Supporting information

Engineering Nanoscopic Hydrogels via Photo-Crosslinking Salt-Induced Polymer Assembly for Targeted Drug Delivery

Materials. Poly(ethylene glycol) (PEG) with dihydroxyl end groups (Mw = 3 400 and 6 000), 6-diphenyl-1,3,5-hexatriene (DPH), phosphodiesterase I from Crotalus atrox and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. The cytocompatible UV photoinitiator Irgacure 2959 (I2959, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone) was obtained from Ciba-Geigy Chemical Co. (Tom River, NJ). Ethyl ethylene phosphate (EEP) was synthesized as previously described in literature and purified with two consecutive vacuum distillations before use.1 Stannous octoate (Sn(Oct)2, Sinopharm Chemical Reagent Co., China) was purified according to a method described in the literature.1 Triethylamine (Sinopharm Chemical Reagent Co., China) was refluxed with phthalic anhydride, then with potassium hydroxide, and distilled. Dichloromethane was refluxed with CaH2 for 48 h and distilled out before use. Acryloyl chloride and p-toluenesulfonyl chloride were distilled twice before use. Doxorubicin hydrochloride (DOX) was purchased from Zhejiang Hisun Pharmaceutical Co. N-Hydroxysulfosuccinimide sodium (Sulfo-NHS) and 1-ethyl-3-(3-dimet hylaminopropyl)carbodiimide (EDC)  were obtained from Shanghai Medpep Co. and used as received. All other reagents were purchased from Sinopharm Chemical Reagent Co. and used without purification.

Synthesis of Triblock Copolymer of Poly(ethyl ethylene phosphate) and PEG (HO-PEEP150-b-PEG6k-b-PEEP150-OH)

PEG6k (Mw = 6 000) was dried by azeodistillation of toluene and dried at vacuum overnight. PEG6k (0.6 g, 0.1 mmol) and EEP (6.0 g, 40.0 mmol) were added into a fresh flamed and
nitrogen-purged round-bottomed flask. After melting at 90 °C, Sn(Oct)_2 (0.03 g, 0.075 mmol) was added quickly. The reaction was carried out at 90 °C for an additional 2 h and the product was precipitated in cold ethyl ether/isopropyl alcohol (5/1, v/v) twice. The supernatant was removed and the product was dried under vacuum to a constant weight at room temperature to obtain the product. ^1H NMR (CDCl_3), δ (ppm): 4.29 (m, -PO\_CH\_2CH\_2O-), 4.15 (m, -OCH\_2CH\_3), 3.65 (s, -OCH\_2CH\_2O-), 1.29 (t, -OCH\_2CH\_3) 3.85 (t, -POCH\_2CH\_2OH). The degree of polymerization of PEEP was 150 for each block as calculated on the basis of the integration ratio of the protons of PEEP at 1.29 ppm and protons of PEG at 3.65 ppm from its ^1H NMR.

Figure S1. ^1H NMR spectrum of triblock copolymer of HO-PEEP\textsubscript{150}-b-PEG\textsubscript{6K}-b-PEEP\textsubscript{150}-OH (ppm, in CDCl_3).

**Synthesis of PEEP\textsubscript{150}-PEG\textsubscript{6K}-PEEP\textsubscript{150} Diacrylate (Acr-PEEP\textsubscript{150}-b-PEG\textsubscript{6K}-b-PEEP\textsubscript{150}-Acr)**

HO-PEEP\textsubscript{150}-b-PEG\textsubscript{6K}-b-PEEP\textsubscript{150}-OH was dried in vacuum for 48 h. HO-PEEP\textsubscript{150}-b-PEG\textsubscript{6K}-b-PEEP\textsubscript{150}-OH (5 g) and triethylamine (0.1 g, 1.0 mmol) were dissolved in 150 mL of CH\_2Cl\_2 in a round-bottomed flask and cooled to -5 °C. Then, acryloyl chloride (0.1 g, 1.1 mmol) dissolved in 10 mL of CH\_2Cl\_2 was slowly dropped to this solution. The mixture was further reacted for 12 h at -5 °C and 24 h at room temperature. Triethylamine hydrochloride formed was removed by filtration, and then the solution was concentrated and precipitated in cold ethyl ether twice. The residue was redissolved in CH\_2Cl\_2 and passed by a column filled with silica gel (200 mesh) for further purification using CH\_2Cl\_2 as the eluent. The solvent was removed and the product was dried under vacuum for 24 h. ^1H NMR (CDCl_3), δ (ppm): 4.29 (m,
-POCH2CH2O-, 4.15 (m, -OCH2CH3), 3.65 (s, -OCH2CH2O-), 1.29 (t, -OCH2CH3) 6.16 (t, -CH=CH2), 5.87 and 6.43 ppm (t, -CH=CH2)

Figure S2. 1H NMR spectrum of triblock copolymer Acr-PEEP150-b-PEG6K-b-PEEP150-Acr (ppm, in CDCl3).

Synthesis of N3-PEG3.4K-OH

HO-PEG3.4K-OH (50 g, 0.015 mol) and triethylamine (2.98 g, 0.03 mol) were dissolved in 200 mL of CH2Cl2 in a 500 mL round-bottomed flask and cooled to 0 °C. Then, p-toluenesulfonyl chloride (5.61 g, 0.03 mol) dissolved in 50 mL of CH2Cl2 was slowly dropped to this solution. The mixture was further reacted for 8 h at 0 °C and 12 h at room temperature. Triethylamine hydrochloride formed was removed by filtration, and then the solution was concentrated and precipitated in cold hexane twice. The residue, which was the mixture of HO-PEG3.4K-OH, HO-PEG3.4K-Tos, and Tos-PEG-Tos, was redissolved in CH2Cl2 and isolated via silica gel chromatography using 89:10:1 CHCl3/CH3OH/NH4OH as eluent. Subsequently, HO-PEG-Tos (2 g, 0.56 mmol) was dissolved in DMF (50 mL) at 85 °C, and then sodium azide (0.35 g, 2.24 mmol) was added. The mixture was stirred at 80 °C for 12 h then cooled to room temperature. The resulting solution was precipitated into cold hexane twice and the product as a white powder was dried under vacuum for 24 h. 1H NMR (CDCl3), δ (ppm): 3.65 (s, 310H, -OCH2CH2O-), 3.86 (t, 2H, N3CH2CH2O-), 3.38 (t, 2H, -OCH2CH2OH).

Synthesis of Heterofunctional Diblock Copolymer Acr-PEEP172-b-PEG3.4K-Lac
N₃-PEG₃.₄K-OH was used as the initiator to synthesize the block copolymer of N₃-PEG₃.₄K-b-PEEP₁₇₂-OH, and then N₃-PEG₃.₄K-b-PEEP₁₇₂-OH was reacted with acryloyl chloride to obtain N₃-PEG₃.₄K-b-PEEP₁₇₂-Acr in a similar procedure as described above. The resulted polymer (1.2 g, 0.04 mmol) was then dissolved in 10 mL of tetrahydrofuran, and reduced to amino groups with triphenylphosphine (25 mg, 0.1 mmol) at 25 °C for 4 h, followed by addition of water (200 µL) and stirring overnight. NH₂-PEG₃.₄K-b-PEEP₁₇₂-Acr was obtained by precipitation in cold diethyl ether (100 mL) twice. In the next step, the resulted amino functionalized block copolymer (500 mg, 0.017 mmol) was dissolved in 50 mL of 2-(4-morpholino)ethanesulfonic acid buffer (MES buffer, 0.1 M, pH 6.5), to which was added sulfo-NHS and EDC·HCl activated lactobionic acid (5 mmol, Sigma) in 50 mL of MES buffer. The reaction was performed at 4 °C for 12 h and room temperature for another 12 h. The product was purified by membrane dialysis (Mw cutoff 2,000, Spectrum® Laboratories) against Milli-Q ultrapurified water at 4 °C for 2 days, and lyophilized overnight. ¹H NMR (CDCl₃, δ (ppm)): 4.29 (m, 4H, -PO(CH₂CH₂O-), 4.15 (m, 2H, -OCH₂CH₃), 3.65 (s, 31H, -OC₆H₄O-), 1.29 (t, 3H, -OCH₂CH₃), 6.16 (t, 1H, -CH=CH₂), 5.87 and 6.43 ppm (t, 2H, -CH=CH₂), 3.0~3.5 (c, H of galactose group). The degree of polymerization of PEEP was 172, calculated as stated above.

Figure S3. ¹H NMR spectrum of triblock copolymer Acr-PEEP₁₇₂-b-PEG₃.₄K-Lac (ppm, in
Characterization of Polymer

Bruker AV300 NMR spectrometer (300 MHz) was used for NMR measurement. Deuterated chloroform containing 0.03% tetramethylsilane was used as the solvent. Number and weight average molecular weights (Mn and Mw) and molecular weight distributions (polydispersity index, PDI=Mw/Mn) were determined by gel permeation chromatography (GPC) measurements on a Waters GPC system, which was equipped with a Waters 1515 HPLC (high performance liquid chromatography) solvent pump, a Waters 2414 refractive index detector, and three Waters Styrage high resolution columns (HR4, HR2, HR1, effective molecular weight range 5,000–500,000, 500–20,000, and 100–5,000, respectively). Chloroform (HPLC grade, J.T. Baker, stabilized with 0.75% ethanol) was used as eluent at 40 °C, delivered at a flow rate of 1.0 mL min⁻¹. Monodispersed polystyrene standards with a molecular weight range of 1,310–5.51×10⁴ were used to generate the calibration curve.

Transmittance Measurements

Optical transmittances of aqueous polymer solution were measured at 500 nm with a UV-Vis spectrophotometer (UV-2802 PC, UNICO, China). Samples were thermostated at 35 °C with a temperature-controller. The sample was equilibrated at various concentration of NaCl for 20 min before recording the data.

UV-Vis Determination

To 4 mL of aqueous solution of Acr-PEEP₁₅₀-b-PEG₆₆-b-PEEP₁₅₀-Acr (10 mg mL⁻¹), 40 μL of methanol containing 0.4 mmol L⁻¹ DPH was added. The mixture was left in the dark for 2 h. The UV-vis absorption spectra of the mixture from 300 to 550 nm were recorded at various concentration of NaCl using a UV-Vis spectrophotometer (UV-2802 PC, UNICO, China).

Preparation of Targeted Nanogels (NG-LA) and Non-Targeted Nanogel (NG)

In general, triblock copolymer at desired concentration was dissolved in 4 mL of Milli-Q ultrapurified water and the solution was incubated at 4 °C for at least 30 min in glass vials.
Subsequently, various concentrations of NaCl were added. The solution was stirred at 35 °C and photo-crosslinked using I2929 as the initiator. The photopolymerization reaction was allowed to proceed for 10 min at 365 nm with a light density of 8 mW/cm². All nanoparticles were purified by membrane dialysis (Mw cutoff 12,000, Spectrum® Laboratories) against Milli-Q ultrapurified water. The nanogel was lyophilized for further use.

To synthesize the nanogels for drug loading, Acr-PEEP150-b-PEG6K-b-PEEP150-Acr at 5 mg mL⁻¹ with 5 mg mL⁻¹ of NaCl was used. On the other hand, the mixture of Acr-PEEP150-b-PEG6K-b-PEEP150-Acr at 4 mg mL⁻¹ and Acr-PEEP172-PEG3.4K-Lac at 1 mg mL⁻¹ with 5 mg mL⁻¹ of NaCl were used.

**Dynamic Light Scattering**

The size and size distribution of particles or nanogels in aqueous solutions were measured by dynamic light scattering carried out on a Malvern Zetasizer Nano ZS90 with a He-Ne laser (633 nm) and 90° collecting optics. The data were analyzed by Malvern Dispersion Technology Software 4.20.

**Transmission Electron Microscopy (TEM)**

TEM was performed on a JEOL-2010 transmission electron microscope with an accelerating voltage of 200 KV. The samples were prepared by pipetting a drop of nanogel solution (0.1 mg mL⁻¹) onto 230 mesh copper grids coated with carbon and allowing the sample to dry in air before measurements.

**Loading of DOX into Nanogels**

The nanogels (100 mg) were allowed to stir in water (1 mL) for 2 days to become fully swollen, and then samples were mixed with DOX (10 mg). The resulting mixtures were stirred at 4 °C in dark for 3 days, and passed through CL-4B column (Sepharose®) to remove free DOX. The loading amount of DOX was calculated by subtracting the amount of DOX after treatment by an acidic hydrolysis in 2.0 M HCl solution at 80 °C for 15 min. The amount of DOX was determined by HPLC analyses using a Waters HPLC system consisting of Waters 1525 binary pump, Waters 2475 fluorescence detector, 1500 column heater and a Symmetry C18 column. HPLC grade
acetonitrile-water (50:50, v/v) with pH 2.7 adjusted by HClO₄ was used as the mobile phase at 30 °C with a flow rate of 1.0 mL min⁻¹. Fluorescence detector was set at 460 nm for excitation and 570 nm for emission and linked to Breeze software for data analysis.

**Determination of DOX Release from Nanogels**

The release of DOX from nanogels was carried at different conditions. DOX-loaded micelles prepared above was diluted with phosphate buffered saline (PBS, 0.01 M, pH 7.4) to 2 mg mL⁻¹ and transferred to a 1 mL dialysis membrane tubing (Mw cutoff 14,000, Spectrum® Laboratories) with or without phosphodiesterase I (10 unit L⁻¹). It was immersed in a tube containing 15 mL of PBS in a shaking water bath at 37 °C. At predetermined time intervals, the external buffer was withdrawn and it was replaced with equal volume of PBS. The release medium was freeze-dried, dissolved in acetonitrile-water (50:50, v/v), and the concentration of DOX was analyzed by HPLC analyses as described above.

**Cell Internalization Studies**

HepG2 cells were cultured in 24-well plates (1×10⁵ cells per well) overnight, then the culture medium was replaced with fresh complete culture medium containing DOX-loaded nanogel, which were precooled to 4 °C. The final concentration of DOX cultured with cells was all at 20 µg mL⁻¹. In the competitive inhibition assay, lactobionic acid at a final concentration of 40 mM was added to the culture medium. The cells were cultured at 4 °C for 2 h, and then washed twice by 4 °C PBS (0.01 M, pH 7.4). Fresh complete culture medium at 37 °C was added and the cells were cultured for additional 4 h in a humidified atmosphere of 5% CO₂. The cells were finally trypsinized, washed with PBS twice, resuspended in 200 mL of PBS and subjected to flow cytometric analysis using a Becton Dickinson FACS Calibur flow cytometer. Cells without treatment were used as the control.

**Viability of HepG2 Cells Treated with DOX-Loaded Nanogels**

The cytotoxicity of DOX-loaded nanogels to HepG2 cells was evaluated in vitro by MTT assay. HepG2 cells were seeded in 96-well plates at 1×10⁴ cells per well in 100 µL of complete RPMI 1640 medium, and incubated at 37 °C overnight. The culture medium was then replaced with 100
μL of complete culture medium containing free DOX-loaded nanogels with an equivalent of DOX content (0.15 - 40 μg mL⁻¹). Cells were incubated at 37 °C for 48 h. In another experiment, cells were incubated at 4 °C with DOX-loaded nanogels for 2 h to allow binding, washed with PBS twice, replaced with fresh culture medium and further cultured at 37 °C for additional 48 h. At the end of the culture, MTT stock solution (25 μL, 5 mg mL⁻¹ in PBS) was added to cells to achieve a final concentration of 1 mg mL⁻¹. After incubation for an additional 2 h, 100 μL of the extraction buffer (20% sodium dodecylsulfonate in 50% N,N-dimethylformamide, pH 4.7, prepared at 37 °C) was added to the wells and incubated overnight at 37 °C. The absorbance of the solution was measured at 570 nm using a Bio-Rad 680 microplate reader and cell viability was normalized to that of HepG2 cells cultured in the culture medium without DOX. The solution containing 100 μL of complete RPMI 1640 medium, 25 μL of MTT stock solution and 100 μL of extraction buffer was used as the blank.

Figure S4. GPC curves of the polymers (a) PEG₃.₄k (b) PEG₆k (c) HO-PEEP₁₇₂-b-PEG₃.₄k-N₃ (d) HO-PEEP₁₅₀-b-PEG₆k-b-PEEP₁₅₀-OH. The polydispersities of Acr-PEEP₁₇₂-b-PEG₃.₄k-Lac and HO-PEEP₁₅₀-b-PEG₆k-b-PEEP₁₅₀-OH are 1.39 and 1.56, respectively.
Figure S5. Cell viability of HepG2 cells after incubation with blank nanogels at 1 or 2 mg mL\(^{-1}\) for 48 h. NG-LA: targeted nanogel; NG: non-targeted nanogel.