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Supporting Information

A novel nanoassembled doxorubicin prodrug with a high drug loading for anticancer drug delivery

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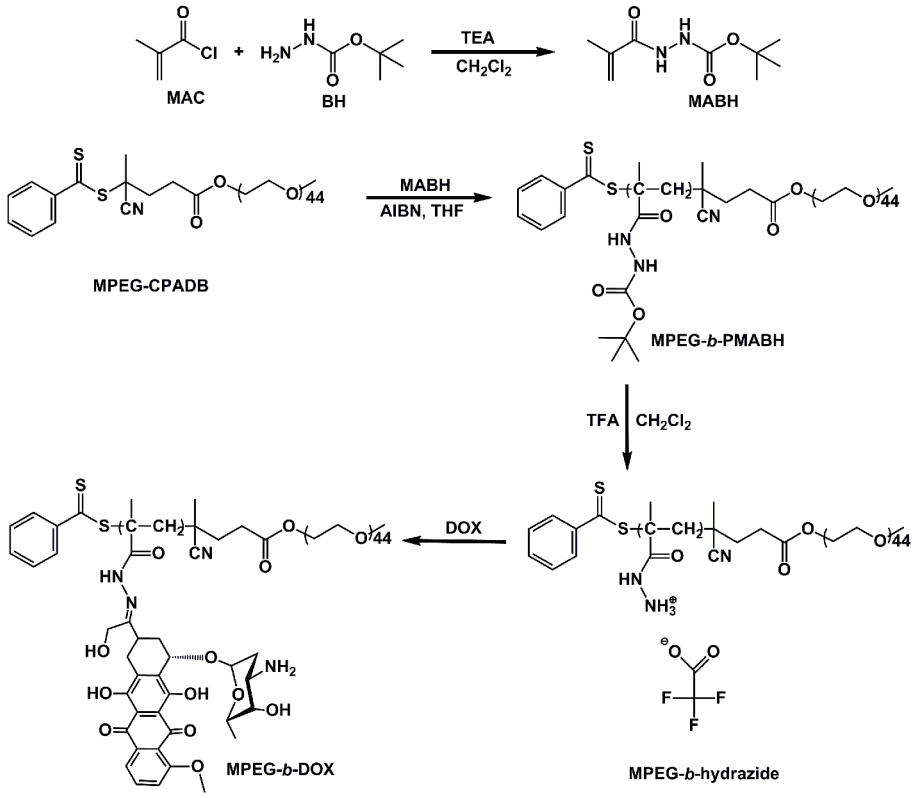

Figure S1. Synthesis of MABH monomer and MPEG-b-DOX prodrug.

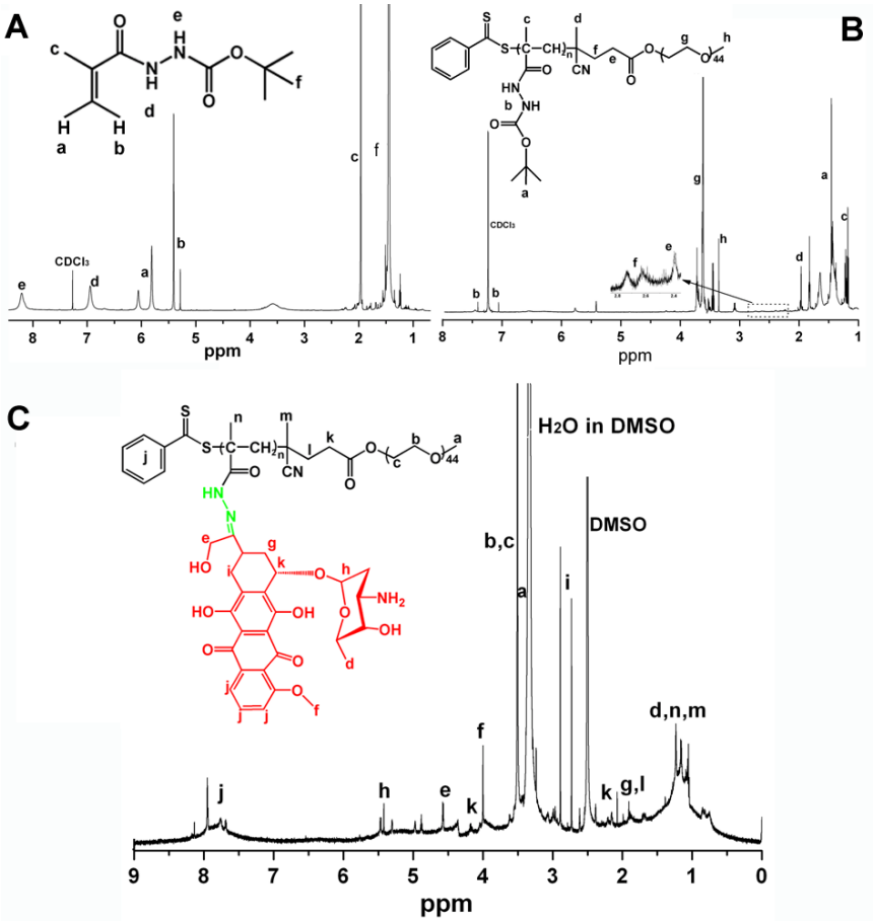

Figure S2. ^{1}H NMR spectra of (A) MABH; (B) MPEG-b-PMABH and (C) MPEG-b-DOX.

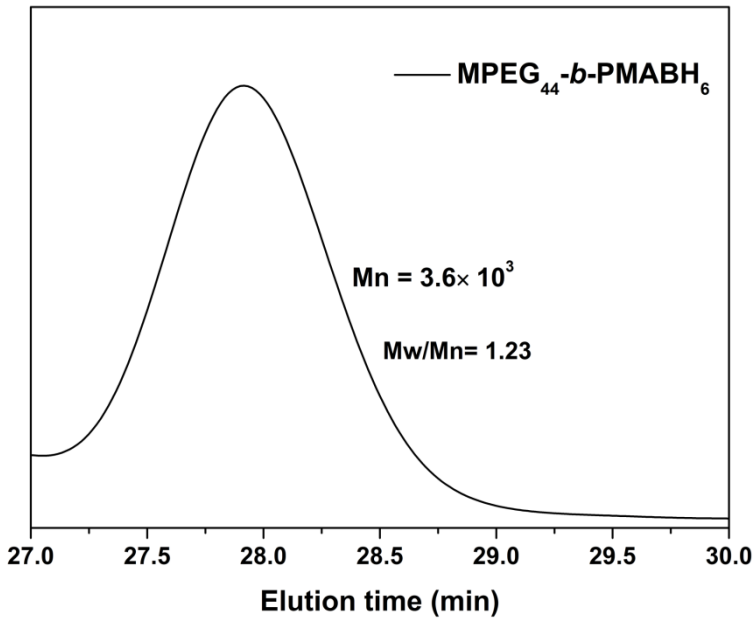

Figure S3. GPC traces of PEG_{44}-b-$PMABH_6$ copolymer in THF. The corresponding molecular weights by ^1H NMR was 3300.

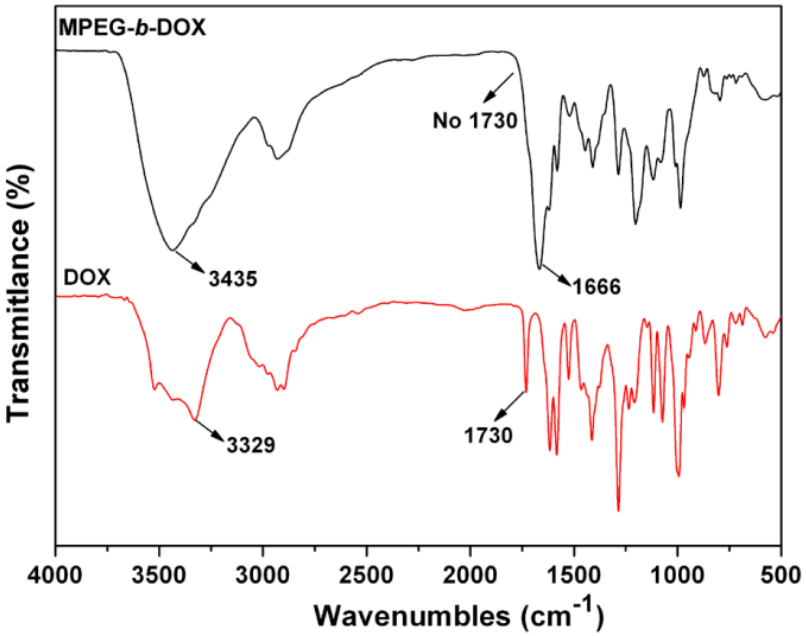

Figure S4. FT-IR spectra of free DOX and MPEG-b-DOX

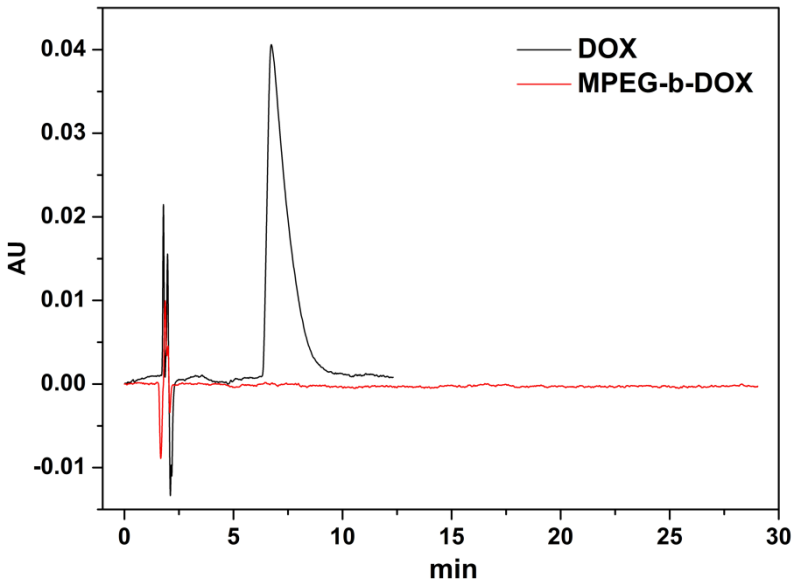

Figure S5. The HPLC curves of DOX and PEG-b-DOX under the given HPLC conditions

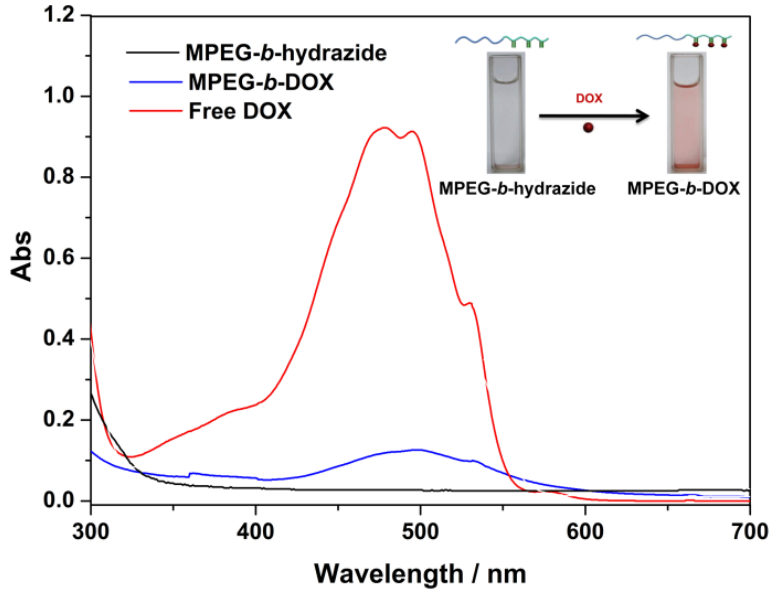

Figure S6. UV−visible spectra of PEG-b-hydrazide, PEG-b-DOX and free DOX dissolved in methanol with same concentrations of 60 μsg /mL

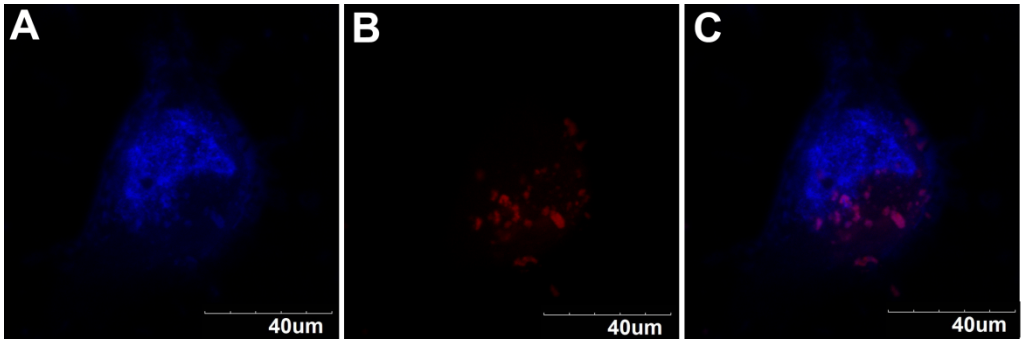

Figure S7. High magnification images in observation of subcellular localization of MPEG-b-DOX prodrug by laser scanning confocal microscope. For each panel, the images from left to right show DOX fluorescence in cell nuclei stained by DAPI (blue, A), cells (red, B) and overlays of the two images (C).

2. Experimental Section

2.1. Reagents and Materials

Polyethylene glycol monomethylether (MPEG, Mn～1900, alfa aesar) were dehydrated by azeotropic distillation of water in toluene, precipitated with cold ethyl ether, filtered, washed with ether, and vacuum-dried. Dimethylaminopyridine (DMAP, 99%, Alfa Aesar), N,N'-dicyclohexylcarbodiimide (DCC, 99%, Tianjin Chemical Reagent, China), Trifluoroacetic acid (TFA, 99.5 %, Tianjin Chemical Reagent, China), tert-Butyl carbazate (BH, 98%, Alfa Aesar) and Methacryloyl chloride (MAC, 98%, Alfa Aesar) were used as received. Triethylamine (TEA, 99%, Tianjin Chemical Reagent, China) was used after being stirred overnight over CaH_2 and distilled under reduced pressure. Doxorubicin hydrochloride (DOX·HCl) was obtained from Beijing HuaFeng United Technology CO. Ltd. All these reagents were distilled before use. The macro radical addition−fragmentation chain transfer (RAFT) agent MPEG-CPADB was prepared by an esterification reaction. The 2, 2-Azobisisobutyronitrile (AIBN, 98%, Alfa Aesar) was purified by recrystallization from methanol. Deionized (D.I.) water was prepared from Millipore (Bedford, MA, USA).

2.2. Synthesis of macro RAFT agent

This macro MPEG-CPADB agent were synthesized according to our previous report.[S1] The details procedure were shown as follow:

*Bis(dithiobenzoyl) Disulfide***.** Bromobenzene (11.30 g, 72 mmol) in 30 mL of dry THF was added into the mixture of magnesium turnings (2.0 g, 82 mmol), a catalytic amount of iodine (250 mg), and bromobenzene (1.26 g, 8 mmol) in THF (10 mL) over a period of 30 min at 0 °C. The reaction was started and then kept for 1 h with mild heating (no more than 20 °C). Then CS_2 (6.10 g, 80 mmol) was added dropwise to the reaction mixture below 20 °C for another 1.5 h. The Grignard product was hydrolyzed with 100 mL of cold, distilled water, and the formed salts, together with excess Mg turnings, were removed by filtration. After removal of THF under reduced pressure

the reaction mixture was acidified to pH of 1.0 by the dropwise addition of fuming HCl. The color changed from brown-red to a permanent deep purple. The mixture was extracted three times with 200 mL of diethyl ether. After drying the organic phase over $MgSO_4$, the ether was removed under vacuum. The red oil so obtained was dissolved in 100 mL of absolute ethanol and reacted with DMSO (12.50 g, 0160 mmol) in the presence of a catalytic amount of iodine (250 mg) at room temperature for 1 h. After keeping the reaction mixture at 0°C for 12 h, the resulting precipitate was filtered, redissolved in ethanol at 40 °C, and recrystallized. After drying, bis-(dithiobenzoyl) disulfide (4.90 g, 77%) was obtained as a purple, crystalline product.

*4-Cyano-4-((thiobenzoyl)sulfanyl)pentanoic Acid***.** 4,4'-azobis(4-cyanopentanoic acid) (1.69 g, 6 mmol) and bis(thiobenzoyl) disulfide (1.22 g, 4 mmol) were dissolved in ethyl acetate (23 mL), and then the solution was heated at reflux for 18 h. After removal of ethyl acetate under reduced pressure, the crude product was subjected to silica column chromatography with ethyl acetate/n-hexane (v/v, 2/3) as eluent, to afford the compound as red oil (3.36 g, 67 % yield). On keeping in a freezer at –20 °C about 10 h, the product turned into a red solid, denoted as CPADB. ^{1}H NMR (Figure S1, 600 MHz, $CDCl_3$)/δ ppm: 1.95 (s, 3H, CH_3); 2.43-2.77 (m, 4H, CH_2CH_2); 7.39-7.41 (m, 2H, m-ArH); 7.56-7.59 (m, 1H, p-ArH) and 7.90-7.92(m, 2H, o-ArH). FTIR, Figure S2: (KBr disc): 3300-2510 (a broad band, COO-H); 2230.8.5 (CN); 1707.1 (C=O); 1047.9 (C=S).

*Synthesis of macro RAFT agent: MPEG-CPADB***.** To a solution of MPEG (1.9 g, 1.0 mmol) and CPADB (0.48 g, 2.1 mmol) in 50 mL of anhydrous DCM was added DCC (0.48 g, 2.1 mmol). The reaction was allowed to proceed in the dark at 25 °C for 48 h. The reaction mixture was filtered to remove insoluble byproduct. After removal of 80% DCM under reduced pressure, then the resulting macro RAFT agent was precipitated in cold anhydrous ether (1.89 g, 77.1 % yield). ^{1}H NMR (Figure S3, 600 MHz, $CDCl_3$): δ 3.38 (s, CH_3O-), 3.65 (s, MPEG, -CH_2CH_2O-), 4.22 (m, MPEG-CH_2OCO), 2.41−2.75 (m,-$OCOCH_2CH_2$-), 1.96 (s, −CH_3), 7.39-7.41 (m, 2H, m-ArH); 7.56-7.59 (m, 1H, p-ArH) and 7.90-7.92(m, 2H, o-ArH).

2.3. Synthesis of Monomer methacrylamide butyl carbazate with hydrazine bond

To an anhydrous dichloromethane (DCM) solution (75 mL) of tert-butyl carbazate (3.96 g, 30 mmol) and Triethylamine (5.46 mL, 36.0 mmol) at 0 ℃ were added dropwise an anhydrous DCM solution (30 mL) of methacryloyl chloride (3.07 mL, 30 mmol) simultaneously. The reaction was stirred at 25 ^{o}C for 12 h after completion of addition. The solution was then filtered to remove the salt, and the crude product was recovered by evaporation of DCM. The product was denoted as MABH and purified by silica column chromatography. (eluent: n-hexane/EA = 80/20). Then, the product was concentrated and dried in vacuo. Yield: 4.8 g, 80%. ^{1}H NMR (600 MHz, $CDCl_3$): δ8.22, 6.96 (s, 2H, -NH), 5.42, 6.07(s, 2H, CH_2=), 1.96 (q, 3H, -CH_3), 1.45-1.53 (q, 9H, -$(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 300 MHz): δ (ppm) 167.8, 155.9, 137.6, 121.5, 81.7, 28.29, 18.36.

2.4. Synthesis of MPEG-b-PMABH by RAFT Polymerization

The diblock copolymer was synthesized by RAFT of MABH using MPEG-CPADB as a macroRAFT agent. Typically, under a nitrogen atmosphere, MPEG-CPADN (140.0 mg, 0.06 mmol), MABH (1.55 g, 4.8 mmol), AIBN (3.6 mg, 0.02 mmol), and 3 mL of tetrahydrofuran (THF) were added into a 10 mL Schlenk flask. The flask was sealed and placed in an oil bath thermostated at 60 ℃. The polymerization proceeded with stirring for 24 h. The resulting copolymer was isolated by precipitation in cold diethyl ether. The as-synthesis product was further treated with an excess of AIBN for removing the terminal trithiocarbonate moieties. After stirring at 60 °C for 8 h, the solution was precipitated cold diethyl ether, and then drying in vacuo oven overnight at room temperature. Yield: 65 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.38 (s, CH_3O-), 3.65 (s, MPEG, -CH_2CH_2O-), 4.22 (m, MPEG-CH_2OCO), 2.43−2.76 (m,-$OCOCH_2CH_2$-), 1.96 (t, −CH_3), 7.43, 7.26, 1.40-1.49 (m, -$NHNHCO(CH_3)_3$-, MABH).

2.5. Synthesis of MPEG-b-DOX Prodrug

In a typical procedure, 500 mg of MPEG-b-MABH was dissolved in 5.0 mL of DCM at room temperature. Trifluoroacetic acid 6 mL was charged into the reaction mixture. Reaction mixture was allowed stirring for 1 h at 25 ^{o}C. The reaction mixture was concentrated to pasty mass and charged diethyl ether to obtain the resulting deprotection product (MPEG-b-hydrazide) by suction filtration, washed with 10 ml diethyl ether and dried at 30 ℃ under vacuum (350 mg, 70 % yield). Then MPEG-b-hydrazide (150 mg, 0.25 mmol of hydrazide) and DOX (60 mg, 0.1 mmol) were dissolved in 10 mL of anhydrous methanol. Trifluoroacetic acid (3 μL) was added to the reaction mixture. The reaction mixture was stirred at room temperature for 48 h, while being protected from light. The resulting prodrugs were isolated by extensive dialysis against anhydrous methanol (MWCO 3500). Then the prodrug solution in dialysis was concentrated to a volume of 1 mL and added to diethyl ether (10 mL) dropwise with stirring. The resulting solution was allowed to stand at 4 ℃. The DOX prodrugs were isolated by centrifugation, washed with diethyl ether, and dried under vacuum to yield the hydrazone linker of DOX prodrug (90 mg, 43% yield).

2.6. Micelle Formation

MPEG-b-DOX micelles were prepared as following. Typically, MPEG-b-DOX (5.0 mg) was dissolved in PBS (pH 7.4, 5 mL), and stirred for 12 h at25 ^{o}C in the dark. Thereafter, the resulting micelles were obtained by centrifugation and were redispersed in fresh PBS solution for further use.

2.7. Drug Loading and Release in Vitro

To determine DOX content in MPEG-b-DOX, 1.0 mg of MPEG-b-DOX was treated with 1.0 M HCl at 25 ^{o}C for 24 h, and the resulting solution was diluted with DI water. The amount of free DOX was determined using UV-visible absorption spectrum at 480 nm.

The release of DOX from MPEG-b-DOX prodrug was evaluated by the dialysis method. First, a dialysis bag (MWCO 3500) was filled with a 5 mL MPEG-b-DOX buffer solution (1.0 mg/mL) and soaked in 50 mL of 0.05 M buffer solution with varying pH values at 37 ± 1 °C in a water bath with gentle shaking. The released DOX outside of the dialysis bag was sampled at a predetermined time and measured using fluorescence spectroscopy. The release amount was obtained by a formula as described. [33] Above release experiments were tested in triplicate.

$$m_{\text{t-act}} = (C_{\text{t}} + \frac{\text{v}}{\text{V}}\sum_{0}^{\text{t-1}} C_{\text{t}})\text{V}$$

Where $m_{t\text{-act}}$ is the actual quantity of DOX released at time t, C_t is the drug concentration in release fluid at time t measured on fluorescence spectrometer, v is the sampled volume taken at a predetermined time interval, and V is the total volume of release fluid.

2.8. Cell Culture and Viability Assay

The human tongue squamous cell carcinoma (TCA8113) cell line and rat adrenal pheochromocytoma (PC12) cell line were provided by the Biology Preservation Center in Shanghai Institute of Materia Medica and maintained with RPMI 1640 medium containing 10% fetal bovine serum (FBS), and 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Then the in vitro cell cytotoxicity of MPEG-b-hydrazide, free DOX and MPEG-b-DOX prodrug were evaluated by Sulforhodamine B (SRB) assay. TCA8113 cells and PC12 cells were all seeded in a 96-well plate (Costar, USA) (3.5×10^4 cells/mL) overnight. Then culture medium was replaced with fresh medium containing MPEG-b-hydrazide with a varied concentrations ranging from 100 to 800 μg/mL and for free DOX and MPEG-b-DOX with varied concentrations ranging from 0.125 to 10.0 μg/mL for 24 h,

respectively. Then cells were fixed by addition of 100 μL cold 10 % trichloroacetic acid (TCA, 4 °C) for 1 h in each well. The wells were gently washed five times with deionized water and then stained with 0.4 % SRB solution (150 μL per well) for 30 min at room temperature. At the end of the staining period, unbound SRB was rinsing out with 1 % acetic acid. As the plate air dried again, 150 μL 10 mM aqueous tris base [tris(hydroxymethyl) aminomethane] was added into each well to solubilize the SRB dye. When the crystals were dissolved, the absorbance values were read on a micro-plate reader (Huake, Shanghai, China) at 570 nm. This procedure was repeated three times.

2.9. Cell Uptake Studies

The ability of MPEG-b-DOX micelles to enter cancer cells were observed by a laser scanning confocal microscope (LSCM, ZEISS, LSM 510 Meta, Germany). In a typical procedure, the TCA8113 cells (3.5×10^4 cells/well) were seeded on a 6-well plate (Costar, USA) at 37 ℃ overnight. After that free DOX, MPEG-b-DOX (at 5 μg DOX/mL) were added, respectively. The cell nucleus was stained by 4′, 6-Diamidino-2-phenylindole (DAPI) (Sigma aldrich). After further 3 h or 24 h incubation, the cells were washed by PBS three times to remove the dead cells and drugs adsorbed on the outer surface of cell membrane and the MPEG-b-DOX prodrug uptake was visualized at excitation wavelength of 488 nm.

2.10. HPLC Analysis

The DOX and MPEG-b-DOX was analyzed by high performance of liquid chromatography (HPLC) method with a C_{18} column (5μm, 4.6×250 mm), mixture of methanol and water (80: 20, *v/v*) with rate of 1mL/min, and detection at 480 nm. 20 μL of each sample solution was injected into the analysis system. The content of free DOX in MPEG-b-DOX was determined based on the peak areas compared with the standard DOX solution.

2.11. Measurements

The 1H NMR spectra of the sample was recorded on a Bruker AVANCE 600MHz spectrometer (Rheinstetten, Germany) using tetramethylsilane as an internal standard at 25 ℃. The Fourier transform infrared (FTIR) spectra were acquired on a Nicolet 20 NEXUS 670 FT-IR spectrophotometer (Ramsey, MA, USA) using KBr pellets. The size distribution of MPEG-b-DOX) micelles were determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90^{o}. The morphology of the samples was recorded by a JEM-1230EX transmission electron microscopy (TEM) (Japan), and samples for TEM measurements were made by casting one drop of the sample's water solution on carbon-coated copper grids. The ζ-potentials of NPs (500 μg/mL) at PBS (pH 7.4) solutions was recorded on a Zetasizer (Nano 3600, Malvern, U.K.) in triplicate.The number-average molecular weight (Mw) and molecular weight distribution (Mw/Mn) were measured by gel permeation chromatography (GPC) system (Waters 1515, USA) equipped with waters 1515 pump and a waters 2414 refractive index detector and a styragel® HT column. Tetrahydrofuran (THF) was used as the eluent (1 mL/min), and polystyrene was used as the standard for calibration. Fluorescence spectra were recorded on a RF-5301PC fluorescence spectrometer (Shimadzu, Japan). Absorbance spectra were carried out using a Puxi UV-1810 visible spectrophotometer (Beijing, China). The cellular images were acquired with a laser scanning confocal microscope (LSCM, ZEISS, LSM 510 Meta, Germany).

References

1 Z. G. Xu, D. D. Wang, S. Xu, X. Y. Liu, X. Y. Zhang, H. X. Zhang,

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