Electronic Supplementary Material (ESI) for Polymer Chemistry

Electronic Supplementary Information

Design and self-assembly of amphiphilic peptide dendron-jacketed polysaccharide polymers into available nanomaterials

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Contents

1 Materials and methods

1.1 Materials

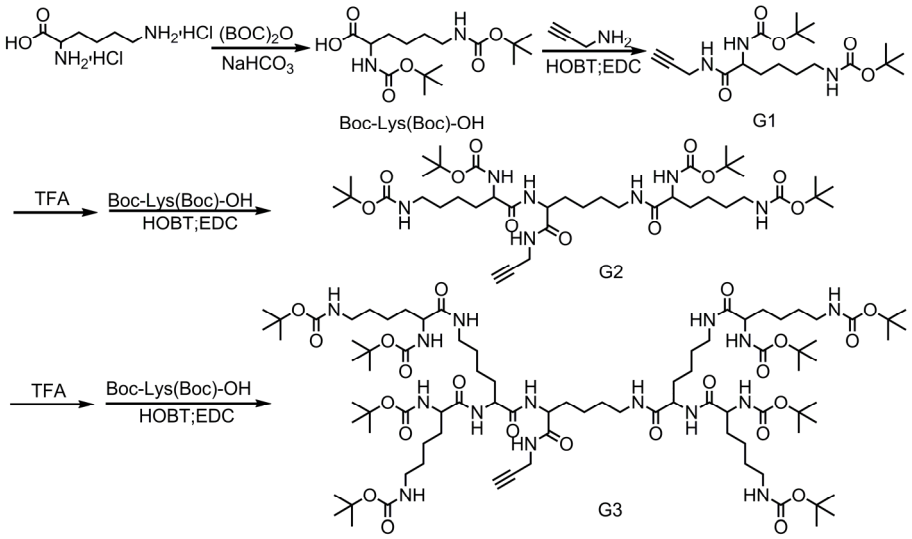
Lysine hydrochloride (H-Lys-OH·HCl), N-(3-dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC·HCl), trifluoroacetic acid (TFA), 1-hydroxybenzotriazole hydrate (HOBT) and N,N-diisopropylethylamine (DIPEA) were purchased from GL Biochem Ltd (Shanghai, China). Di-tert-butyl dicarbonate ($(Boc)_2O$), propargylamine, p-toluenesulfonyl chloride (PTSC), triethylamine (TEA), dioxane, ethyl acetate, n-hexane, dichloromethane, methanol, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), sodium azide (NaN_3), lithium chloride (LiCl), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), sodium ascorbate (SA), sodium bicarbonate ($NaHCO_3$), sodium bisulfate ($NaHSO_4$), sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) were purchased from Asta Tech Pharmaceutical (Chengdu, China). Dextran (MW 20,000) was purchased from Aladdin® Ltd. Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical. All the organic solvents used for reaction in this study were purified using the standard method and distilled before use.

1.2 Methods

^{1}H-NMR spectra were performed on a Bruker Avance II NMR spectrometer at 400 MHz with tetramethylsilane as the internal standard. The molecular weights of dendrons were tested on Autoflex MALDI-TOF-MS (Bruker, USA) with 2,5-dihydroxybenzoic acid (DHB) as matrix. FTIR spectra were recorded on a FTIR PE Spectrometer using the KBr pellet technique. Dynamic light scattering (DLS) experiments were performed on a Malvern Zetasizer Nano ZS at 25 °C. The absorbance of doxorubicin was detected by fluorescence spectrum. The morphologies of nanoparticles were observed on transmission electron microscopy (TEM) (JEM-100CX) and scanning electron microscopy (SEM) (JSM-5900LV). The cellular uptake was observed by confocal laser scanning microscopy (Leica TCP SP5) and flow cytometry (Cytomics FC500, Beckman Coulter).

2 Experimental Parts

2.1 Synthesis of poly(L-lysine) dendron



Scheme S1. Synthetic route of poly(L-lysine) dendron

Synthesis of Boc-Lys(Boc)-OH

Briefly, $NaHCO_3$ (38.32 g, 456.20 mmol) and lysine hydrochloride (10.00 g, 45.62 mmol) were dissolved in 600 mL deionzed water and cooled in an ice-water bath. Then, a solution of $(Boc)_2O$ (29.84 g, 136.86 mmol) in 100 mL dioxane was added dropwise with stirring. After 24 h at room temperature, the mixture was washed with diethyl ether for three times and the pH value was adjusted to 2~3 with dilute hydrochloric acid, then extracted with ethyl acetate for three times. The organic phase was dried with $MgSO_4$ for 12 h, concentrated and dried under vacuum. The product was obtained as white solid. Yield: 13.48 g (85%).

Synthesis of Generation 1 poly(L-lysine) dendron (G1)

Typically, the dendrons were synthesized by divergent approach from a propargyl core. For the first generation, propargylamine (0.50 g, 9.07 mmol), Boc-Lys(Boc)-OH (3.46 g, 9.98 mmol), EDC (1.91 g, 9.98 mmol), HOBT (1.35 g, 9.98 mmol) and 40 mL CH_2Cl_2 were added into a 100 mL flask

under N_2 atmosphere, then DIPEA (6.00 mL, 36.30 mmol) was added. The mixture was stirred in an ice-water bath for 30 min before it was warmed to room temperature and stirred for 24 h. The solution was respectively washed twice with saturated $NaHCO_3$, $NaHSO_4$ and NaCl solutions. The organic phase was dried, concentrated, and purified by column chromatography using ethyl acetate/n-hexane (4:1, v/v) as eluent. The product was obtained as white solid. Yield: 2.84 g (81%).

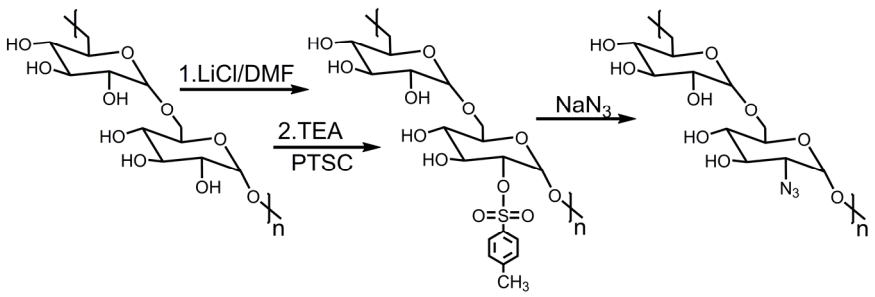
Synthesis of Generation 2 poly(L-lysine) dendron (G2)

First, G1 (1.50 g, 3.91 mmol) was treated with CH_2Cl_2 (6.00 mL) and TFA (6.00 mL) for 4 h to remove tert-butoxycarbonyl groups. Then, CH_2Cl_2 and TFA were removed under vacuum and the product was precipitated under stirring with anhydrous diethyl ether for 4 h. White powder was obtained after the removal of diethyl ether. Then, Boc-Lys(Boc)-OH (2.98 g, 8.60 mmol), HOBT (1.16 g, 8.60 mmol) and EDC (1.65 g, 8.60 mmol) were added together. CH_2Cl_2 (40 mL) and DIPEA (5.20 mL, 31.29 mmol) were added under N_2 atmosphere. The reaction was stirred in an ice-water bath for 30 min and at room temperature for 24 h, and underwent the same purification process as G1. The product was obtained as white solid. Yield: 2.64 g (82%).

Synthesis of Generation 3 poly(L-lysine) dendron (G3)

G3 was synthetized from G2. The synthesis and purification of G3 were similar to G2, using CH_2Cl_2/MeOH (10:1, v/v) as eluent. The product was obtained as white solid. Yield: 2.35 g (75%).

2.2 Synthesis of azido-functionalized dextran



Scheme S2. Synthetic route of azido-functionalized dextran

Considering both the steric hindrance of hydrophobic dendron and the activity sequence of

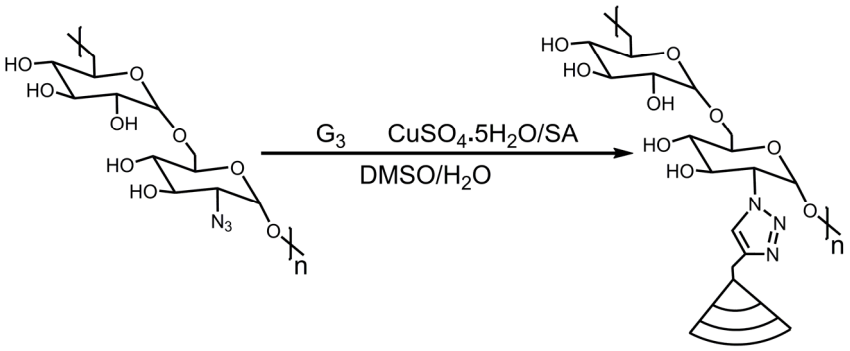
hydroxyl groups in dextran, it's difficult and unnecessary to graft more dendrons on each anhydroglucose unit of dentran. Therefore, the typical procedure employed for the synthesis of Dextran-Tos in this study was as follows. Dextran (5.00 g, 30.86 mmol) was added to 100 mL DMF and kept at 130 °C for 1 h. When the solvent cool to 100 °C, anhydrous LiCl (2.00 g, 20 g/L) dried at 150 °C for 2 h was added and stirred at room temperature to make dextran completely dissolved. After standing overnight, TEA (9.0 mL, 61.73 mmol, 2 mol per mol tosyl chloride) and *p*-toluenesulfonyl chloride (5.88 g, 30.86 mmol, 1 mol per mol anhydroglucose unit) were dissolved in 20 mL DMF respectively and added to the reaction system in turn. The mixture was stirred at 10 °C for 24 h. After the removal of DMF under vacuum, the polymer was precipitated in acetone, filtered off, washed with isopropanol, acetone for several times, and dialyzed (MWCO=2000) for 12 h. The collected product was dried by freeze-dry, getting white solid. Yield: 3.72 g (66%).

The degree of substitution (DS) of tosyl (defined as the number of tosyl groups per one hundred anhydroglucose units) was evaluated from ^{1}H-NMR spectra by calculating the ratio of peak areas of the tosyl peak at 7.4 ppm relative to the anomeric proton of dextran at 4.7 ppm.[1] According the equation, in which A refers to the integral area, the DS of tosyl groups was 12.5%.

$$\text{DS (Dextran-Tos)} = \frac{\text{A[toysl - H @ 7.4 ppm]/2}}{\text{A[anomeric - H @ 4.7 ppm]}} \times 100\% \qquad \text{equation (1)}$$

To a solution of tosyl-functionalized dextran (3.00 g, 16.61 mmol) in 50 mL DMF, NaN_3 (3.24 g, 49.83 mmol, 3 mol per tosyl group) was added. The mixture was heated to 100 °C and reacted for 48 h. The purification process was the same as tosyl-functionalized dextran, getting white solid (2.2 g, yield: 80%). According to the ratio of integral area, the DS of tosyl nearly reduced to zero, indicating almost complete transformation of toysl to azide.

2.3 Synthesis of peptide dendron-jacketed dextran



Scheme S3. Synthetic route of peptide dendron-jacketed dextran

The synthesis of peptide dendron-jacketed dextran was accomplished via the "click" reaction of azide groups with excess propynyl groups. Azido-functionalized dextran (0.40 g, 2.42 mmol) and G3 (1.73 g, 1.00 mmol) were dissolved in DMSO under nitrogen protection. Sodium ascorbate (SA) (79.24 mg, 0.4 mmol) and $CuSO_4 \cdot 5H_2O$ (50.00 mg, 0.2 mmol) were dissolved in deionized water separately and added into the flask in sequence ($DMSO/H_2O$=3:1, v/v). The mixture was stirred for 48 h at 45 °C. Then it was precipitated with acetonitrile and washed with acetone for several times. Finally, the product was dialyzed (MWCO=2000) for two days to get rid of inorganic ions completely. The product was obtained by freeze-dry. Yield: 0.60 g (66%).

The degree of substitution (DS) of dendron (defined as the number of dendron groups per one hundred anhydroglucose units) was evaluated from ^{1}H-NMR spectra by calculating the ratio of peak areas of ε protons in lysine at 2.8-3.0 ppm relative to the anomeric proton in dextran at 4.7 ppm.[2] According the equation, in which A refers to the integral area, the DS of dendron groups was 12.4%.

$$\text{DS (Dextran-dendron)} = \frac{A[\varepsilon\text{-H @ 2.8-3.0 ppm}]/14}{A[\text{anomeric-H @ 4.7 ppm}]} \times 100\% \qquad \text{equation (2)}$$

2.4 Critical aggregation concentration (CAC)

The self-aggregation behavior of peptide dendron-jacketed dextran was investigated using pyrene as a fluorescence probe. The concentration of peptide dendron-jacketed dextran varied from 0.00001 to 125 μg/mL. The CAC (9.70 μg/mL) was estimated as the cross-point when extrapolating the intensity ratio I_{338}/I_{334} at low and high concentration regions. The two linear equations were: y=0.006x +0.887;

y=0.081x+0.813.

2.5 In vitro drug release

Hdrophobic doxorubicin (DOX) was obtained from doxorubicin hydrochloride (DOX.HCl) with excess TEA. TEA (3× DOX·HCl) was added into aqueous solution of DOX.HCl (2.5 mg/mL) stirring overnight. The DOX was got by centrifugation and freeze-dried into powder.

With ultrasonic method, DOX-loaded PDDNs were prepared by dropwise addition of DMSO solution of peptide dendron-jacketed dextran and doxorubicin by a weight ratio of 4:1 followed by extensive dialysis against deionzed water for 24 h. After removal of insoluble and free DOX, the DOX-loaded PDDNs was obtained by freeze-dry.

The amount of DOX was determined using fluorescence measurement (excitation at 485 nm and emission at 585 nm). DOX-loaded PDDNs were dissolved in DMSO and analyzed with fluorescence spectroscopy. The DOX loading was calculated from a calibration curve of DOX absorbance created by measuring the absorbance of different DOX concentrations. The release of DOX from the DOX-loaded PDDNs at different pH (pH 5.0, 6.8, 7.4) was studied using a dialysis method. Freeze-dried DOX-loaded PDDNs at a concentration of 1mg/mL were poured into a dialysis bag (MWCO=2000). The dialysis bags were suspended in 25 mL of phosphate buffer solution (PBS) at 37.0 ± 0.5° C in a shaking bed maintained at 120 rpm. The samples (1 mL) were withdrawn at designated intervals up to 24 h and replaced with an equal quantity of fresh corresponding medium to maintain the sink condition. Sample analysis was carried on fluorescence detector at 485 nm to determine the amount of released doxorubicin. The drug loading content (10.76%) was calculated using the following equation,[3] in which the “weight of DOX-loaded nanoparticles” refers to the total weight. (DOX + dendronized denxran)

$$\text{DOX loading content} = \frac{\text{weight of DOX encapsulated in nanoparticles}}{\text{weight of DOX - loaded nanoparticles}} \times 100\% \qquad \text{equation (3)}$$

2.6 In vitro cytotoxicity and cellular uptake

The cytotoxicity of the DOX-loaded PDDNs to tumor cells was measured by CCK-8 assay using

free DOX as a control to evaluate in vitro antitumor activity. HepG2 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 8×10^3 cells/well and incubated in 100 μL DMEM for 24 h. The medium was then replaced by DMEM containing the DOX-loaded PDDNs or free DOX at various DOX concentrations from 0.001 μg/mL to 100 μg/mL. After 24 h incubation, the cells were washed with PBS (pH=7.4). 10μL CCK-8 solution in culture media was added and the plate was incubated for another 2 h at 37 °C. Finally, the absorbance of each well was measured at 450 nm by a microplate reader. Besides, the IC_{50} values of DOX and DOX-loaded PDDNs and cytotoxicity of blank nanoparticles to NIH/3T3 and HepG2 cells were investigated in the same way.

The cellular uptake was followed with confocal laser scanning microscope (CLSM) and flow cytometry using HepG2 cells. For CLSM observations, HepG2 cells were seeded on 35 mm diameter coverslips at a density of 1×10^4/well. After 24 h adherence, DOX and DOX-loaded PDDNs (DOX concentration: 10 μg/mL) in culture media were added. After incubating for 1 h, the culture media were removed and the cells were rinsed two times with PBS (pH=7.4) before the observation. DOX was excited at 485 nm with emissions at 585 nm. For flow cytometry observations, HepG2 cells were seeded into 6-well plates with a density of 2×10^5 cells/well. DOX and DOX-loaded PDDNs (DOX concentration: 10 μg/mL) in culture media were added into each well. After 24 h incubation, cells were washed with PBS and collected, centrifuged at 1000 rpm for 3 min and washed with PBS for another time. The cells were resuspended with 0.5 mL PBS and tested on flow cytometry.

3 Characterization

3.1 ^{1}H-NMR spectra of compounds

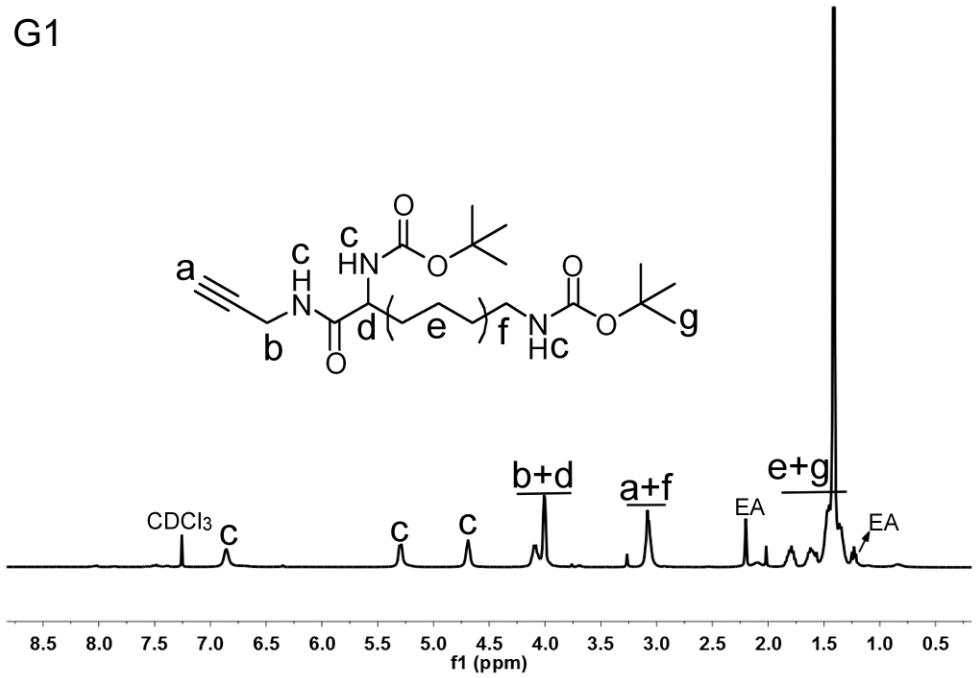


Figure S1. ^{1}H-NMR spectrum of G1 dendron in $CDCl_3$

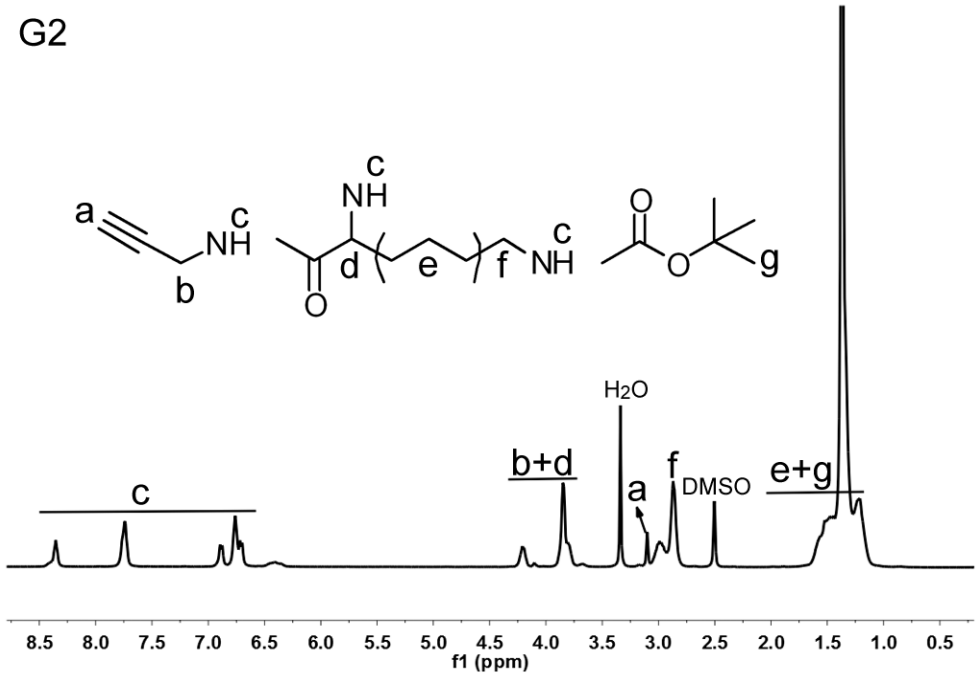


Figure S2. ^{1}H-NMR spectrum of G2 dendron in DMSO-d_6.

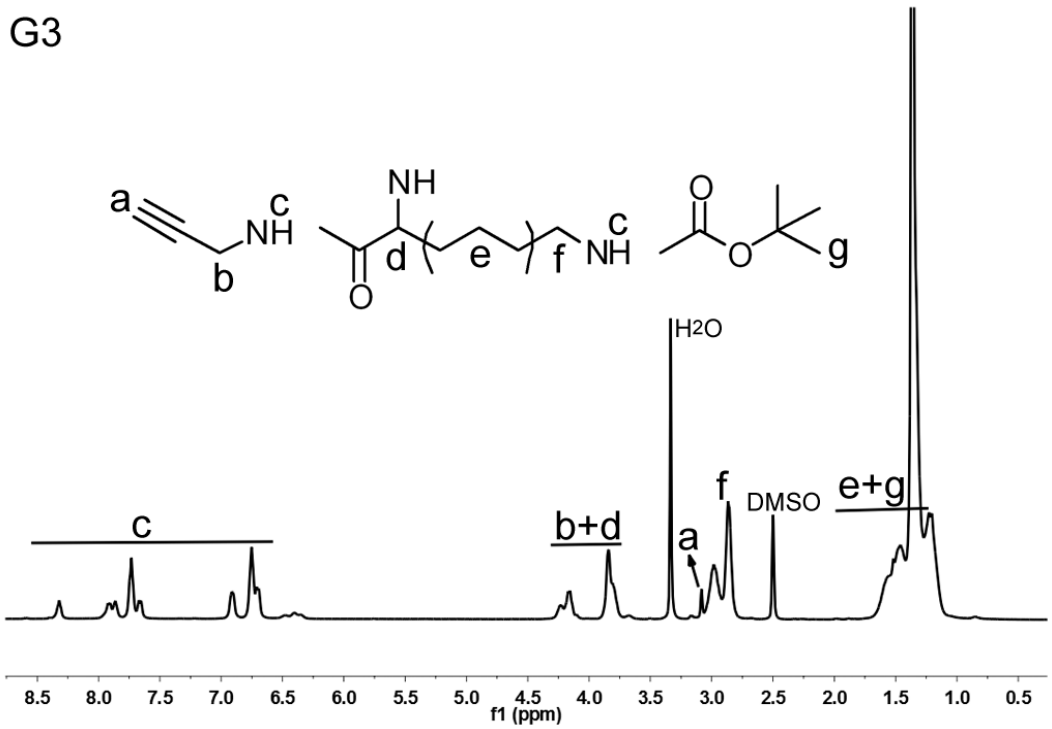


Figure S3. ^{1}H-NMR spectrum of G3 dendron in DMSO-d_6.

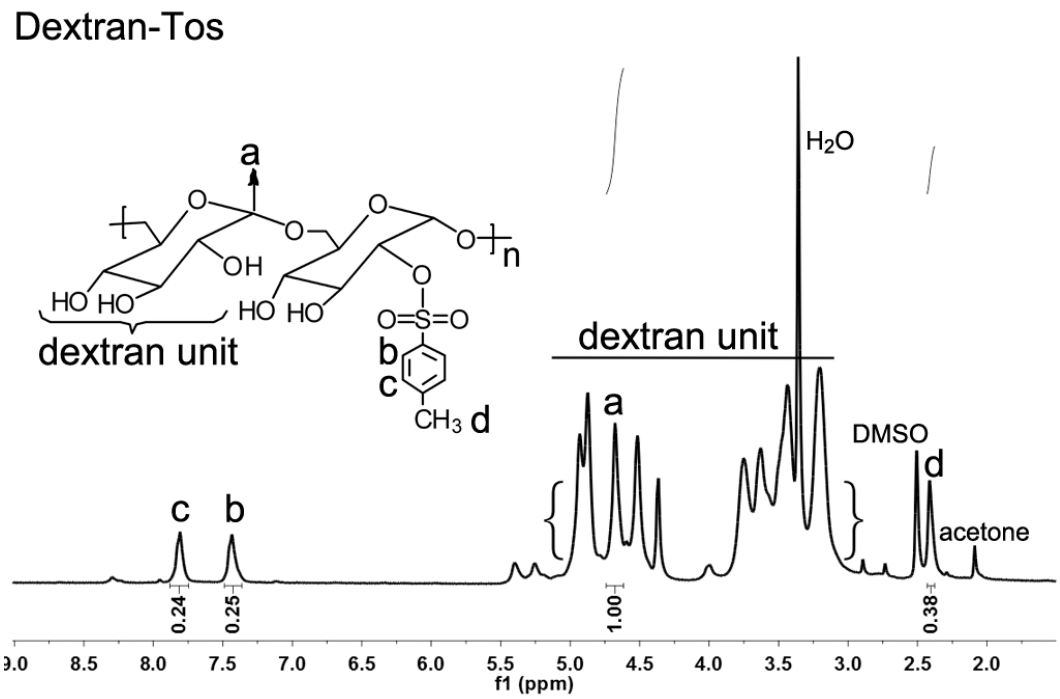


Figure S4. ^{1}H-NMR spectrum of tosyl dextran in DMSO-d_6.

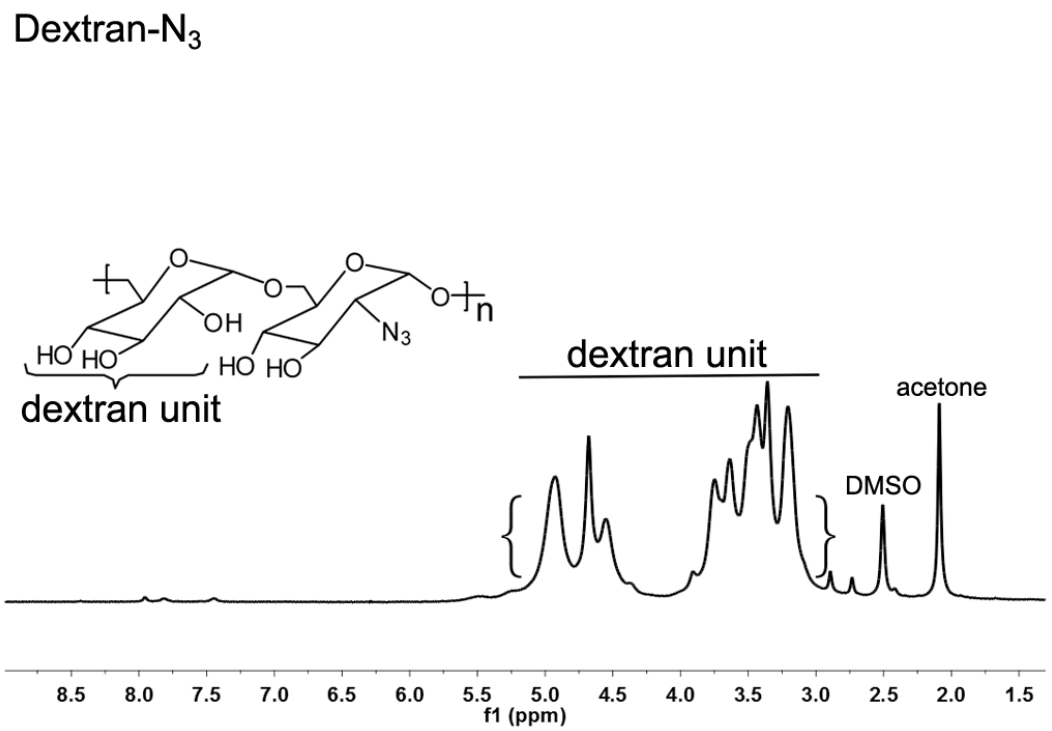


Figure S5. ^{1}H-NMR spectrum of azide dextran in DMSO-d_6.

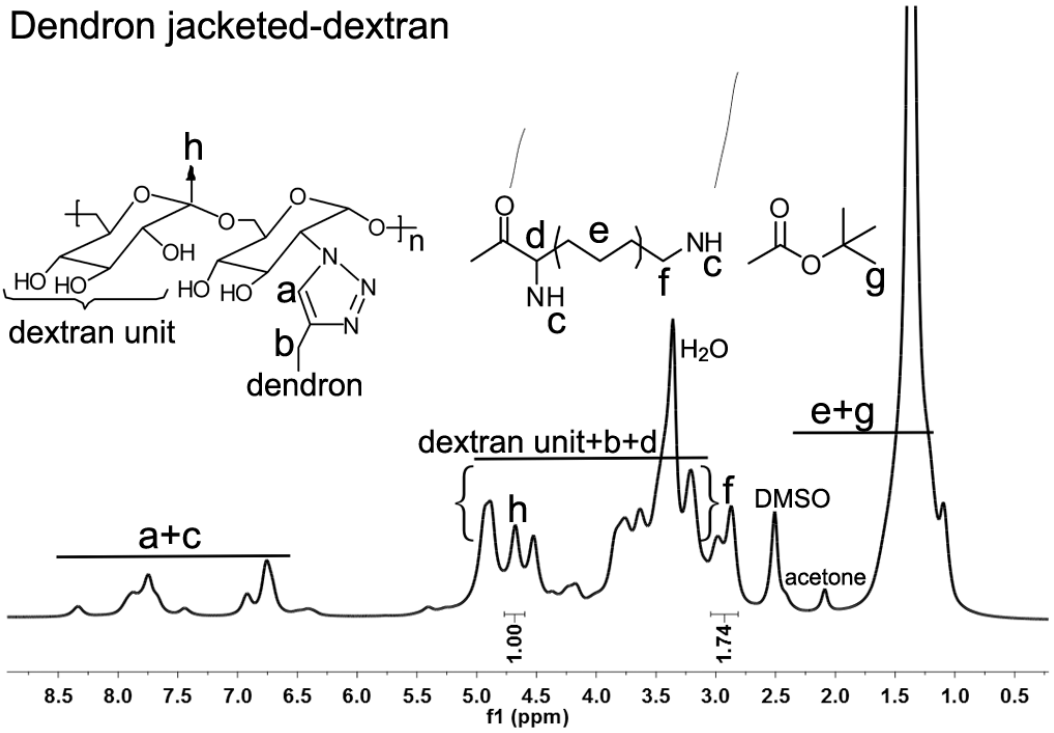


Figure S6. ^{1}H-NMR spectrum of dendron jacketed-dextran in DMSO-d_6.

3.2 Mass spectra of peptide dendron

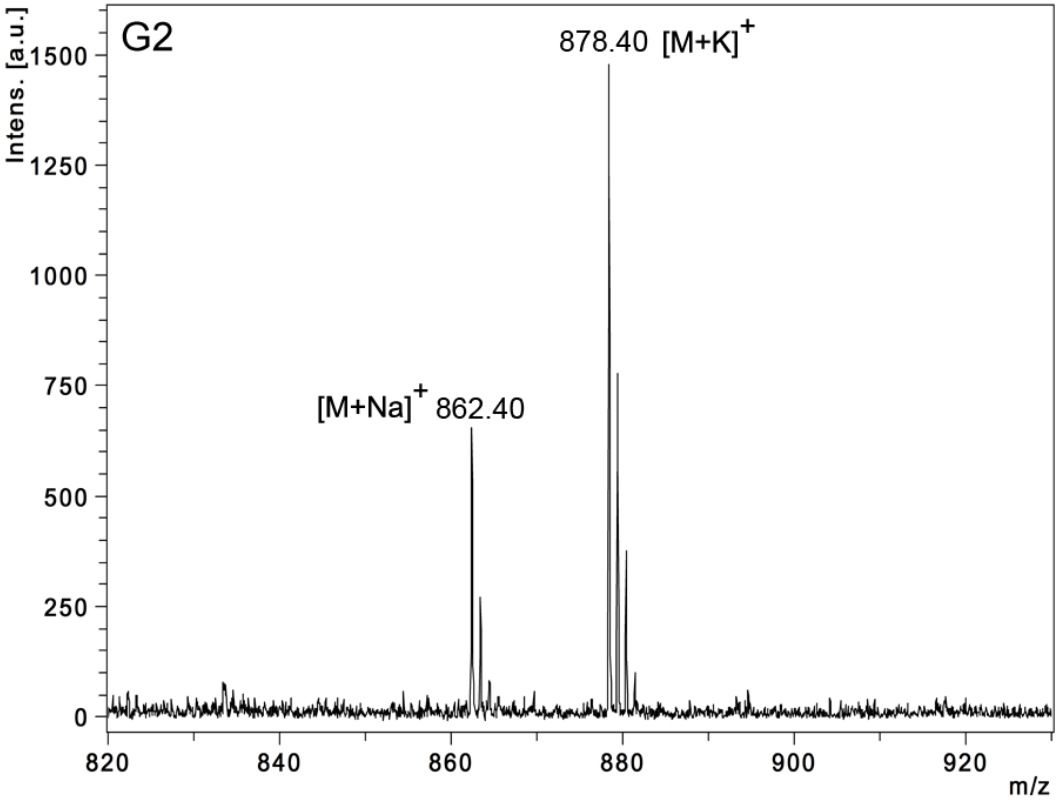


Figure S7. MALDI-TOF-MS spectrum of G2 dendron.

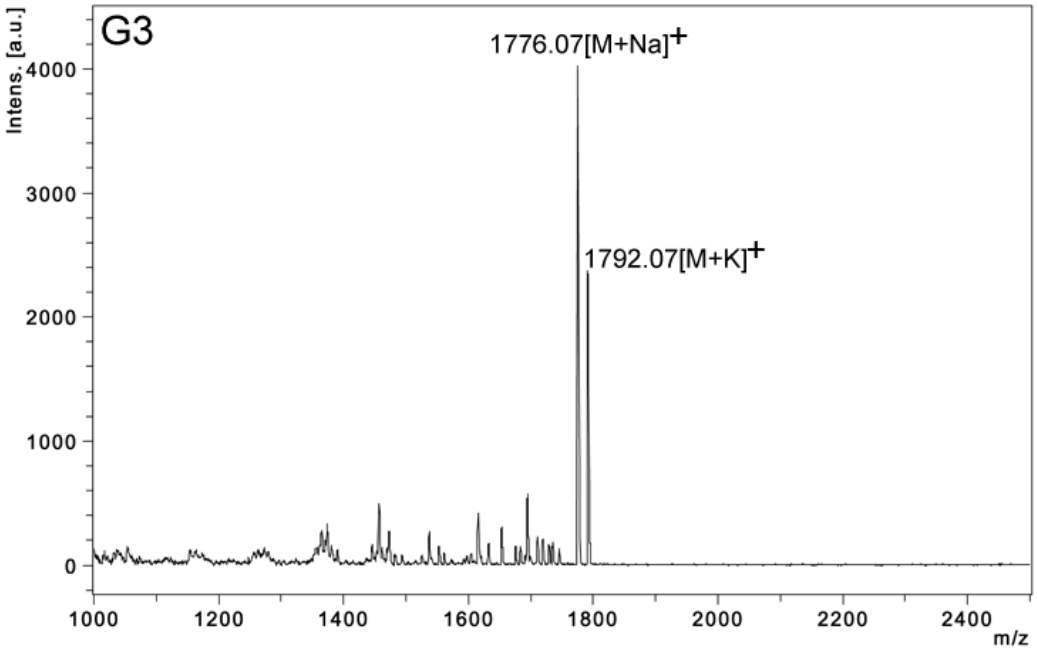


Figure S8. MALDI-TOF-MS spectrum of G3 dendron.

3.3 Morphology and size distribution of DOX-loaded PDDNs

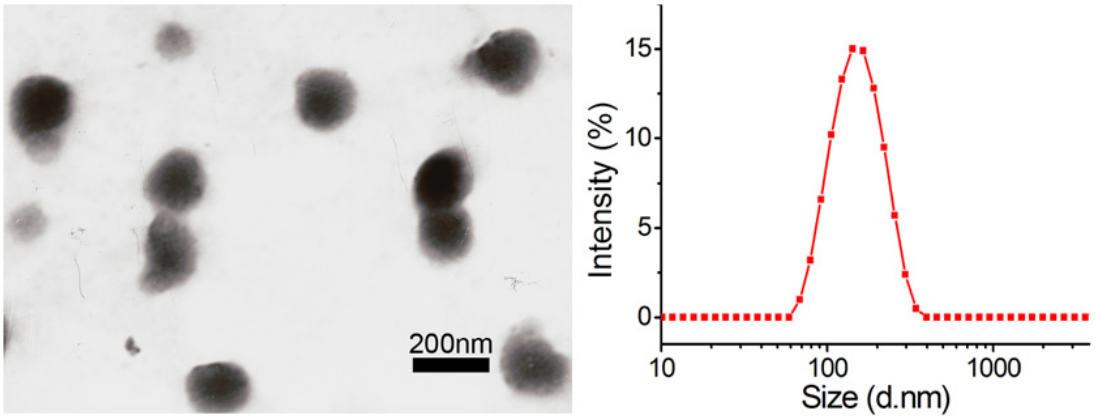


Figure S9. The morphology and size distribution of DOX-loaded PDDNs.

3.4 Cytotoxicity of DOX and DOX-loaded PDDNs

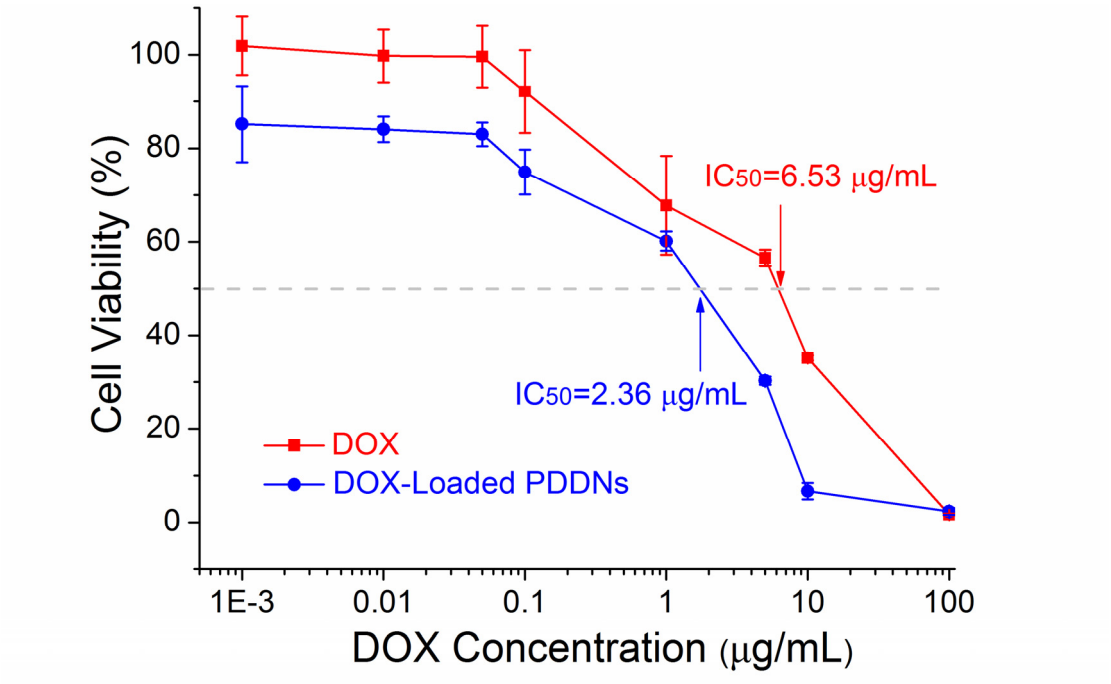


Figure S10. The IC_{50} of DOX-loaded PDDNs and free DOX to HepG2 cells at 37 °C for 24 h.

3.5 Confocal laser scanning microscopy (CLSM) images

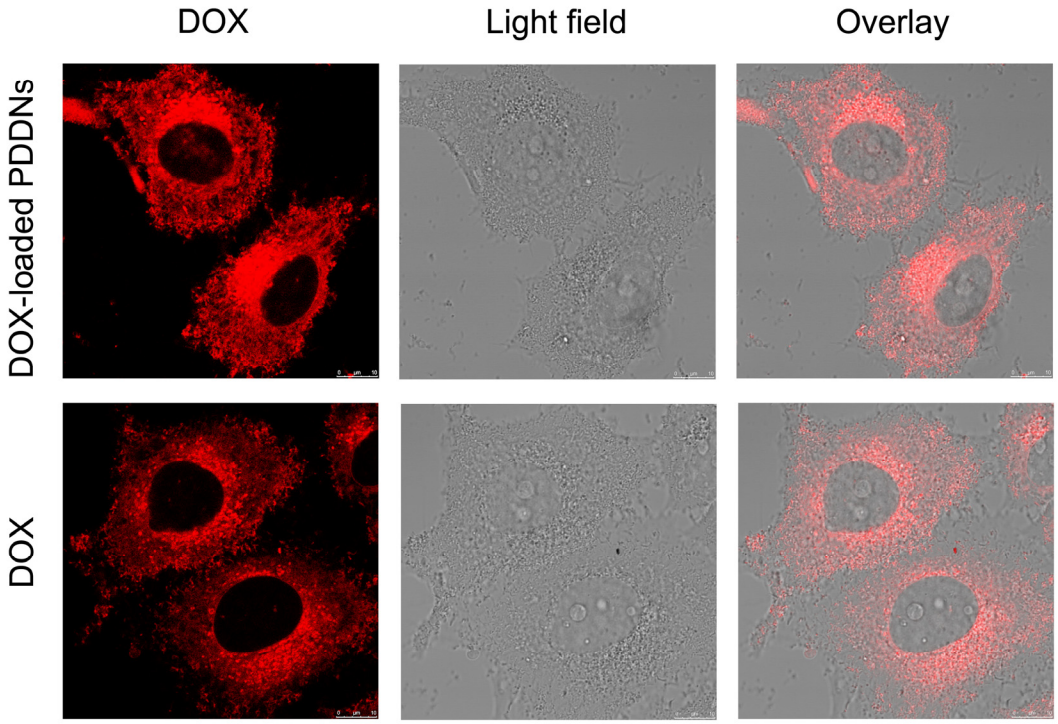


Figure S11. CLSM images for cellular uptake of HepG2 cells incubated with DOX-loaded PDDNs and DOX for 1 h, including DOX channel, and overlay of previous images. The scale bars correspond to 10 μm.

References

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