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Synthesis and Drug Delivery of Novel Amphiphilic Block Copolymers Containing Hydrophobic Dehydroabietic Moiety

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Supplementary Information

Materials

Toluene and tetrahydrofuran (THF) were refluxed with sodium and distilled. Dehydroabietic acid was obtained from Wuzhou Chemicals, China. CuBr, *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA) and poly(ethylene glycol) (PEG, M_n=5000 g/mol) were purchased from Sigma-Aldrich and used as received. PEG-Br and DAEMA were prepared following earlier reports. Piperlongumine (PLGM) was obtained from Mai Ruier Co., Ltd. (Shanghai, China). Taxol® was purchased from Bristol-Myers Squibb (Princeton, NJ, USA). NIR-797-isothiocyanate and fluorescein isothiocyanate (FITC) were bought from Sigma Chemical Co. Male ICR mice (6-8 weeks old and weighing 30-35 g) were purchased from Animal Center of Drum Tower Hospital (Nanjing, China). 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) was purchased from Aldrich. All other reagents were of analytical grade and used without further purification. Human neuroblastoma cell line SH_SY5Y and a murine hepatic cell line H22 were purchased from Shanghai Institute of Cell Biology (Shanghai, China).

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Polymer Characterization.

^{1}H NMR (300 MHz) spectra were recorded on a Varian Mercury spectrometer with tetramethylsilane (TMS) as an internal reference. Gel permeation chromatography (CPC) was tested at 25^{o}C on a Varian system equiooed with a Varian 356-LC refractive index detector and a Prostar 210 pump. The columns were STYRAGEL HR1, HR2 (300×7.5 mm) from Waters. HPLC grade THF was used as eluent at a flow rate of 1 mL/min. THF and polymer solutions were filtered over microfilters with a pore size of 0.2 μm (Nylon, Millex-HN 13 mm Syringes Filters, Millipore, USA). The columns were calibrated against polystyrene standards.

Preparation of PEG-*b*-PDAEMA Block Copolymers

The preparation of PEG-*b*-PDAEMA block copolymers was carried out by ATRP. Macroinitiator PEG-Br and monomer DAEMA were synthesized according to procedures reported earlier. A typical synthetic procedure is as follows: CuBr (5.2 mg, 0.036 mmol) and macroinitiator PEO-Br (125.1 mg, 0.024 mmol) were dissolved in 0.5 mL dry toluene and added into a polymerization tube. The tube was tightly sealed and purged with nitrogen for 10 min. DAEMA (1.0 g, 2.4 mmol) and PMDETA (8.4 mg, 0.048 mmol) were dissolved in 1 mL dry toluene in a small round bottom flask and purged with nitrogen for 10 min. The above mixture of monomer and ligand was then transferred to the polymerization tube under nitrogen atmosphere by a syringe. The tube was placed into a preheated oil bath set at 90 ^{o}C. After 24 hours, the tube was opened to air and the reaction was quenched by addition of large excess of THF. The polymer was precipitated and washed by methanol twice and dried under vacuum to constant weight.

Preparation of PLGM-loaded nanoparticles

PLGM-loaded PEG-*b*-PDAEMA nanoparticles were prepared by a nanoprecipitation method. Briefly, 5 mg of PLGM and 33 mg of PEG-*b*-PDAEMA diblock copolymer were dissolved in 2 mL of THF, and 8 mL of deionized water was added quickly to this solution. The solution turned into opalescent immediately due to the formation of nanoparticles. Following by rotary vacuum evaporation, the solution was dialyzed against deionized water for 12 h using a dialysis membrane bag (14 kDa cut off), and the resultant solution was

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filtrated through a microfilter with a pore size of 5 μm to remove non-incorporated drug crystals as well as copolymer aggregates. Finally, the dispersed solution was lyophilized for further use. Non-drug containing nanopaticles were prepared in a similar manner omitting the drug.

Characterization of PLGM-loaded nanoparticles

Mean diameter and size distribution of the PLGM-loaded PEG-*b*-PDAEMA nanoparticles were determined by dynamic light scattering (DLS) using a Brookhaven BI 9000AT system (Brookhaven Instruments Corporation, US). All DLS measurements were done with a laser wavelength of 633.0 nm at 25 ℃ with a detection angle of 90°. All analyses were triplicated and the results were the average of three runs.

Transmission electron microscopy (TEM) (JEOLTEM-100, Japan) was used to observe the morphology of the PLGM-loaded PEG-*b*-PDAEMA nanoparticles. One drop of nanoparticle suspension was placed onto copper grill covered with nitrocellulose and dried at room temperature before examination with TEM.

Scanning electronic microscope (S-4800 HITACHI, Japan) was also employed to study the morphology of the drug loaded nanoparticles. One drop of nanoparticle suspension was placed on the surface of a clean silicon wafer and dried at room temperature. The sample wafer was coated with a thin layer of gold prior to observation.

HPLC analysis was performed on a Shimadzu LC-15A (Shimazu, Japan) HPLC system equipped with a Shimadzu UV detector and a C-18 Wondasil-HPLC analysis column at 25 °C. The mobile phase was consisted of 50/50 double-distilled water (Millipore, Milford, USA)/acetonitrile (HPLC grade, Merck). The flow rate was set to 1.0 mL/min, and UV detection wavelength was 325 nm. The retention time of PLGM was at 5.5 min. A calibration curve was prepared with the standard PLGM solution under the same conditions.

Drug loading content and encapsulation efficiency measurement

Drug loading content and encapsulation efficiency was determined by HPLC and using a pre-established calibration curve. PLGM-loaded PEG-*b*-PDAEMA nanoparticles with different drug feedings were prepared and lyophilized. The lyophilized nanoparticles were

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dissolved in acetonitrile, a PLGM good solvent, sonicated for 20 min, and centrifuged at 1000 rpm for 10 min, then the supernatant fraction was injected into the HPLC system. The drug loading content and encapsulation efficiency were calculated by Eqs. (1) and (2), respectively. All analyses were triplicated and the results were the average of three runs.

$$\text{Drug loading content \%} = (\text{mass of PLGM in the sample})/(\text{mass of sample}) \times 100\ \% \quad (1)$$

$$\text{Encapsulation efficiency \%} = (\text{mass of PLGM in the sample})/(\text{mass of PLGM in feed}) \times 100\ \% \quad (2)$$

In vitro PLGM release from the nanoparticles

Predetermined amount of lyophilized drug-loaded nanoparticles or free PLGM were redispersed in 1 mL of phosphate buffer solution (PBS, 0.01 M, pH 7.4) and put into a pre-swelled dialysis bag (14 kDa cut-off). Then the bag was immerged into 5 mL release medium (PBS, 0.01 M, pH 7.4), and kept in an incubator with gentle agitation at 37 °C in the darkness. Periodically, the release medium was withdrawn for HPLC analysis of PLGM concentration, and equivalent fresh PBS was added to the system. All the measurements were performed in triplicate. The cumulative amount of PLGM released from the nanoparticles was plotted against time.

In vitro cytotoxicity assay

The *in vitro* cytotoxicity of free PLGM, PLGM-loaded nanoparticles and Taxol® were determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The SH_SY5Y cells were seeded to 96-well plate to ensure 5,000 cells per well and then co-incubated with a serial of various concentrations of PLGM, PLGM-loaded nanoparticles and Taxol® at 37 °C for 24 h. Free PLGM was dissolved in water containing 1% DMSO. One row of 96-well plates was used as control with fresh culture medium only. Next, 20 μL of MTT solution and 180 μL of fresh medium were added to each well, allowing the viable cells to reduce the yellow MTT into dark-blue formazan crystals. After incubation for 4 h, the solution was removed, leaving the precipitate. 200 μL of dimethylsulfoxide (DMSO) was then added to each well, and after 15 minutes, the absorbance of individual well was measured at 570 nm by an ELISA reader. The cytotoxicity of non-drug containing

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nanoparticles was also monitored to evaluate the toxicity. Cell viability was determined by the following formula:

Cell viability (%) = (Abs test cells)/(Abs reference cells) × 100%

where Abs is absorbance.

Preparation of FITC-labeled PLGM-loaded PEG-*b*-PDAEMA nanoparticles

First, the bromine end group of PEG-*b*-PDAEMA block copolymer was hydrolyzed into hydroxyl end group in a basic condition. Then, 3 mg of fluorescein isothiocyanate (FITC), which was dissolved in 1 mL of anhydrous THF, was dropwise introduced into the solution of PEG-*b*-PDAEMA block copolymers (40 mg) to allow the reaction between isothiocyanate group of dye and hydroxyl end group of PDAEMA block for 12 h. The FITC-labeled PEG-*b*-PDAEMA block copolymers were precipitated with 40 mL anhydrous ethanol and the precipitate was washed with anhydrous ethanol three times. Finally, these FITC-labeled PEG-*b*-PDAEMA block copolymers were used for the preparation of FITC-labeled PLGM-loaded PEG-*b*-PDAEMA nanoparticles.

***In vitro* cellular uptake**

For cell uptake, 1.9 mL of RPMI 1640 medium and 100 μL of the FITC-labeled PLGM-loaded PEG-*b*-PDAEMA nanoparticle suspension (1 mg/mL) were added successively into 6-well plate containing with a density of 5,000 cells per well. After 4 h incubation at 37 °C, the cells were washed three times with PBS at 4 °C to remove free FITC-labeled nanoparticles. The nucleus was stained with Hoechst 33258 and the sample was observed by confocal laser scanning microscopy (CLSM).

Real-time near-infrared fluorescence imaging

All animal studies were performed in compliance with guidelines set by Animal Care and Use Committee, Nanjing University. To establish subcutaneous tumor model, H22 tumor cells ($5\text{-}6\times10^6$ cells for per mouse) were inoculated subcutaneously to ICR mice (30-35 g) at the right axilla. The mice were kept 7 days with free access to food and water. NIR-797-isothiocyanate-labeled PLGM-loaded PEG-*b*-PDAEMA nanoparticles were prepared

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as the preparation of FITC-loaded PLGM-loaded PEG-*b*-PDAEMA nanoparticles described above. After that, 0.2 mL of NIR-797 labeled nanoparticles (3 mg/mL) was injected into H22 tumor bearing mice through a tail vein. After *i.v.* administration, the time-dependent biodistribution in tumor bearing mice was imaged using the MaestroTM EX fluorescence imaging system (Cambridge Research & Instrumentation, CRi, USA) on predetermined time.

***In vivo* antitumor efficacy of PLGM-loaded nanoparticles**

The tumor models were established by inoculating subcutaneously H22 tumor cells (5-6×10^6 cells per mouse) to ICR mice at the right axilla as mentioned above. When the tumor volume reached a mean size of about 50-100 mm^3, the mice were randomly divided into six groups and each group contains ten mice. This day was designated as "day 1". On day 1, the mice were administered *via* a tail vein with a single dose of non-drug containing nanoparticles, free PLGM (6 mg/kg), Taxol® (6 mg/kg), PLGM-loaded nanoparticles (6 mg/kg PLGM eq.) and three doses of PLGM-loaded nanoparticles with a four day interval (18 mg/kg PLGM eq.), respectively. Saline was used in control experiments. Free PLGM was dissolved in water containing 5% DMSO and injected into the tumor bearing mice. Tumor were measured by a vernier calipers on alternate day and the volume (V) was calculated as $V = W^2 \times L/2$, where W and L are width and length of the tumor, respectively. Animals were also weighed every other day, and the survival rates were monitored throughout the study.

***In vivo* biodistribution evaluation**

For the biodistribution study, the H22 tumor-bearing ICR mice as described above were randomly divided into 10 groups (3 mice per group). The mice of 5 groups were intravenously (i. v.) injected with PLGM-loaded nanoparticles at an equivalent PLGM dose of 4 mg/kg body weight, while the remaining mice of 5 groups were *i.v.* injected with PLGM at a dose of 4 mg/kg. At different time intervals, blood samples were collected *via* eye puncture, and plasma was obtained by centrifuging whole blood samples at 5000 rpm for 8 min. Subsequently, the mice were humanely killed, and their tumors, hearts, livers, spleens, lungs and kidneys were harvested and weighed. The plasma and tissues were homogenized with acetonitrile. After a further centrifugation step, the PLGM concentrations in the homogenized plasma and tissues

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were measured by HPLC at the wavelength of 325 nm, as described in Section 2.4. The data were normalized to the tissue weights.

Blood biochemistry

Following the antitumor experiments described above, the mice were sacrificed on the 15th day and serum was collected by centrifuging the whole blood at 5000 rpm for 8 min. The biochemical parameters were assayed.

Statistical analysis

Data are expressed as means ± S.D. with statistical significance of the differences in the tumor volume and drug biodistribution. Significant differences in the mean values were evaluated by Student's unpaired t-test. The differences were considered significant for P values.