Supplementary information for:

A cooperative polymeric platform for tumor-targeted drug delivery

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SUPPLEMENTED EXPERIMENTAL

Materials. DMXAA (5,6-dimethylxanthenone-4-acetic acid, ASA404) was a gift from Prof. Wu Zhong (Laboratory of Computer-Aided Drug Design and Discovery; Beijing Institute of Pharmacology and Toxicology; Beijing, China). The FXIIIa peptide substrate (A15, GN***Q***EQVSPLTLLKXC, X=6-aminohexanoic acid linker) was custom-made by ChinaPeptides Co. Ltd. (Shanghai, China). The fibrinogen was purchased from Solarbio (Sigma-F8630) and the fluorescein isothiocyanate (FITC) was bought from Aladdin Reagent Co., Ltd., China. 4,6-diamidino-2-phenylindole (DAPI) was bought from Sigma-aldrich. Human liver cathepsin B was purchased from Sigma-aldrich. We purchased the cisplatin from the Yunnan Guiyan Chemical Company, China and the methoxy poly(ethylene glycol) (mPEG-OH, average M_n = 5000) from Sigma-Aldrich. The maleimide poly(ethylene glycol) (MAL-PEG-OH, average M_n = 5000) was bought from Beijing Jenkem Technology Co. Ltd., China. The BLG-NCA (γ-Benzyl-L-glutamate-N-carboxyanhydride) was bought from ShangHai Yeexin Biochem&Tech Co., Ltd. and recrystallized from an ethyl acetate/n-hexane mixed solution three times. N,N'-diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), N-Hydroxysuccinimide (NHS), dichloroacetic acid (DCA) and 33 wt.% solution of HBr in acetic acid were purchased from Aladdin Reagent Co., Ltd., China. The near infrared dye IR830-B-NH_2 was a gift from Mr. Yuewei Niu, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. The

RhodamineB-NH_2 (RhoB-NH_2) was a gift from Dr. Chunsheng Xiao, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. All the other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. and used as received.

Treatment of C26 tumor-bearing mice with DMXAA for macroscopic observation of hemorrhage. DMXAA was injected into mice bearing C26 tumors via the tail vein at a dose of 0, 10.0, 12.5, 15.0, 17.5 or 20.0 mg/kg. After 4 hrs, the mice were sacrificed. Tumors were removed by dissection and photographed.

Preparation of FITC-labeled fibrinogen (Fibrinogen-FITC). Fibrinogen-FITC was prepared by reacting fibrinogen with FITC (fluorescein isothiocyanate). Briefly, bovine fibrinogen (20 mg, 60 nmol) was dissolved in 6.0 mL PBS by gentle shaking at 37 °C, then FITC (54 μg, 120 nmol) was added. The reaction was performed for 2 hrs then dialyzed extensively at 4 °C against PBS (pH 7.4) to remove the unreacted fluorophores. The obtained solution was stored at -20 °C until use.

Histopathological analysis of fibrin localization in C26 tumors induced by varied doses of DMXAA. Balb/C mice bearing C26 tumors were prepared as described above. DMXAA (0, 10.0, 12.5, 15.0, 17.5, or 20.0 mg/kg, dissolved in water) followed by 0.2 mL (2 nmol) of fibrinogen-FITC (described above) were injected *via* tail vein. After 4 hrs, the mice were sacrificed and the tumors removed by dissection. After staining by DAPI, the cryogenic slices (5

μm, Leica CM 1900) were imaged by confocal laser-scanning microscope (CLSM, Carl Zeiss LSM 710).

Preparation of RhoB-labeled A15-PGA-CisPt. First, RhoB-labeled PGA-*g*-mPEG/MAL-PEG was prepared by reacting the graft copolymer PGA-*g*-mPEG/MAL-PEG with RhoB-NH_2 (**Scheme S1**). The PGA-*g*-mPEG/MAL-PEG (250.0 mg) was dissolved in 5.0 mL DMF. After complete dissolution, EDC·HCl (3.8 mg, 20.0 μmol) and NHS (1.2 mg, 10.0 μmol) were added and the solution was stirred for 1 hr. Then, RhoB-NH_2 (2.5 mg) was added to the solution. The mixture was allowed to react at 0 °C for 24 hrs, dialyzed against distilled water, and then freeze-dried to give the RhoB-labeled PGA-*g*-mPEG/MAL-PEG (yield: 96%). The RhoB-labeled A15-PGA-CisPt were prepared from RhoB-labeled PGA-*g*-mPEG/MAL-PEG and cisplatin in a similar method to that of A15-PGA-CisPt. The resulting RhoB-labeled A15-PGA-CisPt had a size of 59.0 ± 10.3 nm in aqueous solution, surface zeta potential of -10.5 ± 1.3 mV and maximum absorbance at 563 nm in water, measured by UV-Vis spectrometry (UV-2401PC, SHIMADZU).

Preparation of IR830-labeled A15-PGA-CisPt. IR830-labeled A15-PGA-CisPt were prepared by a method similar to that of the RhoB-labeled A15-PGA-CisPt, with the IR830-labeled PGA-*g*-mPEG/MAL-PEG synthesized by reacting the PGA-*g*-mPEG/MAL-PEG with IR830-B-NH_2 (**Scheme S2**). In the first step, PGA-*g*-mPEG/MAL-PEG (250.0 mg) was dissolved in 5.0 mL DMF, then EDC·HCl (3.8 mg, 20.0 μmol) and NHS (1.2 mg, 10.0 μmol)

were added. After stirring for additional 1 hr, IR830-B-NH_2 (4.0 mg) was added. The mixture was allowed to react at 0 °C for 24 hrs, dialyzed against distilled water, and then freeze-dried to give the IR830-labeled PGA-*g*-mPEG/MAL-PEG (yield: 94%). Then, IR830-labeled A15-PGA-CisPt were prepared from IR830-labeled PGA-*g*-mPEG/MAL-PEG and cisplatin similar to the methods described above. The resulting IR830-labeled A15-PGA-CisPt had a size of 68.3 ± 13.3 nm in aqueous solution with a surface zeta potential of -9.5 ± 0.8 mV. The maximum absorbance of the IR830-labeled A15-PGA-CisPt in water was 817 nm, measured by UV-Vis spectrometry.

Characterization of the prepared A15-PGA-CisPt conjugates. Transmission electron microscope (TEM) images of A15-PGA-CisPt were taken from KEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV. The sizes and pdi in aqueous solution were measured by dynamic light scattering (DLS, DAWN EOS, Wyatt Technology) at 90° collecting optics. The zeta-potential was measured with a Zeta Potential/BI-90 Plus particle size analyzer (Brookhaven, USA) at room temperature. XPS (X-ray photoelectron spectroscopy) spectra were recorded on a ESCALAB MK II XPS spectrometer (VG Scientific, UK). Number-average molecular weight (M_n) and PDI (M_w/M_n) of the conjugates were measured by GPC, with 0.1 M phosphate buffer as the eluent. Pt content was determined by inductively coupled plasma mass spectrometry (ICP-MS, Xseries II, Thermoscientific, USA). The total drug loading content (DLC%) was calculated by the following equation:

$$\mathrm{DLC\%} = \frac{\text{weight of cisplatin in conjugates}}{\text{weight of conjugates}} \times 100\% \qquad (1)$$

For free drug content measurement, 2 mL of the A15-PGA-CisPt conjugates were added to a dialysis bag (MWCO = 3500 Da), and then incubated in 28 mL MillQ water and placed at 37 °C with a shaking rate of 100 rpm. After 2 hrs, 3mL of the medium was withdrawn and the Pt content was measured by ICP-MS. The free drug content was calculated by the following equation:

$$\text{Free drug content\%} = \frac{\text{Pt amount in the medium}}{\text{Pt amount fed}} \times 100\% \qquad (2)$$

***In vitro* release profiles.** The *in vitro* release of cisplatin from A15-PGA-CisPt in pH 7.4 phosphate buffered saline (10 mM, PBS 7.4) and pH 5.5 acetate buffered saline (25 mM, ABS 5.5) were evaluated by dialysis method. Briefly, 2mL of the prepared A15-PGA-CisPt solution was added to dialysis membrane with 3 mL PBS 7.4 or ABS 5.5 (MWCO = 3500 Da), which was then incubated in 35 mL respective buffered solution and placed at 37 °C with a shaking rate of 100 rpm. At predetermined time intervals (8 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs), 3 mL of the incubation solution was withdrawn and replaced with an equal volume of fresh solution. The concentration of Pt in the solutions were measured by ICP-MS.

The release of Pt in the presence of cathepsin B was also conducted by dialysis method, and cathepsin B was handled following a method reported by D. Chu.. [1] Briefly, 18.2 μL human liver cathepsin B (0.351 mg/mL stock) was added to 36.4 μL activation buffer (30 mM DTT, 15 mM EDTA) and incubated at 37 °C for 15 min. Then the activated cathepsin B, 2 mL of the prepared A15-PGA-CisPt solution, 3 mL ABS 5.5 were added to dialysis membrane, and

incubated in 35 mL acetate buffered saline (25 mM acetate, 1 mM EDTA, 130 mM NaCl, pH 5.5) and placed at 37 °C with a shaking rate of 100 rpm. At predetermined time intervals (8 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs), 3 mL of the incubation solution was withdrawn and replaced with an equal volume of fresh solution. The concentration of Pt in the solutions were measured by ICP-MS.

***In vitro* cytotoxicity test and cellular uptake.** The *in vitro* cytotoxicity test of DMXAA, cisplatin and A15-PGA-CisPt to tumor cells and endothelial cells were assessed by MTT assay. Firstly, C26 cells and HUVEC cells were seeded in 96-well plates at a density of 1.0×10^5 cells per well in 200 μL DMEM. After 24 hrs, DMXAA, cisplatin and A15-PGA-CisPt at different concentrations were added to the wells. The cells were subjected to MTT test after being incubated for another 24 or 48 hrs. The absorbance was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell survival rate (%) was caculated according to the following equation: Survival rate (%) = $(A_{sample}/A_{control}) \times 100\%$, where A_{sample} and $A_{control}$ were the average absorbances of the sample well and control well, respectively.

For cellular endocytosis, C26 and HUVEC cells were seeded in 6-well plates at a density of 2.0×10^5 cells per well, cultured for 24 hrs, and then incubated at 37 °C with RhoB-labeled A15-PGA-CisPt. After 4 hrs, the culture medium was removed and the cells were washed with PBS thrice, fixed with 4% paraformaldehyde for 20 mins, counterstained with DAPI according to the standard protocols provided by the suppliers. After mounting, the CLSM images were taken through the confocal microscope (Carl Zeiss LSM 710).

Stability test of A15-PGA-CisPt after incubation in plasma. Rabbit blood plasma was collected from the whole blood after centrigfugation at 4 °C and 2500 g for 10 mins. Then 0.05 mL of the prepared A15-PGA-CisPt solution was added to 1.5 mL EP tubes each with 0.5 mL plasma inside and placed under 37 °C at a shaking rate of 100 rpm. At predetermined time points (30 mins, 1 h, 3 hrs, 6 hrs, 24 hrs), one of the EP tubes was taken out and the solution was transfered to another EP tube with 0.5 mL 0.7 M sucrose cushion, and the mixture was centrifuged for 20 mins at 4 °C and 15300 g. The supernatant was discarded and the pellet was washed with PBS twice. Finally, 0.1 mL of the solution was maintained in the tubes.

50 μL of the obtained solution was added to 5 mL millQ water, and the sizes of the seperated conjugates were measured by DLS.

For surface protein measurement, firstly, the proteins were eluted from the A15-PGA-CisPt conjugates by adding 10 μL of the obtained solution with 20 μL SDS sample buffer and incubated for 5 mins at 95 °C. Then the conjugates were pelleted by centrifugation for 15 mins at 15300 g at RT, and the supernatant containing eluted corona proteins were transfered to a fresh tube. 10 μL of the eluted corona proteins solution was loaded on a 12% SDS gel, with 10 μL of the original used plasma as a control. The SDS-PAGE gel was runed at RT at 100 V for 1.5 hrs, stained by coomassie staining and photographed.

Phamacokinetic profiles. 3 Wistar rats (male, average body weight 300 g) were administered via tail vein of A15-PGA-CisPt at a dosage of 4.0 mg CDDP equivalent/kg. At defined time points (3 mins, 15 mins, 30 mins, 1 hr, 2 hrs, 4 hrs, 8 hrs, 24 hrs), 10 drops of blood

were collected from the orbital cavity, heparinized and centrifuged at 10000 rpm for 3 mins to obtain the plasma. The plasma samples were decomposed by heating with nitric acid and the Pt contents were measured by ICP-MS.

REFERENCES AND NOTES

1. D. S.H. Chu, R. N. Johnson, S. H. Pun, *J. Control. Release*, 2012, **157**, 445-454.

Figure caption

Scheme S1. Synthesis of RhoB-labeled PGA-*g*-mPEG/MAL-PEG.

Scheme S2. Synthesis of IR830-labeled PGA-*g*-mPEG/MAL-PEG.

Fig. S1. Macroscopic observation of hemorrhages occurring in C26 colon carcinoma tumor-bearing Balb/C mice 4 hrs following injection of saline and different dosages of DMXAA.

Fig. S2. Histopathological analysis of fibrin localization in C26 tumors at 4 hrs after co-administration of fibrinogen-FITC with saline or different dosages of DMXAA (scale bar = 100 μm).

Fig. S3. H&E analysis of heart, liver, spleen, lung and kidney of blank C26 tumor bearing mice or at 4 hrs after administration of 15.0 mg/kg DMXAA and fibrinogen-FITC (scale bar = 200 μm) and the coresponding histopathological results (scale bar = 100 μm). No obvious hemorrhage or fibrin localization was observed in these vital organs.

Fig. S4. ^{1}H NMR (400 MHz) spectrum of PGA-*g*-mPEG/MAL-PEG in D_2O.

Fig. S5. GPC curves of mPEG-OH, MAL-PEG-OH, PGA, and PGA-g-mPEG/MAL-PEG, measured in phospate buffer (0.1 M, pH 7.4).

Fig. S6. TEM and DLS images of A15-PGA-CisPt. The mean size obtained from TEM is 42.7 ± 5.1 nm (n = 15), and 63.5 ± 12.0 nm from DLS.

Fig. S7. XPS analysis of Cisplatin-NPs and A15-PGA-CisPt. Changes in C1s and the appearance of S2p indicate the presence of the A15 peptide GN***Q***EQVSPLTLLKXC.

Fig. S8. *In vitro* release of cisplatin from A15-PGA-CisPt in PBS7.4, ABS5.5 and ABS5.5+cathepsin B (n = 3).

Fig. S9. Cytotoxicity assay of cisplatin and A15-PGA-CisPt to C26 (a) and HUVEC (b) cells after incubation for 24 and 48 hrs (n = 3).

Fig. S10. CLSM images of C26 and HUVEC cells after incubation with RhoB-labeled A15-PGA-CisPt for 4 hrs. Scale bar = 50 μm.

Fig. S11. Sizes and surface bound proteins measurement of A15-PGA-CisPt after incubation in plasma for 30 mins, 1 h, 3 hrs, 6 hrs and 24 hrs. (a) Sizes meastured by DLS. (b) SDS-PAGE analysis of the absorbed plasma proteins.

Fig. S12. Time profiles of platinum concentration in plasma after i.v. administration of A15-PGA-CisPt at a dosage of 4.0 mg/kg based on CDDP. The results are listed as mean ± SD (n = 3).

Fig. S13. UV-spectrum of IR830-labeled A15-PGA-CisPt in water, with maximum absorption at 817 nm.

Fig. S14. Biodistribution of platinum (Pt) after administering free cisplatin, A15-PGA-CisPt or DMXAA+A15-PGA-CisPt at 4 hrs (a) and 24 hrs (b) in the heart, liver, spleen, lung, kidney and tumor from C26 tumor bearing mice. Injected dosage: 4.0 mg cisplatin equivalent/kg body weight, DMXAA 15.0 mg/kg (n = 4).

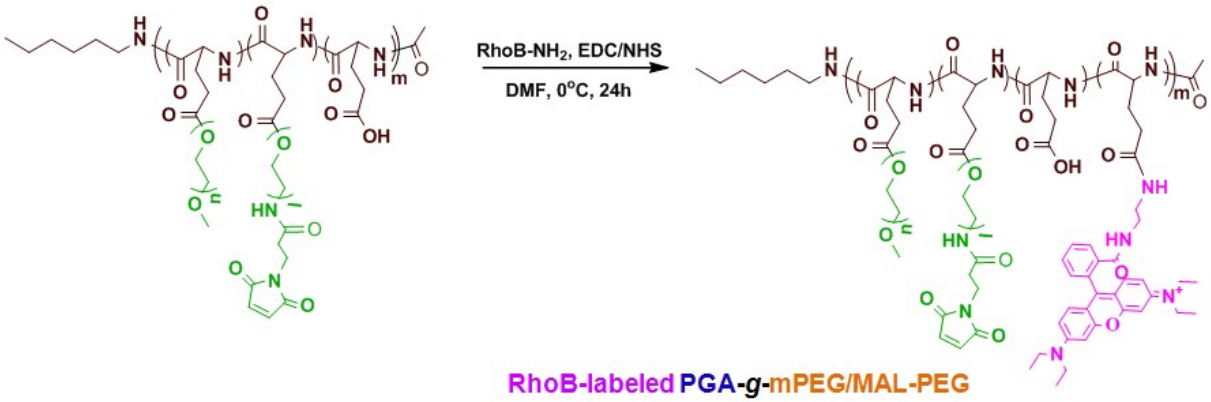
Table S1. Parameters of the applied A15-PGA-CisPt conjugates.

Table S2. IC_{50} values of cisplatin and A15-PGA-CisPt to C26 and HUVEC cells.

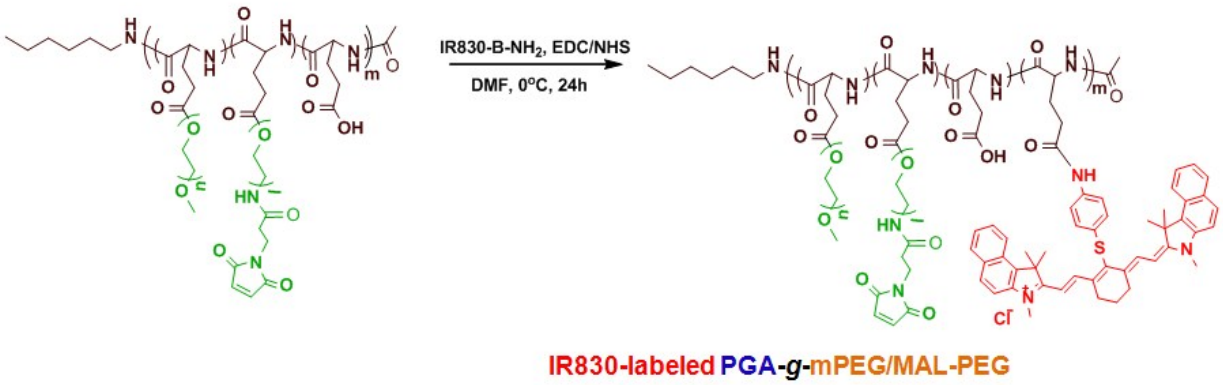
Table S3. Pharmacokinetic parameters of A15-PGA-CisPt in Wistar rats.

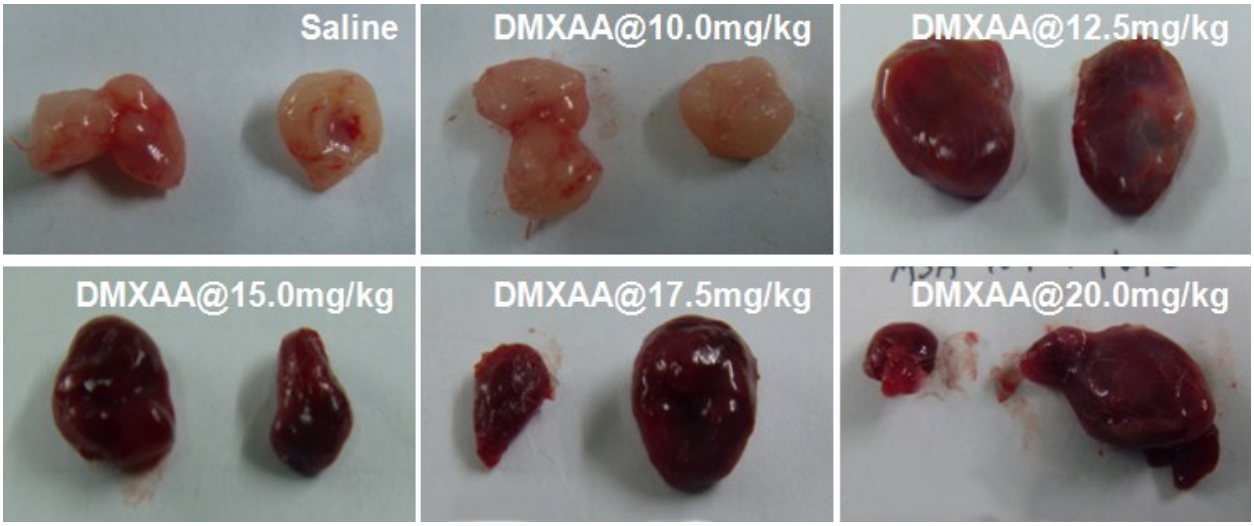
Table S4. Accumulation ratios between tumor and normal organs at 4 hrs and 24 hrs after administration of free cisplatin, A15-PGA-CisPt and DMXAA+A15-PGA-CisPt. Dosage: 4.0 mg cisplatin equivalent/kg body weight, DMXAA 15.0 mg/kg (n = 4).

Scheme S1. Synthesis of RhoB-labeled PGA-*g*-mPEG/MAL-PEG.



Scheme S2. Synthesis of IR830-labeled PGA-*g*-mPEG/MAL-PEG.





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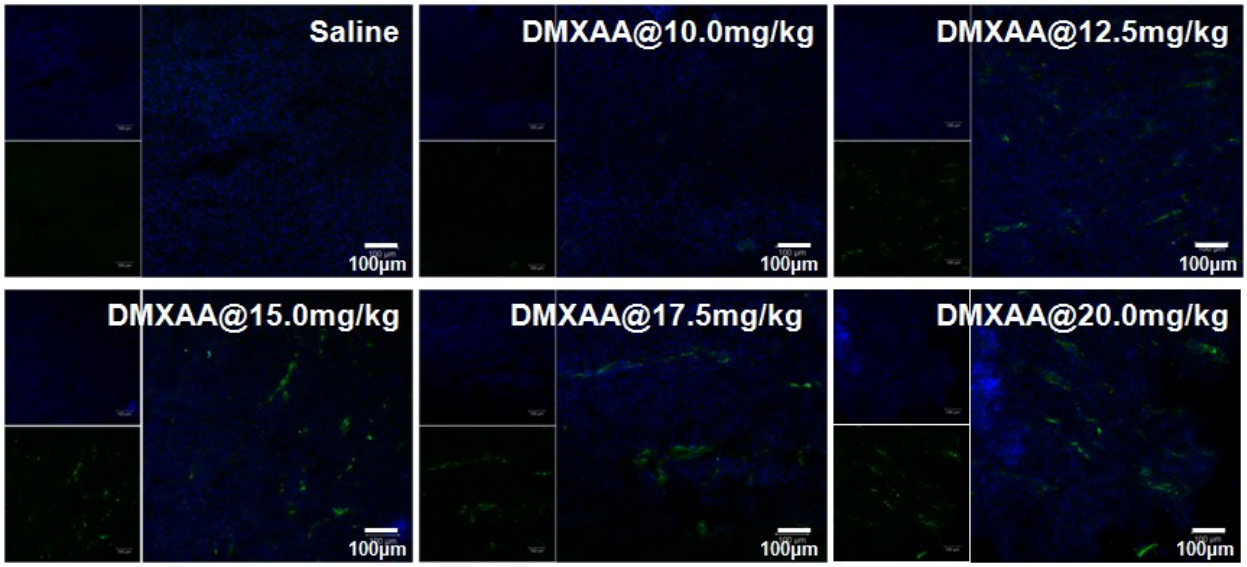
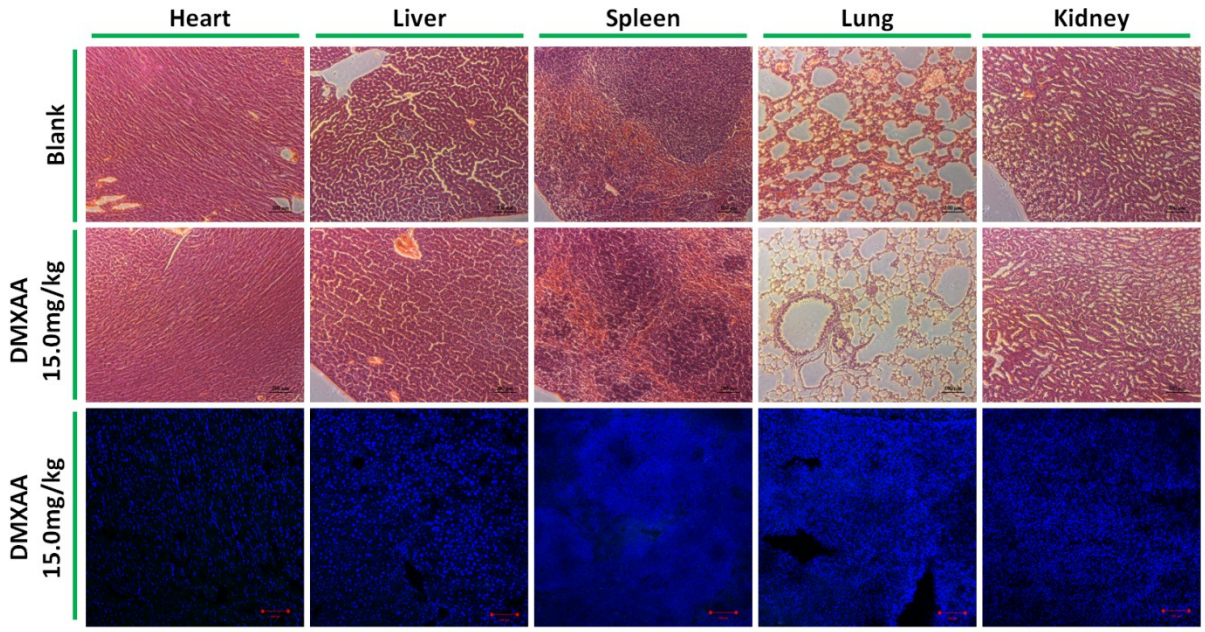


Fig. S2. Histopathological analysis of fibrin localization in C26 tumors at 4 hrs after co-administration of fibrinogen-FITC with saline or different dosages of DMXAA (scale bar = 100 μm).



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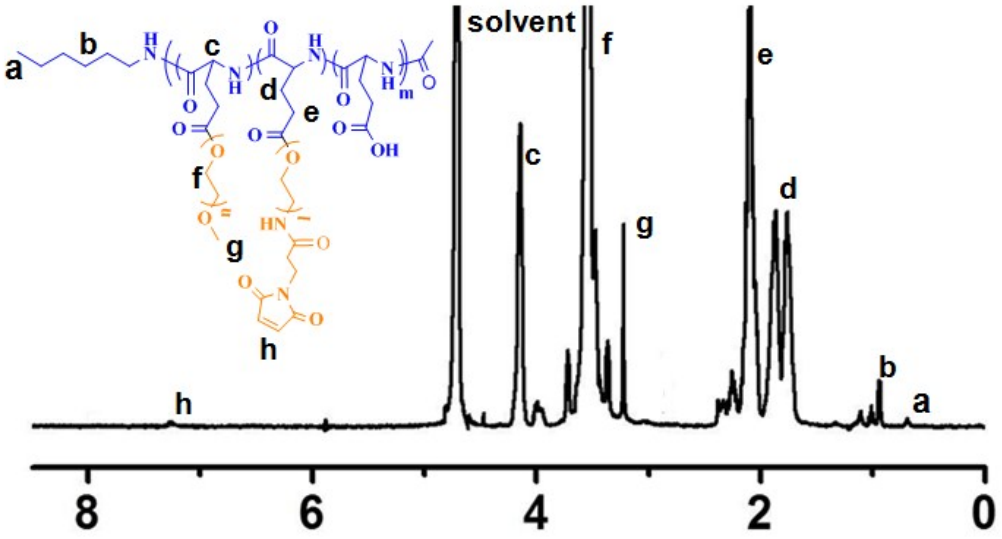

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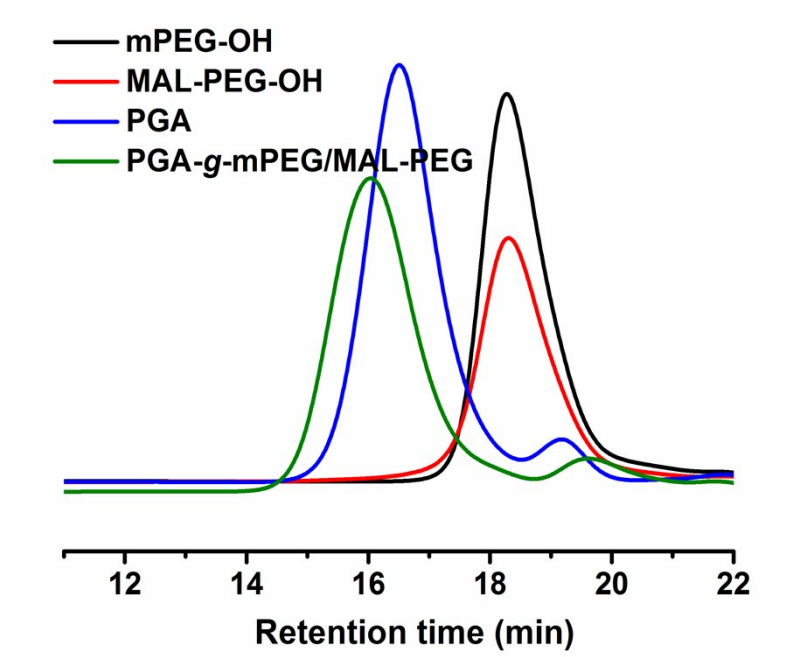


Fig. S5. GPC curves of mPEG-OH, MAL-PEG-OH, PGA, and PGA-*g*-mPEG/MAL-PEG, measured in phospate buffer (0.1 M, pH 7.4).

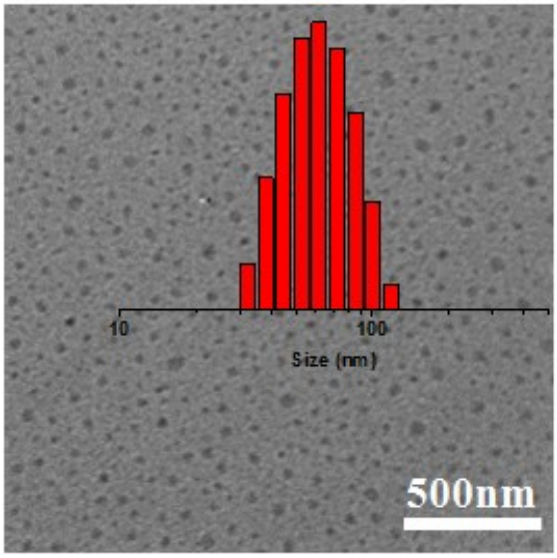
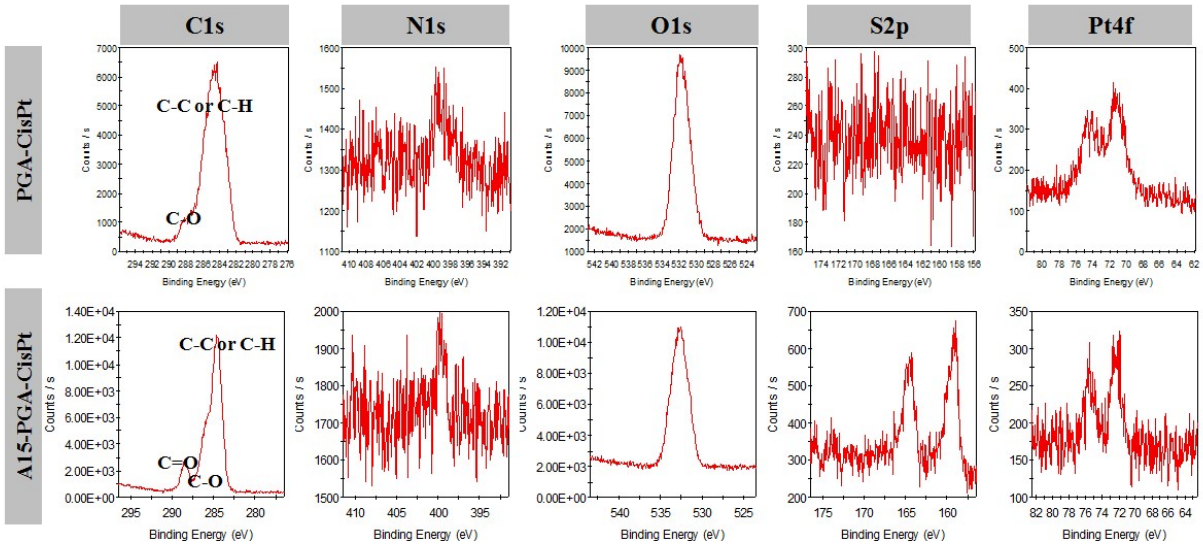


Fig. S6. TEM and DLS images of A15-PGA-CisPt. The mean size obtained from TEM is 42.7 ± 5.1 nm (n = 15), and 63.5 ± 12.0 nm from DLS.



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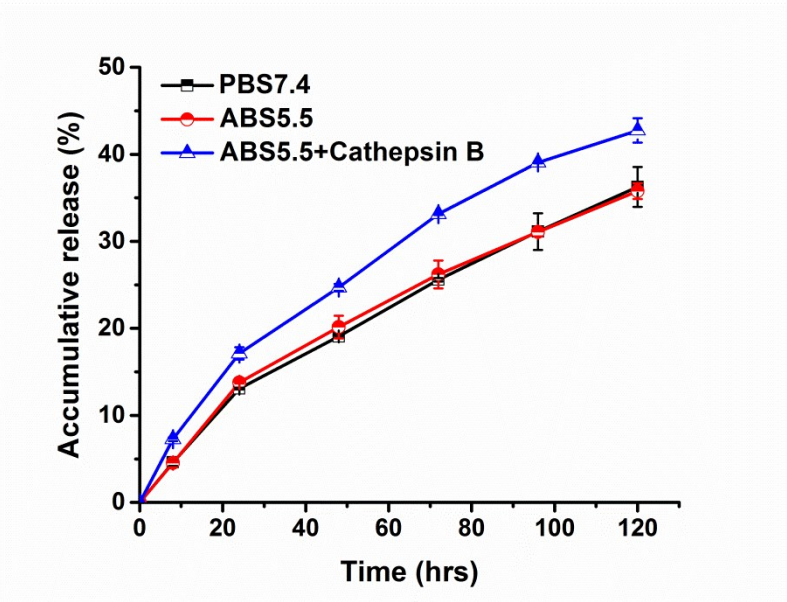


Fig. S8. *In vitro* release of cisplatin from A15-PGA-CisPt in PBS7.4, ABS5.5 and ABS5.5+cathepsin B (n = 3).

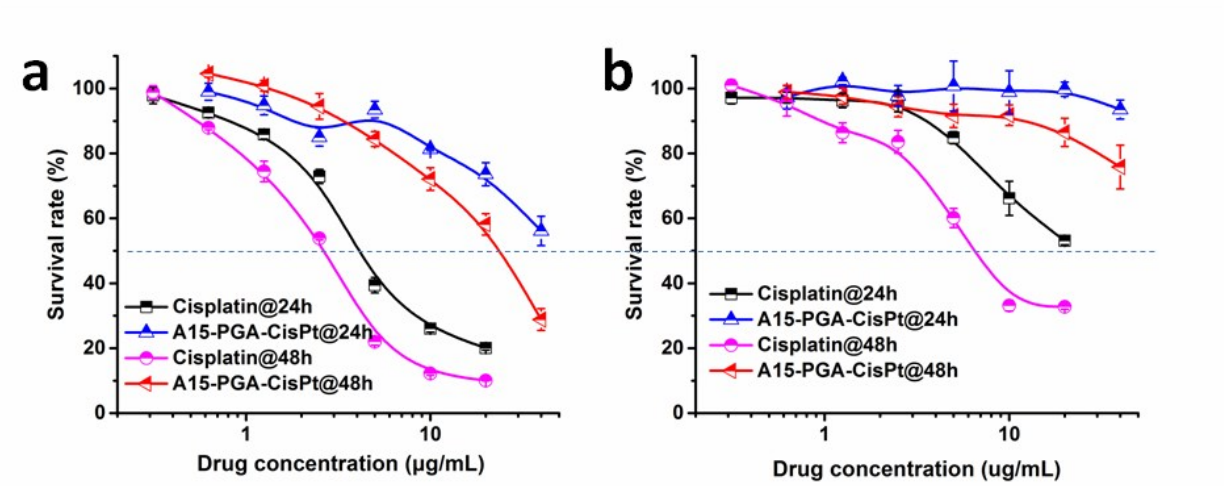
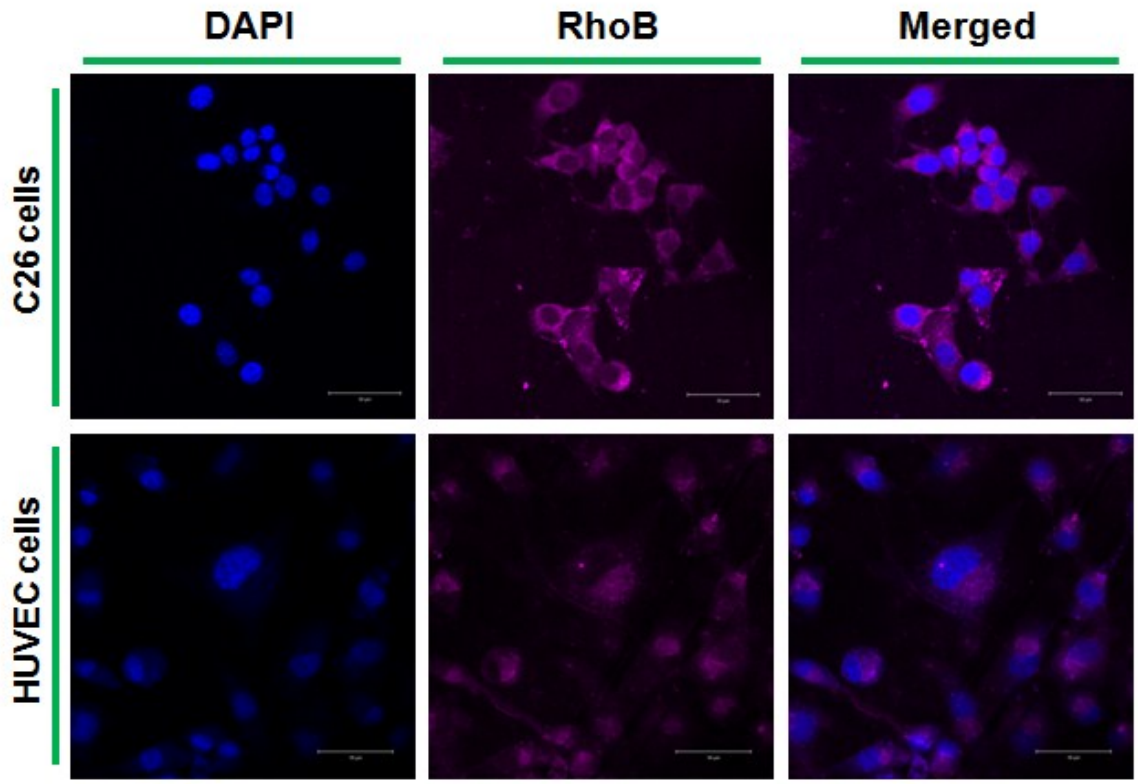


Fig. S9. Cytotoxicity assay of cisplatin and A15-PGA-CisPt to C26 (a) and HUVEC (b) cells after incubation for 24 and 48 hrs (n = 3).



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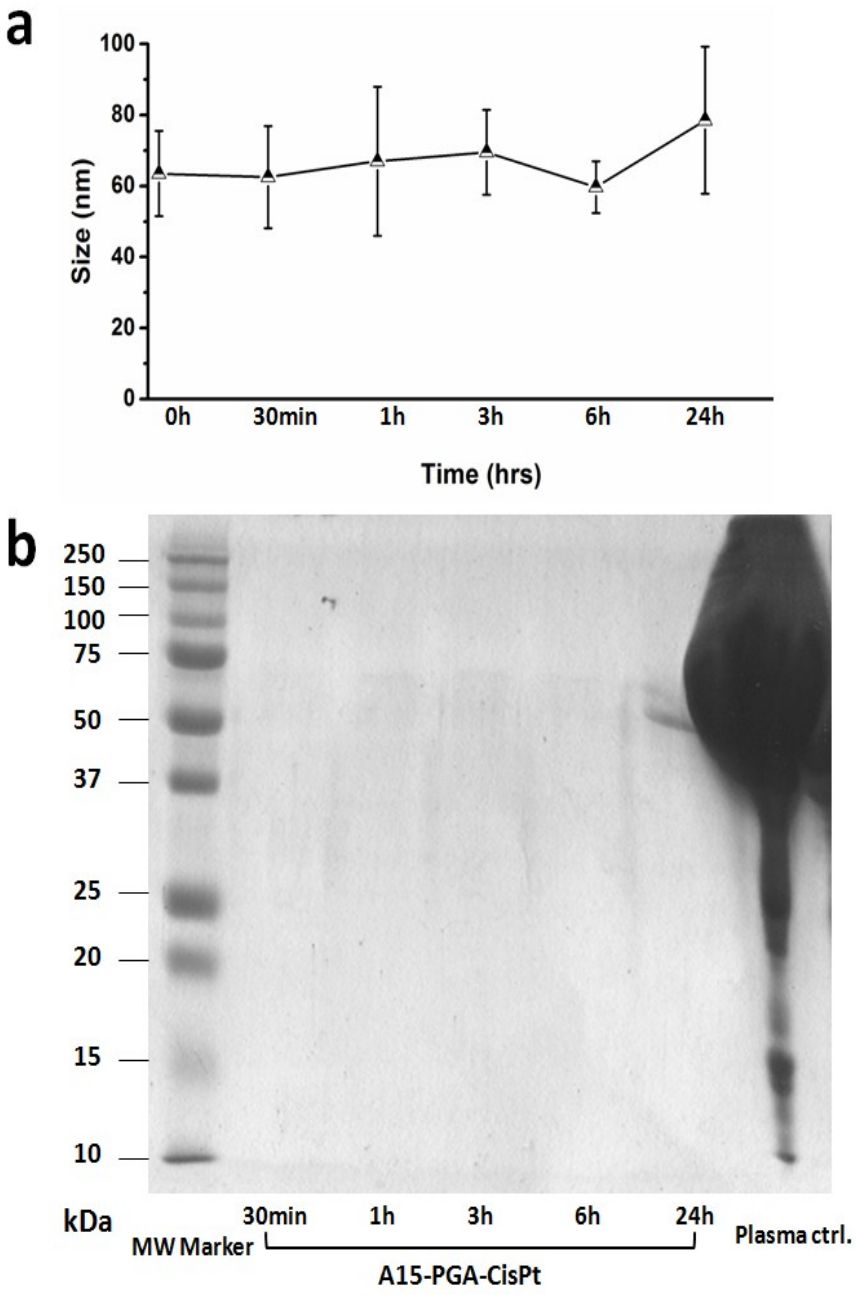

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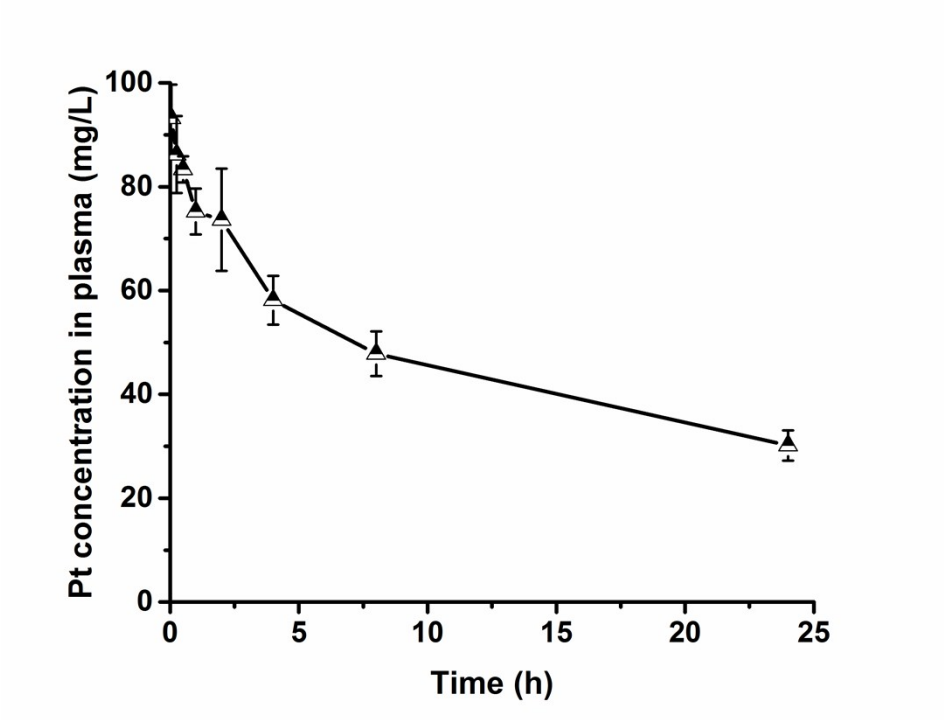

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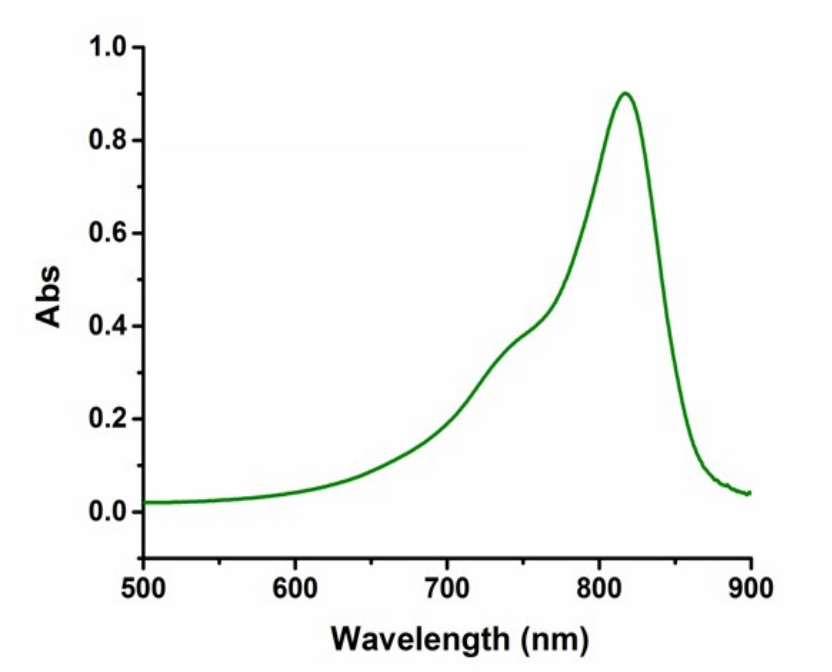

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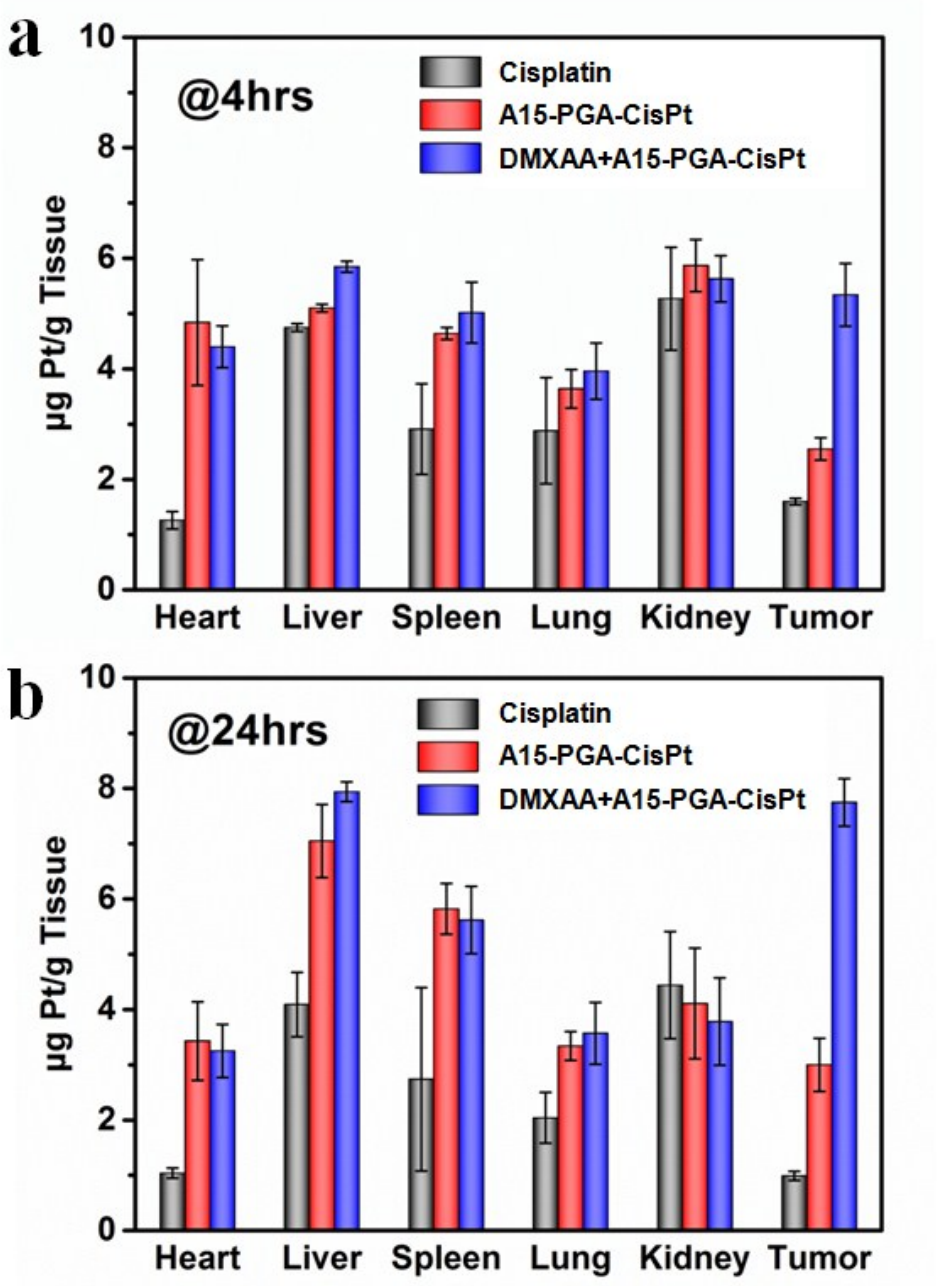

Fig. S14. Biodistribution of platinum (Pt) after administering free cisplatin, A15-PGA-CisPt or DMXAA+A15-PGA-CisPt at 4 hrs (a) and 24 hrs (b) in the heart, liver, spleen, lung, kidney and tumor from C26 tumor bearing mice. Injected dosage: 4.0 mg cisplatin equivalent/kg body weight, DMXAA 15.0 mg/kg (n = 4).

Table S1. Parameters of the applied A15-PGA-CisPt conjugates

	DLC%[a]	Free drug content%[a]	M_n /10^4 Da[b]	M_w/M_n[b]	Zeta potential /(mV)[c]	Size /(nm)[d]	Pdi[d]
A15-PGA-CisPt	15.3	1.6	21.2	1.48	-9.8±1.9	63.5	0.203
RhoB-labeled A15-PGA-CisPt	15.5	1.2	21.5	1.62	-10.5±1.3	59.0	0.212
IR830-labeled A15-PGA-CisPt	14.7	1.5	23.5	1.61	-9.5±0.8	68.3	0.230

a) Measured by ICP-MS, calculated from equation (1) and (2);

b) Mesasured by GPC, with 0.1 M PB buffer (pH 7.4) as the eluent;

c) Measured by Zeta Potential/BI-90 Plus particle size analyzer, in MillQ water;

d) Measured by DLS.

Table S2. IC_{50} values of cisplatin and A15-PGA-CisPt to C26 and HUVEC cells

	C26 cells		HUVEC cells	
	Cisplatin /μg/mL	A15-PGA-CisPt /μg/mL	Cisplatin /μg/mL	A15-PGA-CisPt /μg/mL
24h	4.3	>40.0	>20.0	>>40.0
48h	2.6	23.9	7.0	>>40.0

Table S3. Pharmacokinetic parameters of A15-PGA-CisPt in Wistar rats

Parameter	$T_{1/2}z$ (h)	T_{max} (h)	C_{max} (mg/L)	AUC_{0-t} (mg/L*h)	Vz (mL/kg)	CL_{obs} (mL/h/kg)
	21.9±1.4	0.05	94.8±6.4	1125.5±83.1	61.1±3.6	2.0±0.2

$T_{1/2}z$, terminal half-life; T_{max}, time reach maximum concentration; C_{max}, maximum concentration; AUC_{0-t}, area under curve from 0 to 24 hrs; Vz, apparent volume of distribution; CL_{obs}, observed clearance rate.

Table S4. Accumulation ratios between tumor and normal organs at 4 hrs and 24 hrs after administration of free cisplatin, A15-PGA-CisPt and DMXAA+A15-PGA-CisPt. Dosage: 4.0 mg cisplatin equivalent/kg body weight, DMXAA 15.0 mg/kg (n = 4).

	4hrs			24hrs		
	Cisplatin	A15-PGA-CisPt	DMXAA+A15-PGA-CisPt	Cisplatin	A15-PGA-CisPt	DMXAA+A15-PGA-CisPt
Tumor/Heart	1.27±0.10	0.53±0.11	1.21±0.05	0.95±0.09	0.87±0.07	2.38±0.05
Tumor/Liver	0.34±0.04	0.50±0.01	0.91±0.02	0.24±0.06	0.43±0.07	0.98±0.02
Tumor/Spleen	0.55±0.05	0.55±0.01	1.06±0.06	0.36±0.17	0.52±0.05	1.38±0.06
Tumor/Lung	0.56±0.06	0.70±0.04	1.35±0.06	0.49±0.05	0.90±0.03	2.17±0.06
Tumor/Kidney	0.30±0.06	0.42±0.05	0.95±0.08	0.22±0.10	0.73±0.10	2.05±0.08