Supporting Information
for
Peptide-based Autophagic Gene and Cisplatin Co-delivery Systems Enable Improve Chemotherapy Resistance

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METHODS

1. Materials

Cisplatin, and Annexin V-FITC/PI were purchased from Aladdin, (China), Beclin1 siRNA and TRIZOL Reagent were purchased from Thermo Fisher Scientific, Inc. 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethy-luronium hexafluorophosphate (HBTU) were purchased from GL Biochem (China). DSPE-PEG-COOH 2000 obtained from Seebio Biotech, Inc. LC3 II and β-actin antibody were purchased from Beyotime Institute of Biotechnology, China. Beclin1 antibody was purchased from Abcam. Cell counting kit-8 assay (CCK-8) was purchased from Beyotime Institute of Biotechnology, China. FAM FLICA™ Poly Caspase Kit was purchased from Bio-Rad. A549 were purchased from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Cisplatin-resistant A549 cell lines is a gift from Prof. Xingjie Liang at NCNST. Other solvents and reagents were used as received.

2. Synthesis of Pt(IV)-peptide-bis(pyrene) and cRGD-PEG2000-DSPE polymers

Firstly, Pt(IV) prodrugs were prepared in followed method: cis,cis,trans-[Pt(NH3)2Cl2(OH)2] (400 mg, 1.33 mmol) was oxidized by hydrogen peroxide (30%, 20 mL) at 60 °C for 4 h. After the bright yellow solution was cooled at 4 °C, yellow crystals of c,c,t-[$Pt(NH_3)_2Cl_2(OH)_2$] was collected by centrifugation (yield: 56.5%). The crystals (200 mg, 0.6 mM) were then dissolved in DMSO (0.5 mL) and followed by dropwise adding with succinic anhydride (60 mg, 0.6 mmol) and stirred overnight. The final yellow yield was collected after filtrating and washing. Next, the Fmoc solid-phase peptide synthesis method was used to prepare the gene delivery peptide (KTGRKKRRQRRRG) and the cRGD peptide (KFDGR). In brief, 200 mg Wang resin (K loading ~0.3 mmol/g) was dissolved in HPLC-grade DMF at room temperature. After 2 h, it should be treated with piperidine/DMF (v/v = 1/4) so that Fmoc group was removed, then washed with HPLC-grade DMF and DCM three times. The mixture of amino acids (0.6 mM), HBTU (0.35 M) and NMM (0.4 M) was added and shaken at room-temperature for 2 h before ninhydrin test (ninhydrin, phenol, VC 1:1:1 v/v). bis(pyrene or DSPE-PEG was regarded as an amino acid and coupled to the peptide. When Dde group of Lys was removed by hydrazine/DMF (v/v = 1/19), Pt(IV) was added into the reaction solution. The final products were collected after incubation with a mixture of 95% TFA, 2.5% TIS, and 2.5% H_2O for 3 h at room temperature. Vacuum rotary evaporator was used to remove the residual TFA in the final products.

The chemical structures of Pt(IV)-peptide-bis(pyrene) and cRGD-PEG-DSPE polymers were determined by Bruker ARX 400 MHz spectrometer. 1H NMR spectra of them (10 mg/mL) in *d6*-DMSO were recorded. The molecular weight of Pt(IV)-peptide-bis(pyrene) was detected by MALDI-TOF MS.

3. Preparation and characterization of nanoparticles (NPs)

The formulation for preparing Beclin1 siRNA and Pt(IV) pro-drugs loaded nanoparticles in PBS was as follows: The copolymers (Pt(IV)-peptide-bis(pyrene), cRGD-PEG-DSPE, and PEG-DSPE 1:0.5:0.5, mole ratio) were first mixed in DMSO (200 μL), and then Beclin1 siRNA was added. Under stirring (1000 rpm), the above mixture was dropwise added to the water (2.0 mL). An Amicon Ultra-15 Centrifugal Filter (MWCO 30 KDa, Millipore) was finally used to remove the organic solvent. The final nanoparticles were dispersed in 1 mL of PBS followed by washing with ddH_2O 2 times. The morphology structure and hydrodynamic diameter of

nanoparticles were measured by transmission electron microscopy (TEM, Tecnai G2 20 S-TWIN) and dynamic light scattering (DLS, (Zetasizer Nano ZS)), respectively.

4. In vitro GSH-triggered Pt release. siBec1@PPN (25 μM) were cultured with/without 10 mM glutathione (GSH) and then dialyzed against 20 mL of PBS buffer in a dialysis bag (MWCO: 1000 Da), the dialysis buffer was collected and measured by ICP-MS.

5. Intracellular GSH detection. The A549 normal tumor cells and cisplatin-resistant A549 tumor cells were seeded in 6-well plates (50,000 cells per well) and treated with PBS (control, Ctrl), Pt(IV), PPN, and siBec1@PPN for 48 h, respectively. The cells were collected and homogenized. GSH assay was used to measure the GSH level of the supernatant from treated cells.

6. Confocal laser scanning microscopy (CLSM).

The A549 normal tumor cells were seeded in confocal plates (Costar, United States) (1×10^5 cells/per well) cultured with 1 mL of medium including 10% FBS for 15 h. Thereafter, 1 mL of medium with siRNA-Cy3@PPN (10 μM). The cells were detected by Zeiss LSM710 CLSM with a 63× objective lens.

7. Western Blot

The A549 normal tumor cells and cisplatin-resistant A549 tumor cells first treated with PBS (control, Ctrl), Pt(IV), PPN, and siBec1@PPN, respectively, and then collected and re-suspended with 100 μL of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Triton-X 100 and protease inhibitor). 60 μg of every sample were subjected to SDS-PAGE and then trans-ferried to a nitrocellulose membrane blots. Next, a blocking buffer (5% (wt/v) non-fat milk, 0.1% (v/v) Tween 20 in 0.01 M TBS) was used to block nitrocellulose membrane blots, and thereafter incubated with primary antibodies at 4 °C overnight and with a secondary antibody with HRP at room temperature for 2 h. Finally, the nitrocellulose membrane blots were detected by Typhoon Trio Variable Mode Imager. NIH ImageJ software was used to analysis the band density of proteins.

8. Real-time PCR.

The total RNA was extracted from A549 tumor cells using TRIZOL Reagent according to the manufacturer's instruction. cDNA was generated from total RNA using QuantiTect Reverse Transcription Kit and mRNA level determined using Rotor-Gene SYBR® Green PCR Kit. All the experiments were performed in triplicate. The fold-change in gene expression relative to the control was calculated by 2^ΔΔCT. Primers for genes:

ULK1: 5′-TCGAGTTCTCCCGCAAGG-3′ and 5′-CGTCTGAGACTTGGCGAGGT-3′

TFEB: 5′-CGG CAG TGC CTG GTA CAT-3′ and 5′-CTG CAT GCG CAA CCC-3′

Beclin1: 5′-GGCTGAGAGACTGGATCAGG-3′ and 5′-CTGCGTCTGGGCATAACG-3′

GPX4: 5′-GCCTTCCCGTGTAACCAGT-3′ and 5′-GCGAACTCTTTGATCTCTTCGT-3′

9. *In vitro* cell proliferation

A549 normal tumor cells and cisplatin-resistant A549 tumor cells with 100 μL of medium containing 10% FBS were seeded and cultured in 96-well plates (5,000 cells per well) for 16 h. Thereafter, 100 μL of medium containing PBS (control, Ctrl), Pt(IV), PPN, and siBec1@PPN at different concentrations were added and incubated with 48 h. The CCK-8 assay was finally utilized to evaluate the cytotoxicity of them. The UV-Vis absorptions of all samples were measured by a Microplate reader at 450 nm and a reference wavelength of 690 nm, respectively. Cell viability (%) was equal to $(A_{sample} - Ab_{lank})/(A_{control} - A_{blank}) \times 100$. All

the experiments were performed in triplicate.

Apoptotic process was detected by Annexin V-FITC/PI double-staining by flow cytometry. Briefly, the cells that were treated with different treatments were harvested, washed with PBS one time, and resuspended in binding buffer with Annexin V-FITC and PI solution in the dark for 10–15 min at room temperature, and then Annexin V/PI-stained cells were analyzed by flow cytometry within 1 h.

10. *In vivo* biodistribution of siBec1@PPN

siBec1@PPN was firstly labeled with Cy5. A549 normal tumor-bearing athymic nude mice (3 mice per group) were injected with 6.5 μM siBec1@PPN-Cy5 via an intravenous injection. After 6 h, the mice were sacrificed, tumor and some organ (heart, liver, spleen, lung, and kidney) were excised for *ex vivo* imaging by Maestro 2 *In Vivo* Imaging System (CRi Inc, USA). Image-J software was used to evaluate fluorescence intensity that presents the accumulation of nanoparticles in tissues.

11. *In vivo* antitumor measurement of siBec1@PPN

A549 normal tumor and cisplatin-resistant A549 tumor-bearing athymic nude mice were randomly assigned to 3 groups (n = 5) and intravenously injected with PBS (Ctrl), PPN, siBec1@PPN at 3 mg Pt(II) dose per kg mouse every two days for 9 or 7 times, the first injection day as day 0. Tumor volume: $V = a^2 \times b \times 1/2$, where a and b are the shortest and longest diameters. Mice body weight and their physical performance were recorded as indicators of systemic toxicity.

12. Immunohistochemistry (IHC) and Immunofluorescence staining. After the mice were treated with PBS (Ctrl), PPN, siBec1@PPN, mice were sacrificed, tumor and some organ tissues (heart, liver, spleen, lung, and kidney) were deprived and fixed with 4% paraformaldehyde. The tissues were embedded in paraffin and cut into sections, and finally stained with haematoxylin and eosin (H&E). For tumor tissues, Beclin1 and Lc3 proteins would be detected by immunofluorescence staining. In brief, the tumor slices were heated for 1 h at 60 °C and washed with xylene, ethanol, PBS 3 times, respectively. Next, 10% FBS was used to block for 1.5 h, and then the slices were incubated with Beclin1 or LC3 antibodies (CST) for 1 h at 4 °C. After washed with PBS/0.2% Triton X-100 thrice, the slices were incubated with Alexa Fluor 633 or Alexa Fluor 488-conjugated secondary antibodies (Abcam) for 1 h. When the slides were washed with PBS thrice, they would be stained with Hoechst 33342, and then viewed by Zeiss LSM710 CLSM.

13. Statistical analysis

All the experiments were repeated at least three times and data were expressed as the mean ± standard deviation (SD) unless otherwise noticed. Unpaired student's t-test was used for between two-group comparison, and one-way analysis of variance (ANOVA) for multi-group analysis. Asterisk represented statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supporting Figures and Captions

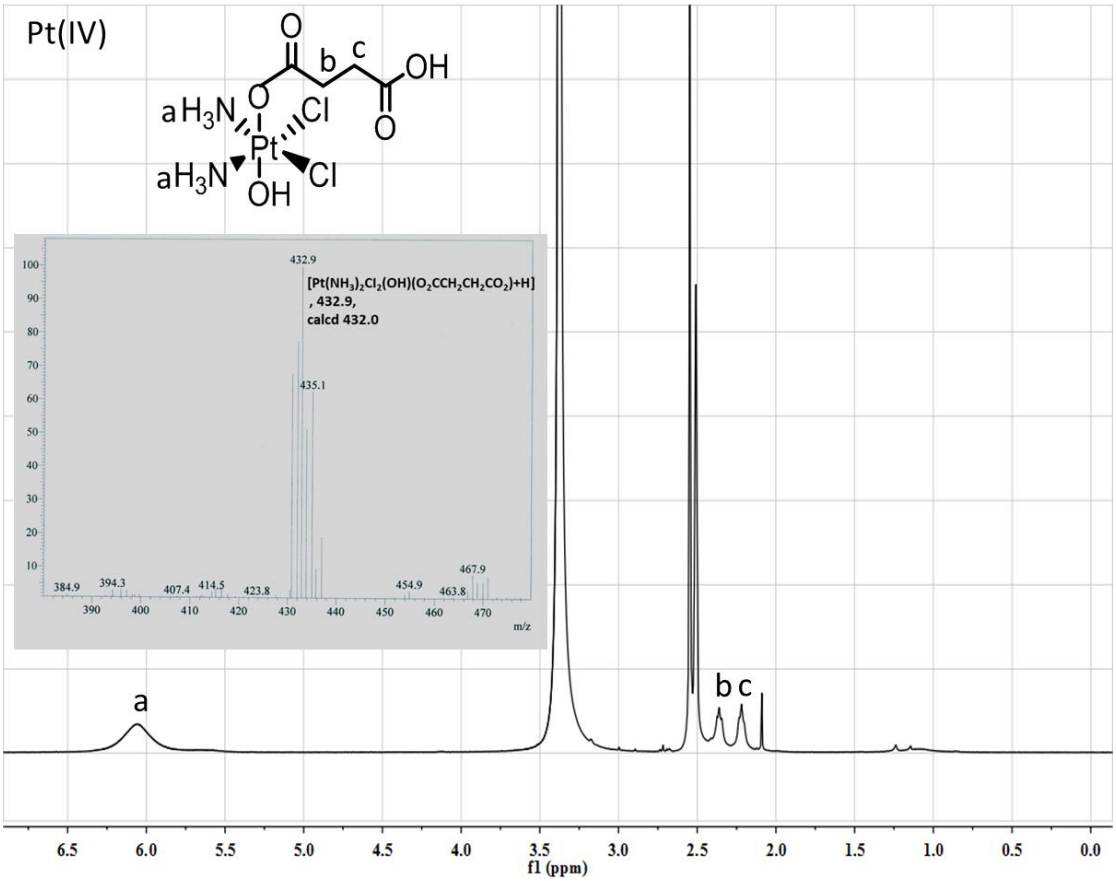

Figure S1. The chemical structures of Pt(IV) were detected by ^{1}H NMR. The inset picture is the molecular weight of Pt(IV) detected by MALDI-TOF MS.

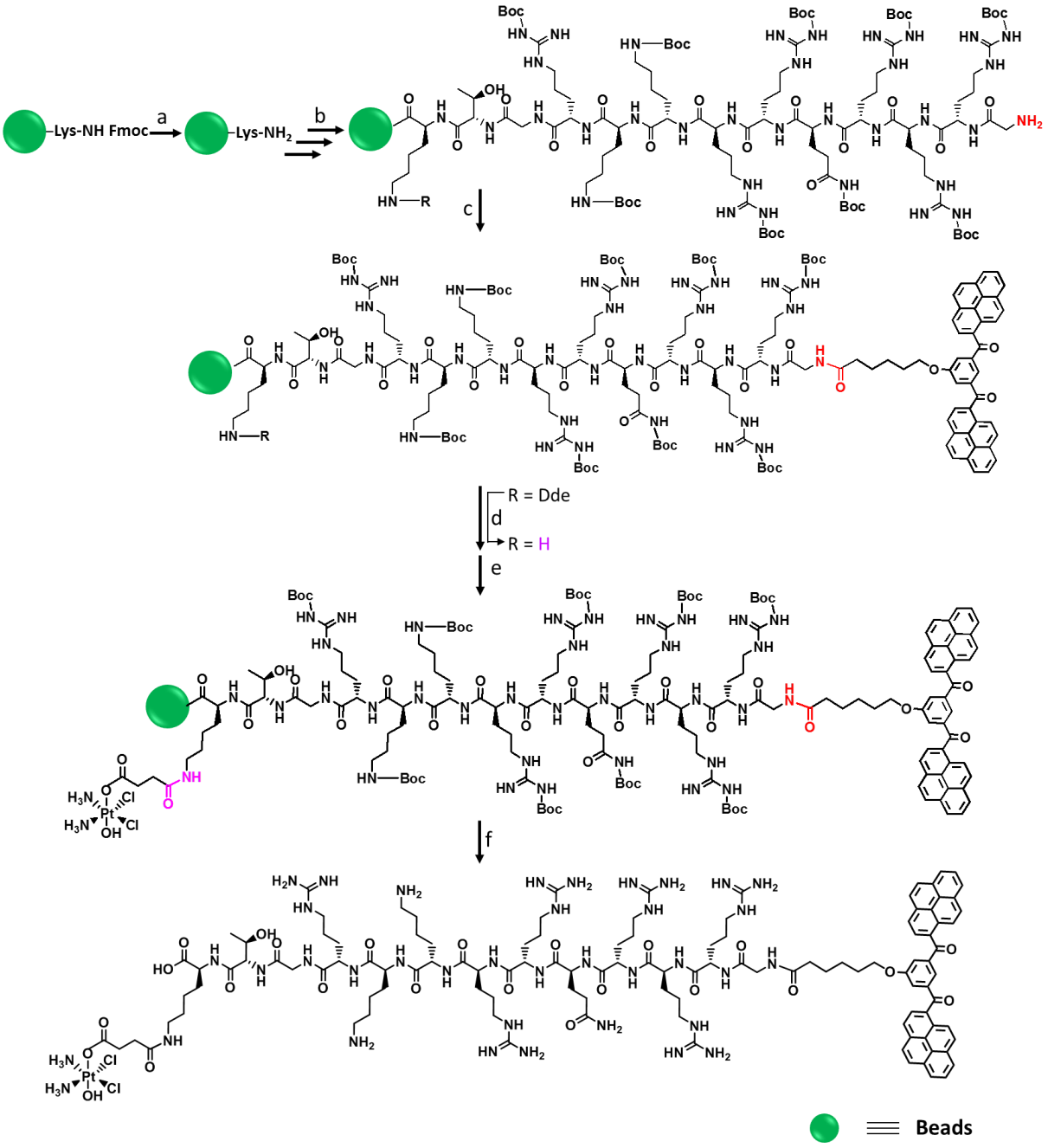

Figure S2. Illustration of synthetic route of Pt(IV)-peptide-bis(pyrene) conjugates. Reagents and conditions: (a) 20% piperidine in HPLC-grade DMF; (b) peptide preparation method based on Fmoc solid-phase peptide synthesis: amino acids with Fmoc, NMM, HBTU, DMF, 2 h; (c); bis(pyrene), NMM, HBTU, DMF, 2 h (d) 5% hydrazine in HPLC-grade DMF; (e) Pt(IV), NMM, HBTU, DMF, overnight; (f) TFA/TIS/H2O (v/v/v = 95:2.5:2.5), 3 h.

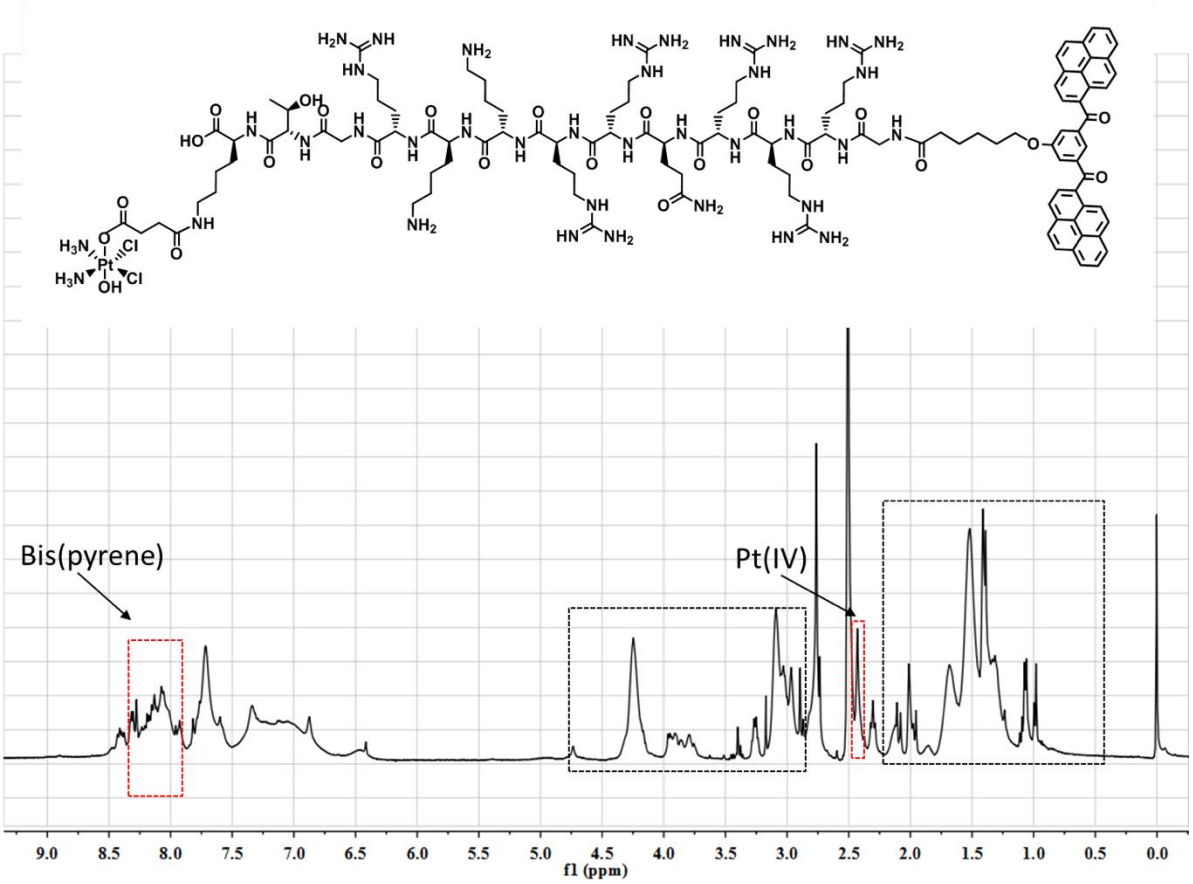

Figure S3. ^{1}H NMR spectra of Pt(IV)-peptide-bis(pyrene) in d_6-DMSO, red dotted boxes represent the bis(pyrene) characteristic peaks, and black dotted boxes represent the Pt(IV) characteristic peaks. Concentration: 10 mg/mL.

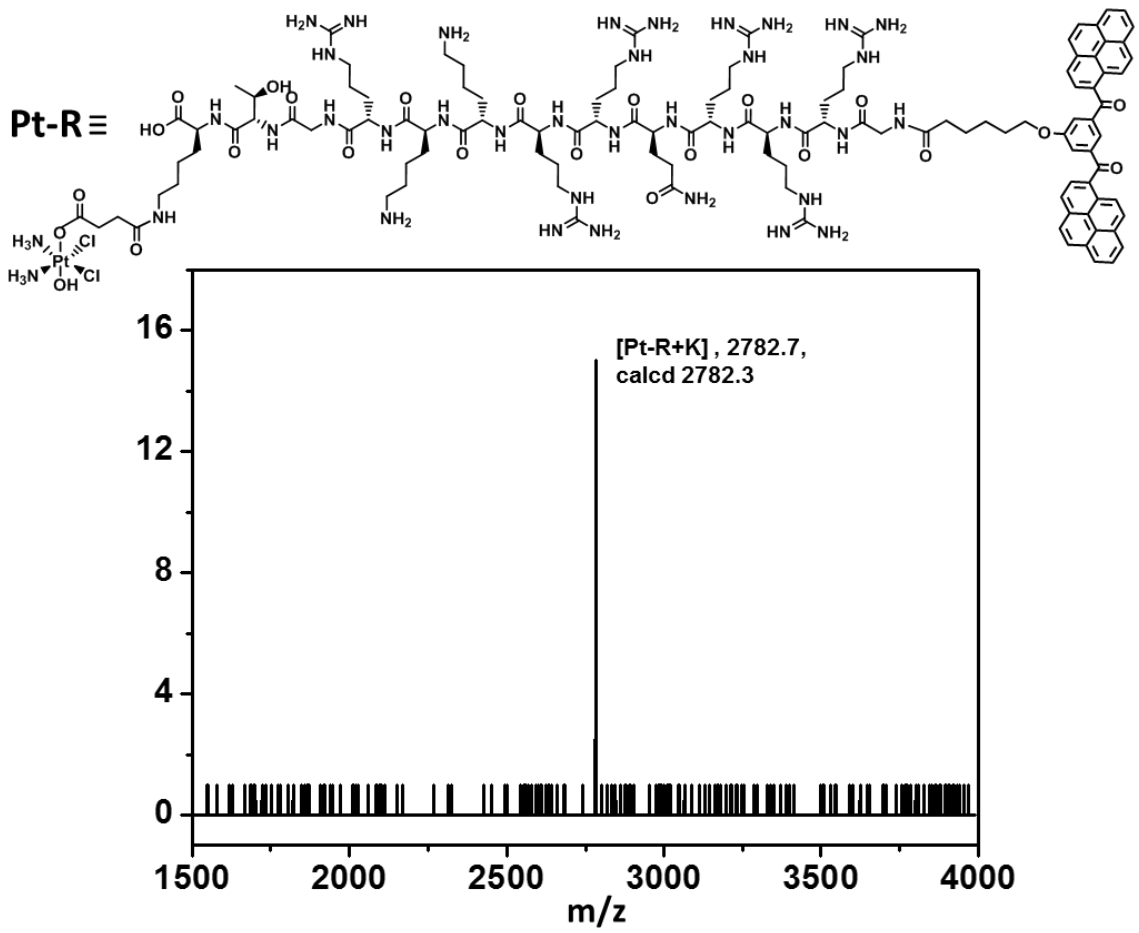

Figure S4. The molecular weight of Pt(IV)-peptide-bis(pyrene) detected by MALDI-TOF MS.

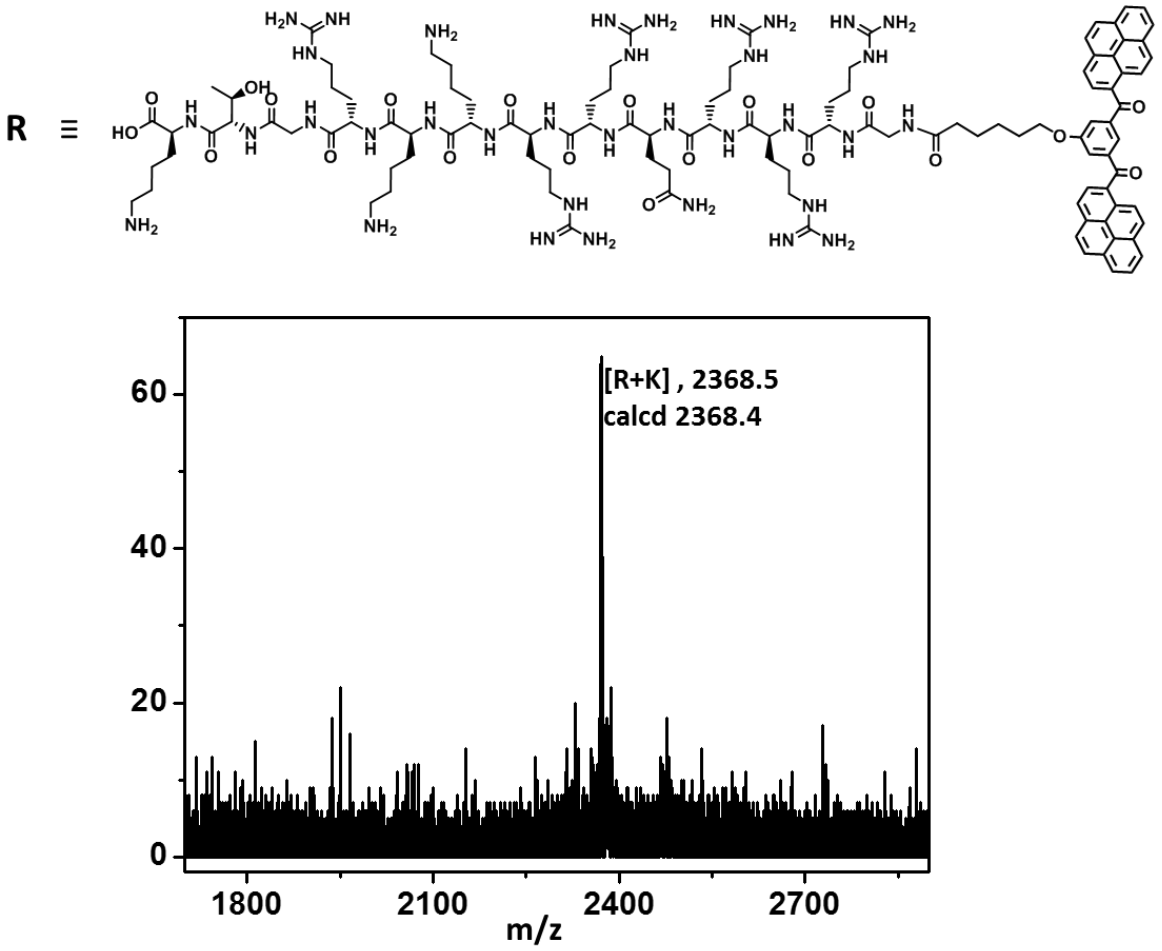

Figure S5. The molecular weight of peptide-bis(pyrene) detected by MALDI-TOF MS.

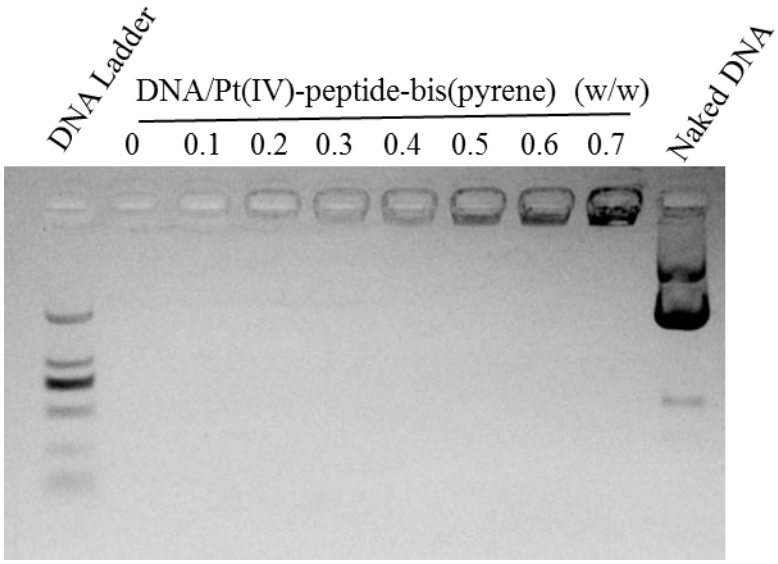

Figure S6. DNA gel retardation assays of gene loading efficiency. Naked DNA was used as a control and the concentration of the Pt(IV)-peptide-bis(pyrene) polymer was at 0.5 mg/mL.

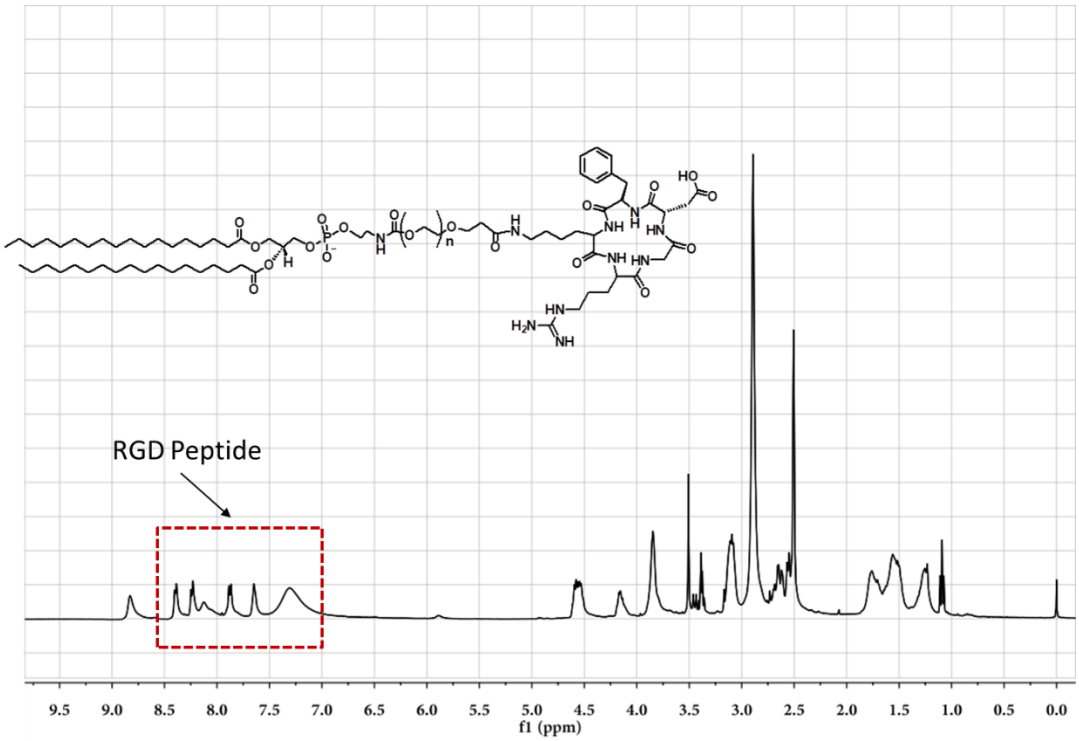

Figure S7. ^{1}H NMR spectra of cRGD-PEG-DSPE in d_6-DMSO, red dotted boxes represent the cRGD characteristic peaks. Concentration: 10 mg/mL.

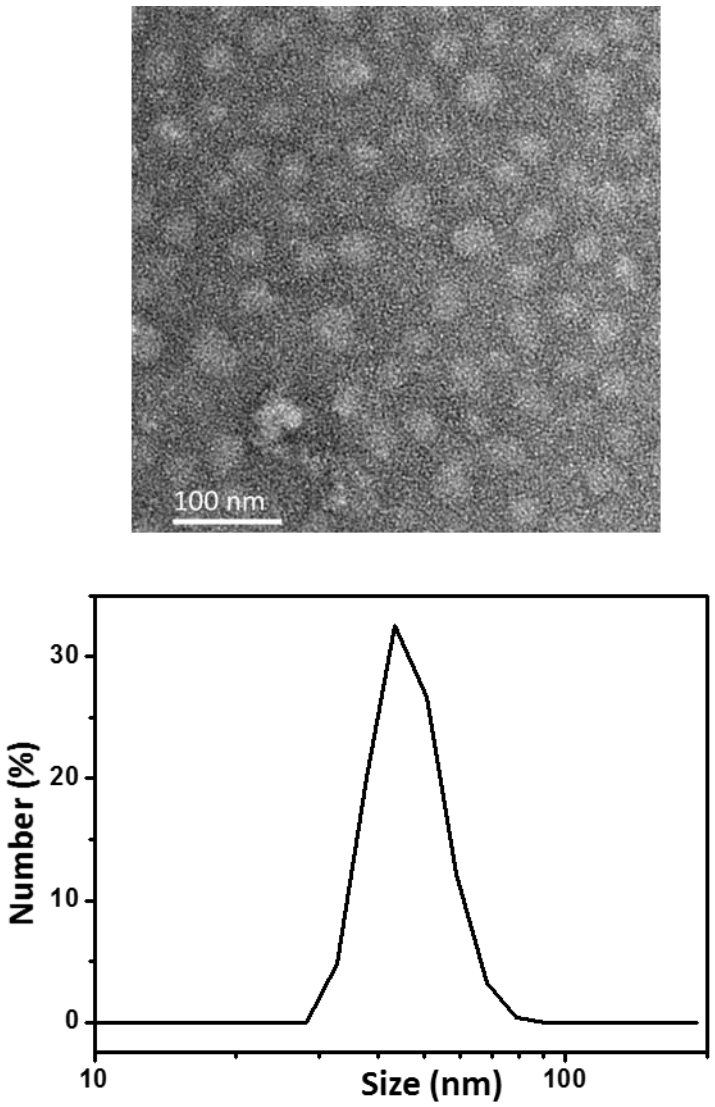

Figure S8. TEM images of PPN and its size distribution detected by DLS.

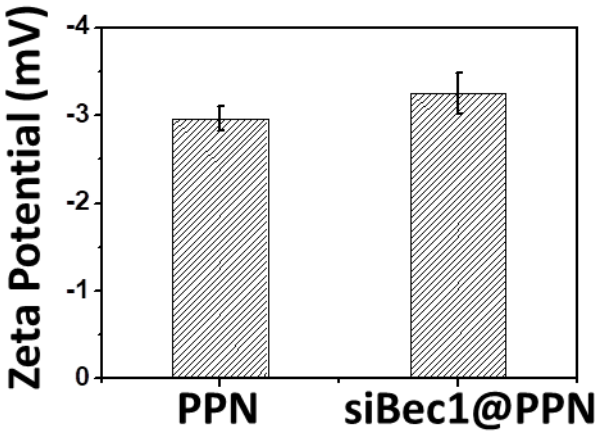

Figure S9. Zeta potential of PPN and siBec1@PPN.

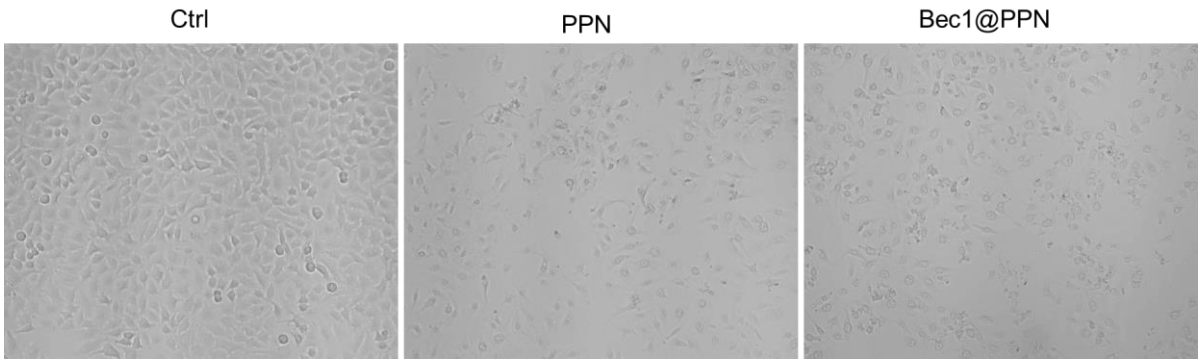

Figure S10. Optical microscopy imaging for the cells that were treated with PBS (Ctrl), PPN, and siBec1@PPN for 48 h.

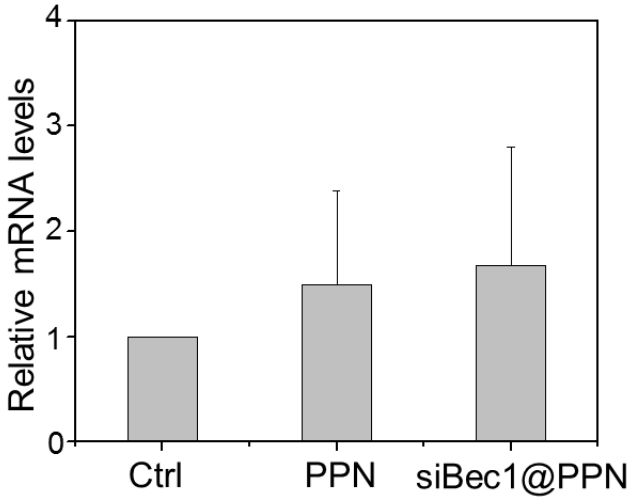

Figure S11. qPCR analysis for the effect of Beclin1 silence on mRNA levels of GPX4 in comparison to GAPDH. A549 cells were treated with PBS (Ctrl), PPN, and siBec1@PPN for 48 h.

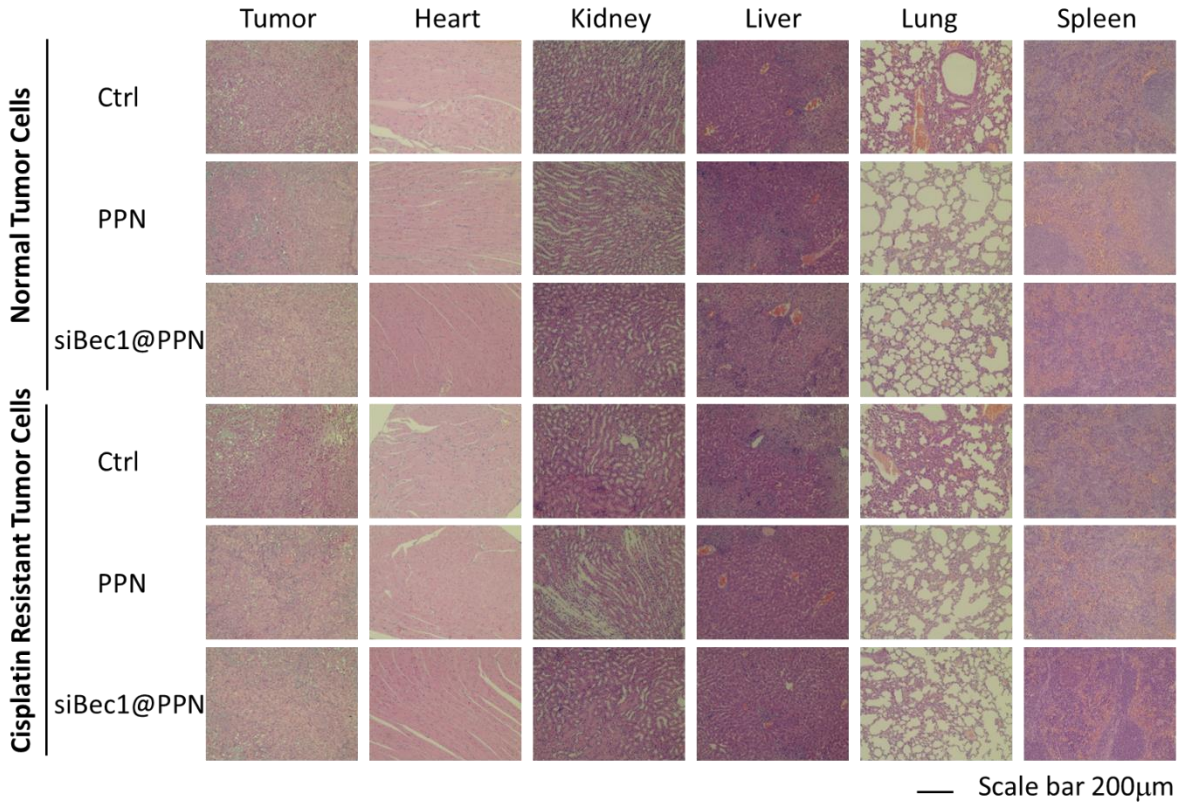

Figure S12. H&E staining images of tumor, liver, kidney, spleen, heart and lung tissues.

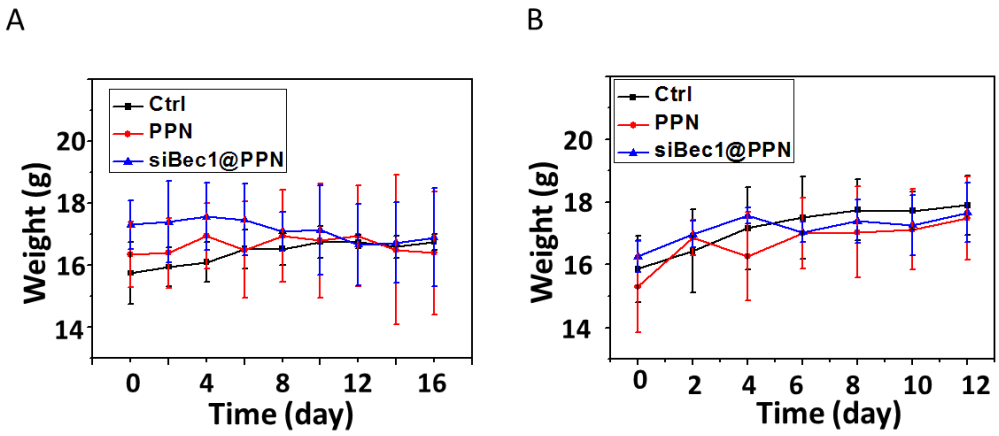

Figure S13. Body weight changes of PBS, PPN, siBec1@PPN in (A) normal A549 tumor- and (B) cisplatin-resistance A549 tumor-bearing athymic nude mice.