Supporting Information

of

Facile Synthesis and Self-assembly Behaviors of Biodegradable Amphiphilic Hyperbranched Copolymers with Reducible Poly(caprolactone) Grafts

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1. Experimental Section

Materials. *N*,*N*-dimethylacetamide (DMAc), triethylamine (TEA, 99%), tin(II)-trifluoromethanesulfonate ($Sn(OTf)_2$, 97%) and 2-hydroxyethyl disulfide were purchased from Sigma-Aldrich and used as received. Oligo (ethylene glycol) monomethyl ether methacrylate (OEGMA, average M_n = 300 and pendent EO units DP ~4.5, Sigma-Aldrich) was purified by passing through a column filled with activated basic Al_2O_3 to remove the inhibitor. ε-Caprolactone (ε-CL, J&K, 99%) was dried over CaH_2 and distilled under reduced pressure prior to use. 2,2′-Azobis(isobutyronitrile) (AIBN, J&K, 99%) was recrystallized twice from ethanol. 2-((2-(acryloyl oxy)ethyl)disulfanyl)ethyl 4-cyano-4-(phenylcarbonothioylthio) pentanoate (ACP) and 2-((2-hydroxyethyl)disulfanyl)ethyl methacrylate (HSEMA) were synthesized according to the references.[1,2] All other chemicals were purchased from Tianjin Chemical Reagent Factory (China) and subjected to further purification following the standard protocols.

***Synthesis of HSEMA-g-PCL*.** HSEMA-g-PCL was prepared by ring-opening polymerization (ROP) of ε-CL using HSEMA as the initiator and $Sn(OTf)_2$ as the catalyst. Briefly, ε-CL (4.1 g, 36.0 mmol), and HSEMA (4.10 g, 18.5 mmol) were placed in a thoroughly dried 25 mL of Schlenk tube with a magnetic stirring bar under nitrogen atmosphere. After three freeze-pump-thaw cycles, a small amount of $Sn(OTf)_2$ (75.0 mg, 0.5mol% vs ε-CL) was added in a nitrogen atmosphere. Finally, the reaction mixture was degassed with another three freeze-pump-thaw

cycles and then sealed followed by immersing the flask into an oil bath preheated to 45 ℃ to carry out the polymerization for 1h. After reacting, the mixture was diluted using tetrahydrofuran, followed by flash column chromatography (ethyl acetate:hexane = 1:3, v/v) to yield the product as light yellow oil (6.2 g, yield: 75.6%). ^{1}H NMR (**Fig. 1**, $CDCl_3$, 400 MHz): δ_{ppm}, 6.11 (s, C**H**$_2$=C), 5.57 (s, C**H**$_2$=C), 4.39 (t, COOC**H**$_2$CH$_2$S), 4.32 (t, SCH$_2$C**H**$_2$OOC), 4.04 (m, OOCCH$_2$CH$_2$CH$_2$CH$_2$C**H**$_2$OOC of PCL), 3.63 (t, CH$_2$C**H**$_2$OH of PCL), 2.95 (t, COOCH$_2$C**H**$_2$S), 2.91 (t, SC**H**$_2$CH$_2$OOC), 2.35-2.27 (m, OOCC**H**$_2$CH$_2$CH$_2$CH$_2$CH$_2$OOC of PCL), 1.93 (s, 3H, C**H**$_3$C=CH$_2$), 1.68-1.52 (m, OOCCH$_2$C**H**$_2$CH$_2$C**H**$_2$CH$_2$OOC of PCL), 1.42-1.32 (m, OOCCH$_2$CH$_2$C**H**$_2$CH$_2$CH$_2$OOC of PCL).

Synthesis of Hyperbranched Statistical Copolymer. The reversible addition-fragmentation chain transfer self-condensing vinyl polymerization (RAFT-SCVP) technique was employed for preparing hyperbranched statistical copolymer. Taking *h*-P(HEMA-g-PCL)-*st*-POEGMA as example. ACP (86.7 mg, 0.185 mmol), HSEMA-g-PCL (382.1 mg, 0.563 mmol), OEGMA (664.1 mg, 2.213 mmol), AIBN (10.1 mg, 0.061 mmol) and DMAc (2.77 mL) were charged into a thoroughly dried 10 mL of Schlenk tube. The solution was deoxygenated via three freeze-pump-thaw cycles and then immersed in a preheated oil bath at 70 °C to start the polymerization. After reaction for 23h the polymerization was stopped by freezing in the liquid nitrogen. After thawing, the polymer solution was diluted with THF and the product was

collected by precipitation in 10-fold ice-cold diethyl ether three times and dried under vacuum until constant weight.

The other hyperbranched statistical copolymer *h*-P(HEMA-g-PCL) were prepared following a similar procedure described above without the addition of OEGMA.

RAFT Synthesis of Hyperbranched Block-Statistical Copolymer, h-P(HEMA-g-PCL)-b-POEGMA Using h-P(HEMA-g-PCL) as a Multimacro-CTA. *h*-P(HEMA-g-PCL)-*b*-POEGMA amphiphilic copolymer was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization using *h*-P(HEMA-g-PCL) as a multimacro-CTA. The typical procedure was as follows: *h*-P(HEMA-g-PCL) (40.0 mg, 0.004 mmol), OEGMA (79.52 mg, 0.265 mmol), AIBN (0.05 mg, 0.3 μmol) and DMAc (2.58 mL) were charged into a thoroughly dried 10 mL of Schlenk tube. The solution was deoxygenated via three freeze-pump-thaw cycles and then immersed in a preheated oil bath at 70 °C to start the polymerization. After reaction for 12h the polymerization was stopped by freezing in the liquid nitrogen. After thawing, the polymer solution was diluted with THF and the product was collected by precipitation in 10-fold ice-cold hexane three times and dried under vacuum until constant weight.

Degradation of Hyperbranched Copolymers. The degradation were realized according to the references with slight modification.[2] Briefly, HP-4 copolymer (30 mg) was dissolved in 3.0 mL of DMF under nitrogen, and about 5.0 μL of Bu_3P was added to the solution at ambient temperature. The mixture was stirred overnight, diluted with DMF and subjected to SEC analysis.

Micelle Preparation. The solution of polymeric micelles was prepared using the classical dialysis method.[3,4] Taking *h*-P(HEMA-g-PCL)-*st*-POEGMA as an example, 10.0 mg polymer was dissolved in 1 mL DMF, the solution was next added dropwise into 7.0 ml of distilled water under vigorous stirring. The mixture solution was next subjected to dialysis (cellulose membrane, molecular-weight cut-off (MWCO), 3.5 kDa) against water for 24 h.

Determination of Critical Aggregates Concentration (CAC). CAC of *h*-P(HEMA-g-PCL)-*st*-POEGMA and *h*-P(HEMA-g-PCL)-*b*-P(OEGMA) was determined using pyrene as the fluorescence probe according to our previous.[5,6] In brief, aliquots of pyrene solutions (3×10^{-6} mol/L in acetone, 120 μL) were added to containers, and the acetone was allowed to evaporate. Six milliliter aqueous polymer solutions at various concentrations were subsequently added to the containers containing the pyrene residue. Note that all the aqueous sample solutions contained excess pyrene residue at an identical concentration of 6×10^{-8} mol/L. The solutions were kept at room temperature for 24 h to reach the solubilization equilibrium of pyrene in the aqueous phase. Excitation was carried at 334 nm, and emission spectra were recorded ranging from 355 to 420 nm. Both excitation and emission bandwidths were 5 nm. From the pyrene emission spectra, the intensity ratio of the third (I_{384}) and first band (I_{373}) were analyzed as a function of the polymer concentrations. The CMC value was

determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

NMR Characterization. ^{1}H and ^{13}C NMR spectra of the synthesized small organic molecules and polymers were analyzed on a Bruker DRX 400 spectrometer at 298 K using deuterated chloroform as solvent and TMS as internal reference.

Molecular Weight Analysis. The size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) analyses were used to determine the molecular weight (MW) and polydispersity (*Đ*) of the synthesized polymers. SEC using HPLC-grade DMF containing 0.1 wt% LiBr at 60 ^{o}C as the eluent at a flow rate of 1 mL/min, Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA, USA) were connected in series to a Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA), an interferometric refractometer (Optilab-rEX, Wyatt Technology, anta Barbara, CA, USA) and a MALLS device (DAWN EOS, Wyatt Technology, Santa Barbara, CA,USA). The MALLS detector was operated at a laser wavelength of 690.0 nm.

Transmission Electron Microscopy (TEM). TEM images were recorded on a JNM-2010 instrument operating at an acceleration voltage of 200 keV. The specimens for TEM observation were prepared by placing a drop of micelle solution onto a carbon-coated copper grid. After deposition, excess solution was removed using a strip of

filter paper. The sample was further stained using phosphotungstic acid (1% w/w) and dried in air prior to visualization.

Size Distribution Measurements. Dynamic light scattering (DLS) was performed on a Zetasizer (Nano ZS, Malvern, Worcestershire, UK) at a fixed detection angle of 173◦.

In Vitro Drug Loading and Drug Release Study. DOX·HCl (2 mg) were dissolved in 1 mL of DMF containing a 3.0 equimolar amount of triethylamine. and stirred overnight in the dark at room temperature to obtain free DOX. Next, *h*-P(HEMA-g-PCL)-*b*-POEGMA (20 mg) in 1.0 ml of DMF was added to the above DOX solution. After stirring at room temperature for 1h, the above mixture was added dropwise into 14.0 ml of distilled water under vigorous stirring. After stirring for 1 h, the mixture solution was later transferred to a dialysis tube with a MWCO of 3.5 kDa and subjected to dialysis against 5.0 L of distilled water for 24 h to remove DMF, TEA and unloaded free DOX. The distilled water was renewed every 3 h during the course of initial 12 h.

For *in vitro* drug release study, the solution of drug-loaded micelles was split in equal volumes into four dialysis tubes with a MWCO of 3.5 kDa, which were then immersed in a Falcon tube containing 25 ml of PBS (pH 7.4, 10mM) buffer solution. The tube was kept in a horizontal laboratory shaker thermostated at a constant temperature of 37 °C and a stirring speed of 120 rpm.[5,7] At predetermined time intervals (0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72h), 3 ml of release medium was sampled and

replenished with equal volume of fresh medium. The sample were analyzed by measuring the absorbance at 499 nm with UV-vis spectrometer. The concentration of released DOX from each cell was calculated based on the standard curves obtained from free DOX·HCl in the corresponding release buffers.

The encapsulation efficiency (EE) and the drug loading content (DLC) of the drug-loaded micelles were determined using the UV-vis spectrometer at 499 nm. The EE and DLC were calculated using the following equations,[3, 4]

$$EE\ (\%) = W_{DOX\ loaded\ in\ micelles} / W_{DOX\ fed\ for\ encapsulation} \times 100\% \quad (1)$$

$$DLC\ (\%) = W_{DOX\ loaded\ in\ micelles} / W_{DOX\ loaded\ micelles} \times 100\% \quad (2)$$

Evaluation of Cellular Uptake by Flow Cytometry (FCM) Analysis. HeLa cells were seeded in 6-well plates at a density of 300 000 cells per well in 1.0 mL of complete growth medium and incubated overnight at 37 °C in 5% CO_2 environment. Then, fresh MEM containing different samples, was added to replace the original medium, and the cells without drug treatment were set as a control. The DOX concentration for free DOX·HCl, and DOX loaded HBP micelles in MEM was set at 23 μg/mL. After incubation for 4 h, the polymer solutions were aspirated, and the cells were rinsed twice with PBS. Cells were then harvested by incubation with 200μL of Trypsin-EDTA, followed by resuspension with 1 mL of complete growth medium. Cells were transferred to 1.5 mL microcentrifuge tubes and pelleted at 300g for 5 min at 4 °C. The supernatant was aspirated, and the cell pellets were resuspended in 500 μL of

PBS. Cells were analyzed for uptake of fluorescent polymer using a flow cytometer (Luminex, GuavaEasyCyte).

Cell Imaging. MCF cells were seeded in 6-well plates at a plating density of 1×10^5 cells per well in 1 mL of complete growth medium and incubated in 5% CO_2, 37 °C environment for 24 h. The DOX concentration for free DOX•HCl, and DOX-loaded HBP micelles in MEM was set at 23 μg/mL and were later added to the wells and incubated at 37 °C for 4 h. After incubation, cells were counterstained with 2-(4-amidinophenyl)-6-indolecarbamidine(DAPI). Coverslips were mounted onto glass slides and imaged using Leica biological microscope.

Cell Viability Study. The cytotoxicity of various formulations was evaluated in vitro using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MCF-7 cells were seeded in 96-well plates at a plating density of 2000 cells per well in 100 μl of complete growth medium and incubated in an incubator maintained at 37 °C and 5% CO_2 environment for 24 h. Free DOX·HCl, blank micelles and HBP@DOX micelles were prepared in serial dilutions in water and then diluted in 10-fold in Opti-MEM medium (Invitrogen). The cells were then rinsed once with PBS and incubated with 100 μl of the sample solutions with different polymer concentrations at 37 °C for 41 h. Cells were next rinsed with PBS and the medium was replaced with 100 μl of culture medium. After 10 μL aliquot of a MTT stock solution (5 mg/mL in PBS) was added to each well, cells were then incubated at 37 °C

and 5% CO_2 for 4 h. Next, the media was removed carefully and then DMSO (150 μL/well) was added to dissolve the precipitated formazan crystals. The absorbance of each well was measured at a wavelength of 570 nm in a micro plate spectrophotometer reader (Thermo Scientific Fluoroskan). Cell viability for each treatment condition was determined by normalizing to the cells only signal.

References

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2 M. Zhang, H. Liu, W. Shao, K. Miao, Y. Zhao, *Macromolecules*, 2013, 46,1325-1336.
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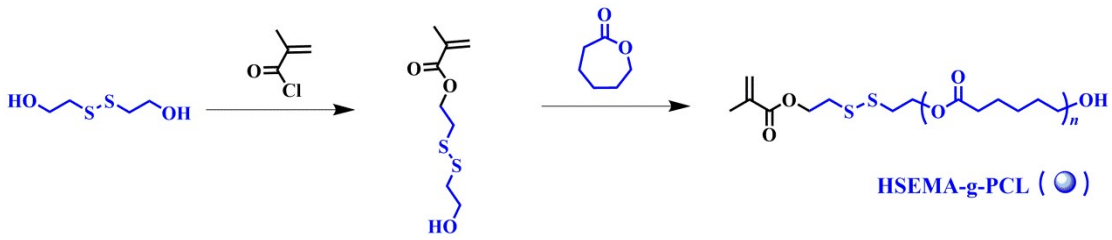
7. J. Yang, L. Yang, Q. Li, L. Zhang, *J. Colloid Interface Sci.* 2022, 626, 719-728.

2. Scheme S1



Scheme S1. Synthesis of HSEMA-g-PCL macro-monomer

3. Figure S1-S10

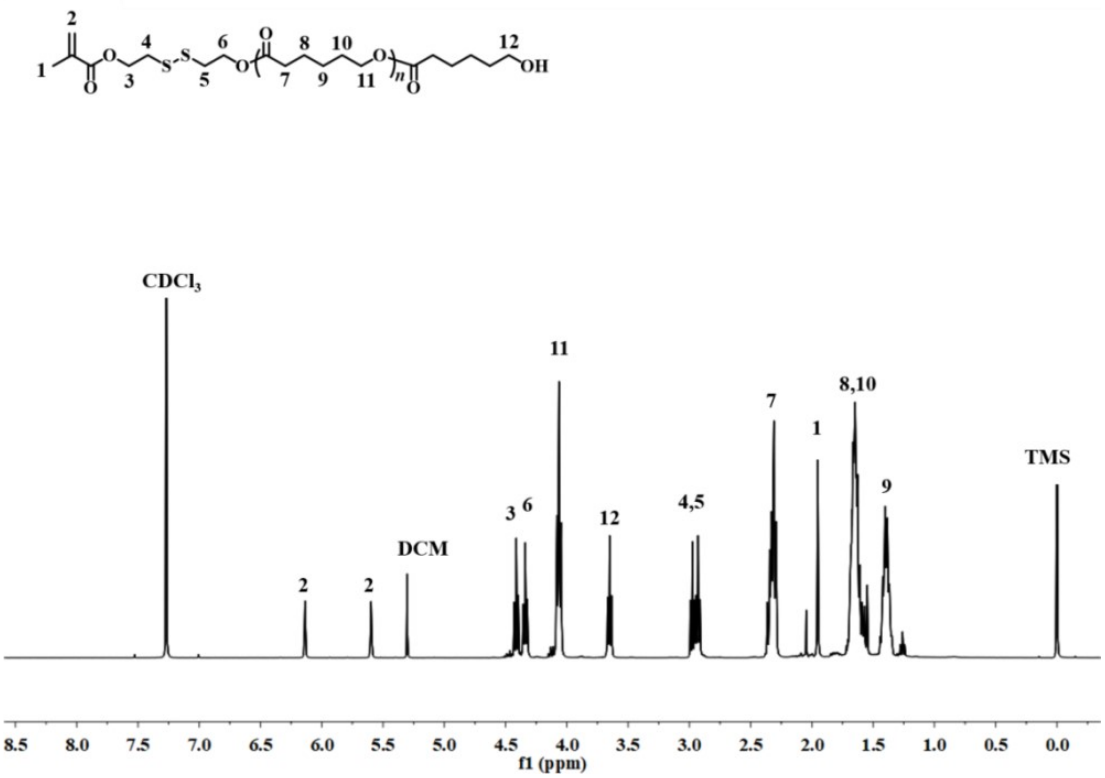


Figure S1. ^{1}H NMR spectra of HSEMA-g-PCL in $CDCl_3$.

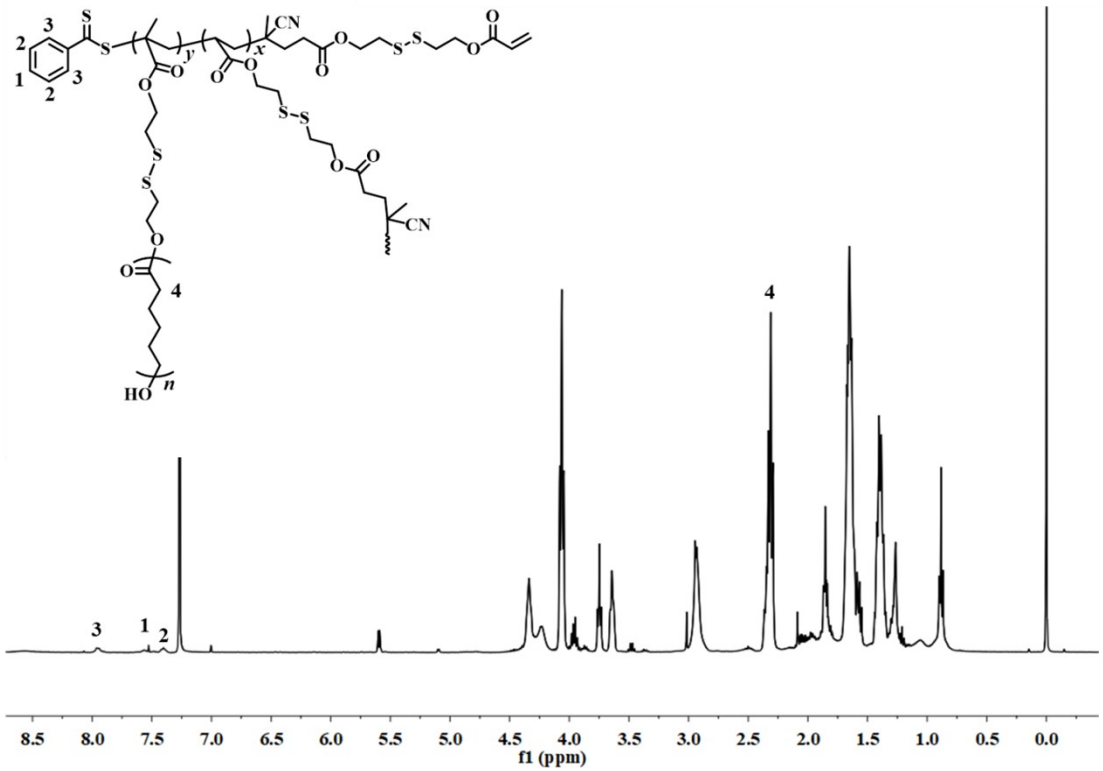


Figure S2. ^{1}H NMR spectra of multimacro-CTA *h*-P(HSEMA-g-PCL) in $CDCl_3$.

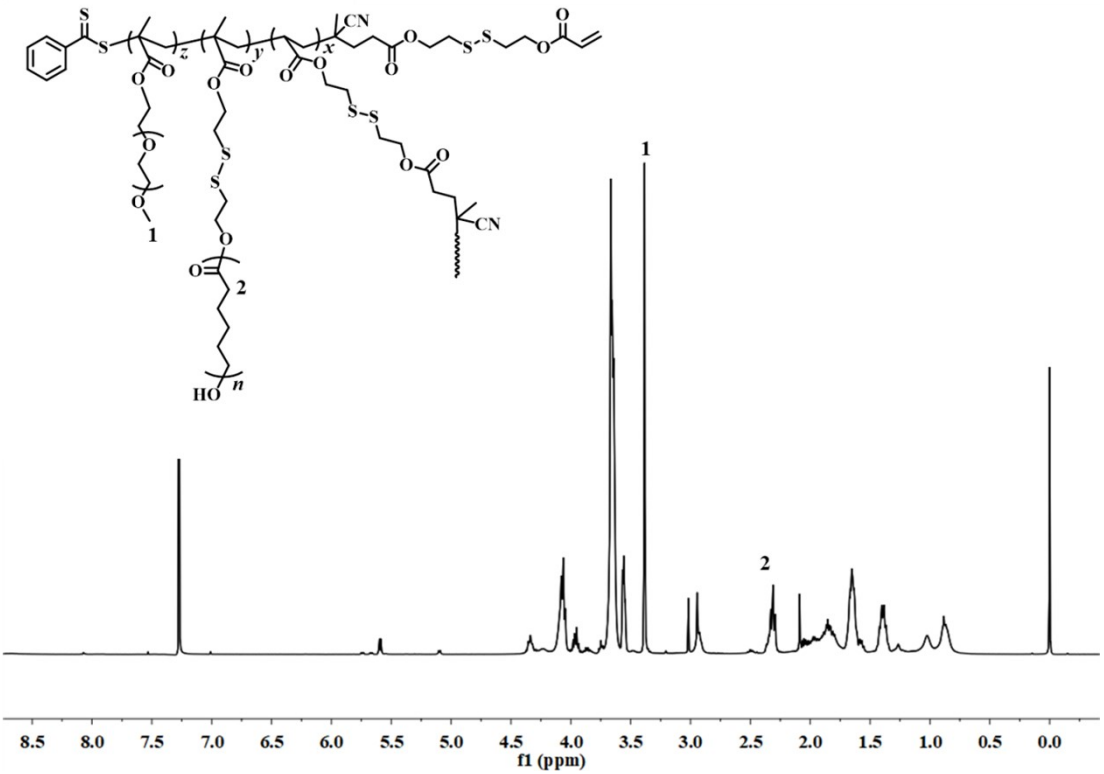


Figure S3. ^{1}H NMR spectra of amphiphilic dendritic toothbrushlike copolymer *h*-P(HSEMA-g-PCL)-*b*-POEGMA in $CDCl_3$.

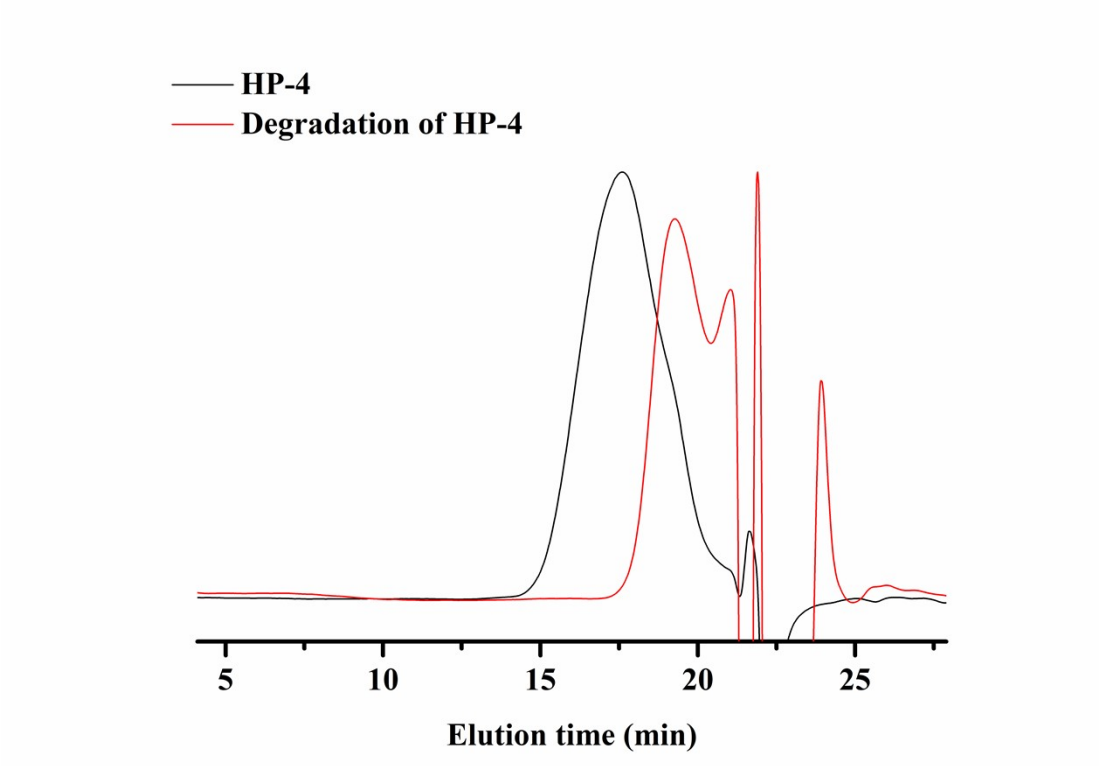


Figure S4. SEC analyses of the degraded products of HP-4.

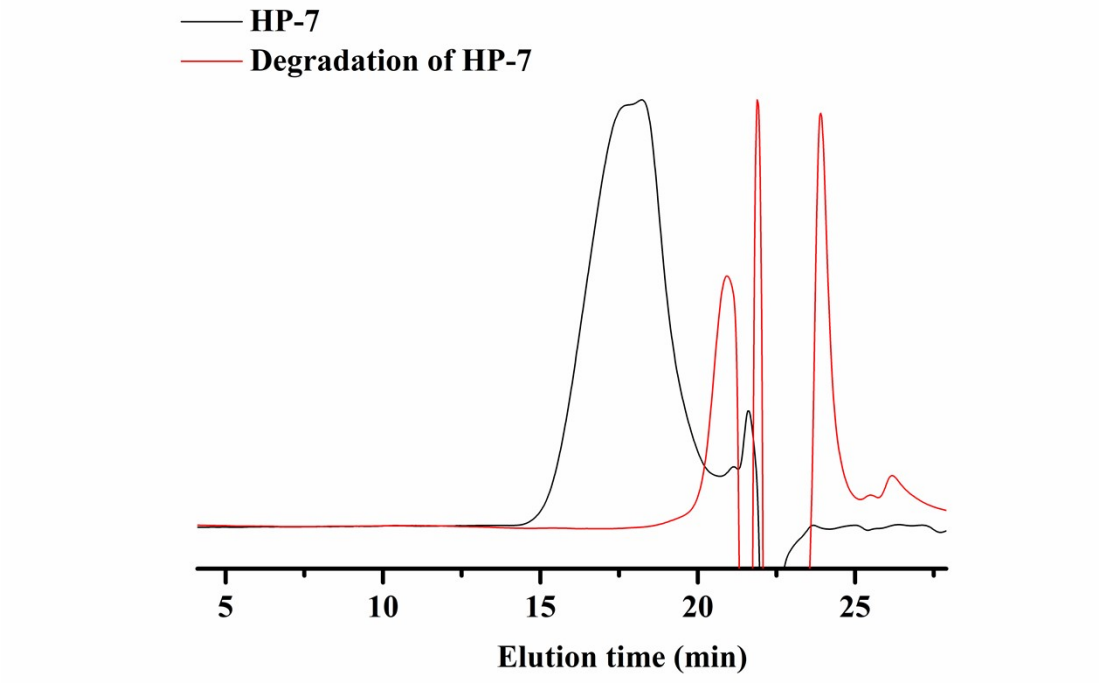


Figure S5. SEC analyses of the degraded products of HP-7.

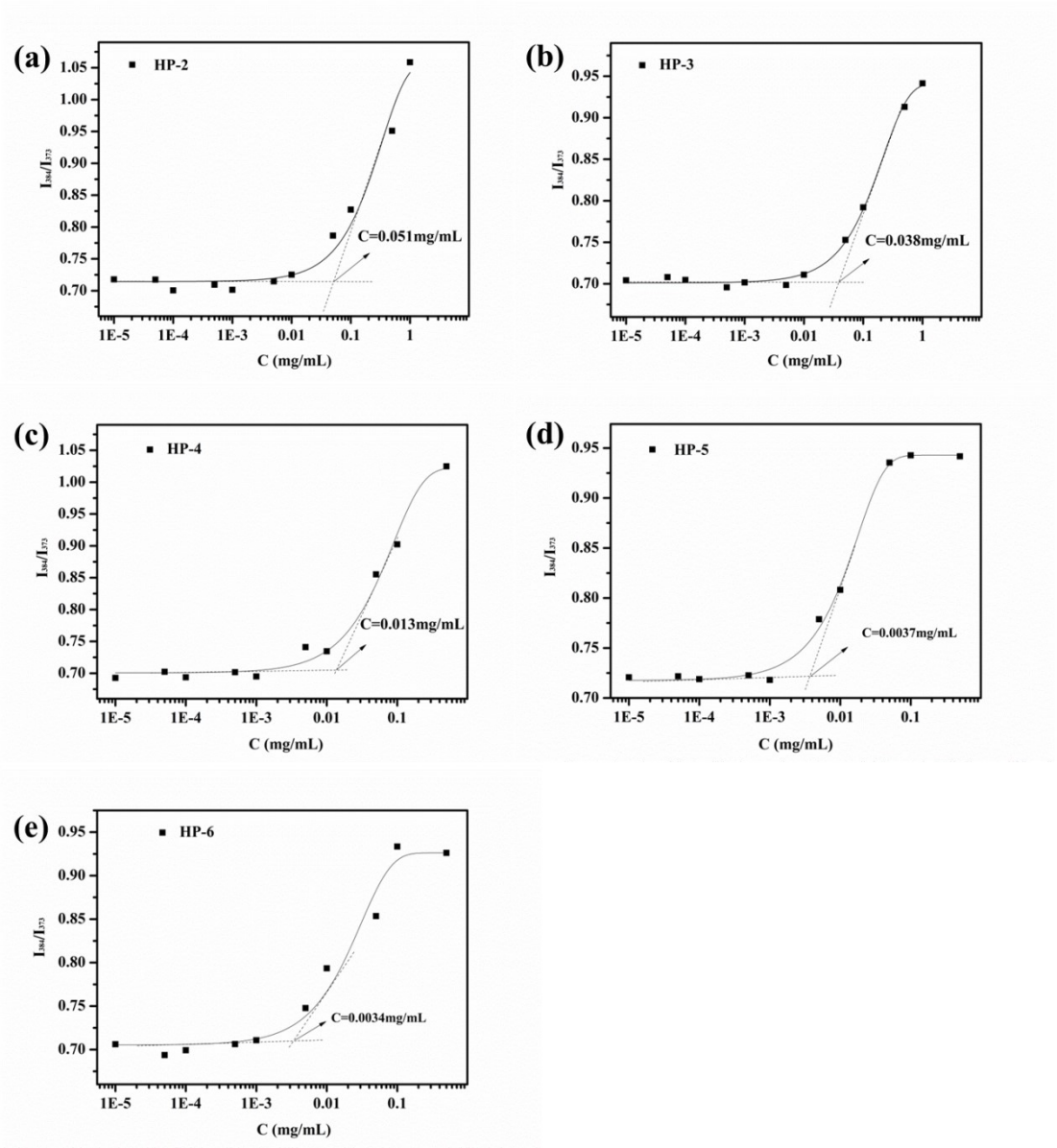


Figure S6. Plots of fluorescence intensity ratios (I_{384}/I_{373}) as function of logarithm of concentrations of amphiphilic hyperbranched statistical copolymers *h*-P(HEMA-g-PCL)-*st*-POEGMA, HP-1.

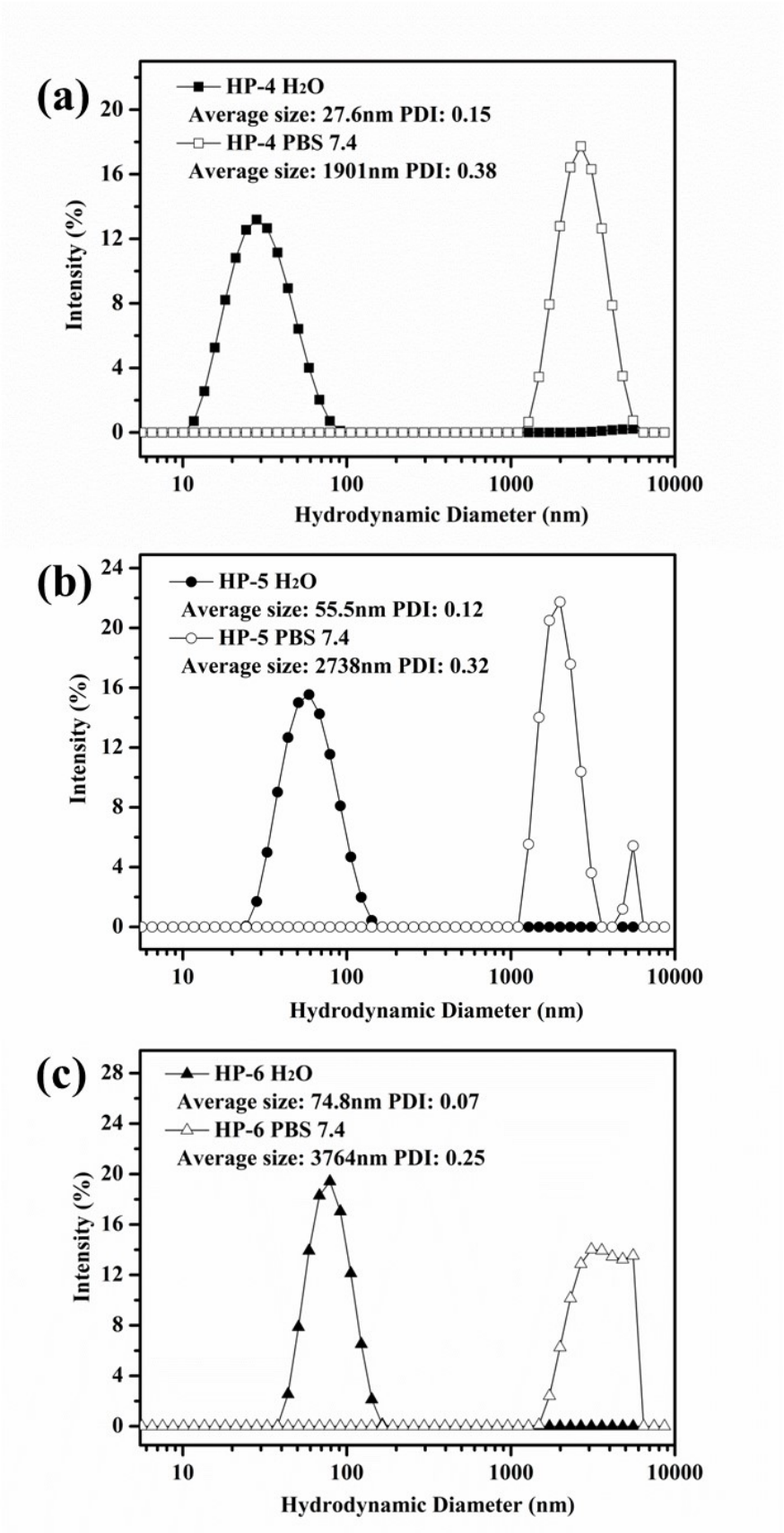


Figure S7. Mean hydrodynamic size and size distribution of the self-assembled dendritic copolymer micelles in water and PBS (7.4, 150 mM): (a) HP-4, (b) HP-5, and (c) HP-6.

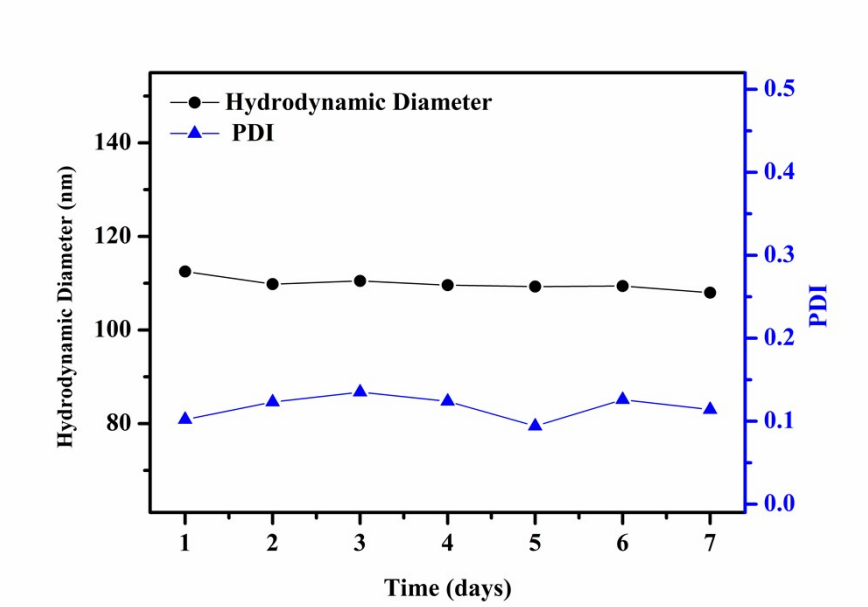


Figure S8. Mean hydrodynamic size and size distribution of HBP-based micelles in PBS (7.4, 150 mM) buffer solution for 7 days

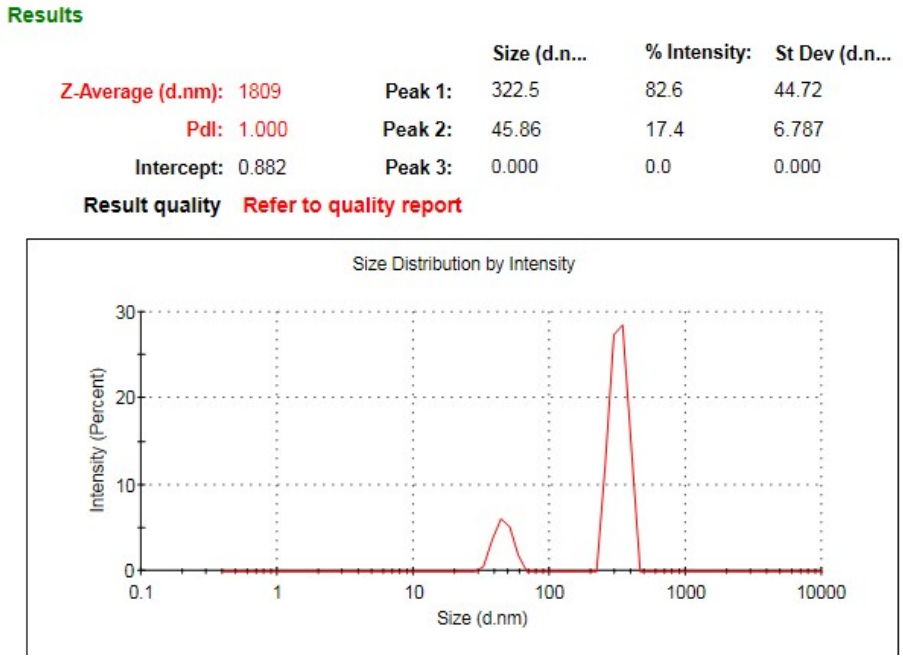


Figure S9. Reduction-triggered size changes of HBP-based micelles at 120h with 10 mM GSH.

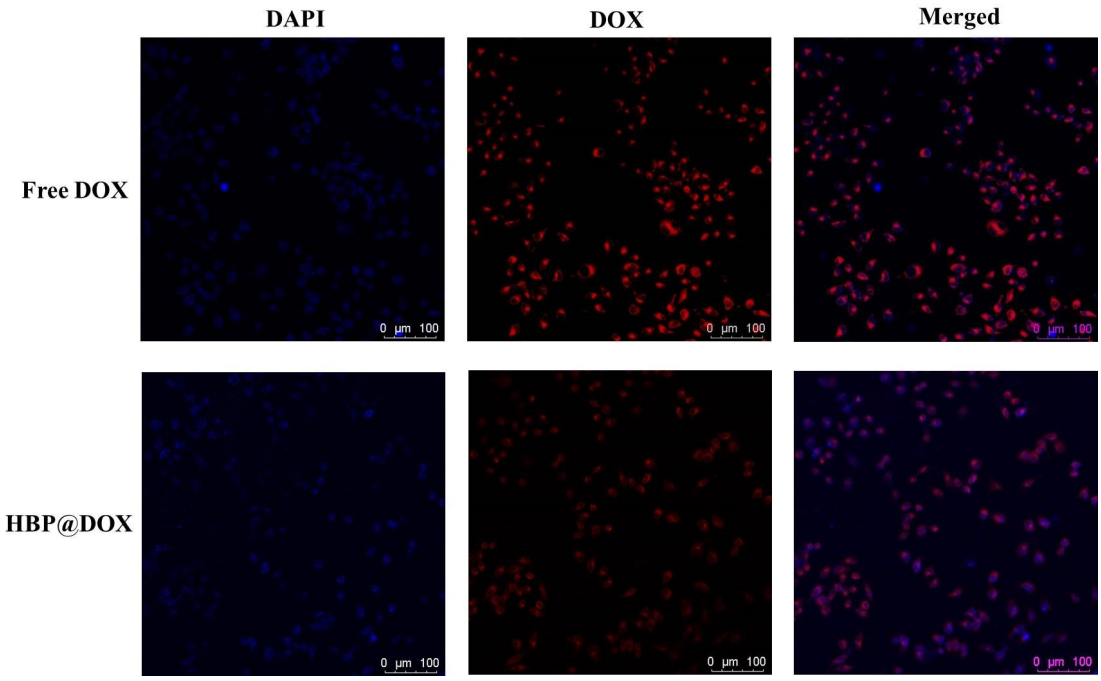


Figure S10. Confocal images of free DOX (red), and HBP@DOX micelle uptake in MCF-7 cells [nuclei stained blue with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI)]. Cells were treated with free DOX or polymer constructs at an equivalent DOX concentration of 23 μg mL^{-1}. The scale bar represents 100 μm. in MCF-7 cells.

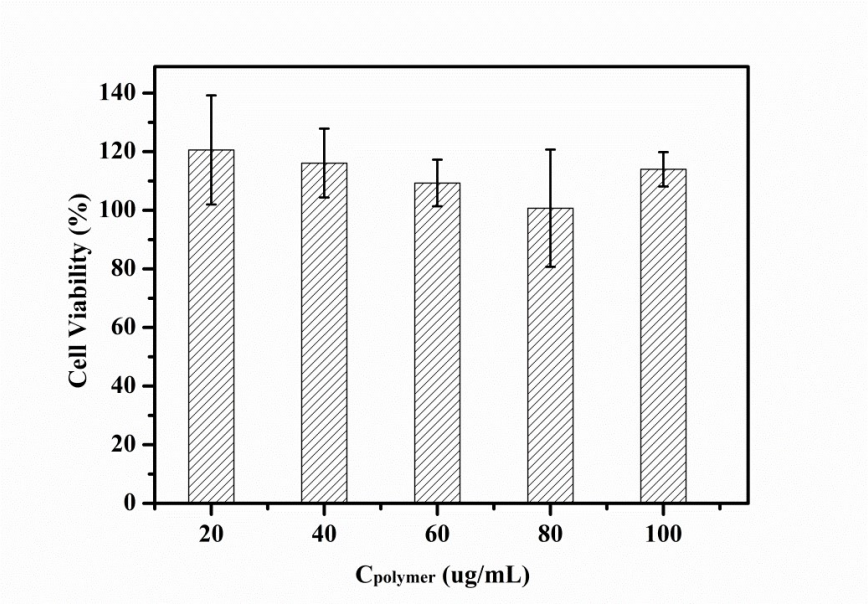


Figure S11. In vitro cytotoxicity of blank micelles of HBP in MCF-7 cells.