Supplementary Information

Integrating encapsulation and self-assembly-induced disulfide cross-linking: A universal strategy for preparing hydrophilic guest-loaded responsive hyperbranched polyglycerol nanogels

Hai Zeng,‡ Qingli Chen,‡ and Li Zhou*

Guangxi Key Laboratory of Optical and Electronic Materials and Devices, Guangxi Colleges and Universities Key Laboratory of Natural and Biomedical Polymer Materials, and College of Materials Science and Engineering, Guilin University of Technology, Guilin 541004, China

∗Corresponding author.

E-mail address: zhouli@glut.edu.cn (L. Zhou)

‡ These authors contributed equally to the work.

1. Materials

Glycidol (96%), lipoic acid (LA, 99%), N,N-dicyclohexylcarbodiimide (DCC, 99%), 4-dimethylaminopyridine (DMAP, ≥98%), dithiothreitol (DTT, 99%), hydrogen peroxide solution (H_2O_2, 35 wt.%), doxorubicin hydrochloride (DOX, 98%), Congo red (CR, 98%), methylene blue (MB, 98%), methyl orange (MO, 98%), tetramethyl azole blue (MTT), and acetone were purchased from Beijing Innochem Technology Co. Ltd. (Beijing, China). Hyperbranched polyglycerol (HPG) with a number average molecular weight (M_n) of 3200 Da and a polydispersity index (Đ) of 1.32 was synthesized using a ring-opening polymerization of glycerol according to literatures and our previous report.[1,2] All used water was ultrapure water (18.2 MΩ).

2. Characterization

Fourier Transform Infrared Spectroscopy (FTIR) was measured under the Thermo Nexus 470 FT-IR instrument using potassium bromide tablet method in the range of 4000-400 cm^{-1}. Nuclear magnetic resonance (^1H NMR) measurements were performed with DMSO-d_6 as solvent on a Varian Mercury Plus 500 MHz NMR instrument. The molecular weight of HPG-LA was measured by gel permeation chromatography (GPC, PE series 200), with 0.01 M LiBr/dimethylformamide (DMF) as the eluent at a flow rate of 1 mL/min. Elementary analysis (EA) of HPG-LA was performed on a Perkin-Elmer 240C Elemental Analytical Instrument. Dynamic light scattering (DLS) measurements were performed in aqueous solution using a Malvern Zetasizer Nano ZS90 apparatus. Scanning electron microscope (SEM) images were taken using a JSM-6380 LV microscope. Transmission electron microscopy (TEM) observation was carried out on a JEOL-2010 TEM with 150 kV accelerating voltage. UV-vis absorption spectra were determined by a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer. Cells were imaged using a confocal laser-scanning microscopy (CLSM, Olympus FV3000).

3. Synthesis of amphiphilic HPG-LA

The synthesis of amphiphilic HPG-LA involved condensation reaction between the carboxylic groups of LA and hydroxyl groups of HPG. To initiate the reaction, 370 mg of HPG, equivalent to 5 mmol of -OH, was dissolved in 10 mL of DMF in a dry flask, followed by addition of 1.03 g LA (5 mmol) and 1.23 g DCC (6 mmol). The mixture was stirred at room temperature for 15 min, and then, 612 mg DMAP (5 mmol) was

added, and the reaction was continued under argon gas protection for 24 h at room temperature. After the reaction was complete, the mixture was filtered, and the filtrate liquid was added to ether to precipitate the product. The precipitate was collected by centrifugation, dissolved in THF, and stored at -4 ^{o}C. It is worth noting that HPG-LA product may form insoluble gels at room temperature. The GPC results of HPG-LA indicated M_n = 9.1 kDa and Đ = 1.58. EA analysis of HPG-LA showed C 49.8 wt%, H 7.1 wt%, S 19.4 wt%, and O 23.7% wt% (calculated). Based on these results, it was calculated that the conversion of hydroxyl groups of HPG to disulfide bonds was 72%.

4. Encapsulation of hydrophilic guest by amphiphilic HPG-LA

We selected four hydrophilic guest molecules, including DOX, CR, MO and MB as representatives, and encapsulated them in amphiphilic HPG-LA by adding the hydrophilic guest molecules to a mixture of water and chloroform containing HPG-LA. The typical experimental procedure is as follows: firstly, 2 mL of an aqueous solution containing the hydrophilic guest (100 mg/L) and 2 mL of a chloroform solution containing HPG-LA (10 mg/mL) were added into a 5 mL glass vial. The mixture was then shaken at room temperature for 2 min and allowed to settle for 1 h, ensuring complete phase separation between the chloroform and water phases. The chloroform phase was collected for subsequent nanogels preparation. Simultaneously, the water phase solution was collected and the content of guest in the water phase was determined using a UV-vis spectrophotometer. The encapsulation efficiency (EE) of HPG-LA for the hydrophilic guest was calculated as follows

EE (%) = (weight of encapsulated guest (mg) / weight of used guest (mg)) × 100 (1).

The encapsulation capacity of HPG-LA for the hydrophilic guest was calculated as follows

Encapsulation capacity (mg/g) = weight of encapsulated guest (mg) / weight of HPG-NGs (g) (2).

5. Preparation of hydrophilic guest-loaded HPG nanogels (HPG NGs)

To create hydrophilic guest-loaded HPG NGs, the hydrophilic guest-encapsulated HPG-LA in chloroform obtained in the previous step underwent rotary evaporation to remove chloroform. The resulting substance was then dissolved in tetrahydrofuran (THF). The following is a typical process for synthesizing hydrophilic guest-loaded HPG NGs: 5 mg of DTT was added to a small beaker containing 2 mL of THF solution that contained hydrophilic guest-encapsulated HPG-LA (about 5 mg/mL). This mixture was stirred for 2 h at room temperature while avoiding exposure to light. The resulting mixture was gradually added to a beaker containing 5 mL of water and 5 mL of acetone while stirring. After stirring for 15 min, the mixture was illuminated using a 365 nm UV lamp (100 W) or 0.2 mL of H_2O_2 (35 wt%) was added while continually stirring for 45 min. The resulting mixture was then dialyzed against water for 24 h to obtain an aqueous dispersion of hydrophilic guest-loaded HPG NGs.

The loading efficiency (LE) of HPG NGs for the hydrophilic guest was calculated as follows

LE (%) = (weight of loaded guest (mg) / weight of used guest (mg)) × 100 (3).

The loading capacity of HPG NGs (mg/g) for the hydrophilic guest was calculated as follows

Loading capacity (mg/g) = weight of loaded guest (mg) / weight of HPG-NGs (g) (4).

6. Cytotoxicity evaluation

The cytotoxicity of HPG-DOX NGs, free DOX and pure HPG NGs was evaluated using the MTT assay. B16 murine melanoma cells were initially seeded in 96-well plates at a density of 1×10^4 cells/mL. After 24 h of incubation, the culture medium was replaced with an aqueous solution containing different concentrations of HPG-DOX NGs (free DOX or HPG NGs). For compassion, the concentration of DOX in HPG-DOX NGs is the same as that of free DOX molecules. The cells were then further incubated for 24 h. Following this, the wells were washed three times with PBS buffer. Subsequently, 100 μL of freshly prepared MTT solution (0.5 mg/mL) in culture medium was added to each well. After incubating for 3 h, the MTT medium solution was carefully removed. Dimethyl sulfoxide (100 μL) was added to each well, and the plate was gently shaken for 20 min. The absorbance of MTT at 570 nm was measured using a microplate reader. Cell viability was determined by comparing the absorbance of the cells incubated with HPG-DOX NGs (free DOX or HPG NGs) to that of cells incubated with only culture medium.

7. Cell imaging

Typically, B16 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium at 37 °C for 12 h in a 5% CO_2 environment. Following the removal of the growth medium, medium containing free DOX and HPG-DOX NGs was added to the B16 cells and incubated at 37 °C for 2 h or 24 h. The B16 cells were then rinsed with phosphate buffer to eliminate free HPG-DOX NGs (or free DOX), and subsequently washed twice with PBS buffer. Finally, imaging of B16 cells was carried

out using a confocal laser-scanning microscopy (CLSM, Olympus FV3000).

8. In vitro drug release

The in vitro release properties of HPG-DOX NGs were evaluated using the dynamic dialysis method. In a typical procedure, HPG-DOX NGs (10 mg in 10 mL) were placed in a cellulose dialysis bag with a molecular weight cut-off of 3500 Da. The dialysis bag was then immersed in 100 mL of the release medium, with pH value of 5.0 or 7.4, and placed on a thermostatic shaker at 100 rpm. The release of DOX was carried out at 37 °C and dark condition. At predetermined time intervals, samples were withdrawn from the release medium and replaced with an equal volume of fresh release medium. The amount of released DOX was quantified using a UV-vis spectrophotometer by measuring the absorbance at a wavelength of 480 nm and comparing it to a calibration curve.

Table S1. Elemental analysis and GPC results of HPG and HPG-LA samples.

Sample	f_{mole}[a]	C (wt%)	H (wt%)	S (wt%)	O (wt%)[b]	$M_{n,GPC}$ (kDa)	Đ	Con.[c] (%)
HPG	-	48.7	8.3	0	43.0	3.2	1.32	-
HPG-LA	1/1	49.8	7.1	19.4	23.7	9.1	1.58	72%

[a] Mole feed ratio of LA to the hydroxyl group of HPG.

[b] Calculated value.

[c] Conversion of hydroxyl groups of HPG to disulfide bonds.

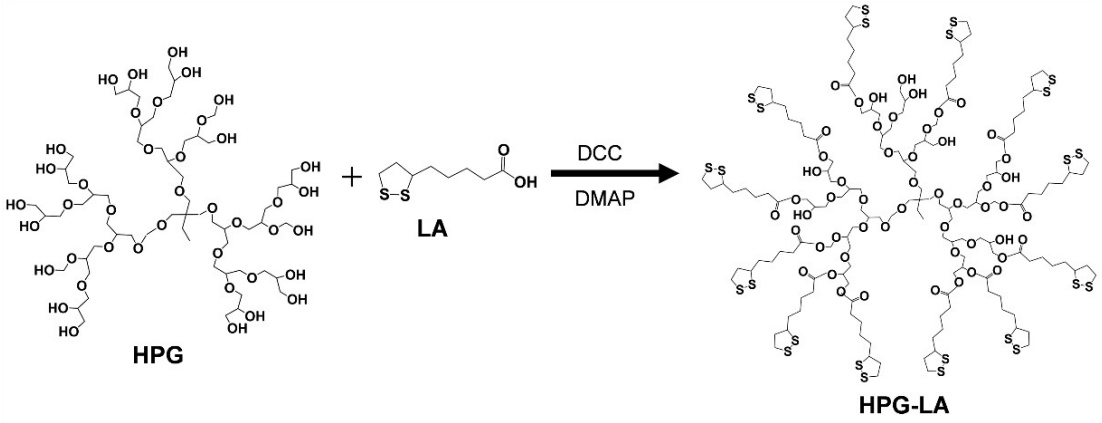

Fig. S1. Schematic illustration of the synthesis of HPG-LA.

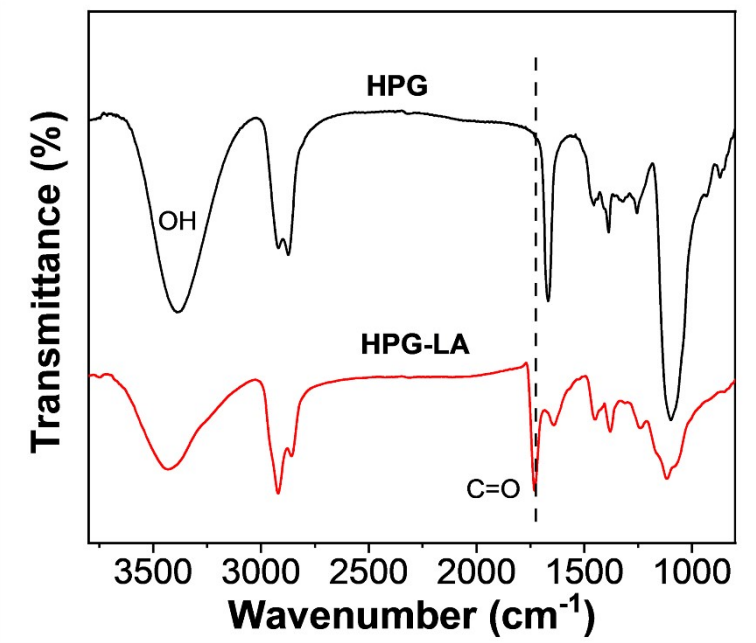

Fig. S2. FTIR spectra of HPG and HPG-LA.

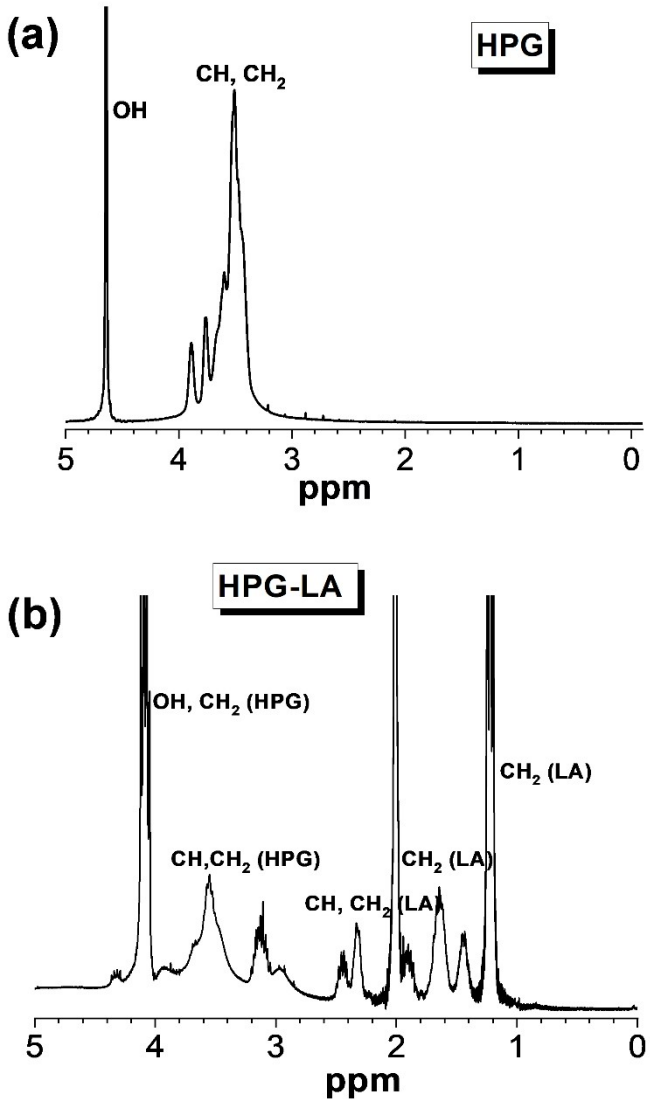

Fig. S3. ^{1}H NMR spectra of HPG (a) and HPG-LA (b).

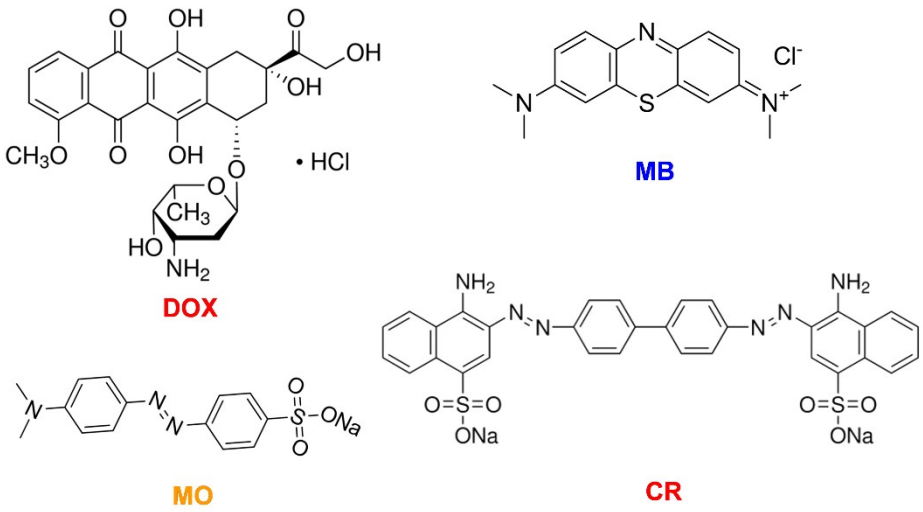

Fig. S4. The molecular structure of the hydrophilic guests used.

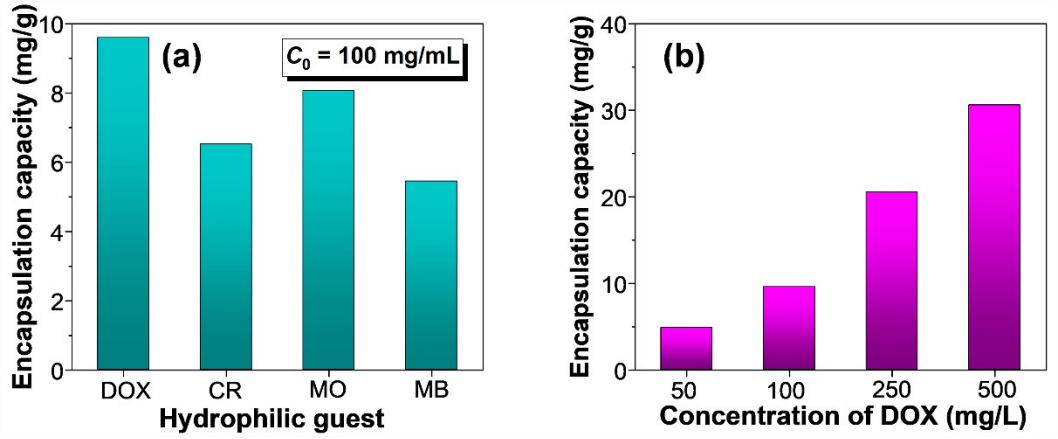

Fig. S5. (a) Encapsulation capacity of HPG-LA for diverse hydrophilic guests. The initial concentration of hydrophilic guest is 100 mg/mL. (b) Encapsulation capacity of HPG-LA for diverse concentrations of DOX.

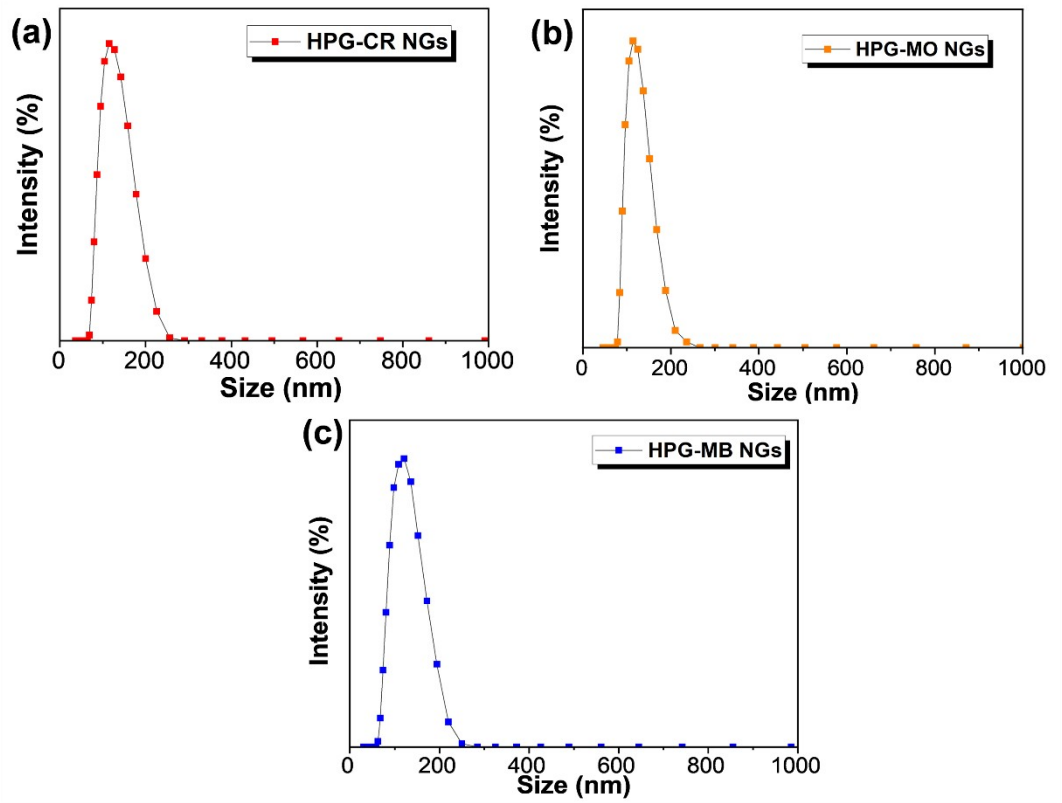

Fig. S6. DLS curves of HPG-CR NGs (a), HPG-MO NGs (a) and HPG-MB NGs