Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

A polymeric IDO inhibitor based on poly(ethylene glycol)-*b*-poly(L-tyrosine-*co*-1-methyl-D-tryptophan) enables facile trident cancer immunotherapy

Yuanyuan Liu, Jiguo Xie, Xiaofei Zhao, Yueyue Zhang, Zhiyuan Zhong*, and Chao Deng*

Biomedical Polymers Laboratory, College of Chemistry, Chemical Engineering and Materials Science, and State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou 215123, China.

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

Materials and methods

Materials

α-methoxy-*ω*-amine-poly(ethylene glycol) (PEG-NH_2, M_n = 5.0 kg/mol, Xiamen Sinopeg Biotech Co., Ltd), acrylate-PEG-NH_2 (AC-PEG-NH_2, M_n = 5.0 kg/mol, Shanghai ToYongBio Tech. Inc.), L-tyrosine (Tyr-OH, GL Biochem (Shanghai) Ltd), 1-methyl-D-tryptophan (1-MT, 99.4%, MedChemExpress (MCE, Shanghai)), (+)-JQ-1 (JQ1, 99.9%, MCE), 2-hydroxy-1-(4-(2-hydroxyethoxy)phenyl)-2-methylpropan-1-one (I2959, 98.0%, Sigma-Aldrich (Shanghai) Trading Co, Ltd), L-tryptophan (TRP, 99%, Meilunbio. Ltd), L-kynurenine (KYN, 98.0%, Meilunbio. Ltd), cyclo(Arg-Gly-Asp-D-Phe-Cys) (cRGD, >98.6%, ChinaPeptides Co., Ltd), and Ehrlich reagent (Shanghai Yuanye Bio-Technology Co., Ltd) were used as received. *N*, *N*-Dimethylformamide (DMF) was dried with $MgSO_4$ and distilled under reduced pressure before use. Other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. and used as received. Enhanced ATP assay kit was purchased from Beyotime Biotech Inc. Mouse interferon-*γ* (IFN-*γ*), tumor necrosis factor-*α* (TNF-*α*), and other cytokine ELISA kits were purchased from Invitrogen. Calreticulin (CRT) antibody was purchased from Abcam. Staining antibodies including PerCP/Cy5.5-*α*CD45, APC-*α*CD3, PE-*α*CD4, FITC-*α*CD8, FITC-*α*CD3, APC-*α*CD80, PE-*α*CD86, FITC-*α*CD11c, Alexa 647-*α*FoxP3, FITC-*α*CD11b, PE/Cy7-*α*Gr-1 and anti-mTOR antibody were purchased from BioLegend. InVivoMab anti-mouse PD-L1 (B7-H1) was purchased from BioXcell.

Characterization

^{1}H NMR spectra were measured with a Unity Inova-400 MHz spectrometer (Agilent) using DMSO-d_6 as a solvent. Chemical shifts were based on solvent signals. Polymer molecular weight and molecular weight distribution index were measured by a Waters 1515 gel permeation chromatograph (GPC) at a flow rate of 0.8 mL/min and a test temperature of 40 °C,

using DMF as the mobile phase and a series of monodisperse poly(methyl methacrylate) (PMMA) as the standard sample. The size and size distribution of nanoparticles were determined by dynamic light scattering (DLS) using Zetasizer Nano-ZS from Malvern Instruments. The microstructure of nanoparticles was characterized by Tecnai G220 transmission electron microscope (TEM, USA) at 200 kV, and the nanoparticle solution (0.5 mg/mL) was stained with phosphotungstic acid (1.0 wt.%). Flow cytometry (Becton Dickinson, FACSVerse, USA) was used to study the endocytic behavior of nanoparticles and analyze the immune cells. The Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) was used for MTT assay and ELISA detection of cytokines. The Agilent Cary Eclipse Fluorescence Spectrophotometer was used to determine the concentration of doxorubicin. The concentrations of 1-MT, JQ1, KYN, TRP were determined by high performance liquid chromatography (ProStar LC240, Waters Alliance HPLC).

Cell culture and animal study

B16F10 mouse melanoma cell line was purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). B16F10 was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 1% streptomycin, 1% penicillin (Hangzhou Jinuo Biomedical Technology Co., Ltd.) and 10% fetal bovine serum (FBS, Gibco, USA). L929 cell culture medium was RPMI-1640 medium (Gibco, USA) containing 1% streptomycin, 1% penicillin and 10% fetal bovine serum. Cells were cultured in a Model 3111 incubator (Thermo Fisher Scientific, Inc., USA) at 37 ºC and 5% CO_2. The 6-week-old female C57BL/6 black mice were purchased from Vital River (Beijing, China). Animal testing procedures were conducted in accordance with agreements approved by Laboratory Animal Center and Animal Care and Use Committee of Soochow University.

Synthesis of 1-methyl-D-tryptophan-*N*-carboxylahydride monomer (1-MT-NCA)

Under a nitrogen atmosphere, 1-methyl-D-tryptophan (1-MT, 4.0 g, 18.8 mmol) and triphosgene (2.7 g, 9.4 mmol) were mixed in tetrahydrofuran solution (THF, 100 mL). The reaction proceeded at 50 ºC until the suspension was completely clear (about 1 h). The reaction solution was concentrated by a rotary evaporator and then precipitated in 20 times excess ice petroleum ether to obtain crude 1-MT-NCA. The crude product was dissolved in THF and recrystallized with petroleum ether twice to obtain light brown 1-MT-NCA. Yield: 81.3%. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 9.08 (-CON*H*-), 7.55, 7.38 and 7.12 (-C_6H_4-), 6.98 (-N(CH_3)C*H*=C-), 4.77 (-COC*H*(NH)-), 3.74 (-NCH_3), 3.15 (-COCH(NH)CH_2-).

Enzyme responsivity and drug release behavior

The enzymatic response of cRGD-NPDJ was monitored against proteinase K (PK) at a concentration of 12.0 U/mL in PB, and the size change was determined by DLS. *In vitro* drug release was performed in two different media: (1) PB buffer (5.0 mM, pH 7.4); (2) PB (5.0 mM, pH 7.4) buffer containing 12.0 U/mL PK. A release bag (MWCO: 3 kDa) loaded with 0.5 mL of cRGD-NPDJ was placed in 25 mL of release medium. At pre-set time points, 5.0 mL of release medium was withdrawn and the same volume of fresh medium was supplemented. The samples were lyophilized and reconstituted with 1.0 mL DMSO to obtain concentrated drug solution, in which DOX was quantified by fluorospectrophotometer (excitation wavelength was 485 nm, and emission wavelength was 560 nm) and JQ1 was measured by HPLC. Experiments were performed three times in parallel, and the final values shown are the mean ± standard deviation (SD) (n = 3). The cumulative release of the drug was calculated by the following formula:

$$E_r = \frac{V_e \sum_1^{n-1} C_i + V_0 C_n}{m_{drug}}$$

E_r: cumulative release of DOX or JQ1, %; V_e: displacement volume of PB medium, 5.0 mL; V_0: total volume of release medium, 25 mL; C_i: the concentration of DOX or JQ1 in the release medium at the i-th time, μg/mL; m_{drug}: total amount of DOX or JQ1 in cRGD-NPDJ used for release, μg; n: number of replacement medium.

The release of 1-MT was similarly investigated in the presence of enzyme. A release bag (MWCO: 3 kDa) loaded with 0.5 mL of cRGD-NP (1.0 mg/mL) was placed in 25 mL of release medium containing 60 U/mL of PK at 37 ° C. At pre-set time points, 5.0 mL of release medium was withdrawn and the same volume of fresh medium was supplemented. The samples were lyophilized, redissolved, and quantified by HPLC at 280 nm (n = 3).

Western blot assay

The expression of mTOR protein in B16F10 cells was investigated using western blot analysis. The cells (2×10^5 cells/well) were treated with 200 μL of 1-MT or cRGD-NP (100 μg/mL of 1-MT) for 48 h. After washing for three times, the cells were lysed for 20 min and centrifuged to obtain the supernatant. The proteins in the supernatant were quantified using BCA protein assay kit, and then boiled with bromophenol blue solution for 5 min. The proteins were separated by electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocking with 5% skimmed milk for 1 h at room temperature, the protein bands were treated with mouse mTOR or GAPDH antibody (1000-fold dilution) overnight at 4 °C, and then incubated with goat anti- mouse antibody (1000-fold dilution) for another 1 h at room temperature. Proteins were observed using a chemiluminescence detection system.

Endocytosis assay

Cy5-labeled PEG-*b*-P(Tyr-*co*-1-MT) prepared through amidation reaction of Cy5-NHS

and terminal amino group of polypeptide block was employed to construct Cy5-adopted nanoparticles (Cy5-cRGD-NP) for endocytosis assay of nanoparticles. B16F10 cells were plated in 6-well plates at a density of 2×10^5 cells/well for 24 h and incubated with 100 μL of Cy5-cRGD-NP (1.0 mg/mL) each well. The cRGD densities of Cy5-cRGD-NP were set at 10 mol.%, 20 mol.%, and 30 mol.%, and the concentration of Cy5-labled polymer was 2.5 μg/mL. Following 4 h incubation, the medium was removed. The cells were washed 3 times with PBS, trypsinized, and centrifuged at 1000 rpm for 3 min. After washing twice with PBS, the collected cells were dispersed in 400 μL of PBS, and their Cy5 fluorescence intensity (Cy5-FI) was detected by a BD FACS Verse™ flow cytometer (FCM).

Cytotoxicity assay

The toxicity of cRGD-NPDJ toward B16F10 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were cultured in a 96-well plate at a density of 5×10^3 cells/well for 24 h with DMEM medium, followed by adding different samples at varying concentrations. After incubating for 4 h, the culture medium was refreshed, and the cells were incubated for an additional 44 h. Then, the above medium was treated with 10 μL of MTT (0.5 mg/mL) for 4 h and replaced with 150 μL of dimethyl sulfoxide to dissolve the purple formazan crystals produced by the living cells. The absorbance of formazan at 570 nm was acquired by a micro-plate reader, and the percentages of viable cells were compared with PBS group to determine the cell viability. The cytotoxicity of blank cRGD-NP in B16F10 and L929 cells were similarly evaluated except that the cells were incubated with nanoparticles for 48 h. The combination index (CI) of DOX and JQ1 was calculated according to the following formula:

$$CI = \frac{(IC_{50\ comb})_{DOX}}{(IC_{50\ \mathrm{alone}})_{DOX}} + \frac{(IC_{50\ comb})_{JQ1}}{(IC_{50\ \mathrm{alone}})_{JQ1}}$$

Here, *CI:* combination index; $(IC_{50\ comb})_{DOX:}$ half lethal concentration of DOX in combined

formulations; $(IC_{50\ alone})_{DOX:}$ half lethal concentration of DOX alone; $(IC_{50\ comb})_{JQ1}$: half lethal concentration of JQ1 in combined formulation; $(IC_{50\ alone})_{JQ1}$: half lethal concentration of JQ1 alone. $CI > 1$ and $CI < 1$ represents antagonism and synergism, respectively.

Table S1. Characterization of nanoparticles with different cRGD densities.

Entry	cRGD (mol.%)	Size[a] (nm)	PDI[a]	ξ[b] (mV)
1	0	62.9	0.10	-3.5
2	10	70.4	0.10	-3.3
3	20	75.1	0.10	-4.1
4	30	77.1	0.11	-4.7

[a]Determined by DLS (1.0 mg/mL, 25 ℃). [b]Determined by electrophoresis in PB (1.0 mg/mL, 25 ℃).

Table S2. Characterization of NPD and NPJ.

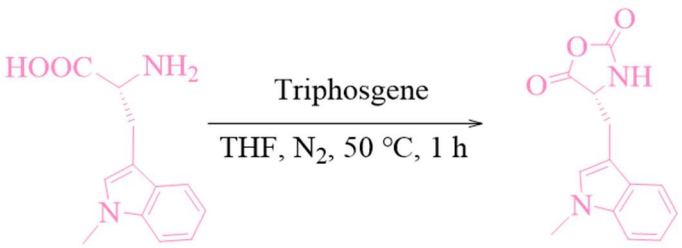
Entry	Drug	DLC (wt.%)		DLE [a]	Size[b]	PDI[b]
		Theo. (wt.%)	Determ. (wt.%) [a]	（%）	(nm)	
1	DOX	10.0	6.6	62.7	60.7	0.09
2		20.0	11.2	50.4	65.4	0.10
3		40.0	24.8	49.5	71.7	0.15
4		60.0	43.9	52.1	78.5	0.13
5	JQ1	10.0	3.1	28.2	57.9	0.15
6		20.0	8.3	36.0	61.5	0.17
7		30.0	14.6	39.7	60.6	0.10
8		50.0	39.8	66.2	81.2	0.18

[a]Dox determined by UV-vis measurement and JQ1 determined by HPLC measurement.

[b]Determined by DLS (1.0 mg/mL, 25 ℃).

Table S3. Summary of IC_{50} (μg/mL) values for different drugs in B16F10 cells.

Entry	Nanodrug (DOX/JQ1)	IC50 (μg/mL)		CI
		DOX	JQ1	
1	NPDJ (1/1)	0.89	0.89	0.70
2	10% cRGD-NPDJ (1/1)	0.54	0.54	0.42
3	20% cRGD-NPDJ (2/1)	1.14	0.57	0.51
4	20% cRGD-NPDJ (1/1)	0.30	0.30	0.23
5	20% cRGD-NPDJ (1/2)	0.14	0.28	0.20
6	20% cRGD-NPDJ (1/4)	0.07	0.27	0.19
6	30% cRGD-NPDJ (1/1)	0.29	0.29	0.23



1-MT 1-MT-NCA

Scheme S1. Synthetic rout of 1-methyl-D-tryptophan-*N*-carboxylahydride monomer (1-MT-NCA).

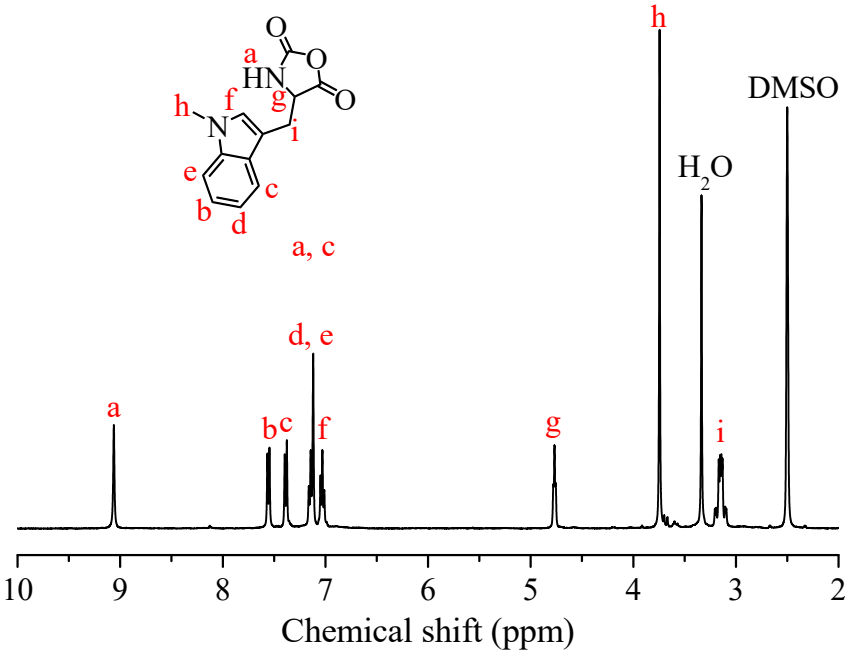


Fig. S1 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of 1-MT-NCA.

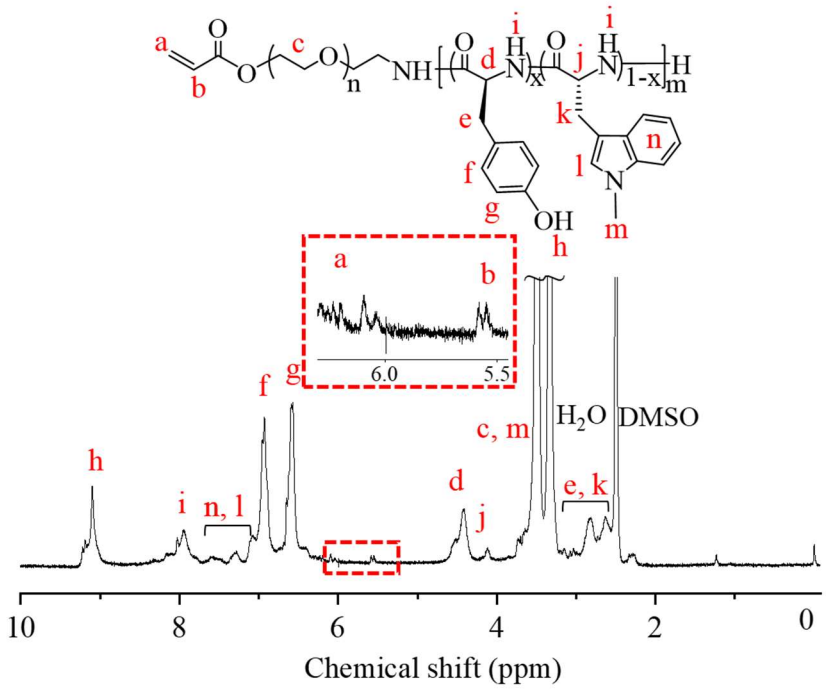


Fig. S2 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of AC-PEG-*b*-P(Tyr-*co*-1-MT).

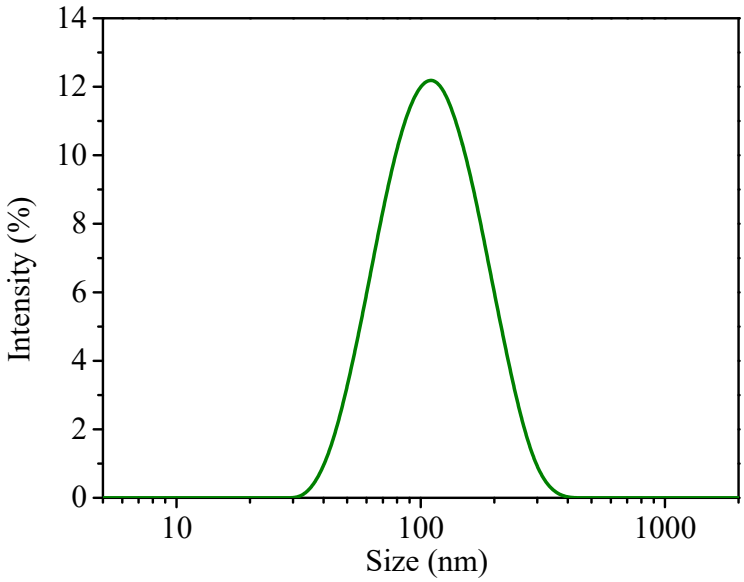


Fig. S3 Size distributions of DOX/JQ1 assemblies at a ratio of 1:1 without copolymers.

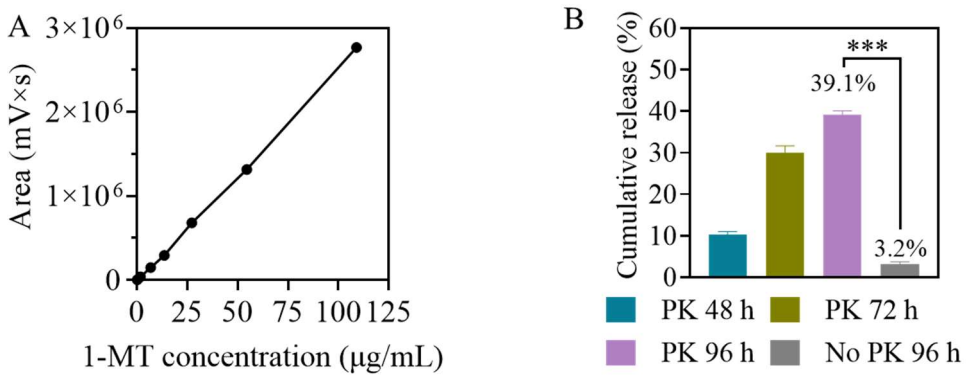
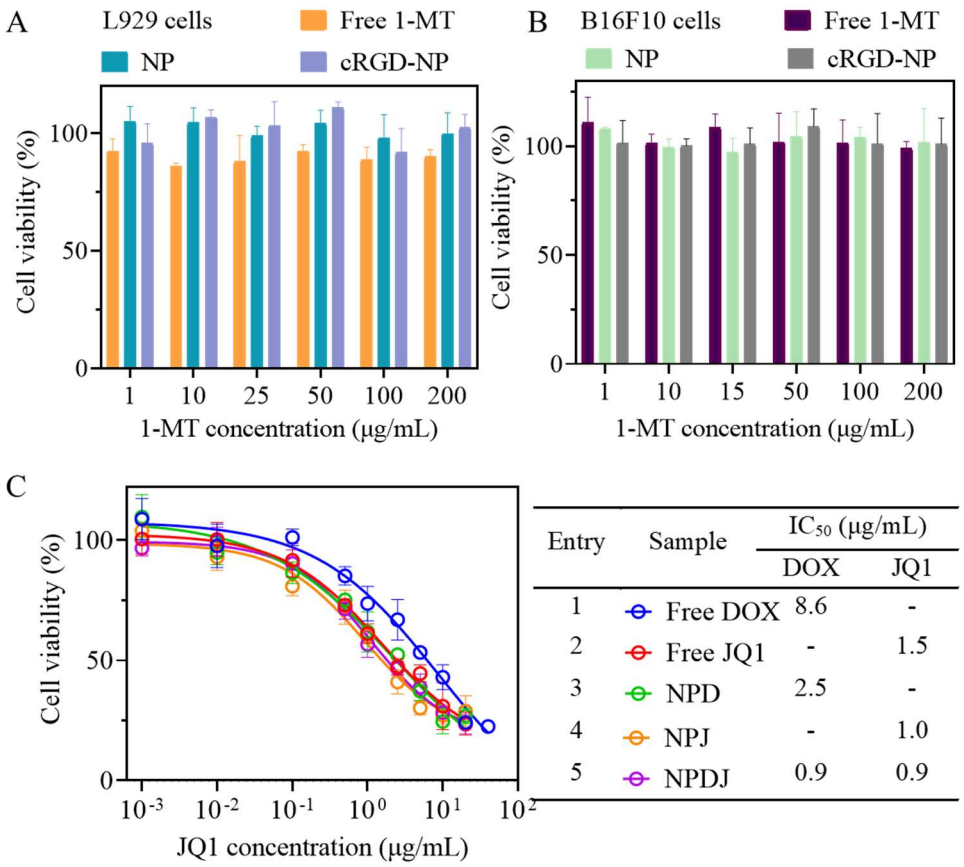


Fig. S4 *In vitro* release of 1-MT from cRGD-NP. (A) Standard curve of 1-MT measured by HPLC at 280 nm; (B) *in vitro* release behavior in the presence of PK (60 U/mL), n = 3.



Entry	Sample	IC_{50} (μg/mL)	
		DOX	JQ1
1	Free DOX	8.6	-
2	Free JQ1	-	1.5
3	NPD	2.5	-
4	NPJ	-	1.0
5	NPDJ	0.9	0.9

Fig. S5 Cytotoxicity assay of different formulation (n = 6). (A) Cytotoxicity assay in L929 cells, the cells were incubated with different formulations for 48 h; (B) cytotoxicity assay of in B16F10 cells, the cells were incubated with different formulations for 48 h; (C) cytotoxicity assay in B16F10 cells. The DOX/JQ1 ratio of NPDJ was 1/1. The cells were incubated with different formulations for 4 h, and fresh medium for another 44 h.

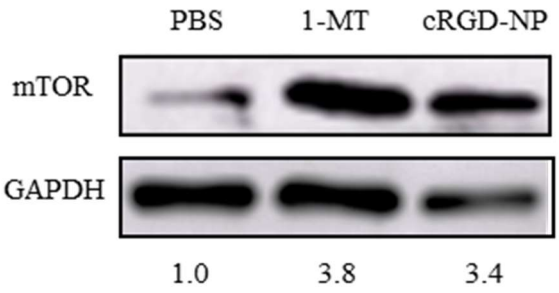


Fig. S6 Western blot assay of the mTOR expression in B16F10 cells treated with different formulations for 48 h.

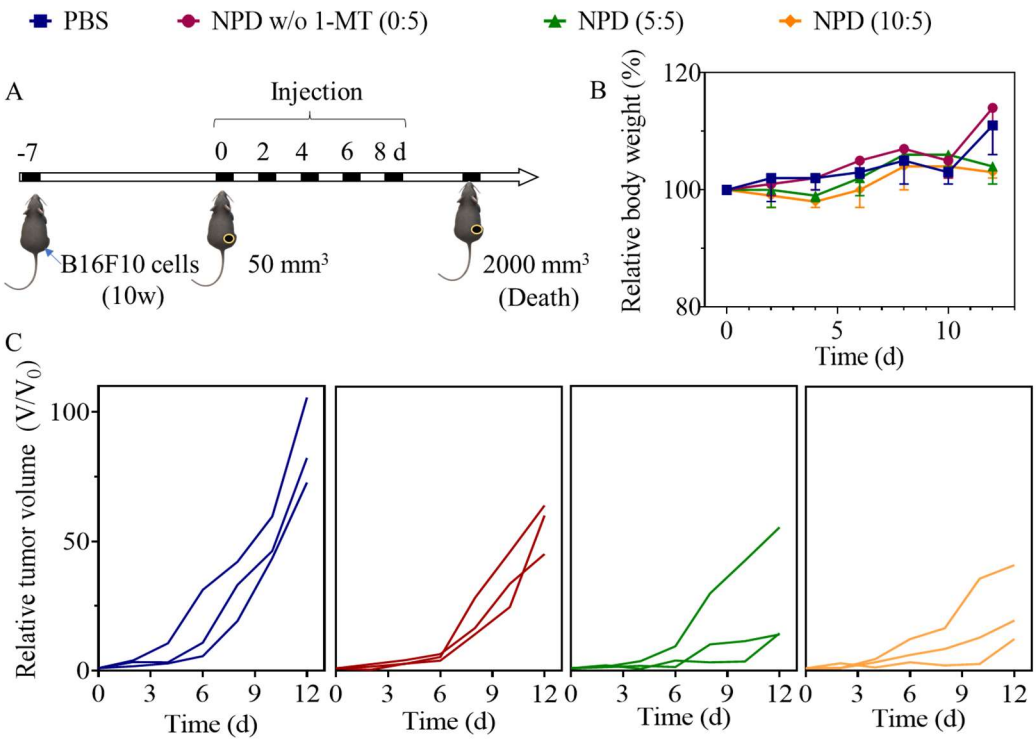


Fig. S7 Evaluation of antitumor potential of polymeric IDO inhibitor in B16F10 melanoma-bearing C57BL/6 mice (n = 3). (A) Schematic diagram of dosing in mice on days 0, 2, 4, 6, and 8. The dose of DOX was fixed at 5 mg/kg and the doses of 1-MT were 0, 5, and 10 mg/kg; (B) changes in relative body weight of mice; (C) changes in relative tumor volume in mice.

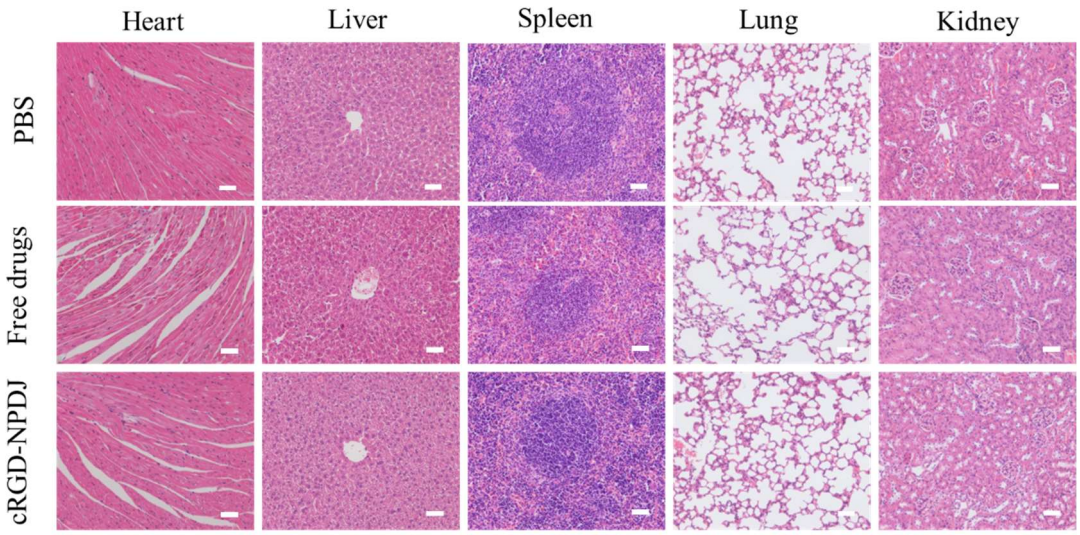


Fig. S8 H&E staining of heart, liver, spleen, lung and kidney of B16F10 melanoma bearing mice following 9 d treatment. Scale bar: 50 μm.

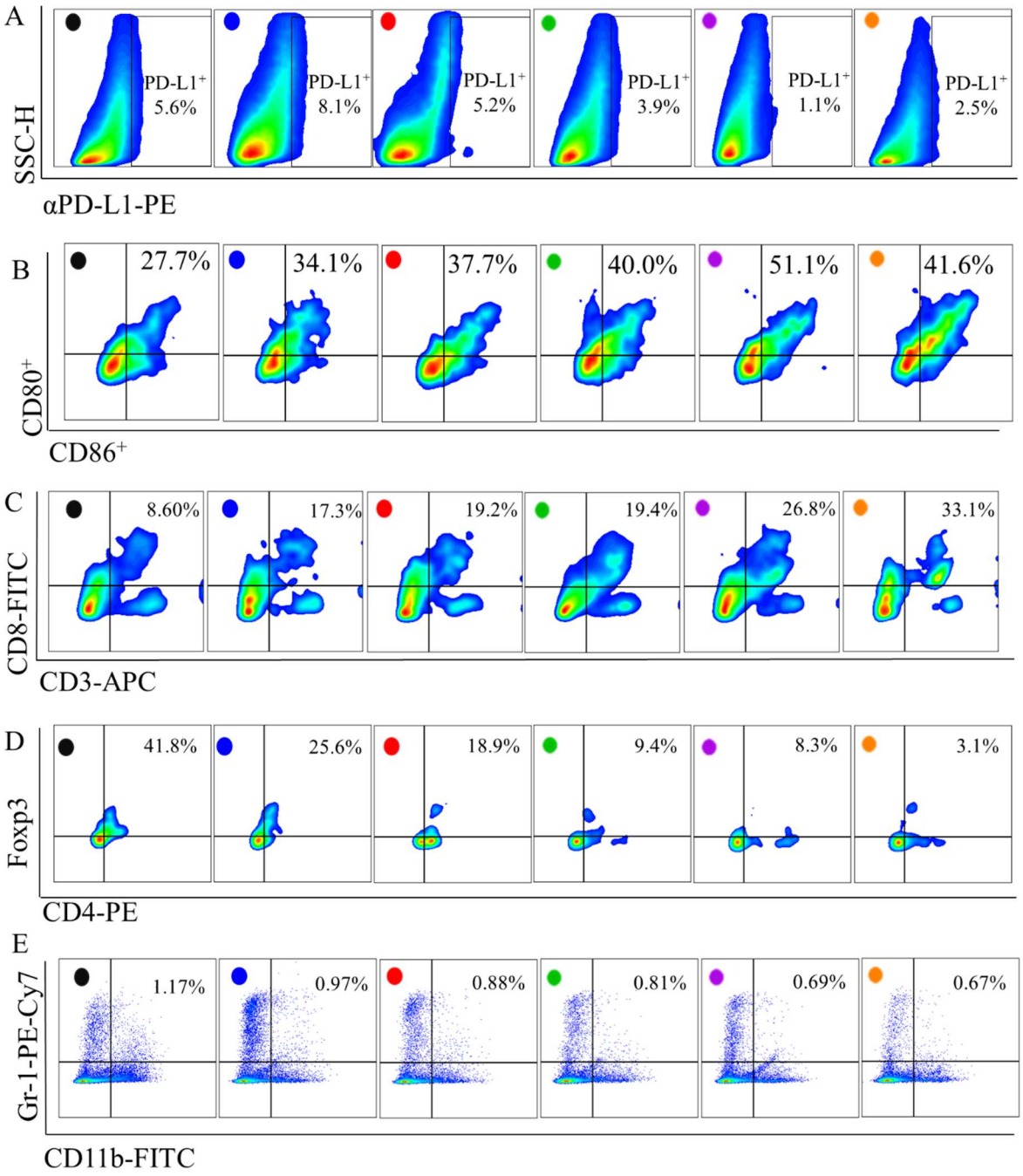


Fig. S9 *In vivo* analysis of tumoral and immune cells measured by FCM. (A) Relative abundance of PD-L1$^+$ tumor cells, PD-L1 was stained with anti-mouse PD-L1 (B7-H1); (B) relative abundance of maturated DC in tumor-draining lymph nodes; (C) relative abundance of CD8$^+$ T cells in the tumor gated on CD3$^+$CD45$^+$ cells; (D) relative abundance of Treg cells in the tumor gated on CD4$^+$CD3$^+$CD45$^+$ cells; (E) myeloid-derived suppressor cells (MDSCs) in the spleen.