Asymmetric copolymer vesicles to serve as a hemoglobin vector for ischemia therapy

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Materials.

ε-Caprolactone (ε-CL, 99%, Alfa Aesar) was dried over CaH_2 and distilled under reduced pressure. L-lactide (L-LA) prepared in our laboratory was recrystallized from ethyl acetate for three times. Toluene, dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were dried according to the conventional procedures. Propargyl alcohol from Acros was further distilled under reduced pressure before use. Tin (II) $_2$-ethylhexanoate (Sn (Oct) $_2$, 90% in 2-ethylhexanoic acid) was purchased from Strem Chemicals. Rhodamine B (RhB, 95%), monomethoxy-poly (ethylene glycol) (mPEG) with a molecular weight of 5000 were obtained from Sigma Aldrich. Dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) were obtained from Acros. α-Carboxy-ω-azide PEG was synthesized according to published procedures.[1] Other solvents were analytical grade and used without further purification.

Characterization.

^{1}H NMR spectra were recorded with a Bruker AV400 spectrometer in $CDCl_3$ or D_2O at 25 ^{o}C. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC instrument equipped with a Waters Styragel HT6E column and a differential refractometer detector. $CHCl_3$ was used as eluent at a flow rate of 1 mL

min^{-1} at 35 ^{o}C. The molecular weights were calibrated with polystyrene standards. Differential scanning calorimetry (DSC) measurements were performed on a Perkin-Elmer Pyris 1 DSC instrument under a N_2 atmosphere. The samples were heated from -80 to 180 ^{o}C and cooled to -80 ^{o}C at a rate of 10 ^{o}C min^{-1} and then reheated to 180 ^{o}C.

Synthesis of mPEG-PCL-PEG.

The mPEG-PCL diblock copolymers were synthesized by ring-opening polymerization of ε-CL using $mPEG_{5K}$ as the macroinitiator and $Sn(Oct)_2$ as the catalyst. Afterwards, the copolymer was reacted with α-carboxy-ω-azide PEG to obtain the final triblock copolymer. Typically, in a dried flask, 4.8g of mPEG-PCL (0.3mmol) and 0.495g of α-carboxy-ω-azide PEG (Mn=1100, 0.45mmol) were dissolved in 50 ml of anhydrous DCM. 0.618g of DCC (3mmol) and 0.0366g of DMAP (0.3mmol) were added at 0 ^{o}C. After stirring for 1h at 0 ^{o}C, the reaction was continued for 24 h at room temperature. The precipitate was filtered out, and the mixture was redissolved in dimethylformamide (DMF) after the evaporation of solvent. Then the solution was dialysed against distilled water (MWCO 3500 Da) for 2 days, and freeze-dried to lead to a white solid (yield 90%). The mPEG-P (CL-*co*-LA)-PEG copolymers were synthesized according to the same procedure except for the random copolymerization of CL and L-LA initiated by mPEG.

Alkynyl-rhodamine B Synthesis.

0.478 g of rhodamine B (1 mmol) and 0.174ml of dried propargyl alcohol (3 mmol) were dissolved in 30 ml of dried DMSO, and then 0.412g of DCC (2 mmol) and

0.122g of DMAP (1 mmol) were added at 0 ^{o}C. After stirring for 1h at 0 ^{o}C, the reaction was continued for 24 h at room temperature. Then the solvent was removed, and the reaction mixture was washed with diethyl ether to remove excess propargyl alcohol. The solid residue was collected and dried under vacuum, yield 70%.

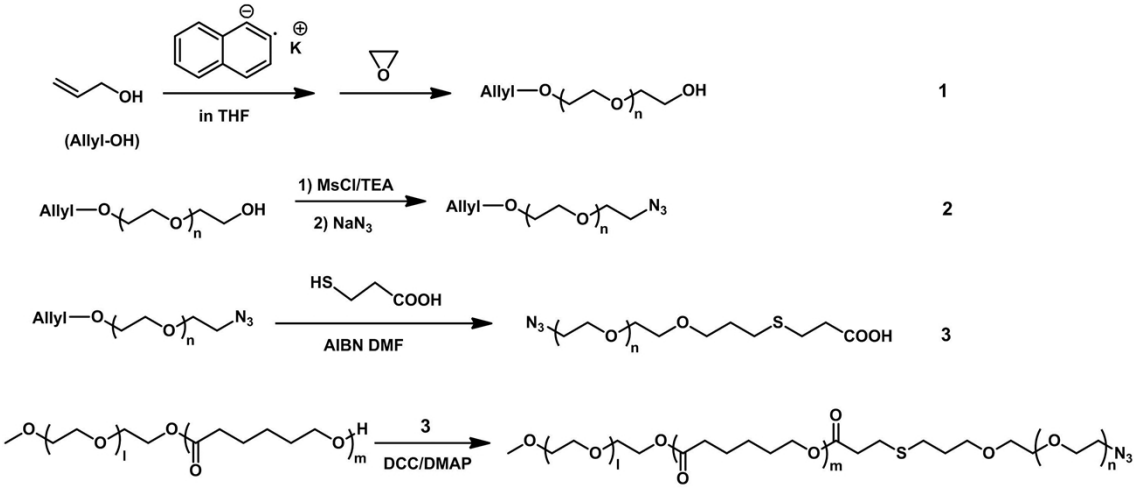
End-group Modification Using "Click" Chemistry.

For a typical procedure of the "click" reaction, 2.55g of mPEG-PCL-PEG-N_3 (0.15mmol) and 0.2327g of alkynyl-RhB (0.45mmol) were dissolved into 20ml of DMF. 0.012 g of copper (Ⅱ) sulfate (0.045mmol) and 0.018g of sodium ascorbate (0.09 mmol) were then added. The reaction mixture was degassed, exchanged with nitrogen for three times, and then stirred at 80 ^{o}C for 5h. Afterwards, the solution was passed through alkaline aluminum oxide column (100-200 mesh) to remove copper salt, precipitated into ice-cooled diethyl ether, and dried in vacuum oven, yield 80%.

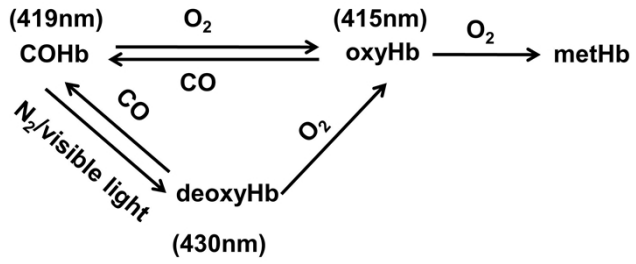
***In vitro* cytotoxicity of HbV**

The cytotoxicity of HbV was evaluated via MTT viability assay against L929 cells. The cells were seeded in 96-well plates at 10^4 cells per well in 100 μL of complete DMEM supplemented with 10% FBS, and incubated at 37 ^{o}C in 5% CO_2 atmosphere for 24 h. Then 100 μL of HbV dispersion in DMEM with a final concentration from 0.125 to 1 mg mL^{-1} was added to the culture medium, and the cells were further cultured for 48 h. Afterwards, 20 μL of MTT solution in PBS with concentration of 5 mg mL^{-1} was added, and the plates were incubated for another 4 h at 37 ^{o}C followed by removal of culture medium containing MTT and addition of 150 μL of DMSO to each well to dissolve formed formazan crystals. Finally, the plates were shaken for

10 min, and absorbance of formazan product was measured at 490 nm by a Bio-Rad 680 microplate reader. Cell viability(%) was calculated from the following equation: $(A_{sample}/A_{control})\times 100\%$, where A_{sample} and $A_{control}$ denoted as absorbance of the sample well and control well, respectively.



Scheme S1. Synthesis of amphiphilic mPEG-PCL-PEG hetero-triblock copolymers



Scheme S2. The state-transition diagram of Hb under different gas atmospheres.

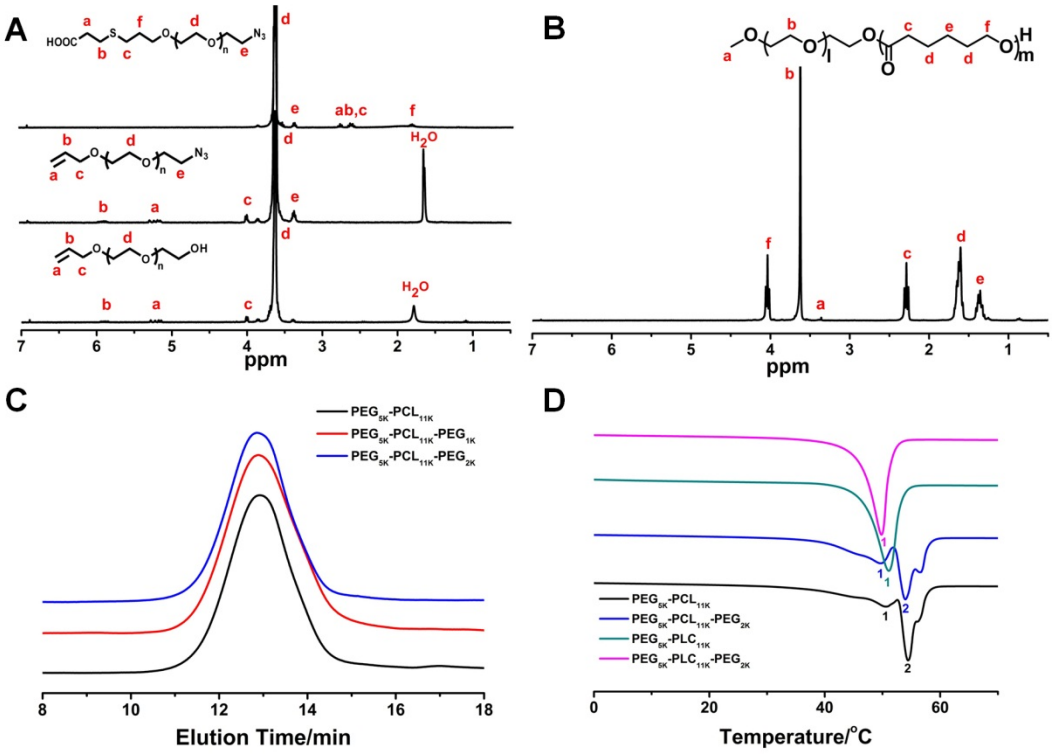


Fig. S1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of heterobifunctional PEG derivative (A). ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of $mPEG_{5K}$-PCL_{11K} (B). GPC curves of $mPEG_{5K}$-PCL_{11K}, $mPEG_{5K}$-PCL_{11K}-PEG_{1K} and $mPEG_{5K}$-PCL_{11K}-PEG_{2K} in $CHCl_3$(C). DSC curves of copolymers with hydrophobic PCL segments or P (CL-*co*-LA) (PLC) segments from the second heating scan (D). The symbols on the curves represented the crystallization transitions of different segments, 1 for PEG and 2 for PCL.

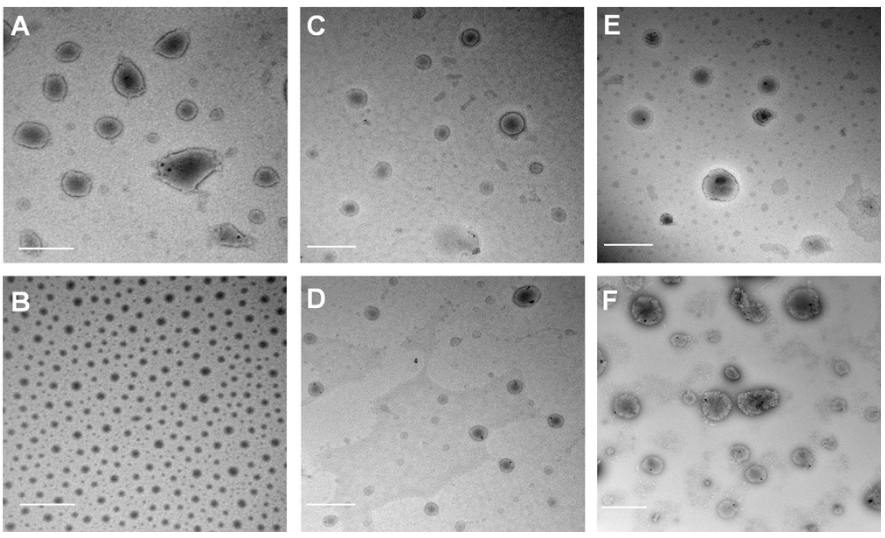


Fig. S2. TEM images of self-assembled copolymers. (A) $mPEG_{5K}$-PCL_{5K}-PEG_{1K}; (B) $mPEG_{5K}$-PCL_{5K}-PEG_{2K}; (C) $mPEG_{5K}$-PCL_{11K}-PEG_{1K}; (D) $mPEG_{5K}$-PCL_{11K}-PEG_{2K}; (E) $mPEG_{5K}$-PCL_{24K}-PEG_{1K}; (F) $mPEG_{5K}$-PCL_{24K}-PEG_{2K}. Scale bars were 500 nm except 2000nm for F.

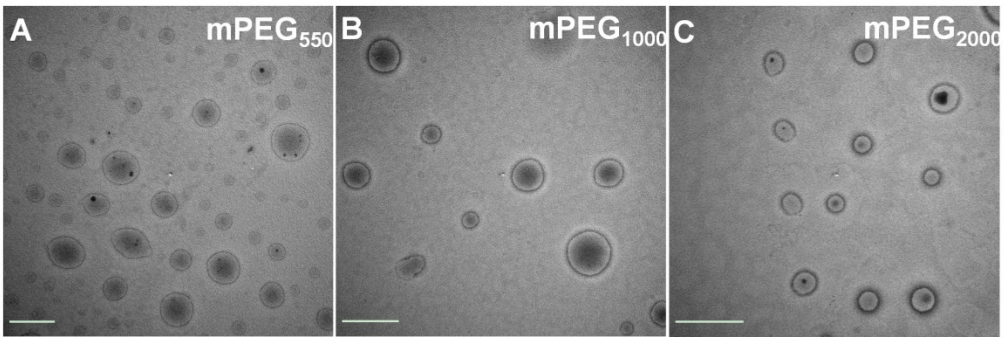


Fig. S3. TEM images of $mPEG_{5K}$-P (CL-*co*-LA) $_{11K}$-PEG_{2K} vesicles with mPEG of different molecular weights as the excipient. (A) $mPEG_{550}$; (B) $mPEG_{1000}$; (C) $mPEG_{2000}$. Scale bars = 500 nm.

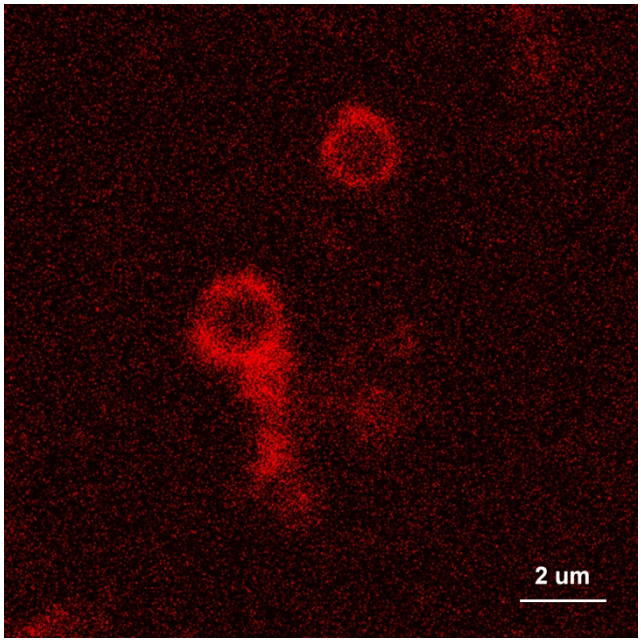


Fig. S4. CLSM image of giant vesicles formed of $mPEG_{5K}$-P (CL-*co*-LA) $_{11K}$-PEG_{2K} conjugated with RhB via click chemistry.

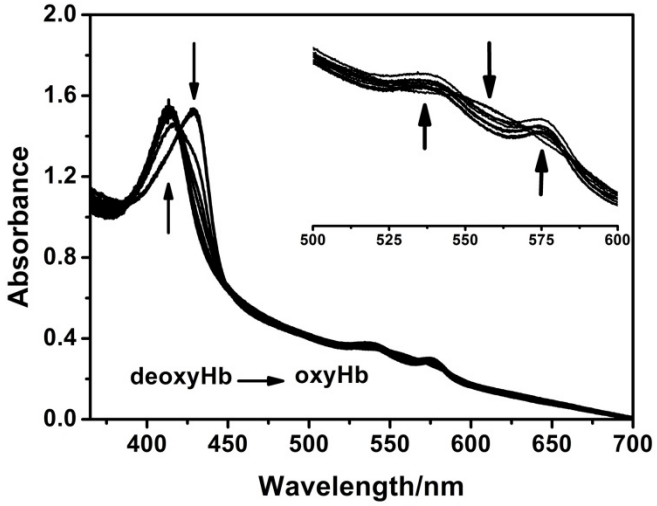


Fig. S5. The evolution of encapsulated Hb from deoxyHb state to oxyHb state in different oxygen partial pressure.

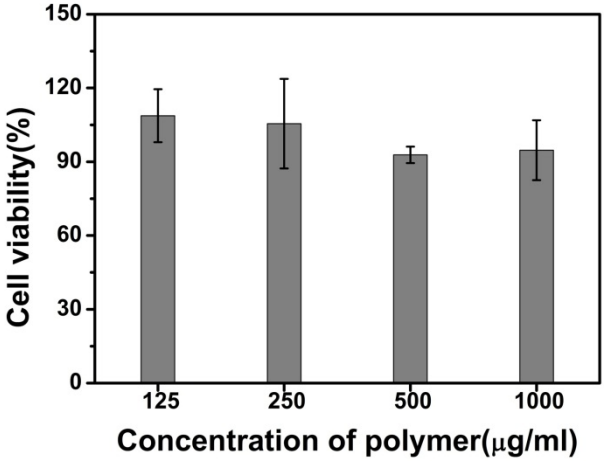


Fig. S6. Cell viability of L929 cells after 48 h incubation with HbV at different polymer concentrations *in vitro*.

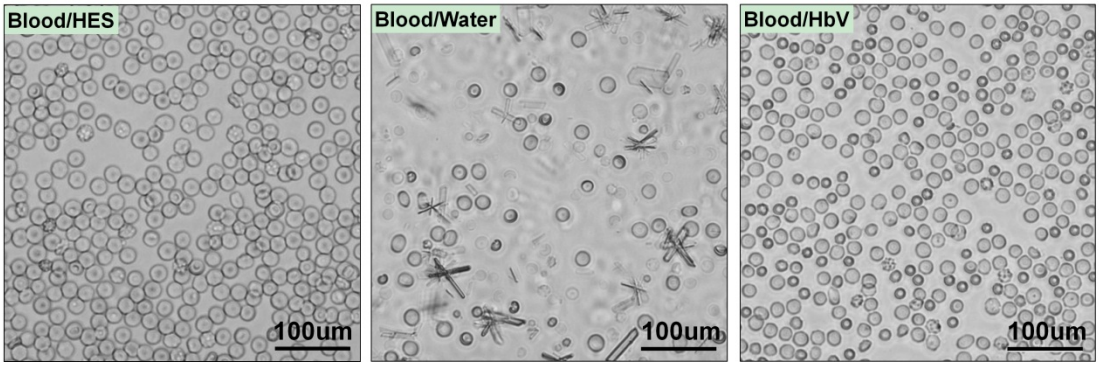


Fig. S7. Optical microscopic observations of RBC morphology of Wistar rat after incubation with different solutions at 37°C for 3 h.

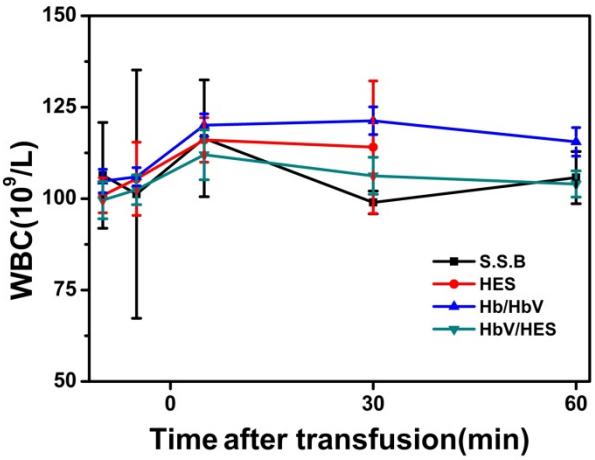


Fig. S8. White blood cell counting during exchange transfusion with infusion of HbV/HES with 10g Hb L^{-1}, Hb/HES with 10g Hb L^{-1}, shed self-blood(S.S.B.) and HES. The time span along the X-axis was labelled after the sample infusion (0 min), and the points before 0 min indicated the data before and after the 30% bleeding, respectively.

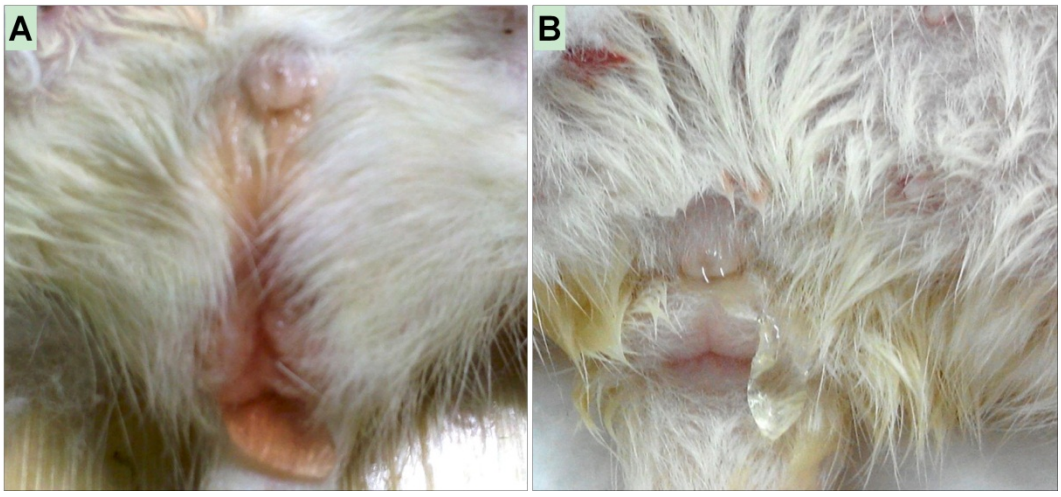


Fig. S9. Typical hemoglobinuria phenomenon observed during the resuscitation of rats transfused with Hb/HES solutions from hemorrhagic shock (A), which was not detected in the HbV/HES groups(B).

Table S1. Characteristics of the hetero-triblock copolymers

Copolymer[a]	Mn[b](Kg mol^{-1})	Đ[b]	$f_{hydrophilic}$[c](wt%)
hydrophobic segments: poly (ε-caprolactone)			
5K-5K-1K	19.1	1.19	54.5
5K-5K-2K	20.3	1.37	58.3
5K-11K-1K	32.0	1.22	35.3
5K-11K-2K	33.5	1.26	38.9
5K-24K-1K	43.1	1.31	20.0
5K-24K-2K	47.7	1.32	22.6
hydrophobic segments: poly (caprolactone-*co*-lactide)			
5K-11K-2K	50.5	1.37	38.9

[a] The numbers denote the molecular weights (Mn) of each blocks of the copolymers determined by ^{1}H NMR with $CDCl_3$ as the solvent. [b] Determined by GPC in $CHCl_3$ using polystyrene standards. [c] Determined by ^{1}H NMR analysis.

Reference

1. S. Hiki and K. Kataoka, *Bioconjugate Chemistry*, 2007, **18**, 2191-2196.