Supplementary Material

Low molecular weight heparin-coated and dendrimer-based core-shell nanoplatfrom with enhanced immune activation and multiple anti-metastatic effects for melanoma treatment

Chunyu Xia, Sheng Yin, Shanshan Xu, Guangyao Ran, Miao Deng, Ling Mei, Xian Tang, Jingdong Rao, Man Li, Zhirong Zhang, Qin He*

Key Laboratory of Drug Targeting and Drug Delivery Systems, West China School of Pharmacy, Sichuan University, No. 17, Block 3, Southern Renmin Road, Chengdu 610041, China.

*Corresponding author: Fax/Tel: 86 28 85502532; Qin He: qinhe@scu.edu.cn
Supplementary methods

Materials

Generation 4 PAMAM dendrimer was purchased from Dendritech, Inc. (Midland, MI). Methoxy PEG-Succinimidyl Valerate (mPEG-SVA, Mw = 5000) was purchased from Laysan Bio Inc. (Arab, USA). Doxorubicin hydrochloride and low molecular weight heparin (LMWH) were purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China). DNA oligonucleotides sequences were as follows: CpG ODNs (1826): 5′-TCC ATG ACG TTC CTG ACG TT-3′; DNA template for Cy5-DNA: 5′-AAG AGA ACC TGG GAG TAG ATA AGG T-3′. Matrigel was purchased from BD Biosciences (San Jose, CA). FITC-label anti-mouse CD4, CD8a, CD40, CD80 and CD86, PE-labeled anti-mouse CD3e, Foxp3 and CD11c were purchased from eBioscience (San Diego, CA, USA). Interferon-γ (IFN-γ) Elisa kit was purchased from Wuhan ColorfulGene Biological Technology (Wuhan, China). All organic solvents used in this study were of analytical grade or better.

Cell lines and animals

Mouse melanoma cell line (B16F10) was cultured in DMEM cell culture medium contain 10% of fetal bovine serum (FBS), 100 μg/mL streptomycin sulfate and 100 U/mL penicillin. Cells were maintained in 37°C incubators under a humidified atmosphere with 5% CO₂. C57 mice (5–8 weeks, 18–22 g, SPF) were purchased from Chengdu Dashuo Biotechnology Co., Ltd (Chengdu, China) and maintained under standard housing conditions. All animal experiments were performed under the guidelines evaluated and approved by the ethics committee of Sichuan University.

Cellular uptake assay

In vitro cellular uptake assay of different formulations on B16F10 cells was investigated by laser scanning confocal microscope. Briefly, B16F10 cells were seeded into 6-well plates at a density of 1× 10⁵ cells/well and cultured for 24 h. After incubation with HEPES, PPD or LMWH/PPD for 2 h, the medium was removed and
the cells were fixed by 4% paraformaldehyde. Then, the cells were incubated with DAPI for 10 min at room temperature and observed under laser scanning confocal microscope (LSM 800, Zeiss, Germany).

**Hemolysis assay**

Hemolysis assay was performed to investigate the hemolysis activities of PP and LMWH/PP. Fresh blood was collected from mice using ACD solution (sodium citrate 1.33 g, citric acid 0.47 g, glucose 3 g, 100 mL ultrapure water) as an anticoagulant. The red blood cells were separated by centrifugation and then resuspended with PBS to a concentration of 2%. Various concentrations of PP and LMWH/PP (the concentrations of PP in PP and LMWH/PP ranged from 10 μg/ml to 600 μg/ml) were incubated with the 2% suspension of red blood cells (mixed with equal volume) at 37°C for 1 h. Then, each sample was centrifuged at 3000 rpm for 15 min. The absorbance of supernatant was analyzed at 540 nm using a Varioskan Flash Multimode Reader (Thermofisher, USA) and the morphology of red blood cells was observed under a microscope. 1% Triton X-100 and PBS-treated suspensions were used as positive and negative controls, respectively. The hemolysis (%) was calculated according to the following formula: \((A_{\text{sample}} - A_{\text{PBS}})/(A_{\text{Triton X-100}} - A_{\text{PBS}}) \times 100\%\).

**Cytotoxicity assay**

The cytotoxicity of different formulations on B16F10 cells was evaluated by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. B16F10 cells were seeded into 96-well plates at a density of 3×10³ cells/well and cultured for 24 h. The cell culture media was removed and different formulations in a series of concentrations were added and incubated for another 24 h. Then, 20 μL MTT solution (5 mg/mL in PBS) was added and the plates were incubated for another 4 h at 37 °C. Finally, the culture media was removed and the residue was dissolved with 150 μL dimethyl sulfoxide (DMSO). The absorbance of each group was measured by
Varioskan Flash Multimode Reader (Thermofisher, USA) at 490 nm. Cell viability (%) = \( \frac{A_{\text{test}}-A_{\text{DMSO}}}{A_{\text{control}}-A_{\text{DMSO}}} \times 100\% \).

Supplementary Figures

![Supplementary Figures](image-url)
Figure S1. $^1$H-NMR spectra of (A) PAMAM (in D$_2$O), (B) mPEG-SVA (in D$_2$O), (C) PEG-PAMAM (in D$_2$O), (D) PEG-PAMAM-MA (in D$_2$O), (E) PEG-PAMAM-MA (in DMSO-d6), (F) PEG-PAMAM-MA-hydrazine (in DMSO-d6) and (G) PPD (in DMSO-d6). Red arrows and circles indicate the changes in the $^1$H-NMR spectra.
Figure S2. IR spectra of (A) PAMAM, (B) mPEG-SVA, (C) PEG-PAMAM, (D) PEG-PAMAM-MA, (E) PEG-PAMAM-MA-hydrazine and (F) PPD.
Figure S3. (A) HRMS of PEG-PAMAM. (B) Enlargement of the mass region of PEG-PAMAM. (C) HRMS of PEG-PAMAM-MA. (D) HRMS of PEG-PAMAM-MA-hydrazine. (D) HRMS of PPD.
**Figure S4.** The serum stability of LMWH/PPD/CpG within 24 h after incubation with fetal bovine serum (50% FBS) at 37°C.

**Figure S5.** Photo images of plastic pipes containing red blood cells after treated with different concentrations of LMWH/PP and PP.
Figure S6. Representative confocal micrographs of B16F10 cells after incubation with different formulations for 2 h and stained with DAPI to label nuclei. The scale bar indicates 20 μm.

Figure S7. (A) Cytotoxicity evaluation of Naked PP and LMWH/PP on B16F10 cells. (B) Cytotoxicity evaluation of free DOX+CpG, PPD, PPD/CpG and LMWH/PPD/CpG on B16F10 cells.
Figure S8. *In vivo* images of B16F10 tumor-bearing mice at 1 h, 2 h, 4 h, 12 h and 24 h after systemic administration of PPD-Cy7 or LMWH/PPD-Cy7, respectively.

Figure S9. CD4$^+$ T$_{eff}$/T$_{reg}$ ratios in B16F10 tumor-bearing C57BL/6 mice upon various treatments (n = 3, mean ± SD). *p < 0.05, **p < 0.01, ***p < 0.001.
Figure S10. H&E staining assays of major organs collected from B16F10 pulmonary metastasis C57BL/6 mice model on day 21 after treatments with HEPES, free LMWH, PPD, PPD/CpG, free DOX+CpG and LMWH/PPD/CpG, respectively. The scale bar indicates 200 μm.