Supplementary information

Preparation and biological characterization of pH-responsive PASP-g-PEG-DDA-Hyd-ADR

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Experimental

Materials

L-ASP (L-aspartic acid), Adriamycin (ADR) and α-methoxy-ω-amino-poly(ethylene glycol) (Mn=2000) (MeO-PEG_{2000}-NH_2) was obtained from the Adrich. 85% phosphoric acid, dodecylamine (DDA), and hydrazine hydrate (80%) (Beijing Chemical works) were commercially available and used without further purification. N,N-dimethyl-formamide (DMF), dimethylsulfoxide (DMSO) were purchased from Beijing Chemical works (China) with further purification. A human liver cancer HepG2 cells was obtained from the second hospital of Jilin University (Changchun, China). 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Beijing CellChip Biotechnology Co. Ltd.

Characterization method

The molecular weights and polydispersity of the polymers (Mw/Mn; Mw=weight-average molecular weight; Mn=number-average molecular weight) were recorded on a gel permeation chromatograph (GPC) equipped with two Mixed-B columns (pore size=10 μm; column size=300 × 7.5 mm) and refractive index detector (Perkin-ElmerSeries 200) using DMF (0.01 mol/L LiBr) as the eluent at 30 ℃ with a flow rate of 1 mL/min. The column system was calibrated by a set of mono-dispersed standard polystyrenes. ^{1}H-NMR spectra were obtained on a 500 Bruker NMR instrument using $CDCl_3$, DMSO-d_6 as the solvent and TMS as a reference standard for chemical shifts.

Hydrodynamic diameters (HD) and size distribution of polymer micelles and conjugate micelles were determined by dynamic light scattering (DLS) using a Brookheaven BI9000AT system Brookheaven Instruments Corporation, USA, with reproducibility verified by collection and comparison of sequential measurements. Shall model was chosen as the test mode. Measurements were performed at a 90° scattering angle at 25 °C. Each sample was measured in triplicate with 7 runs in each measurement and 90 s duration in each run. AFM imaging was used to show the size and the distribution of conjugate micelles. (Digital Instrument, Nanoscope IIIa, Multimode). All the tapping-mode images were taken at room temperature in air with the microfabricated rectangle crystal silicon cantilevers (nanosensor). The topography images were obtained at a resonance frequency of 365 kHz for the probe oscillation. Ultraviolet–visible absorption spectra of probe were measured by a Varian Cary 5000 UV-vis-NIR spectrophotometer. MTT assay was measured by BioTek Elx 800 at a wavelength of 490 nm. CLSM was imaging with Olympus FV1000.

Synthesis of amphiphilic centipede-like polymer-drug conjugate

Synthesis of poly(succinimide) (PSI).
PSI was synthesized according to the method previously reported[30]: Phosphoric acid (85%, 1.0 mL) was dropped into L-ASP (20 g, 150 mmol) and stirred at 180 ℃ under nitrogen atmosphere. Water generated in condensation was continuously removed by Dean-Stark trap. After 4 h, the product was dissolved in DMF. Then the reaction mixture was precipitated in hot water and washed with water until pH of suspension became neutral. After centrifuging and vacuum drying, solids of PSI (23 reapeating units) was obtained, with Mn(GPC)= 2.2×10^3 g/mol and Mn/Mw=1.21. The ^{1}H-NMR (DMSO-d_6, δ/ppm) spectra contained the following peaks: 2.6-3.5 (-C**H**$_2$-CO-), 5.3 (-C**H**-CO-) in Figure 1.

Synthesis of poly(succinimide)-grafted-p(ethylene glycol) (PSI-g-PEG)
PSI (0.5 g, 0.23 mmol) was dissolved in DMF (10 mL), and MeO-PEG_{2000}-NH_2 (3.09 g, 1.55 mmol) was added to the DMF solution. The reaction mixture was stirred at 60 ℃ for 48 h under nitrogen atomosphere. Then the reaction mixture was precipitated in ether. The precipitate was dialyzed against deionized water for 5 days to remove the unreacted PEG (dialysis tube Mw cut-off 8000 Da) and freeze-drized. The product was obtained at 90% yield. with Mn(GPC)=1.58×10^4 g/mol and Mw/Mn=1.34. ^{1}H-NMR (DMSO-d_6, δ/ppm) spectra contained the following peaks: 8.0 (-CO-N**H**-), 3.60 (-O-C**H**$_2$-C**H**$_2$-), 4.6 (-C**H**-CO-NH-), 5.3 (-N-CO-C**H**-), 3.3 (-C**H**$_2$-CO-N-) in Fig. 2(A).

Synthesis of poly(succinimide)-grafted-p(ethylene glycol)-dodecylamine (PSI-g-PEG-DDA)
PSI-g-PEG (500 mg , 0.032 mmol) was dissolved in DMF (5 mL), and DDA (39.8 mg, 0.22 mmol) was added to

the DMF solution. The reaction mixture was stirred at 110 ℃ for 24 h under nitrogen atomosphere. Then the reaction mixture was precipitated in ether. The precipitate was dialyzed against deionized water for 2 days (dialysis tube Mw cut-off 8000 Da) and freeze-dried. The product PSI-g-PEG-DDA (PEG and DDA with 30% and 30% mol substitution with respect to aspartate units, respectively) was obtained at 90% yield. with Mn(GPC)=1.72 $\times 10^4$ g/mol and Mw/Mn=1.42. ^{1}H-NMR (DMSO-d6, δ/ppm) spectra contained the following peaks: 8.0 (-CO-N**H**-), 3.60 (-O-C**H**$_2$-C**H**$_2$-), 4.6 (-C**H**-CO-NH-), 5.3 (-N-CO-C**H**-), 3.3 (-C**H**$_2$-CO-N-) 0.88 (-(CH$_2$)$_{11}$-C**H**$_3$), 1.25 (-(C**H**$_2$)$_{11}$-CH$_3$) in Fig. 2(B). Similary, PSI-g-PEG-DDA (DDA with 20% or 40% mol substitution with respect to aspartate units) were obtained by adjusting the reaction amount of DDA. The yeild were 82% and 89% respectively. GPC results showed the PSI-g-PEG-DDA (DDA 20%) with Mn=1.66$\times 10^4$ g/mol (Mw/Mn=1.31) and PSI-g-PEG-DDA (DDA 40%) with Mn=1.80$\times 10^4$ g/mol (Mw/Mn=1.39).

Preparation and characterizaition of the polymer (PSI-g-PEG-DDA) micelles

PSI-g-PEG-DDA (DDA being 20%, 30% and 40% mol substitution with respect to aspartate units) (50 mg) was dissolved in 4 mL CHCl$_3$, deionized water was added drop by drop to the chloroform solution under ultrasonic wave. After 2 h, chloroform was gradually removed by rotary evaporation. The micelles formed by PSI-g-PEG-DDA with different grafting rates of DDA were successively prepared. The hydrodynamic diameters (HD) and size distribution were analysed by DLS, which can be refer to Fig. 3(B).

Synthesis of amphiphilic centipede-like polymer-drug conjugate

Synthesis of poly(succinimide)-grafted-p(ethylene glycol)-dodecylamine-hydrazide (PASP-PEG-DDA-Hyd)
PSI-g-PEG-DDA (DDA with 30% mol substitution with respect to aspartate units) (200 mg, 0.012 mmol, 0.108 mmol succinimide units) was dissolved in DMSO (5 mL). Excess hydrazine hydrate (250 μL, 5.088 mmol)was added to the DMSO solution above. The reaction mixture was stirred at 60 ℃ for 24 h under argon atomosphere. After the reaction, the solution was transferred into a dialysis tube (Mw cut-off 3500 Da) and dialyzed against 0.025% NH$_3$ aqueous solution and deionized water 3 times respectively, and freeze-dried. The yeild of product was 85%. The ^{1}H-NMR (DMSO-d$_6$, δ/ppm) spectra contained the following peaks: 2.3 (-N-CH-C**H**$_2$), 4.7 (-N-C**H**-CH$_2$), 3.60 (-O-C**H**$_2$-C**H**$_2$-), 0.88 (-(CH$_2$)$_{11}$-C**H**$_3$), 1.25 (-(C**H**$_2$)$_{11}$-CH$_3$), 8.0 (-CO-N**H**-NH$_2$), 10.2 (-CO-NH-N**H**$_2$) in Fig 2(C).
Synthesis of poly(succinimide)-grafted-p(ethylene glycol)-dodecylamine-hydrazone-adriamycin (PASP-PEG-DDA-Hyd-ADR)
PASP-g-PEG-DDA-Hyd (50 mg, 0.027 mmol) was dissolved in DMSO solution (5 mL). 5 mg/mL ADR solution of dry DMSO was added to the solution above under argon atomosphere. The mixture was allowed to react at 40 ℃ for 48 h. After the reaction, the solution was transferred into a dialysis tube (Mw cut-off of 3500 Da) and dialyzed against DMF for 2 days (the DMF was changed every 4 h) to remove the excess amount of unbounded ADR molecules, and then dialyzed against water for 2 days (the water changed every 8 h) to remove DMF. Final products were collected as freeze-dried. The yeild was 91%. The ^{1}H-NMR (DMSO-d$_6$, δ/ppm) spectra contained the following peaks: 2.3 (-N-CH-C**H**$_2$), 4.6 (-N-C**H**-CH$_2$), 3.60 (-O-C**H**$_2$-C**H**$_2$-), 0.88 (-(CH$_2$)$_{11}$-C**H**$_3$), 1.25 (-(C**H**$_2$)$_{11}$-CH$_3$), 8.0 (-CO-N**H**-) 1.23 (C**H**$_3$-CH of ADR sugar ring) in Fig 2(D).

Preparation of the polymer-drug micelles

PASP-g-PEG-DDA-Hyd-ADR (50 mg, 5.52 mg of ADR) was dissolved in 4 mL CHCl$_3$, deionized water was added drop by drop to the chloroform solution under ultrasonic wave. Chloroform was then gradually removed by rotary evaporation. The polymeric micelles were successively prepared. The drug content was 1104 μg/mL. The other micelles solutions with certain ADR concentration was diluted from the solution above.

Stability of the polymer-drug micelles

To evaluate the stability of the polymer micelles here, the micelles (5 mL, 5 mg/mL) were incubated in PBS buffer (10 mL, 5×) at room temperature with measuring the hydrodynamic diameters at a definite time interval.

In vitro drug release

5 mL polymer-drug micelles (5 mg/mL) were transferred into respective dialysis bags (Mw cut-off 3500 Da) and into 45 ml of buffer solution as the pH value decreases from pH 7.4 to 4.5. Aliquots (2 mL) of the buffer solutions were taken at selected time intervals and monitored by UV-Vis spectrometry at 488 nm to determine the rate of drug release. 2 mL fresh buffer was added after each sampling to keep the total volume of buffer solutions constant. In the assessement of drug release, the cumulative amount of released drug was calculated, and the percentages of drug released were plotted against time.

In vitro cytotoxic activity assay

Cytotoxicities of conjugate micelles and free ADR against HepG2 cells were measured by MTT assay. Experiments were conducted in triplicate. The cells were plated in 96-well plates (2$\times 10^4$ cells/well), maintained in 100 μL RPMI 1640 medium supplemented with 10% FBS and incubated for 24 h at 37 ℃ in a humidified

atmosphere with 5% CO_2. After preincubation, the conjugate and free ADR with pre-defined concentrations (ADR concentrations of medium ranged from 0.01 μg/mL to 100 μg/mL) were added into the cell culture medium. After 4 h or 24 h of incubation, 20 μL of MTT (5 mg/mL in PBS) was added to each well and cells were incubated at 37 ℃. After an incubation period of 4 h, the medium was removed and the blue formazan crystals formed inside the cells were dissolved in 100 μL of DMSO. The absorbance was measured in a BioTek Elx 800 at a wavelength of 490 nm.

CLSM imaging

Observations using confocal laser scanning microscopy (CLSM) reveal the intracellular localization of micelles that were incubated with human liver cancer cell HepG2. HepG2 cells were incubated for 2 h in 6-well plates (2×10^4 cells/well) with polymer-drug micelles (ADR concentration: 50 μg/mL) in RPMI 1640 medium supplemented with 10% FBS at 37 ℃. Afterwards, the cells were washed with PBS (pH 7.4), fixed with 4% glutaraldehyde for 10 min and then examined using an Olympus FV1000 CLSM. The excitation wave was chosen as 488 nm.

Flow cytometry analysis

HepG2 cells (3×10^4 cells/well) were washed twice with PBS and then incubated with culture medium containing conjugate micelles or free ADR at an ADR concentration of 10 μg/mL. After exposure for 3 h at 37 ℃, the cells were firstly trypsinized and collected, and then washed with PBS, at last the cells were analyzed using a flow cytometer (Bio-Rad, USA). For detection of ADR fluorescence, excitation was conducted with a 488 nm argon laser, and the fluorescence emission was measured at 595 nm. The other experiment was done under exactly the same experimental condition. The only difference was that after 3 h of incubation, the medium was replaced by the new medium without ADR or conjugate, and that the incubation time was lengthend to 24 h. After 24 h incubation, the cells were tested in the same way.