Supporting Information

"Layer peeling" Co-delivery System for Enhanced RNA Interference-based Tumor Associated Macrophages-specific Chemoimmunotherapy

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Experimental section

Materials and cell lines: CMCS (Mw=50,000 Da) was bought from Haidebei Biological Engineering Co. (Jinan, China). Bifunctional N-hydroxylsuccinimide functioned polyethylene glycol (NHS-PEG-NHS, Mw=2000) was purchased from Biomatrik Technology Co. (Jiaxing, China). α-D-mannopyranosylphenyl isothiocyanate (MPITC) and PEI (Mw=25,000) were obtained from Sigma Aldrich (USA). NGR peptide ($GGCNGRCONH_2$) was synthesized by Shanghai Apeptide Co. (Shanghai, China). Negative control siRNA, Cy3 and Cy5 labeled nonspecific siRNA were obtained from Ribobio Co. (Guangzhou, China). Sense strand: 5'-UGCGCUACGAUCGACGAUGdTdT-3'; antisense strand: 5'-CAUCGUCGAUCGUAGCGCAdTdT-3'. RAW264.7 was bought form ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) media in a 37℃ incubator with 5% CO_2, supplemented with 10 % fetal bovine serum.

Synthesis of MPEI: MPEI was synthesis as the previous method as shown in Figure S1A.[1] 4.0 mg MPITC was dissolved in DMSO and this solution was added dropwise into PEI solution (8.0 mg dissolved in water). The reaction was proceed at room temperature for 24 h. The product was purified by dialysis method and was lyophilized to obtain solid powder. The structure of M-PEI was verified by ^1H NMR (D_2O, 300 MHz) and FTIR.

Synthesis of CPN: DOX-loading shell CDPN was synthesized by conjugating of DOX onto CPN as shown in Figure S1B. The pH-responsive charge reversal polymer CPN was synthesized according to a previously reported method[2, 3]. Briefly, NHS-PEG-NHS (9.0 mg), DMAP (1.5 mg) and NGR (3.0 mg) were mixed in 1 mL distilled water. After 2 h reaction in an ice bath, EDC (1.5 mg) was added and continue the reaction for 2 h. The solution was added to a 2.5 mL CMCS PBS solution (15.0 mg, pH=7.4) and stirring for 24 h. After purified by dialysis method (MWCO: 8,000-14,000), the products were lyophilized. The structure of CPN was verified by ^1H NMR (D_2O, 300 MHz)

In vitro release of siRNA and DOX from CDMPR: In order to evaluate the pH sensitivity of CDMPR, *in vitro* release of siRNA from CDMPR in 50 mM PBS (pH 5.0, 6.5, 7.4) was conducted. The siRNA release profiles of CDMPR were investigated using the dialysis method. Typically, 400 μL of CDMPR solution (equivalent to 8 μg siRNA) was added into the dialysis bags (MWCO=8,000-14,000) and then dialysis against 5 mL of PBS with different pH values in a 37±0.5℃ shaking water bath at 100 rpm. All the 5 mL incubation medium was withdrawn and replaced at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h with the same amount of fresh release

medium to maintain sink conditions. The amount of the released siRNA was analyzed using fluorospectro photometry with Quant-iTTM RiboGreen® RNA Reagent and Kit. The total content of siRNA was quantified by Spectrofluorometer (F-7000, Hitachi, Japan, λ_{ex}=492 nm, λ_{em}=526 nm). The amount of the released DOX was measured by microplate reader (λ_{ex}=480 nm, λ_{em}=620 nm).

In vitro cellular uptake assay of MPR and CDMPR: CLSM (CLSM, Zeiss, LSM-700) was used to investigate the internalization of PR, MPR in RAW264.7 cells and CDMPR in HUVEC cells. To investigate the NGR-targeting ability of CDMPR, 1.5×10^5/well HUVEC cells were seeded overnight. After 12 h, the CPN/M-PEI/Cy3-siRNA (Cy3-siRNA:100 nM) was incubated with the cells for 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h. Then cells were washed with PBS and were observed by CLSM. The mannose-targeting of PR and MPR was evaluated by the cellular uptake in RAW264.7 cells. 1×10^5/well RAW264.7 cells were seeded overnight. 15 μg/mL IL-4 (Peprotech, USA) was added to polarize the cells to M2 phenotype for 12 h. Then, M-PEI/Cy3-siRNA, PEI/Cy3-siRNA (100 nM) were incubated with the cells for 1 h and 4 h, respectively. The cells were fixed with 4% paraformaldehyde and were stained by Alexa Fluor 647™-F4/80 antibody and Alexa Fluor 488™-CD206 antibody (Biolegend, USA), respectively. After that, the sample was observed by CLSM. The competitive inhibition assay of MPR was carried out. The cells were pre-incubated with free mannose (3 mg/mL) for 1 h before incubated with the M-PEI/Cy3-siRNA. The following procedure was same as the cellular uptake of PR and MPR in RAW264.7.

In vitro cytotoxicity assay: The cytotoxicity of CDMPR was studied using MTT method in Hepa1-6 cells. Briefly, 5000/well Hepa1-6 cells were seeded in 96 well plates and incubated for 12 h. The CDMPR was pretreated with pH 6.5 medium for 1 h to mimic tumor microenvironment. Different concentration of free DOX and CDMPR (0.005, 0.05, 0.1, 1.0, 2.0 μg/mL) were added to cells for 48 h. Then, 20 μL MTT (5 mg/mL) was added to the well for 4 h. The supernatant was discard and 150 μL DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Model 680, BIO-RAD, USA). Cells incubate with DMEM were used as control. The relative cell viability (%) was calculated by the formula below:

$$\text{Relative cell viability (\%)} = (A_{sample}/A_{control}) \times 100\%$$

In vivo and ex vivo imaging study: In vivo and *ex vivo* real-time fluorescence imaging study was performed to evaluate the tumor targeting ability of CDMPR in Hepa1-6 bearing female C57BL/6 mice Female Balb/c mice model. When the tumors grew to ≥ 200 mm^3, CDMPR loaded Cy5.5-siRNA and free Cy5.5-siRNA (1.5 mg/kg Cy5.5-siRNA) was injected intravenously to the mice. The real-time fluorescence images

were observed with Xenogen IVIS Lumina system (Caliper Life sciences, USA) at 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after injection. Then the mice were sacrificed and major organs (heart, liver, spleen, lung, kidney and tumor) were excised for *ex vivo* imaging. All experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and the Animal Experiment Ethics Review of Shandong University.

Histological analysis: CDMPR, CDMPnR, mixture of DOX and CMPR, DOX solution and NS were injected into female C57BL/6 mice (siRNA at a dosage of 1.5 mg/kg) through the tail vein every 2 days for four times. 7 days post-injection, the mice were sacrificed and major organs including heart, liver, spleen, lung and kidney were excised and fixed in 4% (w/v) formaldehyde solution. The organs were then embedded in paraffin and stained with hematoxylin and eosin (H&E) for histological analysis. The slides of tumor tissue were tested by TUNEL assay and Ki67 stained for immunohistochemistry evaluation.

Statistical analysis: All studies were repeated at least three times and all data were reported as mean ± standard deviation (SD). Comparison between groups was analyzed by Student's t-test. Differences were considered to be statistically significant when p value was lower than 0.05, and it was considered to be highly significant when p value was lower than 0.01.

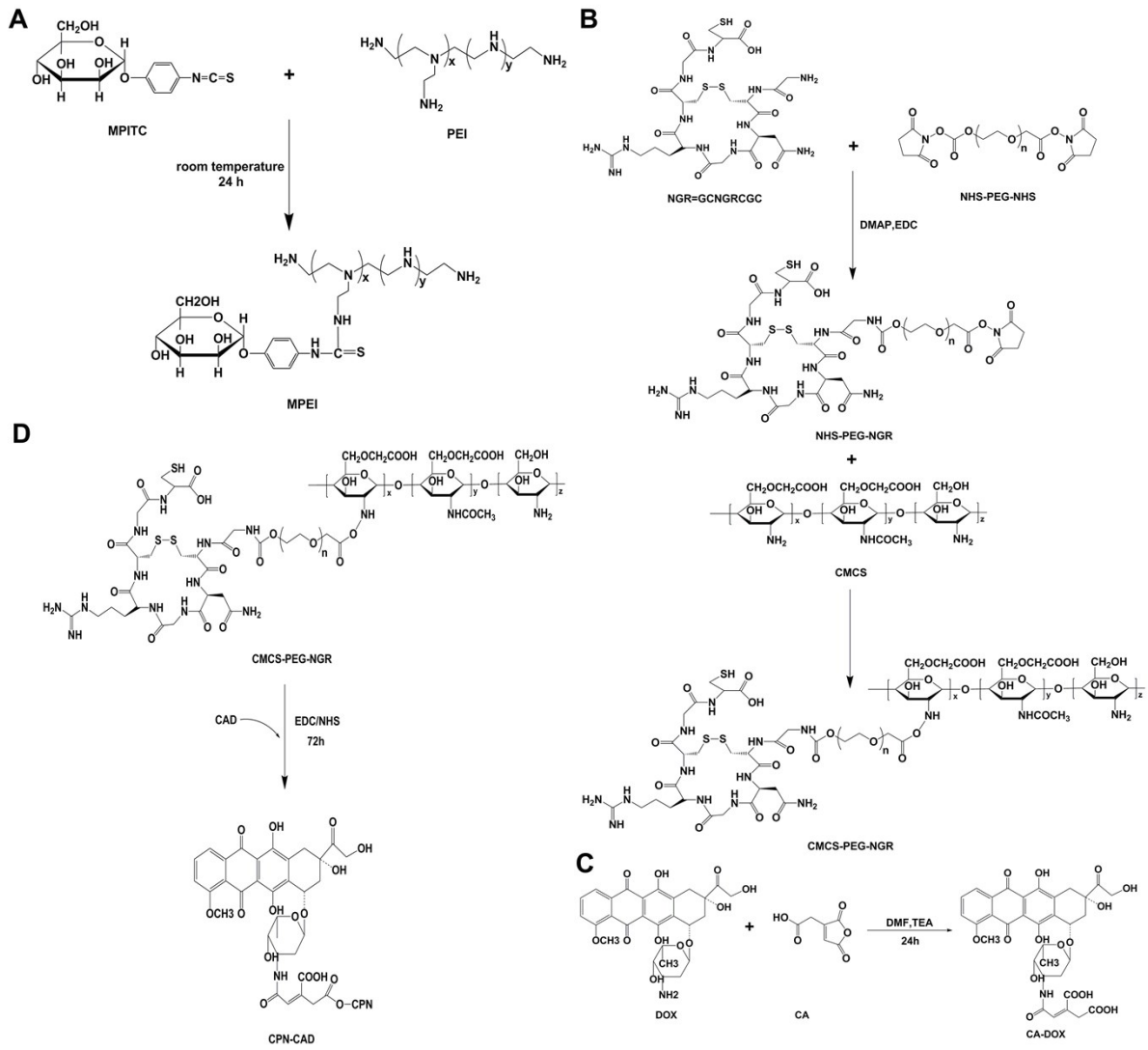

Figure S1 Synthesis route of M-PEI and CDPN. (A) synthesis route of M-PEI (B) synthesis route of CPN (C) synthesis route of CAD (D) synthesis route of CDPN.

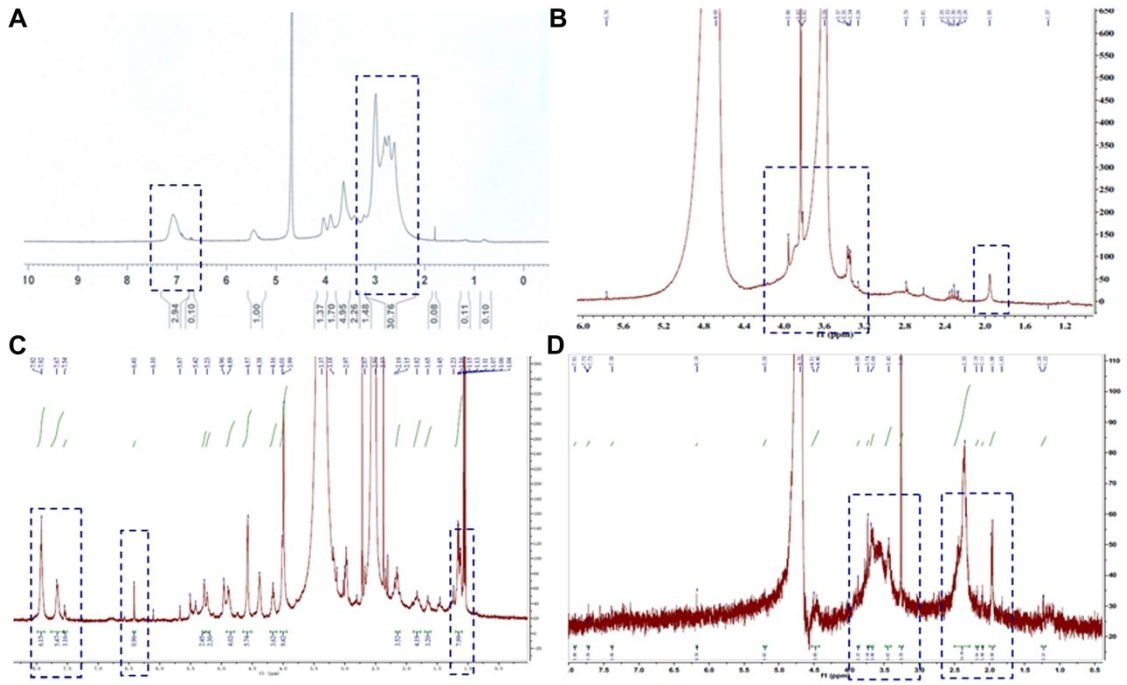

Figure S2 Characterization of M-PEI and CDPN. (A) ^{1}H-NMR spectra of M-PEI (B) ^{1}H-NMR spectra of CPN (C) ^{1}H-NMR spectra of CAD (D) ^{1}H-NMR spectra of CDPN.

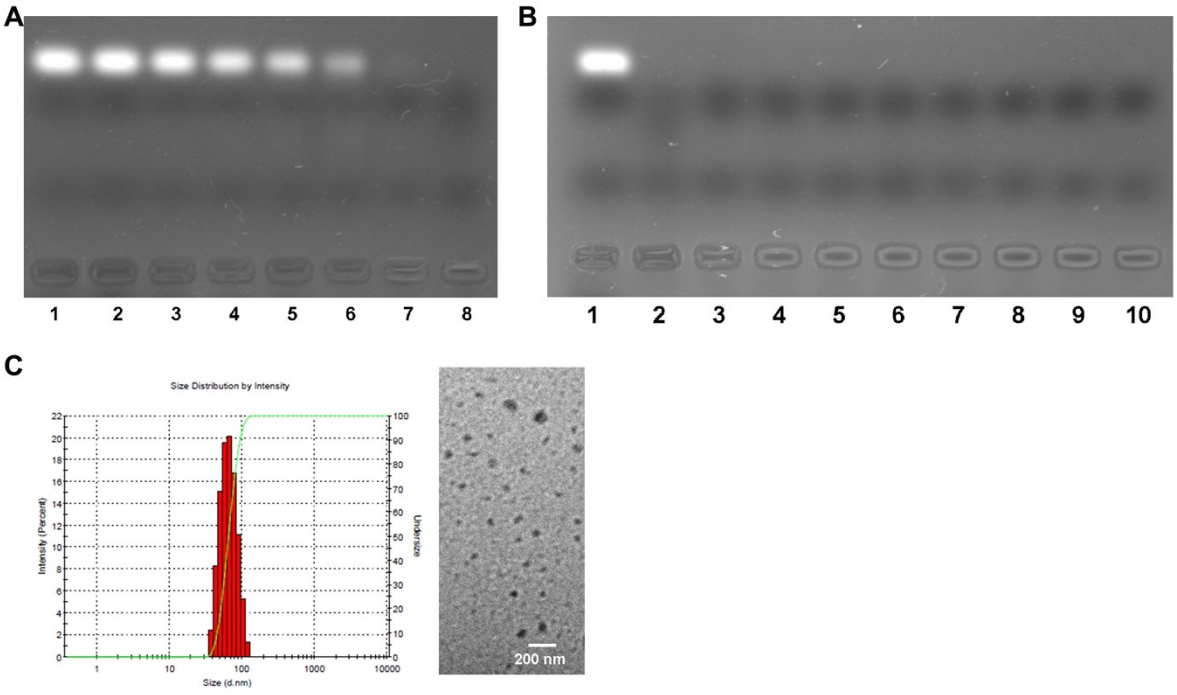

Figure S3 Characterization of MPR and CDMPR. (A) The siRNA retardation assay of MPR at various weight ratios of MPEI to siRNA: 1) naked siRNA ; 2) 1:16 ; 3) 1:8 ; 4) 1:4 ; 5) 1:2 ; 6) 1:1 ; 7) 2:1 ; 8) 4:1 (B) The siRNA retardation assay of CDMPR at various weight ratios of CDPN to siRNA: 1) naked siRNA ; 2) MPR ; 3)1:8 ; 4) 1:4 ; 5) 1:2 ; 6) 1:1 ; 7) 2:1 ; 8) 4:1 ; 9) 8:1 ; 10) 16:1 (C) Size distribution (left) and morphology (right) of MPR.

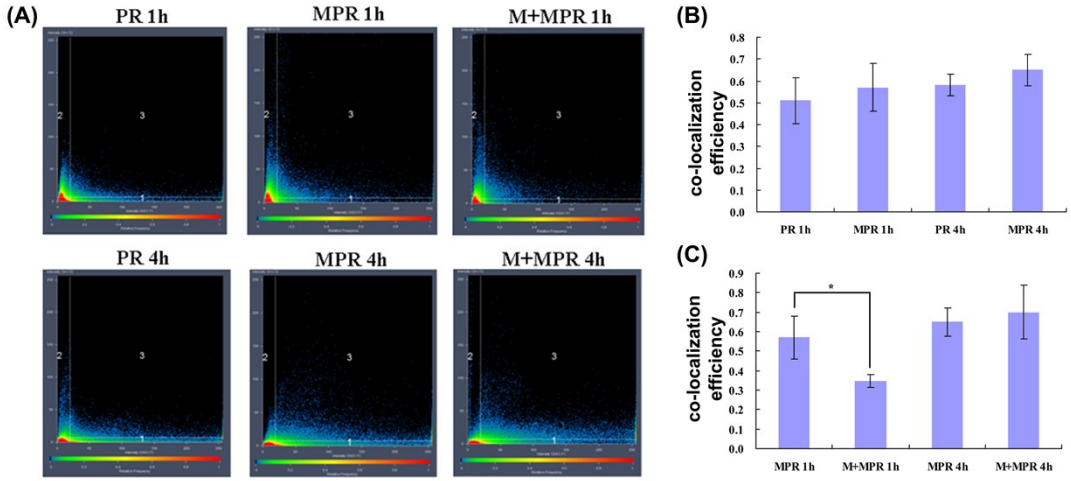

Figure S4 Co-localization efficiency of PR or MPR and M2 type RAW264.7 cells. (A) The density plot of the co-localization. (B) The co-localization efficiency of all the groups (n=3). (C) The co-localization efficiency of MPR and M+MPR (n=3).

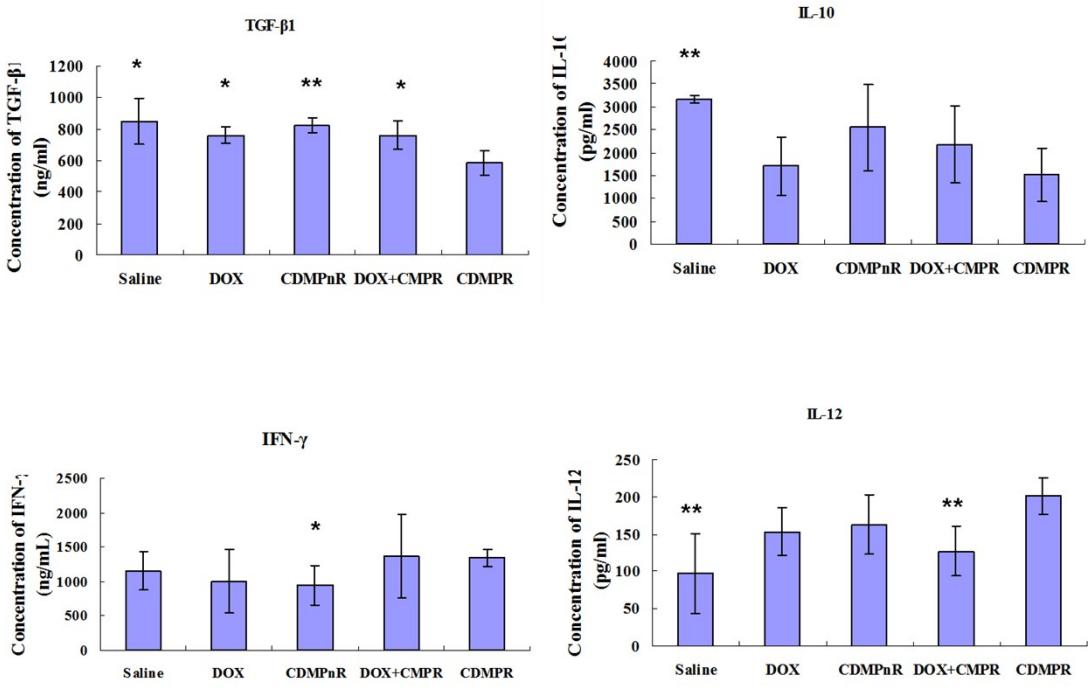

Figure S5 Cytokines evolution *in vivo*. Histograms of cytokines in peripheral blood from Hepa1-6 bearing female C57BL/6 mice after treatment with different formulations (n = 3), *$p < 0.05$, **$p < 0.01$ statistically significant difference compared with CDMPR group.

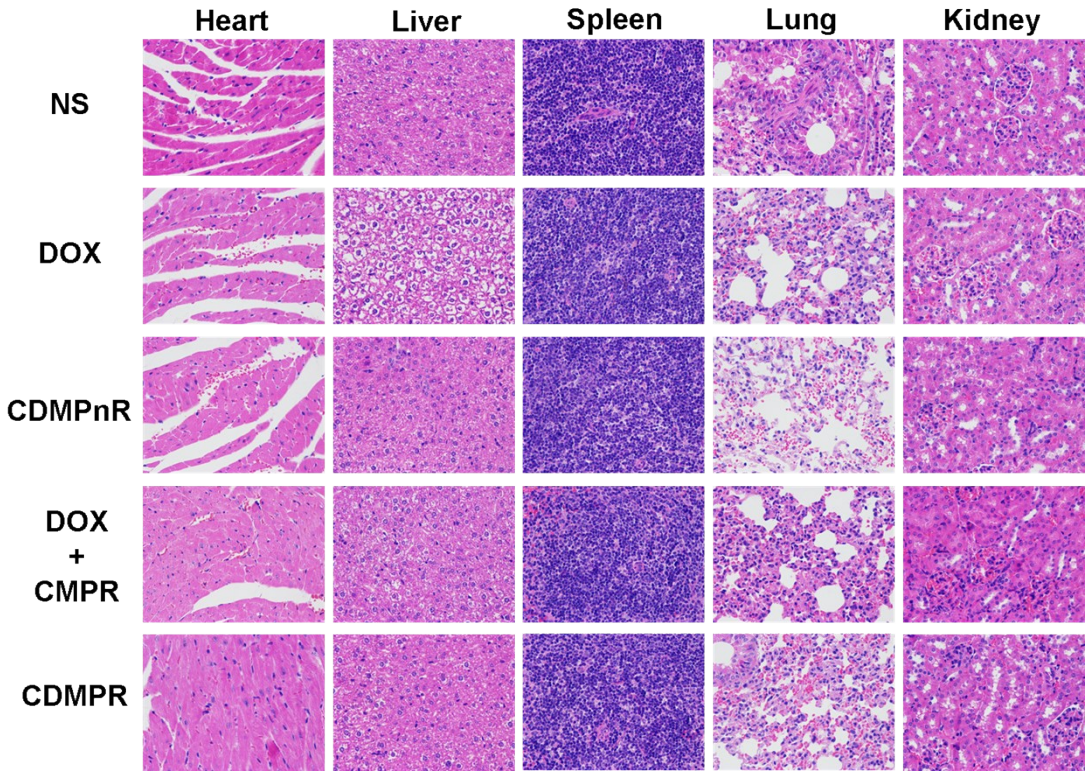

Figure S6 Histological assays. Representative microscopy images of H&E stained histological sections on Hepa1-6 bearing female C57BL/6 mice after treatment with different formulations.

Table S1. Summary of particle size, PDI and zeta potential of MPR (n=3)

	Size (nm)	PDI	Zeta potential (mV)
1:1	124.1±5.8	0.248±0.029	20.8±1.5
2:1	62.3±1.4	0.146±0.011	27.1±2.2
4:1	81.9±6.8	0.392±0.014	28.3±3.1

Table S2. Summary of particle size, PDI and zeta potential of CDMPR (n=3)

	Size (nm)	PDI	Zeta potential (mV)
1:8	165.6±15.0	0.604±0.009	15.7±0.4
1:4	294.5±71.0	0.648±0.213	10.3±2.3
1:2	242.3±48.2	0.512±0.043	11.4±0.8
1:1	156.9±1.0	0.224±0.026	10.9±1.8
2:1	249.2±47.5	0.479±0.030	5.1±1.1
4:1	441.2±24.8	0.435±0.110	1.8±0.4
8:1	168.5±1.0	0.152±0.044	-9.0±2.3
16:1	132.1±14.9	0.379±0.045	-17.8±3.0

Reference

1. Y. Hu, B. Xu, Q. Ji, D. Shou, X. Sun, J. Xu, J. Gao and W. Liang, *Biomaterials*, 2014, **35**, 4236-4246.
2. T. Liu, M. Wang, T. Wang, Y. Yao and N. Zhang, *Colloids and surfaces. B, Biointerfaces*, 2015, **126**, 531-540.
3. M. Wang, T. Liu, L. Han, W. Gao, S. Yang and N. Zhang, *Polymer Chemistry*, 2015, **6**, 3324-3335.