Supporting Information

Hydrogen Peroxide-Responsive Micelles Self-Assembled from Peroxalate Esters-Containing Triblock Copolymer

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1. Materials

ε-Caprolactone (ε-CL, 99%), Triethylamine (TEA, 99%), Stannous Octoate ($Sn(Oct)_2$, 99%) were procured from Sigma-Aldrich (St Louis, MO, USA). Poly (ethylene glycol) (PEG, Mn=4 kg mol^{-1}), Oxalyl Chloride (CP) were obtained from GuangFu Fine Chemical Research Institute (Tianjin, China). Calcium Hydride, Tetrahydrofuran (CP), Ethanediol (CP), Dichloromethane (CP), N,N-Dimethylformamide (CP), Acetone (CP), Methanol (CP), Hexane (CP) were bought from Tianjin Jiang Tian Chemical Technology Co., Ltd. (Tianjin, china). Acetonitrile (GC) and Methanol (GC) used as the mobile phase in high-performance liquid chromatography (HPLC) were purchased from Tianjin Concord Technology Co., Ltd. (Tianjin, China). Phosphate Buffered Saline (PBS) was procured from the Shijiazhuang biotechnology Co., Ltd (Shijiazhuang, People's Republic of China). DMEM/F-12 1:1 (Hyclone), Fetal Bovine Serum (FBS, Solarbio, Beijing Solarbio Science &Technology Co., Ltd); ε-Caprolactone, Dichloromethane, Ethanediol, Tetrahydrofuran were distilled over calcium hydride. Other reagents were used as received.

2. Synthesis and characterization of copolymer

2.1. Synthesis of PEG-PO-PCL-PO-PEG copolymer

The synthetic routes of PEG-PO-PCL-PO-PEG copolymer were shown in scheme S1.

2.1.1. Synthesis of PCL

Firstly, $Sn(Oct)_2$ was dissolved in dichloromethane (CH_2Cl_2) with concentration of 7.64×10^{-3} mol L^{-1}. Mixture of dried ε-CL (10.0 g, 43.8 mmol), $Sn(Oct)_2$ solution (1.92 mL, 0.0146 mmol) and ethanediol (0.054 mg, 0.876 mmol) were added into a clean and dry polymerization flask, then vacuum-pumping and argon-filling alternately three times to remove the oxygen and CH_2Cl_2 in the flask. The polymerization was carried out in a vacuum oven at 180°C for 24 hours. After cooling to room temperature, the crude product was dissolved in CH_2Cl_2 and purified with

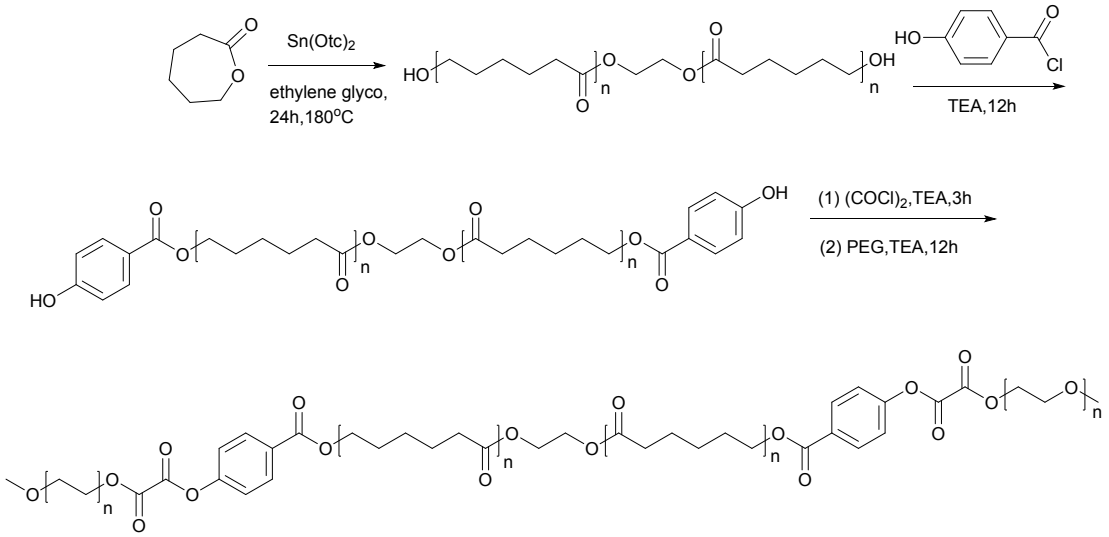
precipitation in cooled hexane for three times. Finally, PCL was dried at room temperature in vacuum until constant weight (8.3 g, 83%). 1H NMR (δ, ppm, $CDCl_3$): 4.073 (t, -OC**H$_2$**$CH_2CH_2CH_2CH_2$COO- in PCL), 2.306 (t, -$OCH_2CH_2CH_2CH_2C$**H$_2$**COO- in PCL), 1.676 (m, -OCH_2C**H$_2$**CH_2C**H$_2$**CH_2COO- in PCL), 1.377 (m, -OCH_2CH_2C**H$_2$**CH_2CH_2COO- in PCL);

2.1.2. Synthesis of PCL-Ar-OH

Initially, p-hydroxybenzoic acid (0.24 g, 1.75 mmol) and a drop of N, N-Dimethyl-formamide were dissolved with 2 mL anhydrous Tetrahydrofuran (THF) in a 50 mL flask. Oxalyl chloride (0.22 g, 1.75 mmol) dissolved in 5 mL anhydrous THF was added dropwise to the above solution under stirring. After reaction for 2 h, THF was removed by evaporation. The residue was re-dissolved with 5 mL anhydrous THF in the flask. Then 15 mL anhydrous THF containing PCL (5 g, 0.438 mmol) and TEA (0.106 g, 1.05 mmol) was added dropwise into the flask under stirring. The reaction was stirred overnight at room temperature under nitrogen atmosphere. The THF was removed by evaporation and the residue was re-dissolved with 5 mL CH_2Cl_2。The organic mixture was washed three times with 1 M HCl and dried over $MgSO_4$ and $NaHCO_3$. The drying agent was removed by filtration. Crude product was obtained while the filtered solution was added to diethyl ether, then, purified with reprecipitation three times. PCL-Ar-OH was dried at room temperature in vacuum until constant weight (4.5 g, 90%).

2.1.3. Synthesis of PEG-PO- PCL-PO-PEG

Oxalyl chloride (0.356 g, 2.80 mmol) was dissolved in 1 mL anhydrous CH_2Cl_2 in a 50 mL flask, and PCL-Ar-OH (4 g, 0.350 mmol) dissolved in 15 mL anhydrous CH_2Cl_2 was added dropwise into the flask under stirring. After reaction for 4 h, CH_2Cl_2 was removed by evaporation. The residue was re-dissolved and re-evaporated for three times to thoroughly remove oxalyl chloride. Then, the residue was dissolved in 10 mL anhydrous CH_2Cl_2, and added dropwise into 15 mL anhydrous CH_2Cl_2 containing PEG (5.6 g, 1.40 mmol) and TEA (0.078 g, 0.771 mmol) under stirring at room temperature. The mixture was further stirred overnight at room temperature under nitrogen atmosphere. The organic mixture was washed three times with 1 M HCl and dried over $MgSO_4$ and $NaHCO_3$. The drying agent was removed by filtration. The filtered solution was precipitated in cool diethyl ether three times and washed by cool methanol to remove the redundant PEG. The product was dried at 40^{o}C in vacuum until constant weight (5 g, 73%). 1H NMR (δ, ppm, $CDCl_3$): 7.949 (d, in benzene ring), 6.867(d, in benzene ring), 4.050 (t, -OC**H$_2$**$CH_2CH_2CH_2CH_2$COO- in PCL), 3.638 (s, -OC**H$_2$C H$_2$**O- in PEG), 2.329 (t, -$OCH_2CH_2CH_2CH_2C$**H$_2$**COO- in PCL), 1.648 (m, -OCH_2C**H$_2$**CH_2C**H$_2$**CH_2COO- in PCL), 1.401 (m, -OCH_2CH_2C**H$_2$**CH_2CH_2COO- in PCL);



Scheme S1 The synthetic route of PEG-PO-PCL-PO-PCL copolymer.

2.2 Characterization of PEG-PO-PCL-PO-PEG

The polymer was analyzed by ^{1}H NMR spectra (400 MHz) spectrometer instrument (Varian Inc, USA) with $CDCl_3$ as the solvent, and the result was showed in Fig. S3.

Infrared spectra were recorded on a Nicolet™ Nexus 470-ESP Fourier-transform infrared (FTIR) spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). As shown in Fig. S1, the peaks at 2946, 2867 cm^{-1}, et al. were characteristic peak of PCL. The peaks at 1625 cm^{-1} were due to the modified benzene ring in terminal hydroxyl of PCL-Ar-OH, which suggest that the PCL-Ar-OH was successfully synthesized. The emerging peaks at 2887 cm^{-1}, 1060 cm^{-1}, 841 cm^{-1} et al. showed that PEG was linked to the PCL-Ar-OH, which suggest that the PEG-PO-PCL-PO-PEG was successfully synthesized. The molecular weight and molecular weight distribution (Mw/Mn) of the copolymer were measured by gel permeation chromatography using a Hitachi L-2490 differential refraction detector and two Agilent PLgel 7.5×300 mm, 10 μm particles columns. Polymethyl methacrylate (PMMA) was used for standard. As shown in Fig. S2, the molecular weight (Mn) of PEG-PO-PCL-PO-PCL copolymer was 18134 g mol^{-1} with a polydispersity index of 1.6, while the molecular weight (Mn) of PCL was 11757 g mol^{-1} with a polydispersity index of 1.51.

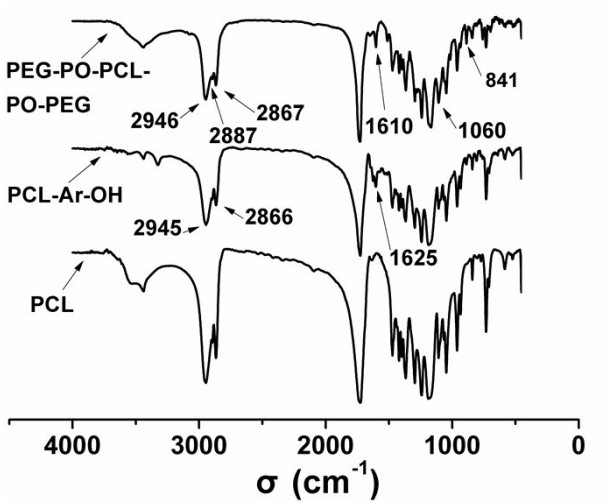


Fig. S1 Fourier-transform infrared spectrum of PCL, PCL-Ar-OH, and PEG-PO-PCL-PO-PEG copolymer.

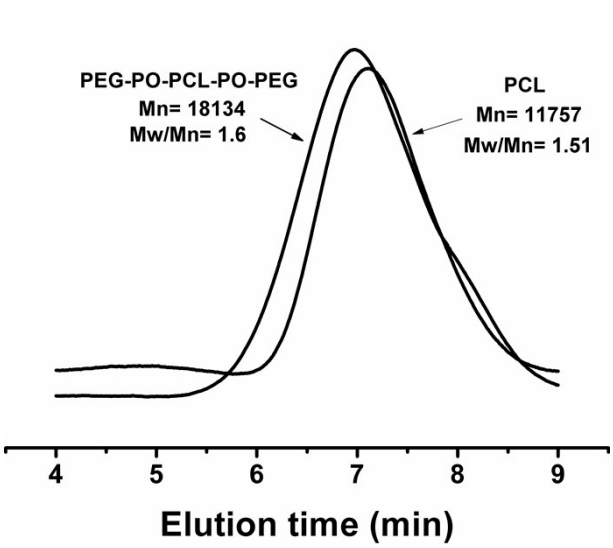
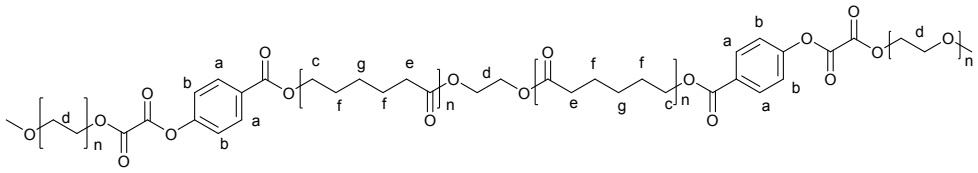


Fig. S2 The GPC of PCL and H_2O_2-responsive PEG-PO-PCL-PO-PEG copolymer.



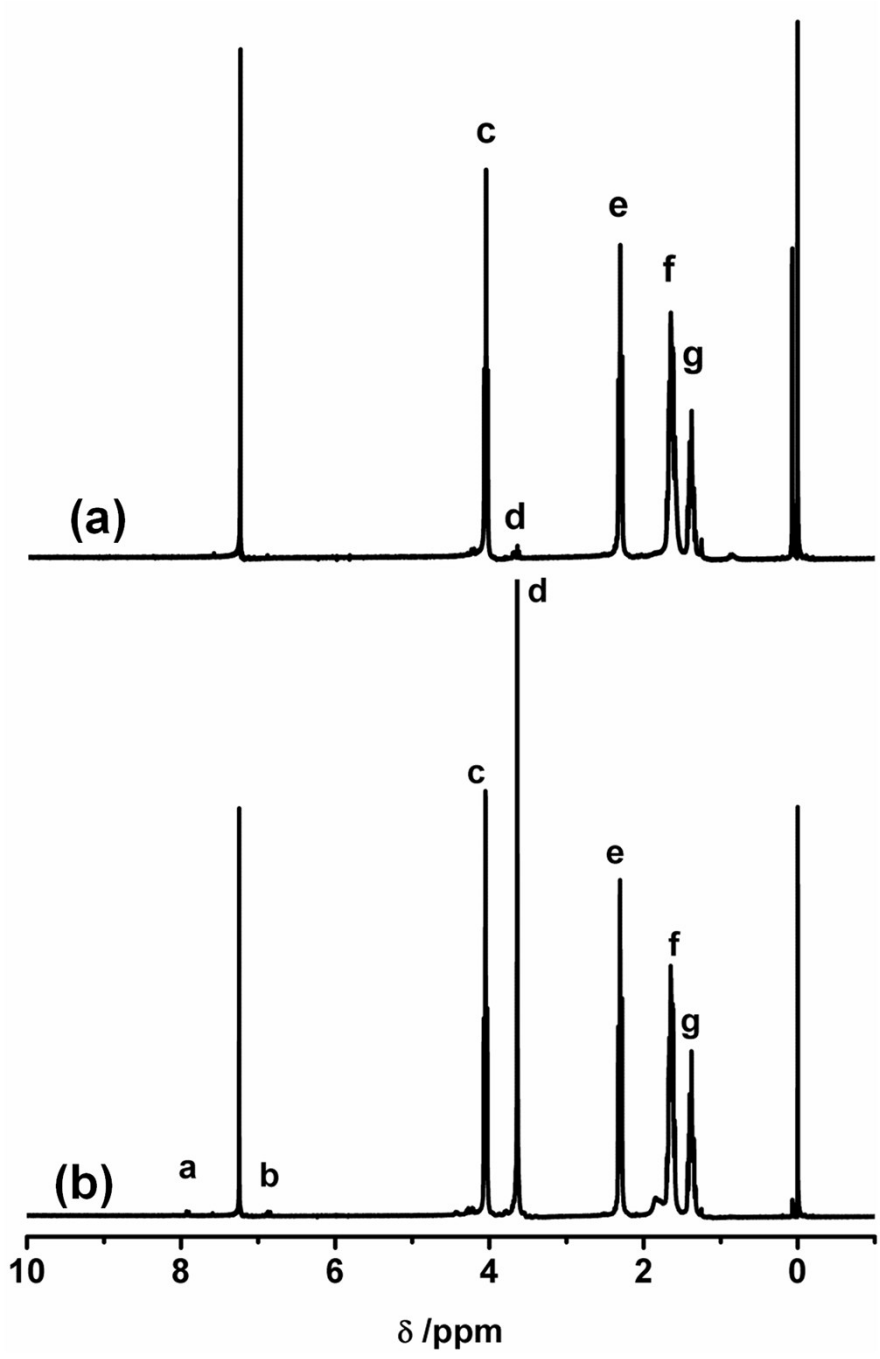


Fig. S3 The ^{1}H NMR of PCL (a) and H_2O_2-responsive PEG-PO-PCL-PO-PEG copolymer (b).

3. Preparation and Characterization of micelles

3.1 Preparation of PEG-PO-PCL-PO-PEG micelles

Micelles were prepared by solvent evaporation method. Briefly, 100 mg PEG-PO-PCL-PO-PEG was dissolved in 4 mL acetone, and added drop-wise slowly into 20 mL distilled water under violent stirring. The solution was stirred with high speed overnight at room temperature. Organic solvent was removed by evaporation. Micelles were washed by ultracentrifugation at 23,000 rpm for 30 min three times. Then, micelles were re-dissolved with 30 mg mL^{-1} PEG aqueous solution and stored in the freezer at -80 ^{o}C for 24 h. The lyophilized powder was obtained by lyophilization. The Rapamycin-loaded micelles were obtained by the same method.

3.2 Size and size distribution of PEG-PO-PCL-PO-PEG micelles

The size and size distribution were evaluated by dynamic light scattering on a Zetasizer NanoZS (Malvern Instruments Ltd, Malvern, UK). The morphology of the prepared micelles was observed under transmission electron microscope (H-6009IV; Hitachi Ltd, Tokyo, Japan). Before the measurement, the micelles were diluted with distilled water and added to a copper grid. The copper grid was dried at room temperature.

3.3 CAC measurements of PEG-PO-PCL-PO-PEG micelles

The method was carried out according to literature[S1]. Micelles were prepared by the same method. 2 mL micelles aqueous solution was taken and diluted to the concentration of 1 mg mL^{-1} for CAC measurement experiments. The critical aggregate concentration (CAC) of PEG-PO-PCL-PO-PEG was measured by the fluorescent probe method. 5 μL of 5×10^{-3} mg mL^{-1} pyrene solution in acetone was added to prepared micelles solutions with different concentration. The solutions were sonicated for 10 min before fluorescence emission measurements. As shown in Fig. S4, the CAC of the copolymer was about 3.98×10^{-6} mg mL^{-1}. (The CAC was chosen as the concentration when pyrene exhibited an apparent decrease in the I1/I3 ratio with an increasing concentration of the copolymer, indicating that the aggregation of the copolymer occurred).

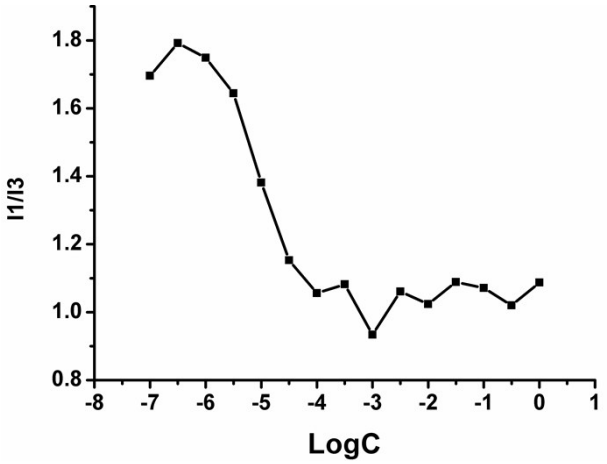


Fig. S4 Determination of CAC for the PEG-PO-PCL-PO-PEG copolymer using fluorescent method with pyrene as a probe, C (mg ml^{-1}).

3.4 Scavenging of hydrogen peroxide by PEG-PO-PCL-PO-PEG micelles

The method was carried out according to literature[S2]. PCL nanoparticles were prepared by ultrasonic emulsion method[S3]. 1 mg PCL nanoparticles, PEG, and PEG-PO-PCL-PO-PEG micelles were dissolved into 1 mL of hydrogen peroxide solution (0.1 μM). The solutions were incubated with mechanical at 37°C for 24 h. After centrifugation for 5 minutes, the hydrogen peroxide concentration of the supernatant was determined using the Amplex Red assay (Invitrogen, US) according to the manufacturer's protocol.

3.5 The release behavior of RPM loaded PEG-PO-PCL-PO-PEG micelles

Firstly, two groups of micelles solution were prepared with 5 mg as-lyophilized micelles dissolved in 4 mL PBS buffer in 15 mL of centrifuge tube. Each group was done in triplicate. The solutions were incubated at 37°C with mechanical. 2 ml of supernatant was taken out from the tubes after centrifugation and the same volumn of PBS was added at the setting time point. Start from the point of 9h, one group of micelles was stimulated by adding H_2O_2 (5 μM). The RPM content of the collection fluid was measured by HPLC using an Agilent LC1100 (Santa Clara, CA, USA). HPLC assay was performed using a reverse-phase diamond C18 column (150.0 × 4.6 mm; Agilent Technologies, Santa Clara, CA, USA), which was eluted with mobile phase (acetonitrile/water, 75/25, v/v) at a flow rate of 1 ml min^{-1}. The ultraviolet detector was used to detect RPM at 277 nm.

3.6 Cytotoxicity assay of PEG-PO-PCL-PO-PEG micelles

Tetrazolium salt reagents (WST-1) reduction assay was performed to evaluate the cytotoxicity of PEG-PO-PCL-PO-PEG micelles. T/G HA-VSMCs cells were incubated at 37 °C in a 5% (v/v) carbon dioxide atmosphere incubator in DMEM/F-12 medium (HyClone) with 10% Fetal Bovine Serum and 1% Penicillin Streptomycin (Gibco). Cells were seeded in 96-well plate with 200 μL culture medium at a density of 5000 cells/well. After incubated for 24 h, cells were treated with various amounts of

micelles (10、50、100 μg mL^{-1}) and incubated for another period of 24 h, 72 h and 120 h. At intervals, each well was given 15 μL water soluble tetrazolium salt reagents (WST-1) solution and incubated for another 4 h. The optical density (OD) was measured at 450 nm with a Varioskan Flash microplate reader (Thermo Fisher Scientific). The cell viability was obtained by comparing the absorbance of micelles-treated cells to that of control groups. All tests were performed in quadruplicate. All these experiments were repeated at least three times.

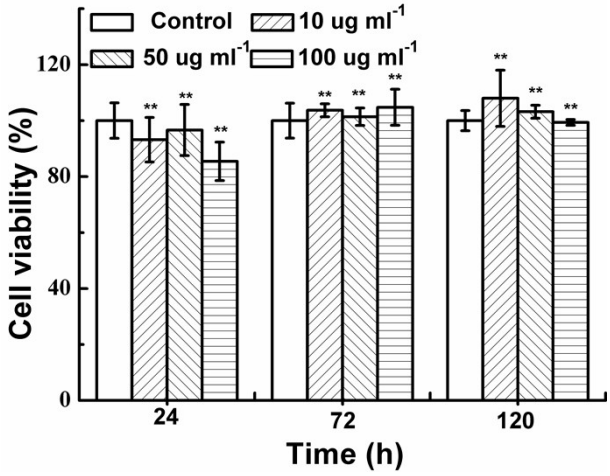


Fig.S5 Cytotoxicity of PEG-PO-PCL-PO-PEG micelles determined by the WST-1 assay **p<0.05 vs. control groups.

References

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