Supporting information for:

Bioreducible large compound vesicles with controlled size prepared via the self-assembly of branched polymer in nanodroplet templates

Long-Hai Wang, Xiao-Man Xu, Chun-Yan Hong, De-Cheng, Wu, Zhi-Qiang Yu, Ye-Zi You

1. Materials

Dithiothreitol (DTT, ≥98%, Sigma), 2-cyano-2-propyl benzodithioate (>97%, Aldrich), fluorescein isothiocyanate isomer I (FITC, 90%, Aladdin), bull serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM, Hyclone), and fetal bovine serum (FBS, Hyclone) were used as obtained. Oligo(ethylene glycol) methacrylate (OEGMA, M_n ~ 475 g/mol) (95%, Aldrich) and 2-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA, M_n ~ 188 g/mol) (95%, Aldrich) were de-inhibited by passing through a column of activated basic alumina. Acryloyl chloride (99%) was freshly distilled before use. Azobisisobutyronitrile (AIBN, 99%, Aladdin) was recrystallized twice from ethanol. Cysteamine hydrochloride (97%), Span 80 (AR), Tween 80 (AR), sodium hydroxide (≥ 96%), dichloromethane (≥ 99.5%), cyclohexane (≥ 99.5%), triethylamine (≥ 99%), n-hexane (≥ 97%), tetrahydrofuran (≥ 99%), and disodium hydrogen phosphate dodecahydrate (≥ 99%) were purchased from Sinopharm Chemical Reagent Co., LTD, and were used as received without purification unless otherwise mentioned. Water was deionized to 18 MΩ•cm resistivity using the Nanopure system.

2. Characterizations.

^{1}H NMR was recorded on a Bruker AV 300 (300 MHz) instrument. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC) performed with three linear Styragel 15 columns and a Waters 2414 differential refractive index (RI) detector (flow rate of 1.0 mL/min, THF as eluent). The cloud points of the polymer solutions were measured using a Beckman DU 640 UV spectrophotometer equipped with a digital temperature controller. Transmission electron microscopy (TEM) was performed on a JEM-2100F field emission transmission electron microscope with an accelerating voltage of 200 KV. The formed large compound vesicles were dripped onto copper grids coated with carbon and allowing the sample to dry in air before measurement. The samples for TEM time trace of the self-assembly of polymer in nanodroplet templates were diluted with heated cyclohexane (50 ºC), then dripped onto copper grids quickly. SEM was performed on a SIRION200 field emission scanning electron microscope. The samples were prepared by pipetting a drop of LCVs in ethanol onto copper plates and allowing the sample to dry in air before measurement. Dynamic light scattering (DLS) was carried out on a Malvern Zetasizer Nano ZS90 with a He-Ne laser (633 nm) and 90^o collecting optics. The rheological measurements for branched copolymer was performed on a rheometer (TAAR2000, TA Instruments) to detect evolutions of storage elastic modulus (G') and loss elastic modulus (G'') versus time at the temperature of 50 ºC at a fixed frequency of 10 rad/s.

3. Synthesis of N,N’-cystaminebisacrylamide (CBA).

Cystamine dihydrochloride (5.8 g, 25 mmol) was dissolved in 50 mL water. Aqueous solution of sodium hydroxide (10 mL, 10 M) and solution of acryloyl chloride (4.7 g, 50 mmol) in dichloromethane (5mL) were added dropwise simultaneously under stirring at 0 ºC. The reaction was performed for 3 h at room temperature after the addition was complete. The reaction mixture was filtered and washed three times with deionized water. The product was obtained by crystallization from ethyl acetate.

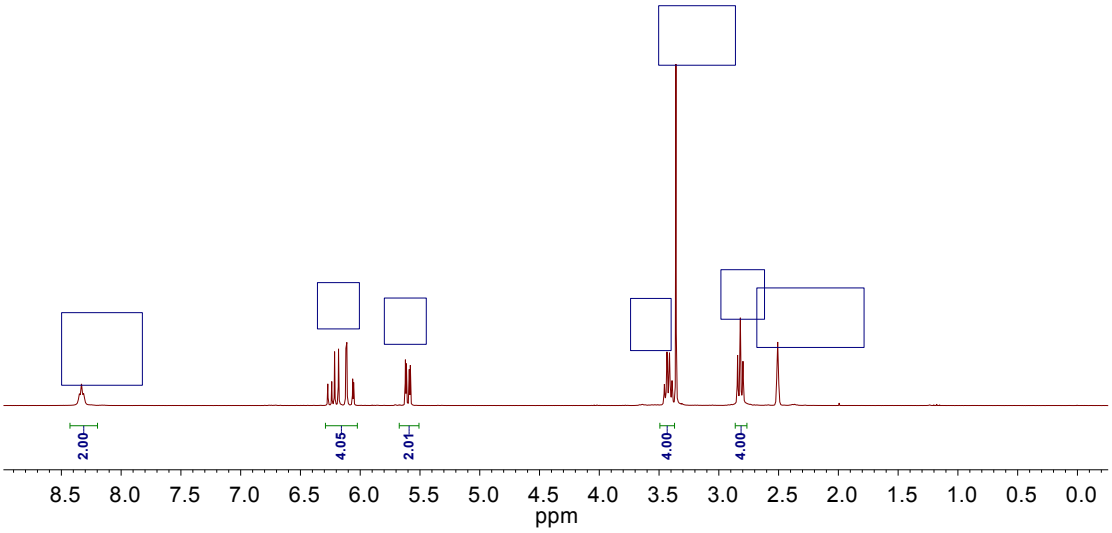


Fig. S1. ^{1}H NMR spectrum of *N,N'*-cystaminebisacrylamide in DMSO-d_6.

4. Synthesis of PEG-based branched copolymer via RAFT Polymerization.

In a typical procedure, MEO_2MA (658.0 mg, 3.5 mmol), OEGMA (712.5 mg, 1.5 mmol), CBA (260.0 mg, 1.0 mmol), CDB (16.6 mg, 0.075 mmol) and AIBN (2.5 mg, 0.015 mmol) were dissolved with THF (8 mL) in a dry glass tube, and then the solution was degassed by three freeze-pump-thaw cycles. After the tube was sealed under

vacuum, it was placed in a preheated oil bath at 60 ºC. After 30 h, the polymerization was terminated by rapid cooling in liquid nitrogen and exposure to air. The resulting polymer (M_n = 28.3 kDa, PDI = 2.0) was isolated and purified by repeated precipitation in hexane and dried under vacuum. The branching degree of the MEO2MA-OEGMA-CBA copolymer was calculated from the integral data of ^{1}H NMR. The double bond content represents the mol percentage of CBA with free vinyl functional groups in the copolymer and the branching degree represents the mol percentage of CBA without vinyl groups in the copolymer. The copolymer composition (m, n, x, y) can be calculated from the integral data S_a, S_b, S_c, and S_h.

$$x = S_h/3,\ m+n = S_a/3,\ y = (S_b/2-m-n-x)/2$$

$$\text{branching degree\%} = y/(m+n+x+y) \times 100 = 9.0\%$$

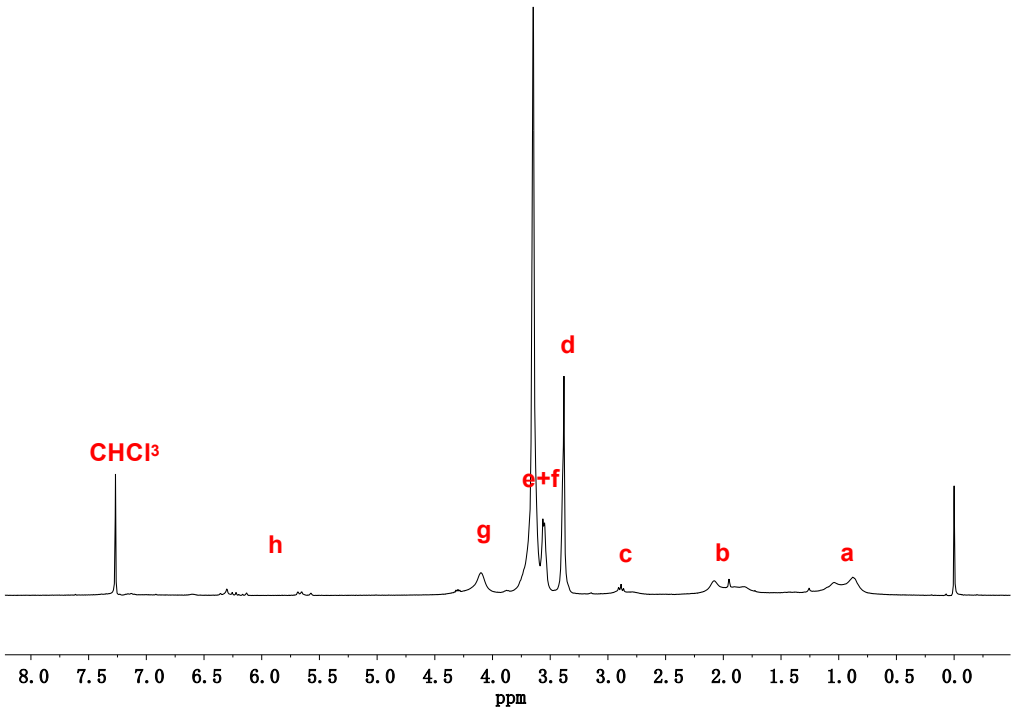


Fig. S2. ^{1}H NMR spectrum of MEO_2MA-OEGMA-CBA copolymer with disulfide linkages in $CDCl_3$.

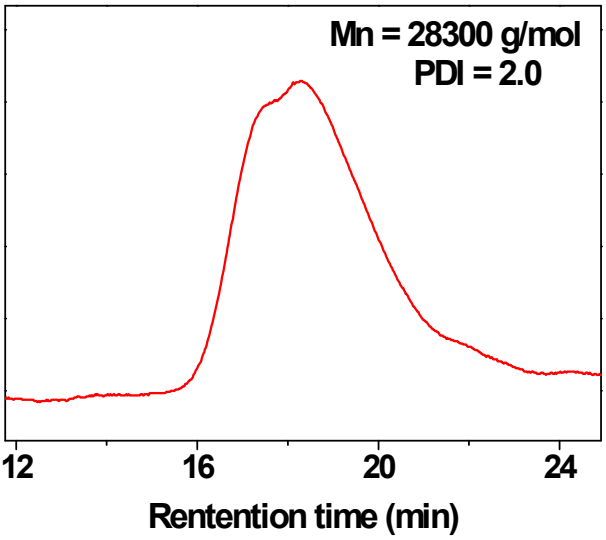


Fig. S3. GPC curve of MEO_2MA-OEGMA-CBA branched copolymer.

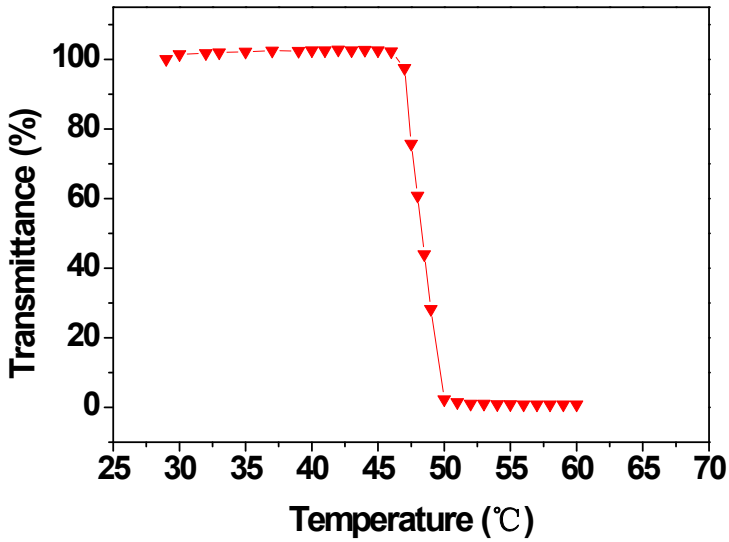


Fig. S4. The change of transmittance of MEO_2MA-OEGMA-CBA branched copolymer aqueous solution (3.0 mg/mL) with temperature.

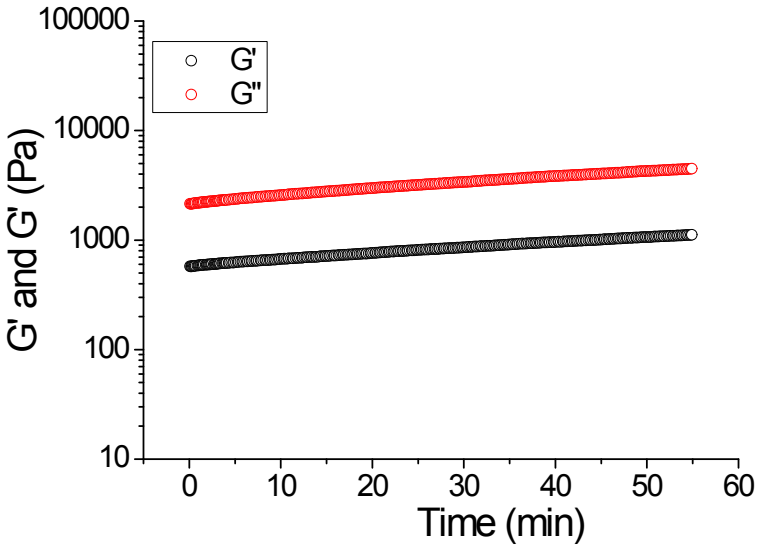
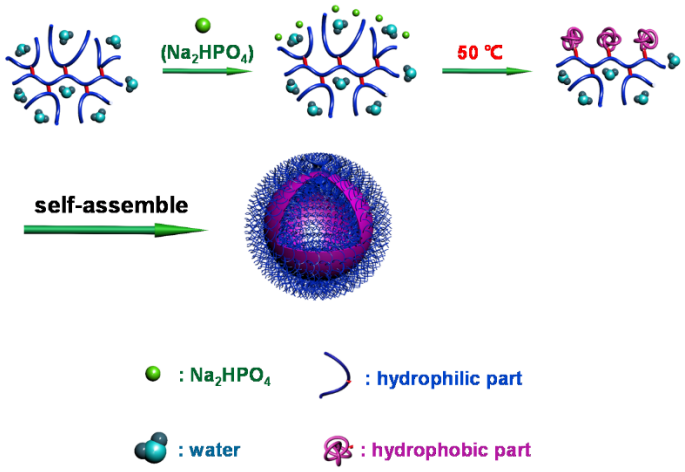


Fig. S5. G' and G'' of the MEO_2MA-OEGMA-CBA branched copolymer varied with time at 25 °C

5. Biodegradable vesicles can form in water.

The solution containing MEO_2MA-OEGMA-CBA copolymer of 3.0 mg/mL and Na_2HPO_4 of 0.03 M was prepared. After uniformly mixed, the mixture was placed in water bath at the temperature of 50 ℃. Then the solution was dripped onto the preheated copper plates using cotton ball in drying oven, and the water was absorbed by filter paper immediately. (The process of sample preparation was carried out in drying oven.)

A



B

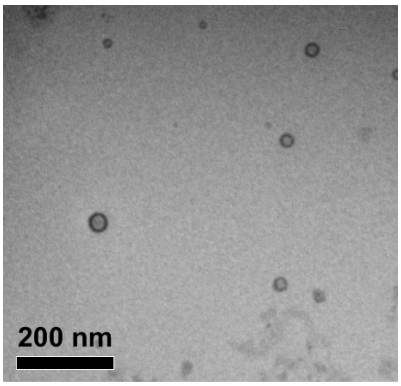


Fig. S6. (A)The mechanism of the formation of vesicles, (B) TEM image of vesicles from branched copolymer in water without surfactant.

6. Preparation of biodegradable large compound vesicles using emulsion nanodroplets as templates.

The surfactant plays an important role in emulsion system. It is closely related to the stability of the emulsion. We chose Span80 and Tween 80 as mixed surfactants since they were turned out not to be interacted with PEG, and suitable to obtain stable emulsion of water-in-cyclohexane system.

In detail, Span 80 and Tween 80 (3/1, w/w) in cyclohexane (15 mL) were vigorously stirred in a cylindrical 60 mL vial for 30 min. Polymer (120 mg) and Na_2HPO_4 (10.0 mg) was first dissolved in water (0.75 mL) to form the aqueous phase. Then the aqueous solution was added to the cyclohexane phase, and the whole was stirred vigorously for 1 hour. The formed macro-emulsion was ultrasonicated with sonicator under ice cooling, forming emulsion nanodroplets. The diameter of the formed emulsion nanodroplets is 103 nm, 151 nm, 203 nm and 305 nm when the total amount of surfactants of 200 mg, 180 mg, 160 mg and 134 mg was used, respectively. The initial emulsion nanodroplets was transferred to a flask and heated with stirring at 50 ºC for 3

h. After cooling to room temperature, the large compound vesicles were separated from the oil phase by centrifugation. The obtained large compound vesicles were washed with cyclohexane, after redispersing in ethanol and centrifuging, the large compound vesicles were further washed with PBS buffer.

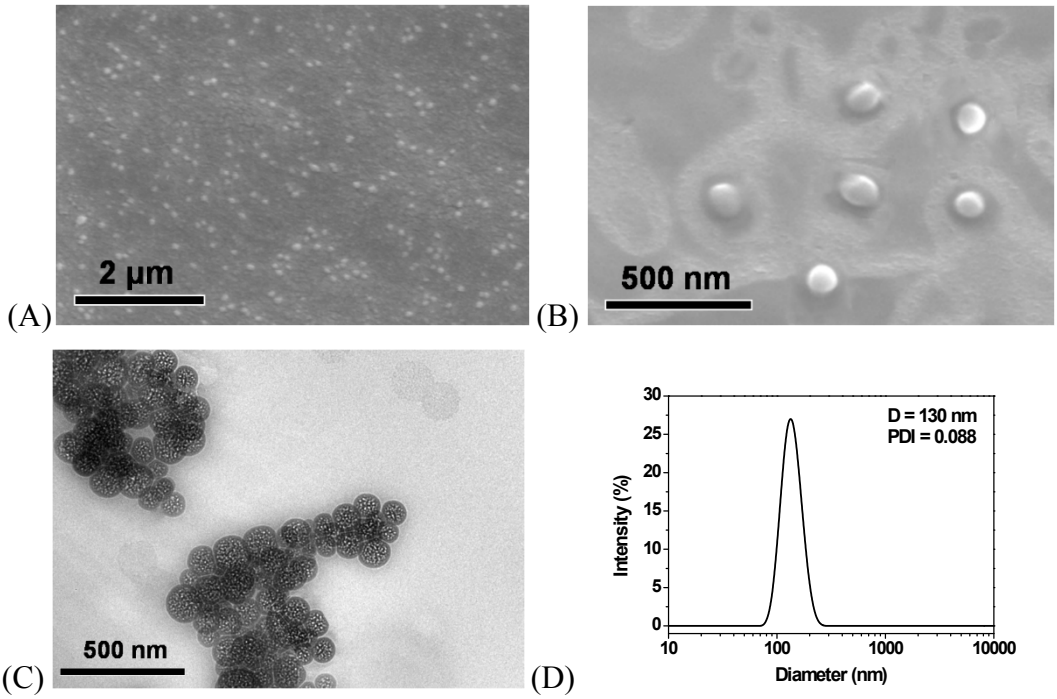


Fig. S7. (A) (B) SEM images and (C) TEM image of cross-linked large compound vesicles prepared from emulsion nanodroplets (the amount of surfactants was 200 mg). (D) The size and size distribution of large compound vesicles obtained from DLS. The low PDI value in DLS result shows the narrow size distribution of LCVs.

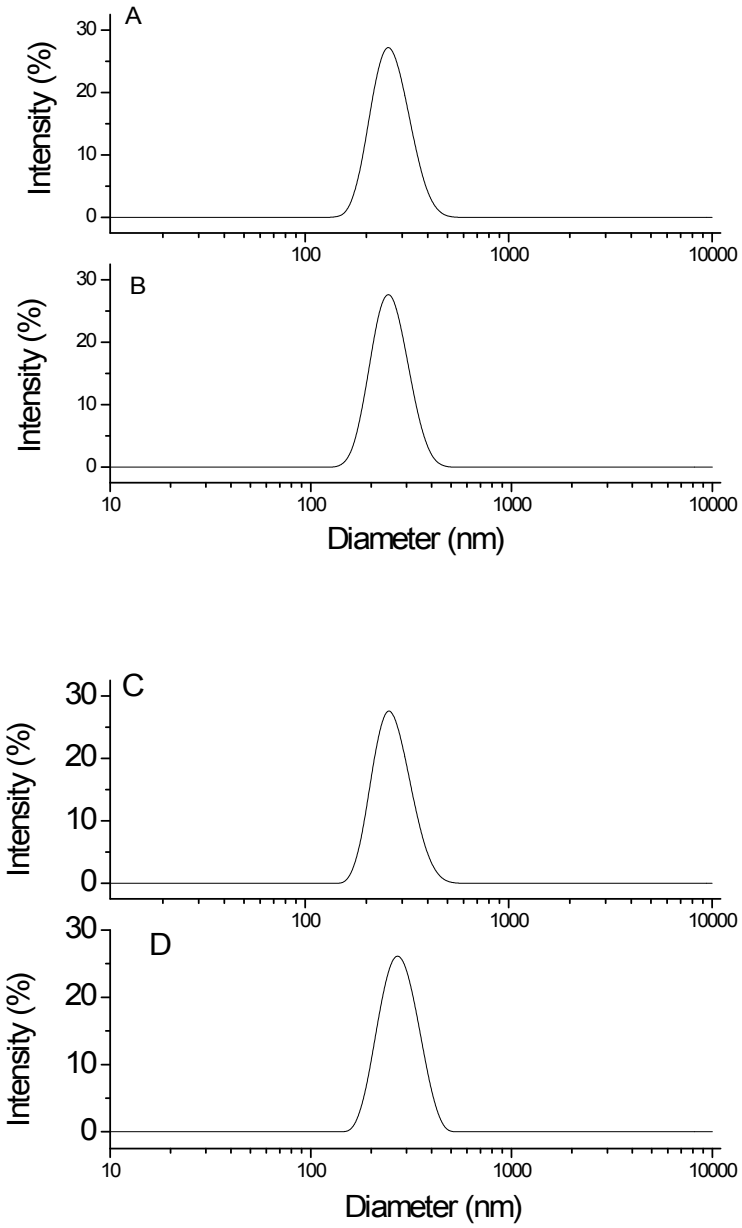


Fig. S8. The prepared LCVs are stable: The size of LCVs almost remained unchanged after being soaked in water for 0 h (A, 255 nm) and 24 h (B, 262 nm), and the size of LCVs after being soaked in ethanol for 0 h (C, 260 nm) and 24 h (D, 272 nm).

7. **Fluorescence labelling of Bull Serum Albumin (BSA).** Fluorescence labeling of Bull Serum Albumin (BSA). Fluorescein isothiocyanate isomer I (FITC, 10 mg) was dissolved in DMSO at concentration of 1 mg/mL. 7.56 g $NaHCO_3$, 1.06 g Na_2CO_3, 7.36 g NaCl were added into volumetric flask and dilute with water to 1000 mL to obtain carbonate buffer solution (pH = 9.0). Then 100 mg BSA was

dissolved in carbonate buffer solution at concentration of 2 mg/mL. FITC solution was slowly added to BSA solution in dark while stirring, then incubated overnight at 4 ℃. The solution was dialyzed (MWCO = 7,000 Da) in dark against de-ionized water and purified solution was freeze-dried to get yellow solid.

8. **Encapsulation of protein into the bioreducible LCVs.** Encapsulation of protein into the bioreducible LCVs. For the encapsulation of protein molecules, the aqueous phase contained polymer (50.0 mg), Na_2HPO_4 (10.0 mg), FITC-BSA (1.0 mg) and deionized water (0.375 mL). The continuous phase consisted of Span 80, Tween 80 and cyclohexane (7.5 mL). Then the aqueous solution was added to the cyclohexane phase, and the whole was stirred vigorously for 1 hour. The formed emulsion droplet was ultrasonicated under ice cooling to form nanodroplet. The initial nanodroplets were transferred to a flask and heated with stirring at 50 °C for 3 hours. After cooling to room temperature, the LCVs were separated from the oil phase by centrifugation. The obtained large compound vesicles were washed with cyclohexane, after redispersing in ethanol and centrifuging, the large compound vesicles were further washed with PBS buffer.

9. **Confocal fluorescence microscopy.** Hela cells were seeded in a 4-chamber glass bottom dish at a density of 2.0×10^4 cells per well and incubated in DMEM supplemented with 10% FBS overnight. After the media was refreshed FITC-BSA loaded LCVs dispersion were added into wells. After the cells were incubated for 4 h, media was removed and the cells were washed three times with PBS. The CLSM observation was performed using a confocal laser scanning microscopy

(Leica TCS SP5 microscope) at excitation wavelength of 488 nm (Ar laser), emission detection channel was set to be 500 ~ 535 nm.

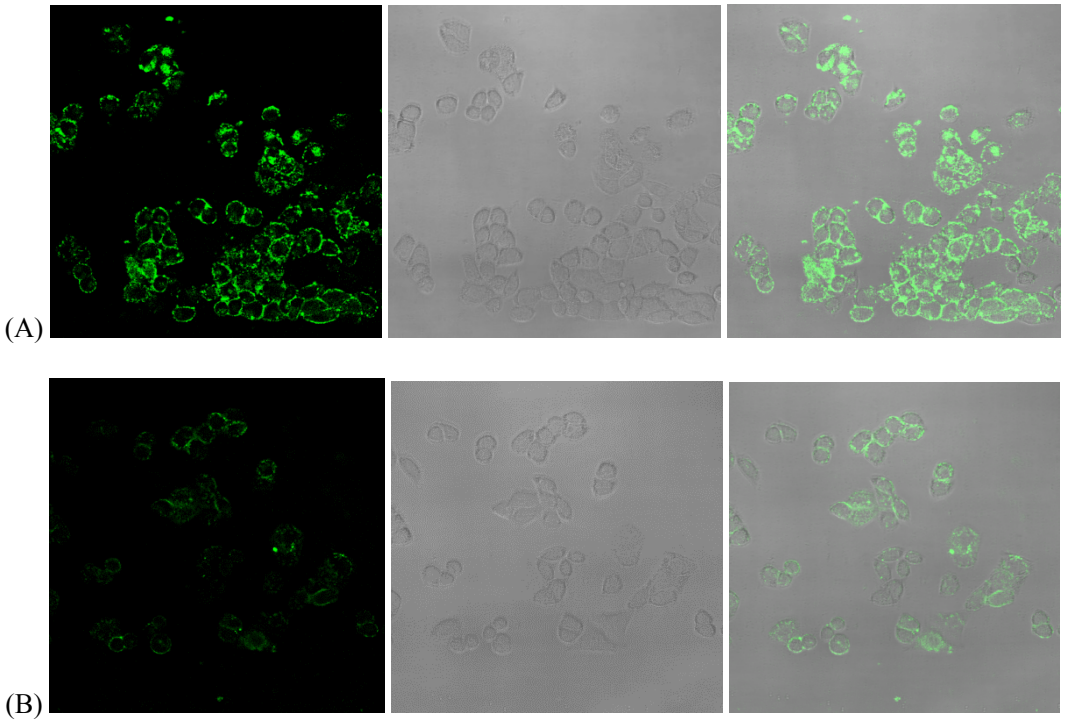


Fig. S9. Confocal fluorescence images of FITC-BSA loaded LCVs with sizes of 100 nm (A) and 310 nm (B) in Hela cells after incubation for 4 h. It shows that 100 nm-sized LCVs have greater cell uptake than 310 nm-sized LCVs.

10. In Vitro Cytotoxicity.

Cell viability was examined by the MTT assay. HeLa cells and HepG2 cells were seeded in a 96-well plate at a density of 8,000 cells per well and incubated in DMEM supplemented with 10% FBS overnight. After the media was refreshed, the cells were treated with PEG-based branched polymer solution at a given concentration. The treated cells were incubated in a humidified environment with 5% CO_2 at 37 ºC for 24 h, then MTT reagent (in 20 μL PBS buffer, 5 mg/mL) was added to each well, and the cells were further incubated for 4 h at 37 ºC. The culture medium in each well was

removed and replaced by 100 μL dimethyl sulfoxide (DMSO). The plate was gently agitated for 15 min, and the absorbance values were recorded at a wavelength of 490 nm upon using a Thermo Multiskan flash. The cell viability is calculated as $A_{490,treated}/A_{490,control} \times 100\%$, where $A_{490,treated}$ and $A_{490,control}$ are the absorbance values with or without the addition of polymer, respectively. Each experiment was done in quadruplicate. The data are expressed as average ± standard deviations (± SD).

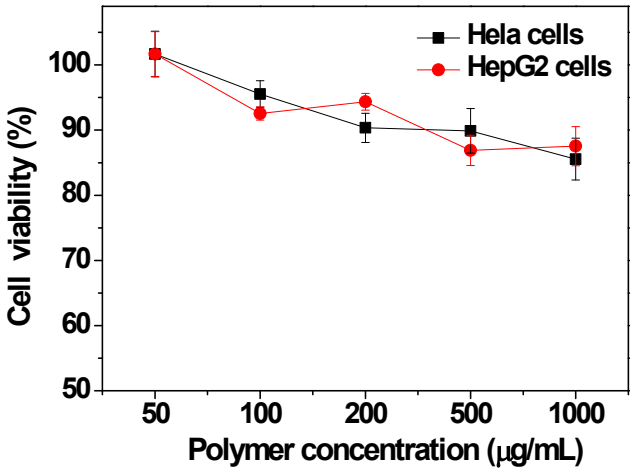


Fig. S10. Cytotoxicity of MEO_2MA-OEGMA-CBA copolymer in HeLa cells and HepG2 cells.

11. Protein BSA Adsorption Assay. The solutions containing BSA of 0.5 mg/mL and MEO_2MA-OEGMA-CBA copolymer of 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL were prepared, respectively. The solutions were mixed and shaken at 37 °C for 0.5 h. Then the mixtures were centrifuged and the supernatants were collected. The quantified results for the amount of BSA were measured by BCA assay. The BSA concentrations in supernatants were determined using a calibration curve obtained from BSA solutions of known concentrations by BCA assay. The protein adsorbed (%) value A was defined as: $A = (C_iV - C_sV) / C_iV$

× 100 %, where C_i and C_s are the initial BSA concentration and the BSA concentration in the supernatant, V is the total volume of the solution.

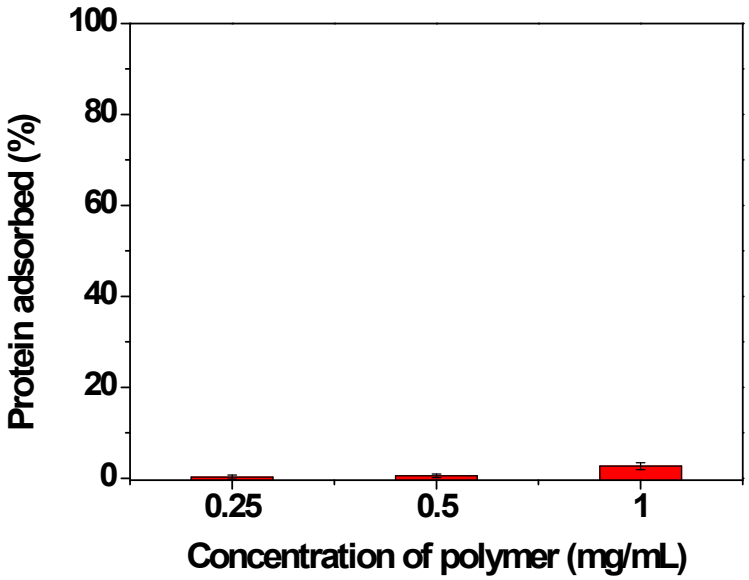


Fig. S11. Protein adsorption for MEO_2MA-OEGMA-CBA copolymer at different concentration.