Electronic Supplementary Material (ESI) for Polymer Chemistry

Pluronic L61 as a long-circulating modifier for enhanced liposomal delivery of cancer drugs

Materials and Methods

1. Materials

Pluronic L61 was a product of BASF (USA). Soybean lipid (SPC) and DSPE-PEG2000 were purchased from Toshisun Enterprise Co., Ltd (Shanghai, China). Paclitaxel was obtained from Zhaolonghaiwang Biotechnology Co., Ltd (Xi'an, China). Cy5 fluorescence dye was acquired from Biotium, Inc. (USA). MCF-7 cell line was a generous gift of Pharmaceutical Institute, Zhejiang University, and was maintained with RPMI 1640 culture medium with 10% fetal bovine serum (FBS).

2. Pluronic L61 modification of paclitaxel loaded liposomes

Liposomes were prepared by using a film hydration method with sonication modified from our previous report. [1] In brief, SPC (2 mg) was dissolved in chloroform in a pear-shaped flask, and a certain quantity of paclitaxel and L61 ethanol solution was added. The organic solvent was removed by reduced pressure evaporation. The lipid/L61/paclitaxel film was then hydrated with 1ml of saline. The resultant suspension was sonicated using a sonifier (200 w, 2 min) equipped with ice-water bath. The final products contained paclitaxel (15 μg/ml) and SPC (2 mg/ml), with L61 concentration varying from 0% to 2% (w/w) in the different formulations.

DSPE-PEG modified liposome was also prepared as described above, with the DSPE-PEG concentration is 0.33 mg/ml (DSPE-PEG: SPC=1:5, w/w). The Cy5-liposomes were also prepared using the same "thin-film" procedures.

3. Particle size and morphology

Particle size of the diluted samples was determined by using a Nano-S90 Zetasizer (Malvern, UK). A transmission electron microscope was utilized for the morphological observation of the liposomes (JEM-1230, JEOL, JP). Negative staining was processed using phosphotungstic acid.

4. Drug release study

Release of paclitaxel from liposome was investigated using the modified method previously discribed by Yang et al. [2] In brief, 3 ml of liposomal solution with aliquots of paclitaxel about 300ug were sealed in dialysis bag (MW: 12,000) and be immersed into 300 ml PBS (pH 7.4) containing 0.1% (v/v) Tween 80. The application were kept on a shaker (180 rpm) at 37°C. With predetermined time interval, 4 ml solution was taken placed by fresh PBS for analysis. Cumulative release of free DOX was also conducted with the same procedure. Paclitaxel was determined by HPLC method (C18, 4.6×250 mm, 5μm, 40% acetonitrile, detection wavelength at 220 nm).

5. Liposome stability test

Turbidity has been well employed as an index to assess serum protein binding efficiency of nanomaterials.[1] In brief, 20% (v/v) fetal blood serum was mixed with equivalent volume of liposome (1mg/ml). Turbidity was determined by UV absorbance under 400 nm at different time interval.

6. The protein-binding resistant effect of L61

To access the effect of L61 on reduction of protein adsorption, the 96-well microplates were coated with different amounts of L 61. Then the FITC-labeled BSA was added and incubated at 37 °C for 2 hours for protein adsorption. The microplates were

washed with water for 5 times to remove unabsorbed protein. To quantify the surface adsorbed protein in the well, 200 μl water was added and fluorescence was detected to quantify the amount of surface absorbed protein.

4. *In vitro* cytotoxicity assay

Human breast carcinoma cells (MCF-7) were cultured in RPMI 1640 medium containing 10% of fetal bovine serum and 2% of penicillin/streptomycin solution (10,000 IU/ml). The cytotoxicity was assessed by a routine MTT method. The cultured MCF-7 cells were exposed to the liposomal samples diluted with 1640 medium (equal to 75 ng paclitaxel per well). At 24 and 48hr after treatment, cell viability was measured by a standard MTT assay. Each sample was run quadruply.

5. *In vivo* imaging study

All the animal experiments were approved by the Animal Care and Use Committee of Zhejiang University. The anesthetized Balb/c nude mice were given 100 μl of Cy5 liposome solution through tail vein injection. *In vivo* fluorescence optical evaluation of intratumor distribution was conducted by using the Maestro I system (CRi, USA) at 2, 5, 15, 30, 60, 180, 360, 720 and 1440 min postdose, respectively. The Cy5 excitation filter: 615–665 nm, emission filter: 700 nm. The data was analyzed with Maestro software (Version 2.5.42), and the autofluorescence of animals was subtracted. The *in vivo* fluorescence in the brain and liver was analyzed with Image J software, and the semi-quantitative comparison of various liposomes accumulated in the organs was conducted.

6. *In vivo* tumor accumulation study

Tumor-bearing animal model were established by injecting 200 μl of MCF-7 cells (1×10^6/ml) suspension to the armpits of the male Balb/c nude mice. The tumor-bearing

nude mice were injected the Cy5-labelled Pluronic L61-liposomes (100 μl) for *in vivo* fluorescence imaging by Maestro I system with a procedure as described above. Liposomes without L61 modification were used as control.

7. Antitumor treatment in Mice

The male ICR mice weighed from 18 to 20 g were randomly divided into 4 groups: i) saline, ii) SPC liposomes, iii) PEG-liposomes, and iv) 0.1% L61-liposomes. Each mouse injected subcutaneously with 100 μl of cell suspension containing 3.26×10^7 H22 cells at the axillary space. The pharmacodynamic test was started when the tumor volumes developed to approximately 1.5 × 1.5 cm (L × W) (L is the long diameter of the tumor and W is the short diameter). Liposomes were prepared and injected at a paclitaxel dose of 6 mg/kg into the mice through the tail vein. The body weight and tumor sizes of the mice were measured daily and the variation ratios were calculated.

8. Data analysis

The results are presented as means ± standard deviations (SD). Where applicable, multiple comparisons were evaluated by one-way ANOVA.

Supplementary Table and Figures:

Table S1. Particle size and PDI of liposomes

Hydrodynamic particle size and PDI of liposome							
L61 Con. (%)	0	0.02	0.05	0.1	0.2	0.5	1
Size/nm	155±14.6	138±10.2	129±14.3	104±11.8	145±13.6	168±17.1	246±18.3
PDI	0.323	0.360	0.371	0.306	0.354	0.328	0.248

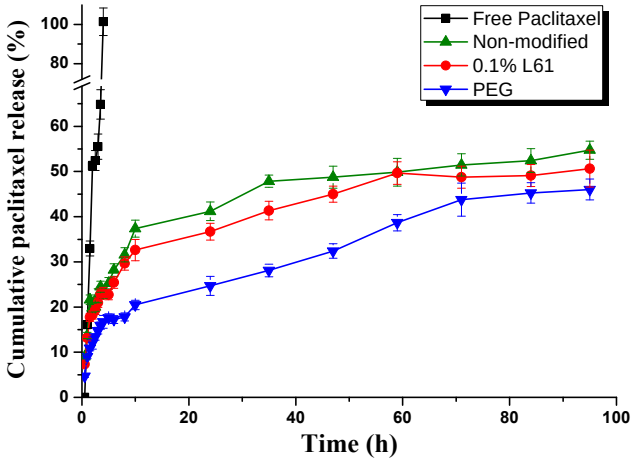

Fig. S1 Drug release profiles from various liposomes

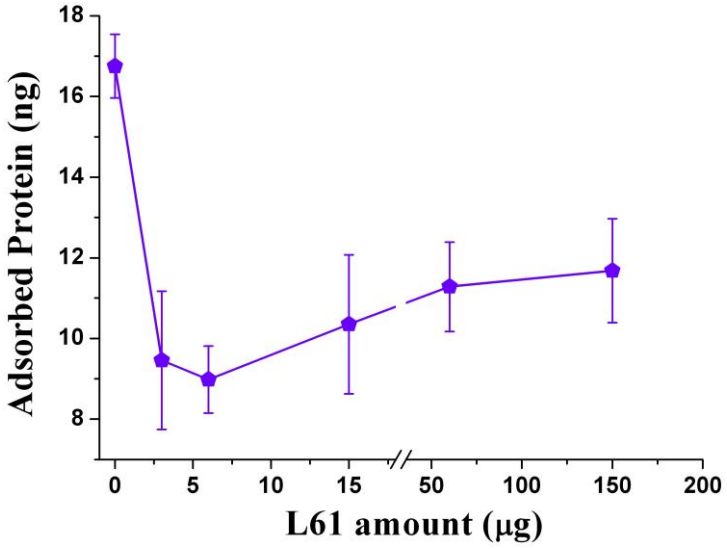

Fig. S2 Protein-binding resistant effect of Pluronic L61 coating

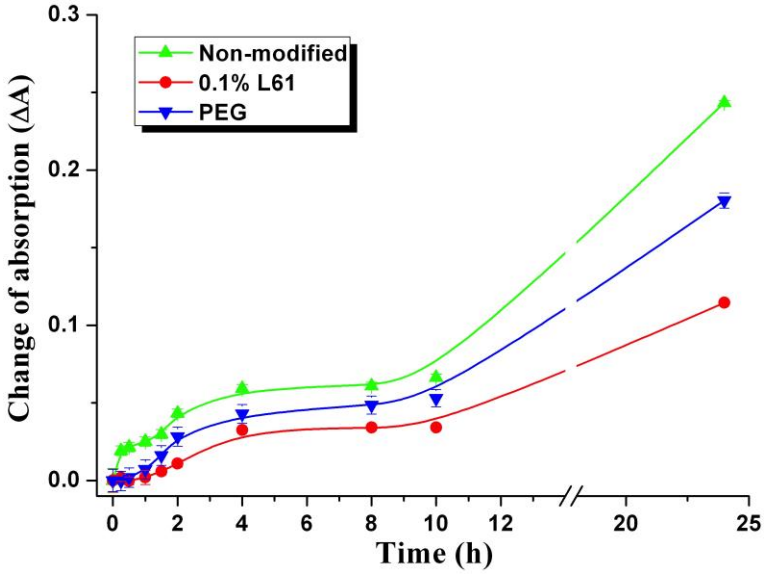

Fig. S3 Turbidity change of the liposomes

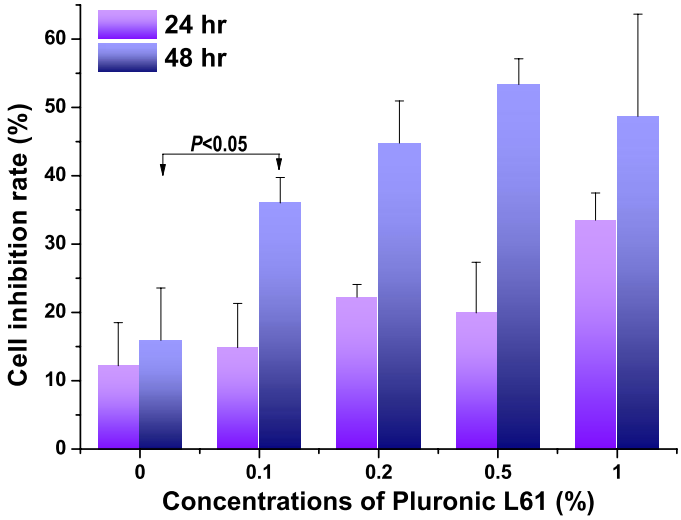

Fig. S4 Cell proliferation studies of paclitaxel L61-modified liposomes. It showed a slight trend that high concentration of L61 brought higher cytotoxicity at 48 h, presumably due to the inhibition effect of L61.

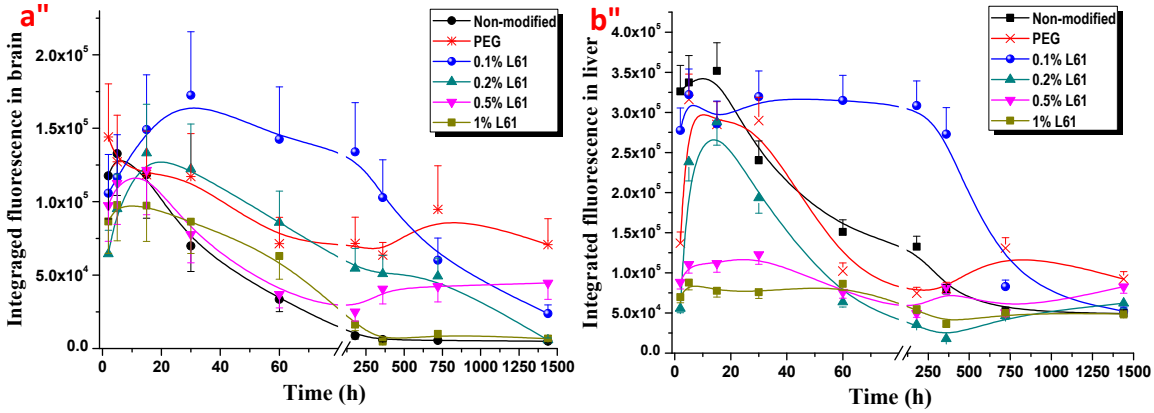

Fig. S5 The *in vivo* fluorescence in the brain (a) and liver (b) was analyzed with Image J software and the semi-quantitative comparison of various liposomes accumulated in the organs.

Reference:

1. Y. Huang, Y. Rao, J. Chen, V. C. Yang and W. Liang, *J Biomed Mater Res A*, 2011, **96**, 513-519.
2. S. Chung, C. Shim, D. Kim, *Int J Pharm,* 2007, **338**, 317-326.