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Electronic Supplementary Information

Amphiphilic hyperbranched polymers with biodegradable hyperbranched poly(ε-caprolactone) core prepared from homologous AB_2 macromonomer

Xiaojin Zhang,[a,*] Juan Cheng[b] and Renxi Zhuo[a]

Experimental Section

1. Materials

Tetrahydrofuran (THF) was distilled over Na–K alloy in the presence of benzophenone before use. Toluene was dried over Na before use. Poly(ethylene glycol) methyl ether ($mPEG^1$ MW 5000 and mPEG MW 2000) was purchased from Acros and used as received. ε-Caprolactone (ε-CL, 99%) was purchased from Acros and purified by distillation under reduced pressure prior to use. Stannous octoate [$Sn(Oct)_2$, 95%] was purchased from Aldrich, purified by distillation under reduced pressure and then dissolved in dry toluene prior to use. Other reagents were purchased from Sinoparm Chemical Reagent Co., Ltd in China and used as received. $mPEG^1$-*b*-PCL were synthesized as described in the literature.[1]

2. Measurements

Proton nuclear magnetic resonance (^1H NMR) analyses were performed on a Mercury VX-300 spectrometer using tetramethylsilane (TMS) as an internal reference and $CDCl_3$ as a solvent. The molecular weight and polydispersity index (PDI) were determined by gel permeation chromatography (GPC) using a Waters high-pressure liquid chromatographic system equipped with a model 2690D separation module, a model 2410 refractive-index detector, and Styragel HR1 THF and HR4 THF columns. THF was used as the eluent at a flow rate of 0.3 mL min^{-1}. Poly(ethylene glycol) standards with narrow distribution were used to generate a calibration curve.

3. Synthesis of amphiphilic hyperbranched polymers mPEG-HPCL

Benzyl-protected carboxyl-terminated poly(ε-caprolactone) (BPCL) was synthesized by ring-opening polymerization of ε-caprolactone with dibenzyl malate[2] as an initiator and $Sn(Oct)_2$ as a catalyst. Typically, a round bottom long-neck flask pretreated with trimethylchlorosilane was charged with dibenzyl malate (0.47 g, 1.5 mmol), ε-CL (2.05 g, 18 mmol or 4.10 g, 36 mmol) and 0.1 M $Sn(Oct)_2$ solution (180 μL or 360 μL) in dry toluene. The flask was evacuated and charged with argon three times, and then sealed under vacuum with a magnetic stirring bar inside. After the mixture was stirred at 110 ºC for 24 h, the polymerization was quenched by immersing the flask in a cool water bath. The product was purified by precipitation from dichloromethane with cold methanol and subsequently dried under vacuum to obtain a white solid in approximately 95% yield.

Carboxyl-terminated poly(ε-caprolactone) (CPCL) was synthesized via the hydrogenolytic deprotection of BPCL with Pd/C as a catalyst under room temperature and normal pressure. Typically, a flask was charged with BPCL (2.00 g), Pd/C (10 wt%, 0.20 g) and 20 mL of dry THF. The flask was evacuated and charged with hydrogen three times. After the mixture was stirred at room temperature for 48 h under normal hydrogen pressure, the Pd/C catalyst was removed by

filtration. The filtrate was evaporated to give a white solid in approximately 96% yield.

Amphiphilic hyperbranched polymers mPEG-HPCL were synthesized by one-pot polyesterification of AB_2 macromonomer CPCL and mPEG. In order to be able to calculate molar ratio and molecular weight of mPEG-HPCL from the ^{1}H NMR spectrum, a small amount of BPCL was added and the integration of the peak areas for the benzyl groups was used as the base. Taking the synthesis of $mPEG_{2.7}$-$(HPCL_{10})_{5.2}$ as an example, a flask was charged with BPCL (0.14 g, 0.1 mmol), CPCL (0.60 g, 0.5 mmol), DMAP (12.2 mg, 0.10 mmol), DCC (0.206 g, 1.0 mmol) and 10 mL of dry THF. After keep stirring at room temperature for 24 h, mPEG (0.60 g, 0.3 mmol), DMAP (7.3 mg, 0.06 mmol), and DCC (0.12 g, 0.6 mmol) were added to the solution. After keep stirring at room temperature for other 24 h, the precipitated DCC-urea was removed by filtration. The filtrate was concentrated under pressure, then transferred into a dialysis bag (MWCO 3500 Da) and dialyzed against deionized water for 2 days. The product was obtained by lyophilization to give a flocculent solid in approximately 85% yield.

4. Micelle preparation and characterization

To a stirred solution of amphiphilic block or hyperbranched polymer (20 mg) with or without methotrexate (2.0 mg) in 5 mL of THF, was added 10 mL of deionized water dropwise. The resulting solution was stirred continuously overnight and then dialyzed against deionized water for 2 days. The micelle solution was obtained after filtration through a membrane filter with a pore size of 0.45 μm.

The micelle size and size distribution were determined by dynamic light scattering (DLS). Measurements were carried out and repeated three times at 25 ºC with a scattering angle (θ) of 90º in optically homogeneous quartz cylinder cuvette by using a Beckman Coulter N4 Plus submicron particle sizer. A JEOL JEM-3011 transmission electron microscope (TEM) was used to characterize the morphology of micelles. The samples for TEM analysis were prepared as follows: one drop of micellar solution was added onto a carbon-coated copper grid. After 3-5 min, most of the solution was removed by touching edge of filter paper until the grid surface is nearly dry. A drop of 1% phosphotungstic acid solution (pH 7.2 adjusted with NaOH) was added onto the copper grid for negative staining. About 1-2 min later, the staining solution was removed by touching on a piece of filter paper. The grid was allowed to dry under ambient conditions.

5. Determination of drug loading content (DLC) and entrapment efficiency (EE)

To determine the DLC and EE, the drug-loaded micelle solution was dried by rotary evaporation and then dissolved in DMF (chromatographic grade). The UV absorbance at 303 nm was measured to determine the drug concentration with a Perkin-Elmer Lambda Bio 40 UV-vis spectrophotometer.

DLC and EE were calculated as follows:

DLC (%) = weight of loaded drug/weight of polymer and loaded drug × 100

EE (%) = weight of loaded drug/weight of drug in feed × 100

6. In vitro drug release behavior

The drug-loaded micelle solution was placed in a dialysis bag (MWCO 3500 Da). The dialysis

bag was sealed and immersed in 40 mL of phosphate-buffered saline (PBS) (pH 7.4). *In vitro* drug release study of drug-loaded micelle was carried out in a shaking water bath at 37 ºC. About 3 mL of solution was taken out and the same volume of PBS solution was added after each sampling at predetermined time intervals. The drug concentration was determined by measuring the absorbance of methotrexate at 303 nm. The rate of drug release was measured by the released concentration of methotrexate at predetermined time intervals according to the calibration curve of methotrexate.

7. In vitro cytotoxicity assay

4.0 mg of polymer was dissolved into 2 ml of cell culture medium (DMEM) containing 2×10^{-3} M glutamine, 10% FBS and 50 units P/S. The solution was filtered through a 0.22 μm membrane filter and diluted in DMEM to different concentrations. COS7 cells were seeded into a 96-well plate, 5000 cells/well, in 100 μL complete DMEM. After the cells have grown at 37 ºC for 24 h in an atmosphere containing 5% CO_2 and 95% air, 100 μL of polymer solution at different concentrations was added to the wells containing cells and complete DMEM (100 μL). Cells were cultured with polymer solutions at 37 ºC for 24 h and then 20 μL of MTT solution (5 mg mL^{-1}) in PBS was added to each well. Cells were cultured at 37 ºC for a further 4 h. 150 μL of DMSO was added to each well to dissolve the formed purple crystals derived from MTT. The absorbance of the solution was measured using microplate reader (Bio-Rad 550, USA) at 570 nm. The percent relative viability related to control well containing complete DMEM without polymer solution was calculated by the following equation:

$$\text{Cell Viability (\%)} = (OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$$

where OD_{sample} is the absorbance of the solution containing cells cultured with copolymer; OD_{blank} is the absorbance of the medium; and $OD_{control}$ is the absorbance of the cells cultured with the medium only.

Table 1 Synthetic results of BPCL[a] and CPCL[b]

Sample[c)]	Feed ratio[d)]	Yield (%)	M_n (predicted)	^{1}H NMR		GPC	
				Composition[d)]	M_n	M_n	PDI
$BPCL_{10}$	1:12	95	1700	1:10	1400	1500	1.16
$BPCL_{22}$	1:24	96	3000	1:22	2800	2900	1.19
$CPCL_{10}$	-	97	1200	-	1200	1400	1.18
$CPCL_{22}$	-	96	2600	-	2600	2800	1.21

[a] Polymerization conditions: [Monomer]/[$Sn(Oct)_2$] = 1000, 110 ºC, 24 h, in bulk;

[b] Pd/C, H_2, THF, room temperature, 48 h;

[c] The subscripted numbers at the lower right corner of a monomer unit represent the degree of polymerization calculated on the basis of ^{1}H NMR spectra;

[d] Molar ratio of dibenzyl malate/ε-CL.

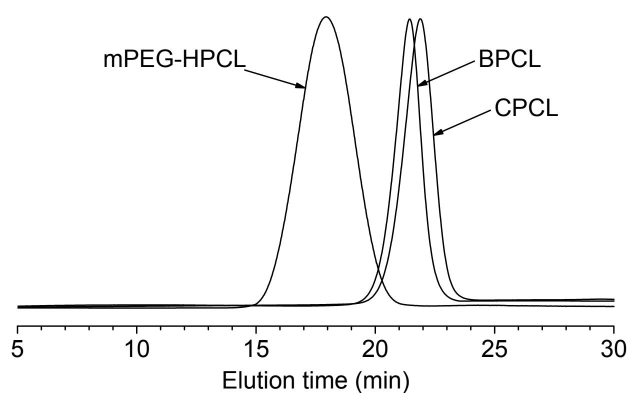

Fig. S1 Normalized GPC curves of $BPCL_{10}$, $CPCL_{10}$, and $mPEG_{2.7}$-$(HPCL_{10})_{5.2}$.

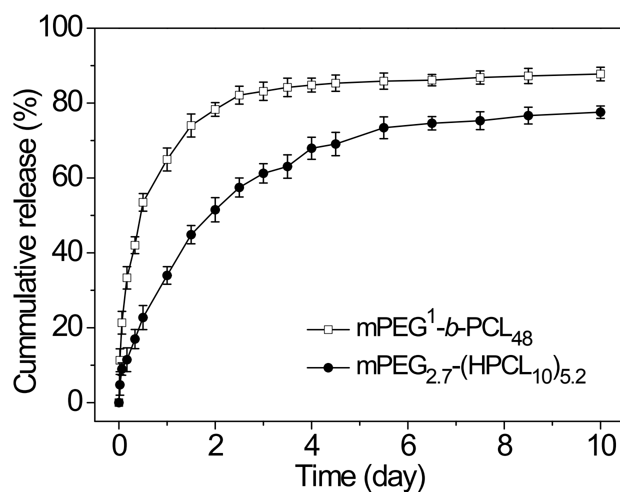

Fig. S2 Release profiles of methotrexate from $mPEG^1$-*b*-PCL_{48} micelles and $mPEG_{2.7}$-$(HPCL_{10})_{5.2}$ micelles (PBS, 0.1M, pH 7.4; 37 ºC).

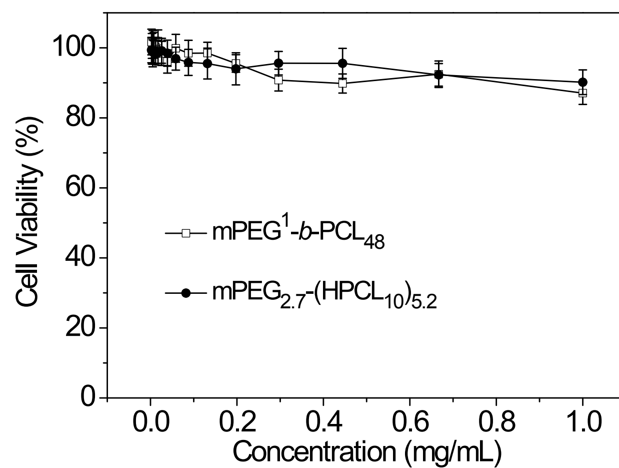

Fig. S3 *In vitro* cytotoxicity of mPEG^1-*b*-PCL$_{48}$ and mPEG$_{2.7}$-(HPCL$_{10}$)$_{5.2}$ (average of four measurements).

References:

1 X. J. Zhang, J. A. Cheng, Q. R. Wang, Z. L. Zhong and R. X. Zhuo, *Macromolecules*, 2010, **43**, 6671-6677.

2 R. H. Lin, J. Castells and H. Rapoport, *J. Org. Chem.*, 1998, **63**, 4069-4078.