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Electronic Supplementary Information

Mesomeric configuration makes polyleucine micelle an optimal nanocarrier

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1 Materials and methods

1.1 Materials

D-Leucine (D-Leu), L-leucine (L-Leu), and cyclo(Arg-Gly-Asp-D-Phe-Cys) (c(RGDfC)) were purchased from GL Biochem, Ltd. (Shanghai, P. R. China). Allyloxy poly(ethylene glycol) (APEG; number-average molecular weight (M_n) = 4600 g mol^{-1}) was obtained from Jiangsu Haian Petrochemical Plant (Nantong, P. R. China). D-Leucine *N*-carboxyanhydrides (D-Leu NCA), L-leucine *N*-carboxyanhydrides (L-Leu NCA), and amino-terminated APEG (APEG-NH_2) were synthesized according to the proposals described in our previous work.[1] 4,4′-Azobis(4-cyanovaleric acid) (ACVA) was purchased from Acros Organics (Beijing, P. R. China). Doxorubicin hydrochloride (DOX·HCl) was got from Beijing Huafeng United Technology Co., Ltd. (Beijing, P. R. China). *N,N*-Dimethylformamide (DMF) was reserved with calcium hydride (CaH_2) for at least 7 days and then gathered by vacuum distillation before use. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (Shanghai, P. R. China). All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, P. R. China) and used as obtained. Clear 6-well and 96-well tissue culture polystyrene (TCP) plates were got from Corning Costar Co. (Cambridge, MA, USA). The deionized water was prepared using a Milli-Q Water Purification equipment (Millipore Co., MA, USA).

1.2 Syntheses of APEG-*b*-PLeu copolymers

As shown in Scheme S1, APEG-*b*-PLeu was synthesized by the ring-opening polymerization (ROP) of Leu NCA in anhydrous DMF with APEG-NH_2 as a macroinitiator. In a typical synthesis procedure of APEG-*b*-PDLeu, a trace of water in APEG-NH_2 was first removed by being azeotropically distilled with toluene at 120 °C for 2 h. Thereafter, D-Leu NCA and anhydrous DMF were added. The polymerization was performed at room temperature for 3 days, and then the reaction solution was precipitated into a large excess of diethyl ether. The obtained solid was dried overnight under vacuum at room temperature. In the same way, APEG-*b*-PLLeu and APEG-*b*-PDLLeu were prepared by the ROP of L-Leu NCA, and equivalent D-Leu NCA and L-Leu NCA initiated by APEG-NH_2 (Scheme 1B and 1C), respectively. All the yields were appropriate 85%.

1.3 Synthesis of cRGD-PEG-*b*-PDLLeu

cRGD-PEG-*b*-PDLLeu was synthesized through the thiol-ene click reaction between the mercapto group in c(RGDfC) and allyloxy group in the APEG of APEG-*b*-PDLLeu (Scheme 1D). Briefly, c(RGDfC), APEG-*b*-PDLLeu, and ACVA were dissolved in DMF in a flask. After deoxygen through three freeze–pump–thaw cycles, the polymerization was performed at 70 °C for 24 h. And then, the solution was precipitated into excess diethyl ether. The obtained solid was redissolved in DMF, dialyzed against water for 3 days (cellulose membrane, molecular weight cut-off (MWCO) = 3500 Da), and then lyophilized. The yield was around 70%.

1.4 Preparations of micelles

Micelles were produced by a commonly used dialysis approach. Concretely, 5.0 mg of APEG-*b*-PDLeu, APEG-*b*-PLLeu, APEG-*b*-PDLLeu, or equivalent APEG-*b*-PDLeu and APEG-*b*-PLLeu was dissolved in 2.0 mL of DMF and kept stirring for 5 h at room temperature. Then 2.0 mL of phosphate-buffered saline (PBS) was added into the above solution under stirring. The obtained copolymer solution was dialyzed with a cellulose membrane against Milli-Q water for 24 h after being stirred for 3 h at room temperature (MWCO = 3500 Da), and Milli-Q water was replaced

every 4 h. Eventually, the volume of the above solution was set to be 5.0 mL to obtain the micelle solution. The above four kinds of micelles with dextrorotatory, levorotatory, mesomeric, and racemic polypeptide cores were referred as PDM, PLM, PDLM, and PD/LM, respectively.

1.5 Characterizations

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AV 600 NMR spectrometer (Billerica, MA, USA) in deuterated trifluoroacetic acid (TFA-*d*). Fourier transform infrared (FT-IR) spectra were recorded on a Bio-Rad Win-IR instrument (Bio-Rad Laboratories Inc., Cambridge, MA, USA) using potassium bromide (KBr) method. Dynamic laser scattering (DLS) measurements were measured on a WyattQELS instrument with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology Corporation, Santa Barbara, CA, USA). Meanwhile, the scattering angle was fixed at 90°. Transmission electron microscopy (TEM) measurements were performed on a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 100 kV. Circular dichroism (CD) spectra were recorded on a Jasco-710 spectropolarimeter (Jasco, Tokyo, Japan).

1.6 Preparations of DOX-loaded copolymer micelles

A general nanoprecipitation technique was employed to encapsulate DOX with micelles. Briefly, 5.0 mL of the DMF solution of APEG-*b*-PDLeu (20.0 mg), APEG-*b*-PLLeu (20.0 mg), APEG-*b*-PDLLeu (20.0 mg), equivalent APEG-*b*-PDLeu (10.0 mg) and APEG-*b*-PLLeu (10.0 mg), or cRGD-PEG-*b*-PDLLeu (20.0 mg) was mixed with 5.0 mL of DOX·HCl solution in DMF (4.3 mg) by stirring at room temperature for 12 h. The mixture solution of 9.0 mL of Milli-Q water and 1.0 mL of phosphate-buffered saline (PBS) at pH 7.4 was added dropwise under stirring at 25 °C for 24 h. And then, the solution was dialyzed with cellulose membrane (MWCO = 3500 Da) against Milli-Q water for 12 h to eliminate DMF and excess free DOX. The Milli-Q water was refreshed every 2 h. The above procedure was performed in the dark. After filtration, the solution was lyophilized. The five drug-loaded micelles were marked as PDM/DOX, PLM/DOX, PDLM/DOX, PD/LM/DOX, and R-PDLM/DOX, respectively.

To determine the drug-loading content (DLC) and drug-loading efficiency (DLE) of DOX-loaded micelles, the loading micelles were dissolved in DMF at 25 °C, stirred for 24 h, and then analyzed by fluorescence spectroscopy on a Photon Technology International (PTI) Fluorescence Master System with Felix 4.1.0 software (PTI, Lawrenceville, NJ, USA; λ_{ex} = 480 nm and λ_{em} = 590 nm). The DLC and DLE of laden micelles were calculated by the below Equations (1) and (2), respectively.

$$DLC \quad (wt.\%) \quad = \frac{amount\ of\ drug\ in\ micelle}{amount\ of\ drug - loaded\ micelle} \times 100 \tag{1}$$

$$DLE \quad (wt.\%) \quad = \frac{amount\ of\ drug\ in\ micelle}{total\ amount\ of\ feeding\ drug} \times 100 \tag{2}$$

1.7 *In vitro* drug release

The release behaviors of DOX-loaded micelles except R-PDLM/DOX were investigated in 0.01 M PBS at pH 7.4. Typically, the weighed freeze-dried DOX-loaded micelles were suspended in 10.0 mL of PBS at pH 7.4 and moved into a cellulose membrane (MWCO = 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag into 100.0 mL of PBS at pH 7.4, 37 °C with continuous swaying of 70 rpm. At predetermined intervals, 2.0 mL of external release medium was taken out, and the same volume of fresh PBS was added. The amount of released DOX was determined by fluorescence spectroscopy as mentioned above.

1.8 Intracellular DOX release

The cellular uptakes and intracellular release behaviors of R-PDLM/DOX and PDLM/DOX were determined by confocal laser scanning microscopy (CLSM) and flow cytometry (FCM) toward human breast cancer MDA-MB-231 cells. MDA-MB-231 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, P. R. China) and cultured in complete DMEM supplemented with 10% (*V*/*V*) FBS, penicillin (50IU mL^{-1}), and streptomycin (50.0 IU mL^{-1}) at 37 °C in a 5% (*V*/*V*) carbon dioxide (CO_2) atmosphere.

1.8.1 CLSM. MDA-MB-231 cells were seeded in a 6-well plate at a density of 1.0×10^5 cells per well in 2.0 mL of complete DMEM. After being incubated at 37 °C for 24 h, the culture medium was replaced with the complete DMEM containing DOX-loaded micelles or free DOX at a final DOX concentration of 2.5 mg L^{-1}, and the incubation was performed for an additional 2 h. Subsequently, the culture medium was removed, and the cells were washed with PBS three times. Afterwards, the cells were fixed with 4% (*W*/*V*) PBS-buffered paraformaldehyde for 30 min at 25 °C, and thereafter cell nucleus was dyed with DAPI. The CLSM microimages of cells were obtained on a CLSM (ZEISS LSM 780, Germany).

1.8.2 FCM. The cells were seeded in a 6-well plate at a density of 1.0×10^5 cells per well in 2.0 mL of complete DMEM. After being incubated at 37 °C for 24 h, the culture medium was removed, the DMEM with DOX-loaded micelles or free DOX at a final DOX concentration of 5.0 mg L^{-1} was supplemented, and the cells were incubated for an additional 2 h. The culture medium was removed, and the cells were washed with PBS three times and treated with trypsin. And then, 1.0 mL of PBS was added to each well to make the cells suspend, and the solution was centrifuged for 5 min at 3000 rpm. After the removal of supernatant, the cells were resuspended in 0.3 mL of PBS. The data for 10,000 gated events were collected, and the analyses were performed using a flow cytometer (Beckman, California, USA).

1.9 *In vitro* cytotoxicity assays

The cytotoxicities of blank micelles, the DOX-loaded micelles, and free DOX were determined by a MTT assay toward MDA-MB-231 cells *in vitro*. The cells were seeded in 96-well culture plates at a density of about 5000 cells per well in 200.0 μL of complete DMEM and incubated for 24 h, followed by removing the culture medium and then adding the blank micelles (0 – 100.0 mg L^{-1}), or DOX-loaded micelles or free DOX as control (0 – 10.0 mg L^{-1} DOX equivalent) in 200.0 μL of complete DMEM. The cells were subjected to MTT assay after being incubated at 37 °C for 72 h (blank micelles) or 24 h (DOX formulations). The absorbance of solution was measured on a Bio-Rad 680 microplate reader (Hercules, CA, USA) at 490 nm. Cell viability was calculated based on Equation (3).

$$Cell\ viability\ (\%) = \frac{A_{sample}}{A_{control}} \times 100\% \qquad (3)$$

In Equation (3), A_{sample} and $A_{control}$ represented the absorbances of sample and control wells, respectively.

1.10 Statistical Analyses

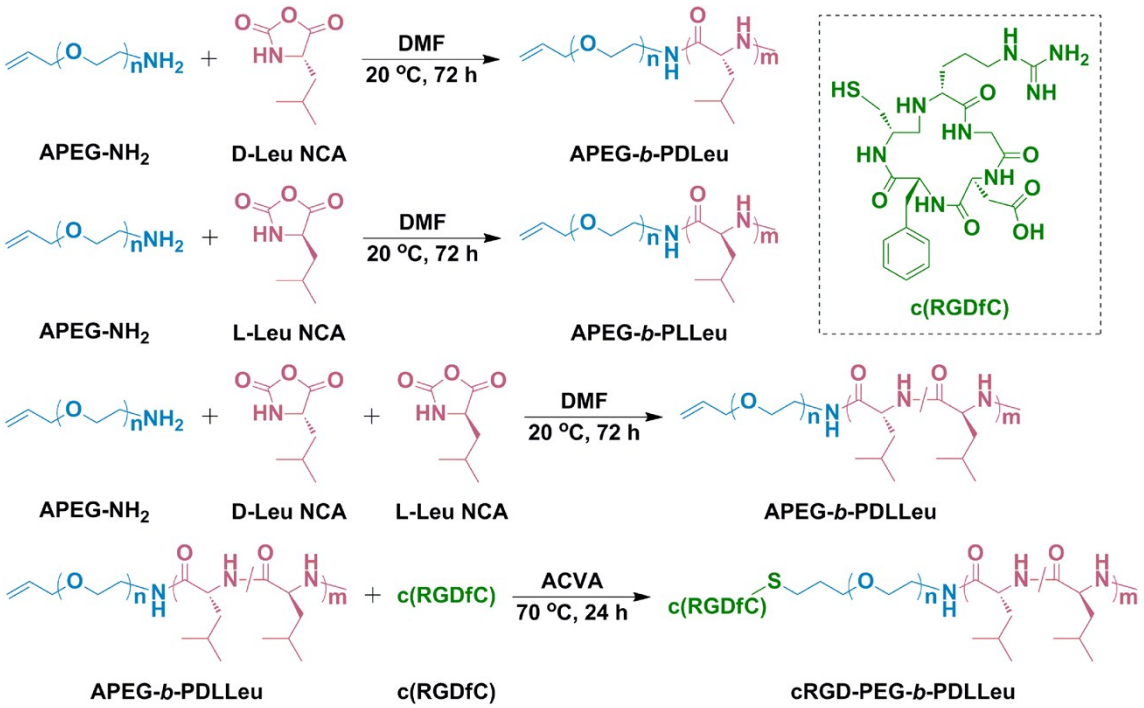
All statistical data were expressed as mean ± standard deviation. All experiments were repeated at least three times. The significance of the results was determined with ANOVA test and repeated measure tests with Bonferroni correction. All data analyses were performed using SPSS statistical software (Version 15.0; SPSS Inc.,Chicago, IL, USA). $P < 0.05$ was considered statistically significant, and $P < 0.01$ and $P < 0.001$ were considered highly significant.

Notes and references

1 J. Ding, C. Li, Y. Zhang, W. Xu, J. Wang and X. Chen, *Acta Biomater.*, 2015, **11**, 346–355.

Table S1 Properties of PLeu-contained copolymer micelles.

Entry	R_h (nm)	DLC (wt.%)	DLE (wt.%)	IC_{50} (mg L^{-1})
PDM	66.5 ± 5.8	3.4	17.4	—
PLM	70.5 ± 6.0	4.6	24.1	—
PDLM	52.9 ± 3.3	6.7	36.0	1.57
PD/LM	59.4 ± 5.8	6.1	32.5	—
R-PDLM	52.3 ± 2.1	8.2	44.8	0.11



Scheme S1 Synthesis pathways for APEG-*b*-PDLeu (A), APEG-*b*-PLLeu (B), APEG-*b*-PDLLeu (C), and cRGD-PEG-*b*-PDLLeu (D).

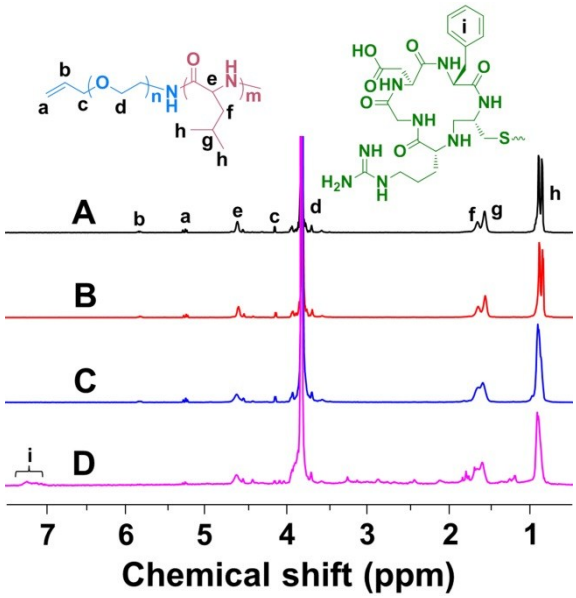


Fig. S1 ^{1}H NMR spectra of APEG-*b*-PDLeu (A), APEG-*b*-PLLeu (B), APEG-*b*-PDLLeu (C), and cRGD-PEG-*b*-PDLLeu (D) in TFA-*d*.

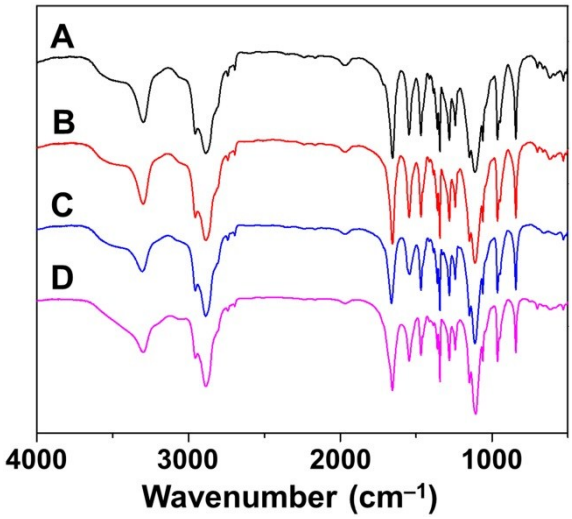


Fig. S2 FT-IR spectra of APEG-*b*-PDLeu (A), APEG-*b*-PLLeu (B), APEG-*b*-PDLLeu (C), and cRGD-PEG-*b*-PDLLeu (D).

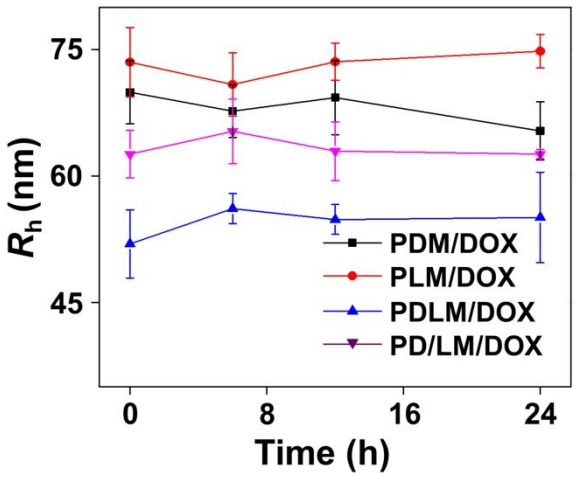


Fig. S3 R_h changes of PDM, PLM, PDLM, and PD/LM *versus* time in PBS at pH 7.4, 25 °C.

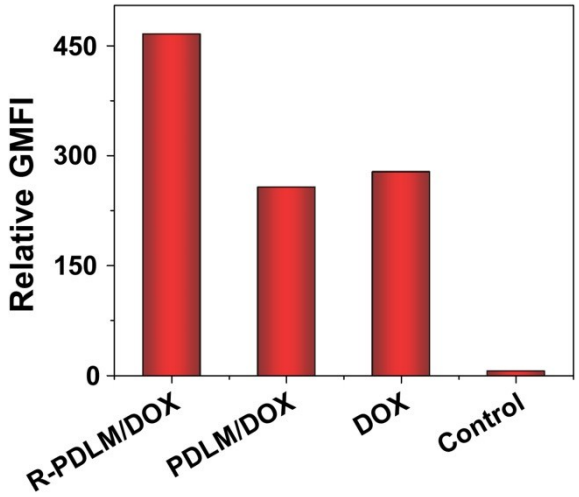


Fig. S4 Relative geometrical mean fluorescence intensities (GMFIs) of MDA-MB-231 cells without treatment or after incubation with R-PDLM/DOX, PDLM/DOX, or free DOX for 2 h.

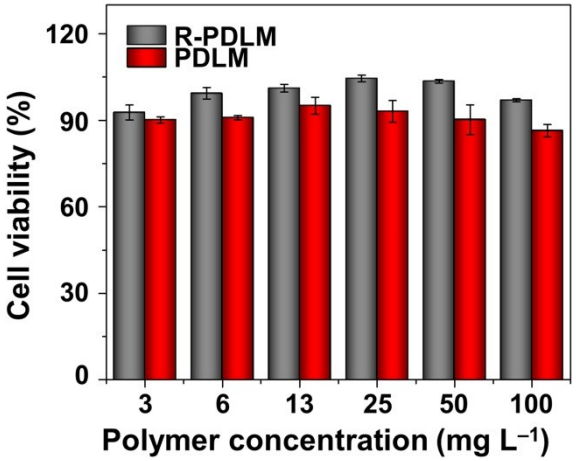


Fig. S5 Survival rates of MDA-MB-231 cells after incubation with R-PDLM and PDLM for 72 h.