYELEC-01 Ir-coated, noncontact mode Si cantilevers, with a resonant frequency of 70 kHz and a spring constant of 2 N/m.

Cryogenic transmission electron microscopy (Cryo-TEM): The sample was diluted to 1 mg/mL by deionized water before imaging. An aliquot of sample (3.5 μL) was placed on a freshly carbon-coated holey TEM grid. The sample was prepared using Vitrobot (FEI) and stored under liquid nitrogen. Then the sample was placed in a FEI Tecnai 20 electron microscope using a cold stage and imaged with an accelerating voltage of 200 kV. Images were obtained on a bottom mounted CCD camera system (Ultrascan 894, Gatan).

Critical aggregation concentration: MCT-free micelles and OCMs with MCT contents of 5%, 10%, 40%, 50% and 80% were prepared by dialysis with 10 mg of block copolymers. Then the micelle solutions were transferred to 10 mL volumetric flasks to give stock solutions at 1 mg/mL block copolymer. A stock solution of pyrene at 6×10^{-5} M in acetone was prepared. Then, 100 μL of this solution was transferred to a series of volumetric flasks (10 mL), and the solvent was evaporated in vacuum. Then aliquots of the block copolymer stock solutions were added into the pyrene-containing volumetric flasks, yielding block copolymer solutions with concentrations

ranging from 1×10^{-6} mg/mL to 0.1 mg/mL. Then the samples were sonicated for 30 min and then equilibrated overnight.

Core polarity: A stock solution of pyrene at 6×10^{-5} M in acetone was prepared. Then, 100 µL of this solution was transferred to a volumetric flask (10 mL), and the solvent was evaporated in vacuum. Aliquots of MCT-free micelles or OCMs were added to volumetric flasks to give a final polymer concentration of 0.5 mg/mL. The samples were sonicated for 30 min and then equilibrated overnight.

Core viscosity: Aliquots of a P3P stock solution in chloroform were transferred to a volumetric flask (10 mL) to obtain final P3P concentration of 2×10^{-7} M^2 upon dilution. The concentration of polymer in all samples was fixed at 0.5 mg/mL.

Methods

Determination of DLC:

HPLC equipment: Hitachi, L-2130 pump, L-2400 UV detector, L-2200 auto-sampler and L-2300 column oven.

Column: Unitary C18 column (5µm, 150×4.6 mm, Acchrom, China).

Samples for HPLC assays were prepared by dilution of 200 µL MCT-free micelles or OCMs to 10 mL with THF followed by passage through a 0.45 µm filter. The mobile phases used were: acetonitrile and water – 65:35 v/v (CTX and TM-2); and methanol and water – 70:30 v/v (DSF). The detection wavelengths of CTX, TM-2 and DSF were 230 nm, 227nm and 254nm, respectively. The flow rate and column temperature for the drugs were set at 1.0 mL/min and 25 ˚C, respectively. Drug loading content (DLC) was calculated using the following equation:

$$\mathrm{DLC}\ (\%) = \frac{\mathrm{Weight\ of\ drug\ in\ micelles}}{\mathrm{Weight\ of\ (drug + polymer + MCT)}} \times 100\%$$

***In vitro* drug release**: The drug-loaded preparations were placed in dialysis bags (MWCO: 14k Da) which were then incubated in 10 mL PBS (10 mM) at pH 7.4 containing 0.5% tween 80 at 37±0.5 ˚C in a horizontal water bath shaker at a shaking speed of 100 rpm. At predetermined time intervals, an aliquot of release medium was taken and centrifuged at 10000 rpm and the supernatant was subjected to HPLC analysis. The remaining release medium was discarded and replaced with fresh medium.

Cell studies:

Cell culture: The PC3 and MCF-7 cell lines were cultured in RPMI 1640 medium and DMEM, respectively, and both were supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and streptomycin). Both types of cell were cultured in a 95% air humidified atmosphere containing 5% CO_2 at 37 ˚C. PC3 or MCF-7 cells were seeded at a density of 1×10^4 cells per well in 96-well plates and allowed to grow overnight.

Cell viability: cells were incubated with 100 μL culture medium containing free CTX or CTX-loaded formulations for 48 h or 72 h. The concentration range of the empty OCMs was 0.1-1000 μg/mL while, for free CTX and CTX-loaded formulations, the range was 1-1000 nM. After incubation, the culture medium was removed and replaced with 90 μL fresh medium with 10 μL MTT solution (5 mg/mL). Then, the cells were further incubated for another 4 h. Cells incubated with medium were used as a negative control. After incubation, MTT was removed and 200 μL DMSO was added to dissolve the formazan. The absorbance was assayed at 492 nm by a microplate reader (SpectraMax M3, Molecular Devices) and the cell viability was calculated using the following equation:

$$\text{Cell Viability}\,(\%) = \frac{A_{\text{formualtion}}}{A_{\text{control}}} \times 100\%$$

Cell uptake: PC3 and MCF- cells were cultured overnight at a density of 1×10^3 cells per well in 96-well plates. Then, the medium was replaced with fresh medium containing coumarin-6 loaded MCT-free micelles or OCMs. The concentration of coumarin-6 in the OCMs or MCT-free micelles was determined by HPLC (mobile phase: methanol and water – 90:10 v/v; detection wavelength: 444 nm; flow rate: 1.0 mL/min; column temperature: 25 ˚C). After further incubation for 4 h, the cells were washed three times with cold PBS and fixed with 4% paraformaldehyde for 20 min and then the nuclei were stained with DAPI. Finally, 70 µL 50% glycerol was added to each well and the accumulation of coumarin-6 was visualized using an Image Xpress® Micro instrument (Molecular Devices, Metaxpress™).

Pharmacokinetics: Fifteen male rats were randomly divided into three groups and were administered intravenously with the three preparations at dosage of 4 mg/kg. At 0.833, 0.25, 0.5, 0.75, 1, 2, 4, 8, and 12h after administration, 0.5 mL of blood samples were collected into heparinized tubes. Then, the blood samples were centrifuged at 4000 rpm for 10 min to separate the plasma. The obtained plasma was stored at -20 ˚C before further treatment. Plasma sample was treated as follows: 200 µL plasma was mixed with 20 µL internal standard solution (larotaxel, 500 ng/mL in methanol) and then extracted by 3 mL methy tert-butyl ether by vortexing for 10 min. After centrifugation for 10 min, 2 mL of the supernatant was collected and evaporated to dryness under nitrogen at 35 ˚C. The residue was reconstituted in 100 µL acetonitrile and vortexed for 5 min, and then centrifuged at 12,000 rpm for 10 min, and an aliquot of 5 µL was used for analysis.

Table S1. Characteristics of the synthesized polymers

Sample	$M_{n,\ theoretical}$	M_n[a]	Hydrophobic ratio[b]	PDI[c]
PCL_{10k}	10,000	10,400	-	1.33
$mPEG_{5k}$-b-PCL_{10k}	15,000	9,900	2	1.20
$mPEG_{5k}$-b-PCL_{13k}	18,000	13,000	2.6	1.19
$mPEG_{5k}$-b-PCL_{16k}	21,000	15,800	3.2	1.36

[a] Calculated from the ^{1}H-NMR spectra;

[b] The hydrophobic ratio was defined as $M_{n,\ hydrophobic}/M_{n,\ hydrophilic}$, and $M_{n,\ hydrophilic}$ was held at 5000;

[c] Determined by GPC.

Table S2. Loading efficiency of samples prepared in maximum drug loading experiment.

	Cabazitaxel	Disulfiram	TM-2
0%	67.87%±3.38%	53.67%±2.13%	76.64%±2.12%
5%	67.36±2.75%	55.21±1.09%	74.86%±3.86%
10%	69.27±1.33%	62.35±2.74%	76.42%±1.97%
25%	74.12%±2.80%	75.82%±1.95%	78.37%±2.46%
50%	79.32%±1.58%	70.64%±2.45%	78.26%±2.65%

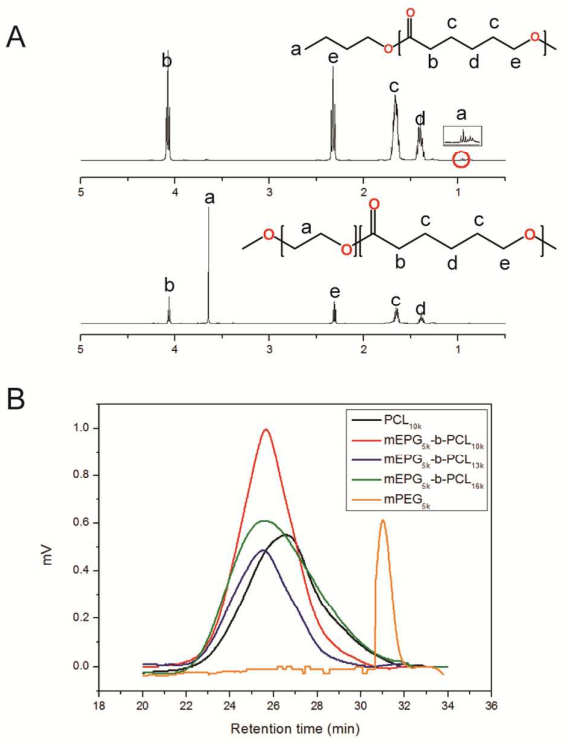

Figure S1. Characterization of synthesized polymers. (A). ^{1}H-NMR spectra of PCL (upper) and mPEG-b-PCL (lower), inset: the proton signal of methyl in n-butanol. (B). GPC traces of mPEG macro-initiator, PCL homopolymer and mPEG-b-PCL copolymers with different molecular weights.

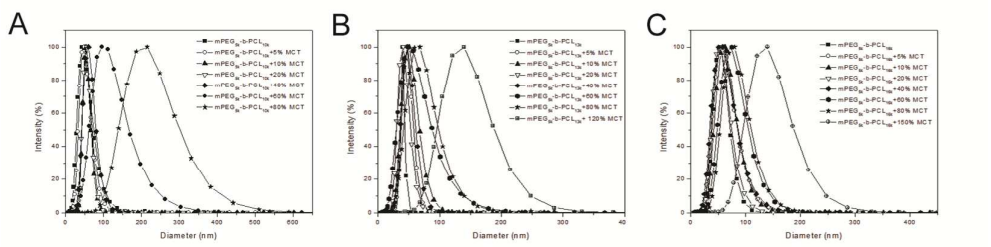

Figure S2. Distribution plots of nano-assemblies composed of block copolymers with different hydrophobic lengths and different MCT contents. (A). $mPEG_{5k}$-b-PCL_{10k}; (B) $mPEG_{5k}$-b-PCL_{13k} and (C) $mPEG_{5k}$-b-PCL_{16k}.

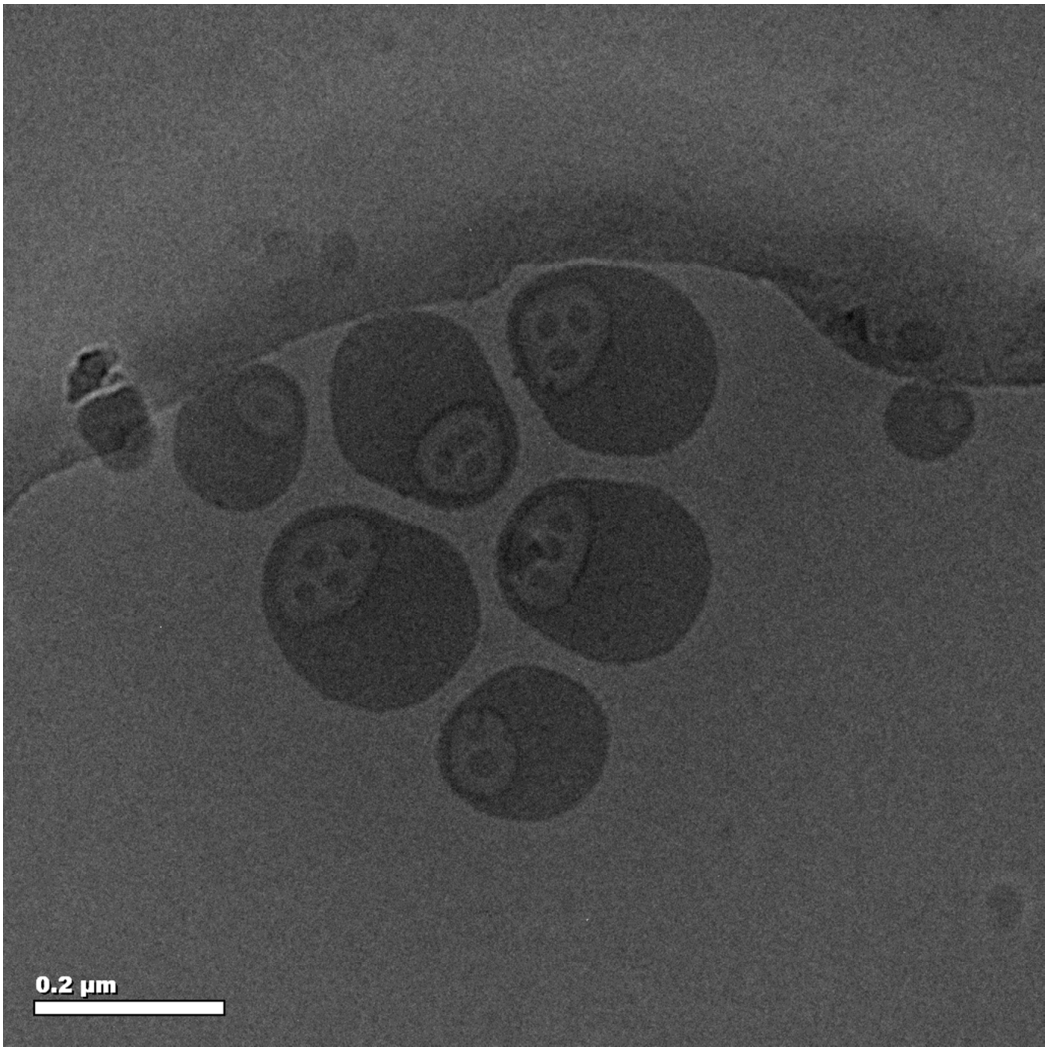

Figure S3. Cryo-TEM image of OCMs with MCT contents of 80%.

References:

1. Zhu, X.; Fryd, M.; Tran, B. D.; Ilies, M. A.; Wayland, B. B., Modifying the Hydrophilic–Hydrophobic Interface of PEG-b-PCL To Increase Micelle Stability: Preparation of PEG-b-PBO-b-PCL Triblock Copolymers, Micelle Formation, and Hydrolysis Kinetics. *Macromolecules* **2012,** 45, (2), 660-665.

2. Vakil, R.; Kwon, G. S., Poly(ethylene glycol)-b-Poly(ε-caprolactone) and PEG-Phospholipid Form Stable Mixed Micelles in Aqueous Media. *Langmuir* **2006,** 22, (23), 9723-9729.