Supporting Information

Polytyrosine nanoparticles enable ultra-high loading of doxorubicin and rapidly enzyme-responsive drug release

Xiaolei Gu, Min Qiu, Huanli Sun, Jian Zhang, Liang Cheng, Chao Deng*, and Zhiyuan Zhong*

Biomedical Polymers Laboratory, and Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, China.

*Corresponding authors. Tel./Fax: +86-512-65880098. E-mail: cdeng@suda.edu.cn (C. Deng), zyzhong@suda.edu.cn (Z. Zhong).

Materials

α-Methoxy-*ω*-amine-poly(ethylene glycol) (PEG-NH_2, M_n = 5.0 kg/mol, Fluka), doxorubicin hydrochloride (DOX·HCl, >99%, Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd.), and doxorubicin hydrochloride PEGlated liposome injection (LP-DOX, 20 mg/mL, Shanghai Fudan Zhangjiang Biomedical Co., Ltd) were used as received. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and 4′6-diamidino- 2-phenylindole dihydrochloride (DAPI) were purchased from Sigma and used as received. Tetrahydrofuran (THF) and petroleum ether (bp 60-90 ^oC) were purified using the solvent purification system (Innovative Technology, USA). *N, N*-Dimethylformamide (DMF) was dried with $MgSO_4$ and distilled under reduced pressure before use.

Characterization

^1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using DMSO-d_6 as a solvent. The chemical shifts were calibrated against residue solvent signals. The molecular weight and molecular weight dispersity of copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with MZ-gel SDplus columns (500 Å, 10E3 Å, 10E4 Å) following a differential refractive-index detector (RI 2414). The measurements were performed using DMF as the eluent at a flow rate of 0.8 mL/min at 25 ºC and a series of narrow polystyrene standards for the calibration of the columns. The size of nanoparticles was evaluated by dynamic light scattering (DLS). Measurements were carried out at 25 ^oC by a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. Zeta potential measurements were carried out using Zetasizer Nano-ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell. Transmission electron microscopy (TEM) was carried out using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 20 μL of 0.5 mg/mL micelle suspension on the copper grid followed by staining with 1 wt.% phosphotungstic acid.

Synthesis of L-tyrosine N-carboxyanhydride (Tyr-NCA)

Under a nitrogen atmosphere, triphosgene (6.56 g, 22.1 mmol) was added to a solution of L-tyrosine (8 g, 44.2 mmol) in dry THF at 50 ^oC. After stirring for 1 h, the reaction solution was concentrated, and precipitated in petroleum ether to give crude Tyr-NCA. The crude product following dissolving in ethyl acetate was washed with cold saturated $NaHCO_3$ aqueous solution and cold water sequentially, and dried with anhydrous $MgSO_4$. Then the

product recrystallized three times with hexane and THF. Yield: 78%. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 9.33 (s, 1H, -O*H*), 9.03 (s, 1H, -CON*H*), 6.97 (s, 2H, -$C_6H_2CH_2$), 6.67 (s, 2H, -C_6H_2OH-), 4.70 (t, 1H, -C*H*NH), 2.91 (d, 2H, -$C_6H_4CH_2$), Anal. Calcd for $C_{10}H_9NO_4$: C, 57.97; H, 4.35; N, 6.76. Found: C, 58.01; H, 4.64; N, 6.72.

Preparation and in vitro SPECT/CT imaging of I-labeled PTNs

For *in vitro* SPECT/CT imaging, ^{125}I–labeled PEG-PTyr nanoparticles (^{125}I-PTNs) were prepared from ^{125}I–labeled PEG-PTyr copolymer that was obtained by the reaction of PEG-PTyr polymers (4 mg) with 300 μCi of Na^{125}I in DMF (100 μL) at r.t. for 15 min. The resulted copolymer solution was purified by dialysis to remove the excess ^{125}I. The amount of ^{125}I in PEG-PTyr copolymers was measured by radioactivity meter (FJ-391A4, Beijing Heng Odd Instrument Co. Ltd., Beijing, China), and the results showed that around 53% of added ^{125}I has been labeled on PEG-*b*-PTyr copolymers. ^{125}I-PTNs were prepared as above description. The ^{125}I-PTNs with ^{125}I concentrations of 10, 20, 40, 80 and 160 μCi were employed as contrast agents for SPECT/CT imaging, and the SPECT/CT images were collected using U-SPECT/CT (MILabs, Netherlands).

DOX Loading in Nanoparticles

DOX loaded nanoparticles (PTN-DOX) were prepared by dropwise addition of HEPES (900 μL, pH 7.4) to a DMF solution of DOX and PEG-*b*-PTyr (100 μL), followed by dialysis against HEPES buffer for 12 h to remove non-encapsulated DOX. The DOX feed ratios ranged from 20 to 70 wt.%. The dialysis medium was changed every hour and all the experiments were performed in the dark. For determination of drug loading content (DLC), the solution of PTN-DOX was diluted with DMF (30-fold) followed by sonification, and analyzed using fluorescence measurement (FLS920) with excitation at 480 nm and emission at 560 nm. Calibration curve of DOX fluorescence was obtained with different DOX concentrations in DMF solution. DLC and drug loading efficiency (DLE) were calculated according to the following formula:

DLC (wt.%) = weight of loaded drug/(total weight of polymer and loaded drug) ×100

DLE (%) = weight of loaded drug/weight of drugs in feed ×100

Determination of π-π stacking in polypeptide nanoparticles (PTNs)

The π-π stacking interaction between PTNs and DOX was characterized by UV-Vis spectrometry. Briefly, 300 μL of PTN-DOX and free DOX (125 μg/mL) were diluted in 2.7

mL of HEPES buffer (10 mM, pH 7.4). Both of them were determined using UV-Vis spectrometry, following setting in a shaking bed at 200 rpm and 37 ^{o}C for a predetermined time.

In vitro cytotoxicity assays

The in vitro cytotoxicity of blank PTNs was evaluated in RAW 264.7 cells. The cells were plated in a 96-well plate (5000 cells/well) with 80 μL DMEM media supplemented with 10% fetal bovine serum, 1% L-glutamine, and antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 24 h. When the cells reached about 70% confluence, blank PTNs in 20 μL HEPES were added to yield final micelle concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. The cells were cultured in an atmosphere containing 5% CO_2 at 37 ^{o}C for 48 h. Then, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) solution (10 μL, 5 mg/mL) in PBS was added to each well. After incubation for 4 h, the supernatant was carefully aspirated and 150 μL DMSO was added into each well to dissolve the formazan crystals generated by live cells for 10 min. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Tek, ELX808IU). The cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. The experiments were performed in quartets. The antiproliferative activity of PTN-DOX against RAW 264.7 cells and HCT-116 human colorectal cancer cells was also examined by MTT assays in a similar way. LP-DOX was used as a control. The cells were treated with PTN-DOX and LP-DOX for 4 h, and then incubated in fresh medium for another 68 h. The final DOX concentrations in PTN-DOX incubated with cells were fixed at 0.01, 0.1, 0.5, 1, 5, 10, 20 and 50 μg/mL.

In vitro confocal microscopy measurements

Confocal microscopy measurements were employed to validate the cellular uptake and intracellular drug release behaviors of PTN-DOX in RAW 264.7 cells and HCT-116 cancer cells. Cells were cultured on microscope slides in a 24-well plate (2×10^4 cells/well) using 400 μL DMEM media containing 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). After 24 h, PTN-DOX in 100 μL HEPES was added to each well (DOX dosage: 10.0 μg/mL), and incubated with cells for 4 h or 8 h. Then, the culture medium was removed, and the cells on microscope plates were washed three times with PBS, fixed with 4% paraformaldehyde solution for 15 min, and washed with PBS for three times. The cell nuclei were stained with 4',

6-diamidino-2-phenylindole (DAPI, blue) for 10 min and washed with PBS for three times. The fluorescence images were obtained using a confocal microscope (TCS SP5).

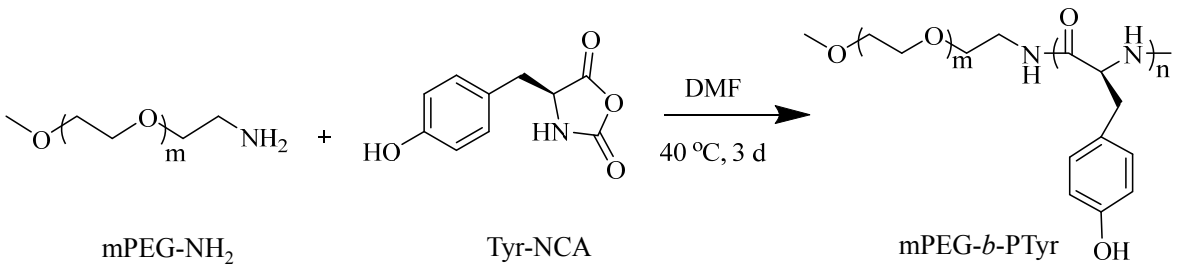
Blood analysis

The mice were handled under protocols approved by Soochow University Laboratory Animal Center and the Animal Care and Use Committee of Soochow University. PEG-*b*-PTyr nanoparticles (dose 50 mg/kg) were injected into healthy Kunming mice via tail vein (n = 3). For blood analysis, mice were sacrificed 1 d post injection and general blood parameters including white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelets (PLT), red cell distribution width (RDW), mean platelet volume (MPV) were analyzed using hematology analyzer evaluate (CELL-DYN 3700).

Table S1. Characteristics of PTN-DTX.

Entry	DLC (wt.%)		DLE[a] (%)	Size[b] (nm)	PDI[b]
	theory	determined[a]			
1	20	11.2	54.5	96	0.16
2	30	17.5	49.3	122	0.18

[a]Determined by HPLC. [b]Determined by DLS analysis using Zetasizer Nano-ZS (Malvern Instruments) at 25 ^{o}C in water.



Scheme S1. Synthesis of PEG-*b*-PTyr copolymers.

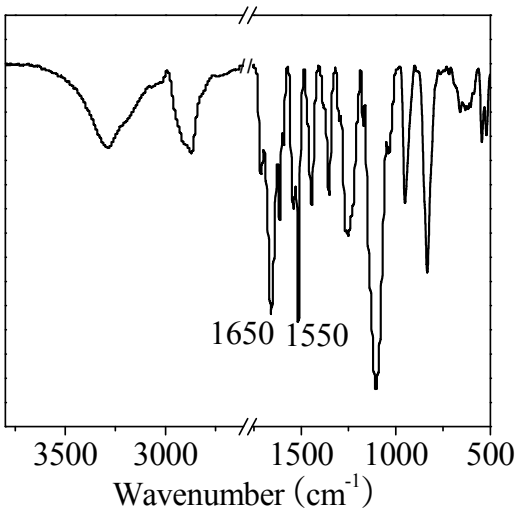


Fig. S1 FTIR spectrum of PEG-*b*-PTyr copolymer (**Table 1**, Entry 1).

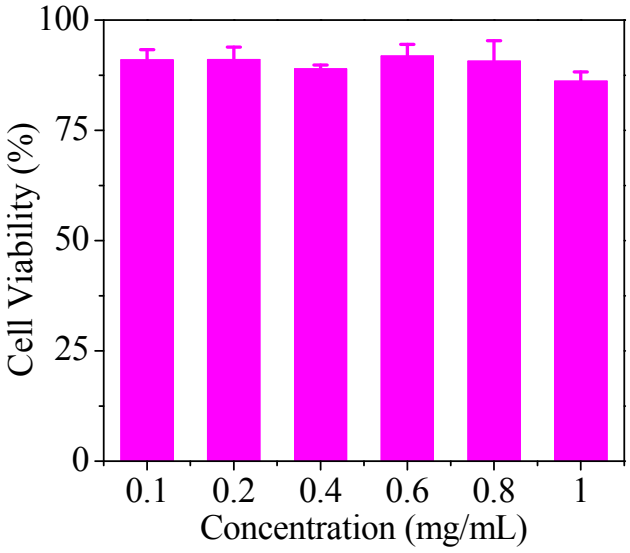


Fig. S2 MTT assays of blank PTNs in L929 cells following 48 h incubation.