Supplementary Information

Supramolecular Self-assembly Nanoparticles for Chemo-Photodynamic Dual Therapy against Cisplatin Resistant Cancer Cells

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Experimental Section

Materials and methods

Cisplatin, succinic anhydride (SA), N,N-diiso-propylethylamine (DIEA) and 2-(1H-7-aza-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU) were purchased from Aladdin Reagent. Meso-tetra(4-pyridyl)porphine (TPyP), 1-adamantyl bromomethyl ketone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Sigma Aldrich. All other chemicals were from commercial sources and used without further purification. Distilled water was purified by passing through a Millipore Milli-Q Biocel purification system (18.2 MΩ) with a 0.22 μm filter.

UV-vis spectra were monitored with a Varian Cary 300 UV/Vis spectrophotometer. The fluorescence emission spectra were obtained using a Shimadzu RF-5301PC spectrofluorophotometer. ESI-MS spectra were performed on a Thremo LCQ-DECA-XP spectrometer. ^{1}H NMR spectra were recorded on a Bruker AVANCE-400 NMR spectrometer. Elemental contents were analyzed by a Perkin-Elemer 240 elemental analyzer. Pt content was measured on a Thermo X Series 2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Morphology of the supramolecular self-assembly nanoparticles was characterized by a Bruker Multimode 8 atomic force microscope (AFM) under ScanAsyst mode.

Crystallographic structure determination

Crystals of TpyP-Ad qualified for X-ray analysis were obtained by slow evaporation of methanol solution of TpyP-Ad. The data were collected at 150(2) K on a Rigaku Pilatus diffractometer equipped with Mo Kα radiation (λ = 0.71073 Å). The empirical absorption corrections were applied using the SADABS program.[5] The crystal structure of TPyP-Ad was solved by direct methods with program SHELXS and refined using the full-matrix least-squares program SHELXL.[6] In the final stage of least-squares refinement, non-hydrogen atoms were refined anisotropically. PLATON SQUEEZE has been applied to calculate the solvent disorder area and remove its

contribution to the overall intensity data. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre. The deposition number is CCDC 1022409.

Synthesis and Characterization

Synthesis of Pt-CD: The complexes *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)_2$] and *c,c,t*-[$Pt(NH_3)_2Cl_2(O_2CCH_2CH_2CO_2H)_2$] were synthesized by literature methods.[1,2] Synthesis of 6-monodeoxy-6-monoamino-β-cyclodextrin was carried out according to previous report.[3] To a solution of *c,c,t*-[$Pt(NH_3)_2Cl_2(O_2CCH_2CH_2CO_2H)_2$] (0.16 g, 0.3 mmol) in N,N-dimethylformamide (DMF, 10 mL) was added a DMF solution (0.5 mL) containing HATU (0.342 g, 0.9 mmol). This mixture was stirred for 10 min at room temperature. To the resulting solution was added a DMF solution containing 6-monodeoxy-6-monoamino-β-cyclodextrin (0.788 g, 0.6 mmol) and DIEA (0.097 g, 0.75 mmol). The mixture was stirred at room temperature for 24 h in the dark. DMF was then removed under vacuum to afford a white solid which was washed repeatedly with acetone. The crude product was recrystallized from ethanol/water (1:1) and then purified by column chromatography over Sephadex G-25 with distilled deionized water as the eluent to give the pure compound, Pt-CD (0.33 g, 36.2%). ^{1}H NMR (400 MHz, d_6-DMSO), δ 7.69 (s, 2H), 6.50 (s, 6H), 5.96-5.60 (m, 28H), 4.83(s, 14H), 4.48 (s, 12H), 3.88-3.43 (m, 56H), 3.33-2.90 (m, 28H), 2.10(d, 8H). ESI-MS: m/z Calcd $[M+H]^+$ 2765.8, Found 2766.2. Elemental Analysis, Found: C 36.24; H 6.09; N 1.85. Calc. for $C_{92}H_{154}Cl_2N_4O_{74}Pt \cdot 15H_2O$: C 36.39; H 6.11; N 1.85 %.

Synthesis of TPyP-Ad: Synthesis of meso-tetra[1-(1-adamantyl methyl ketone)-4-pyridyl] porphyrin (TPyP-Ad) followed the procedures described in the literature. [4] To a solution of TPyP (0.309 g, 0.5 mmol) in DMF (10 mL) was added a DMF solution (20 mL) of 1-adamantyl bromomethyl ketone (0.772 g, 3.0 mmol). The resulting solution was heated to 110 ^{o}C and stirred for 24 h under nitrogen atmosphere. After being cooled to room temperature, the brown precipitate was collected by filtration and washed with diethyl ether to yield crude product. The crude product was

purified by recrystallization from methanol/chloroform (1:2) to give a purple solid, TPyP-Ad (0.62 g, 75.3%). ^{1}H NMR (400 MHz, d_6-DMSO), δ 9.48-9.44 (m, 8H), 9.18 (s, 24H), 6.49 (d, 8H), 2.19-2.07(m, 36H), 1.84 (s, 24H). ESI-MS: m/z Calcd (Found) $[M-4Br]^{4+}$ 331.9 (331.9), $[M-4Br-H]^{3+}$ 442.2 (442.4), $[M-4Br-2H]^{2+}$ 662.9 (663.5). Elemental Analysis, Found: C 58.65; H 6.14; N 6.25. Calc. for $C_{88}H_{94}Br_4N_8O_4 \cdot 9H_2O$: C 58.41; H 6.24; N 6.19 (%).

UV-vis Titration

UV-vis titration was carried out according to a well-established method by Cramer. [7] Pt-CD and TPyP-Ad were dissolved in distilled water to prepare the stock solution. All titrations were carried out at 298 ± 0.1 K. Pt-CD was titrated into a solution of TPyP-Ad (5 μM) in 1 cm cuvette to achieve different ratio. After each addition, the mixture solution stabilized for 5 min before the UV-vis curve was recorded. The absorbance changes at 425 nm (ΔA_{425}) were plotted as a function of the Pt-CD:TPyP-Ad ratio.

Isothermal titration calorimetry (ITC)

ITC was employed to characterize the complexation of Pt-CD (Host) with TPyP-Ad (Guest). The experiments were carried out on the VP-ITC (MicrolCall). All titration experiments were carried out at 298 ± 0.1 K. The guest molecules TPyP-Ad (0.025 mM) were loaded into the 1.40 mL sample cell and the host molecules Pt-CD (0.5 mM) were loaded into the continuously rotating (300 rpm) 280 μL syringe. The titration was conducted by adding 10 μL aliquots of the host solution into the guest solution at an interval of 4 min (25 injections in total). The first 10 μL aliquot injection was ignored to eliminate the effect of solute diffusion across the syringe tip during the equilibration period. The titration data were fitted using an Origin program provided with the calorimeter (Microcal Origin 5.0 for ITC). The molar enthalpy changes (ΔH), entropic changes (ΔS) and the inclusion complex association constants K_a were calculated accordingly. The total heat produced during each injection was

obtained by integrating heat flows over the time. The calculated total heat (μcal) was plotted against the mole ratio of the titrant (host) to the titrate (guest).

Atomic Force Microscopy (AFM) Measurement

The aqueous solution of Pt-CD and TPyP-Ad was mixed together to initial the supramolecular self-assembly process ([Pt-CD] = 50 μM, [TPyP-Ad] = 25 μM). Then the solution was dropped onto newly clipped mica and air-dried. The samples were performed using a Bruker Multimode 8 AFM under ScanAsyst mode in air at room temperature.

Cell Lines and Culture Conditions

A549, A549R cells were obtained from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Cells were routinely maintained in RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium containing 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco BRL). The cells were cultured in tissue culture flasks in a humidified incubator at 37 ^{o}C, in an atmosphere of 5% CO_2. Cisplatin-resistant A549R cells were cultured in medium containing cisplatin to maintain the resistance.

MTT Cell Proliferation Assay

A549 or A549R cells were seeded into 96 well tissue culture plates at a confluence of 2000 cells per well and incubated for 24 h. Then cells were treated with various concentrations of CDDP, Pt-CD/TPyP-Ad, Pt-CD or TPyP-Ad. The plates were incubated for 68 h at 37 ^{o}C, and then 20μL of MTT (5 mg/mL in PBS) was added and incubated for 4 h. The medium was removed, cells were lysed by adding 150μL of DMSO, and the absorbance of the purple formazan was recorded at 550 nm using a BioRad iMark plate reader.

For Chemo-Photodynamic dual therapy, the plates were irradiated with 430 nm LED light (HTLD-4II, Height-LED) for 2 min (10 mW/cm^2) 24 h after the addition of the

compounds. Then the plates were incubated for another 48 h, and treated with the same procedure as described above.

Cellular Uptake

To measure the cellular uptake of the platinum complexes, A549R cells were seeded in 10 cm tissue culture dishes and incubated for 24 h. The medium was removed and replaced with fresh medium containing CDDP, Pt-CD or Pt-CD/TPyP-Ad ([Pt] = 5 μM). After 3 h incubation, the cells were washed with PBS, trypsinized and collected. The cells were counted and digested with HNO_3 (65%, 0.5 mL). The platinum content in cells was determined by ICP-MS.

Reactive Oxygen Species (ROS) Detection in Aqueous Solution

The generation of singlet oxygen ($^1O_2$) was determined following the Kraljic procedure [8]. Solutions of p-nitrosodimethylaniline (RNO, 30 μM), imidazole (0.5 mM) in PBS (10 mM, pH=7.4) was added TPyP-Ad (1 μM), Pt-CD/TPyP-Ad (1 μM) or [Ru(2,2′-bipyridine)]$_3Cl_2$ (Rubpy, 10 μM) and then irradiated with 430 nm light at the power density of 10 mW/cm^2 for different periods of time. The absorbance reduction of RNO at 440 nm was plotted against irradiation time to calculate the singlet oxygen quantum yield (Φ_Δ) using Rubpy (Φ_Δ=0.18) as the standard.

Cellular ROS Detection

When irradiated with visible light, porphyrin molecules can transfer the energy of light to surrounding oxygen molecules, thereby producing highly reactive oxygen species, primarily $^1O_2$. The cellular ROS can be detected by ROS probe DCFH-DA, because cell-permeable non-fluorescent DCFH-DA can be de-esterified intracellularly and turns to highly fluorescent DCFH upon oxidation.

Confocal microscopy. A549R cells were seeded into 35 mm dishes (Corning) and incubated for 24 h. Then the medium was removed and replaced with fresh medium containing Pt-CD/TPyP-Ad ([TPyP-Ad] = 2.5 μM). After 24 h incubation, the cells

were washed with serum-free DMEM and then incubated with 10 μM DCFH-DA in serum-free DMEM at 37 °C for 20 min. After washed twice with serum-free DMEM, cells were irradiated with 430 nm LED light for 2 min, and immediately examined under a confocal microscope (LSM 710, Carl Zeiss, Germany) with excitation at 488 nm and emission at 530±20 nm.

Flow cytometry. A549R cells were seeded into 6-well tissue culture plates (Corning) and incubated for 24 h, then incubated with TPyP-Ad or Pt-CD/TPyP-Ad ([TPyP-Ad] = 2.5 μM) for 24 h. The cells were washed with serum-free DMEM and then incubated with 10 μM DCFH-DA in serum-free DMEM at 37 °C for 20 min. After washed twice with serum-free DMEM, cells were irradiated with 430 nm LED light for 2 min. Then the cells were harvested and the fluorescence intensity of cells was measured immediately by flow cytometry (FACSCalibur™, Becton Dickinson). Green mean fluorescence intensities were analyzed using FlowJo 7.6 software.

Annexin V/ propidium iodide (PI) apoptosis assay

The assay was performed according to the manufacturer's protocol. A549R cells cultured in 6-well plates were exposed to the tested compounds ([Pt] = 5 μM, [TPyP-Ad] = 2.5 μM) for 24 h. For chemo-photodynamic dual therapy group, 2 min light irradiation was introduced at 12 h time point. The cell suspension was stained with 5 μL annexin V and 10 μL PI at room temperature for 10 min in the dark, and analysed immediately by flow cytometry (FACSCalibur™, Becton Dickinson). Data were analyzed by FlowJo 7.6 Software.

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2. K. R. Barnes, A. Kutikov and S. J. Lippard, *Chem. Biol.*, 2004, **11**, 557.
3. S. P. Tang, Y. H. Zhou, H. Y. Chen, C. Y. Zhao, Z. W. Mao and L. N. Ji, *Chem. Asian J.*, 2009, **4**, 1354.

4. S. Yu, Y. Yin, J. Zhu, X. Huang, Q. Luo, J. Xu, J. Shen and J. Liu, *Soft Matter*, 2010, **6**, 5342.
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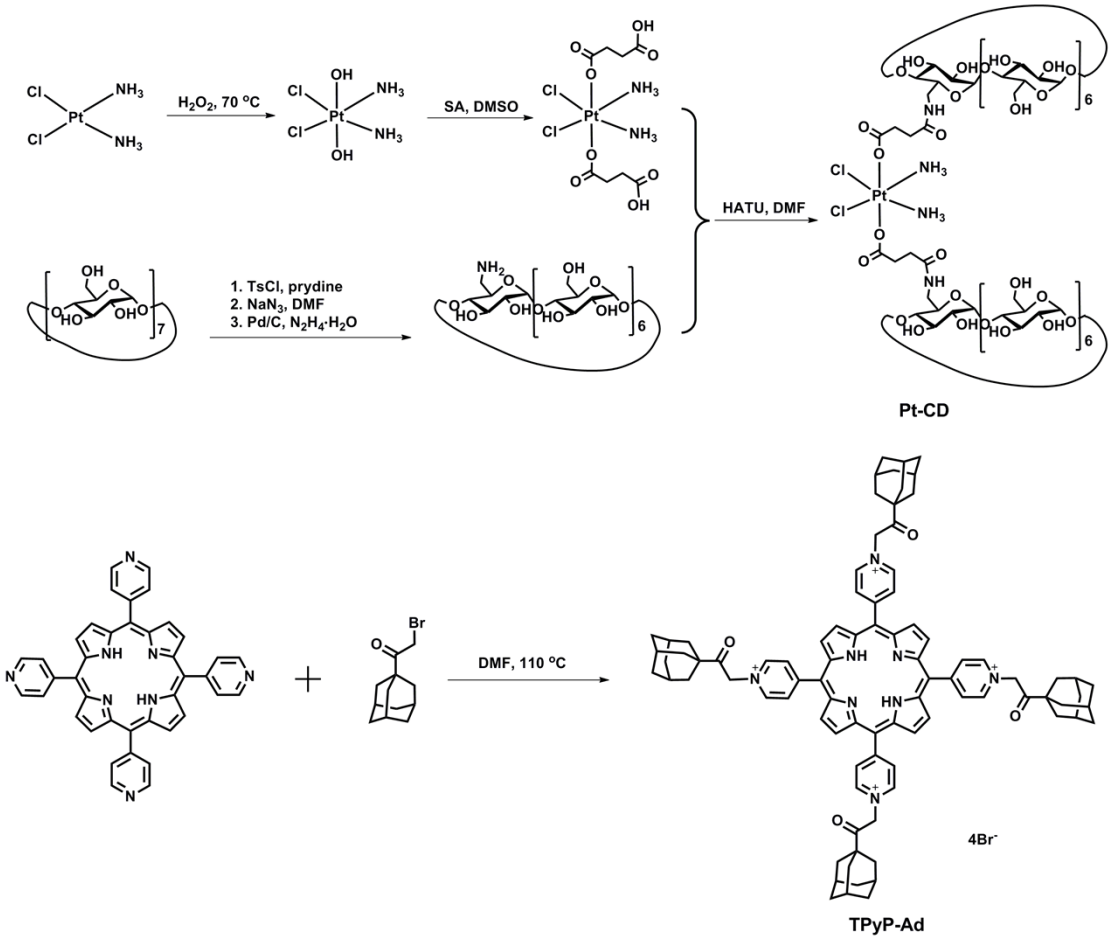
8. I. Kraljić and S. E. Mohsni, *Photochem. Photobiol.*, 1978, **28**, 577.

Supplementary Scheme, Table and Figures



Scheme 1 Synthesis of Pt-CD and TPyP-Ad.

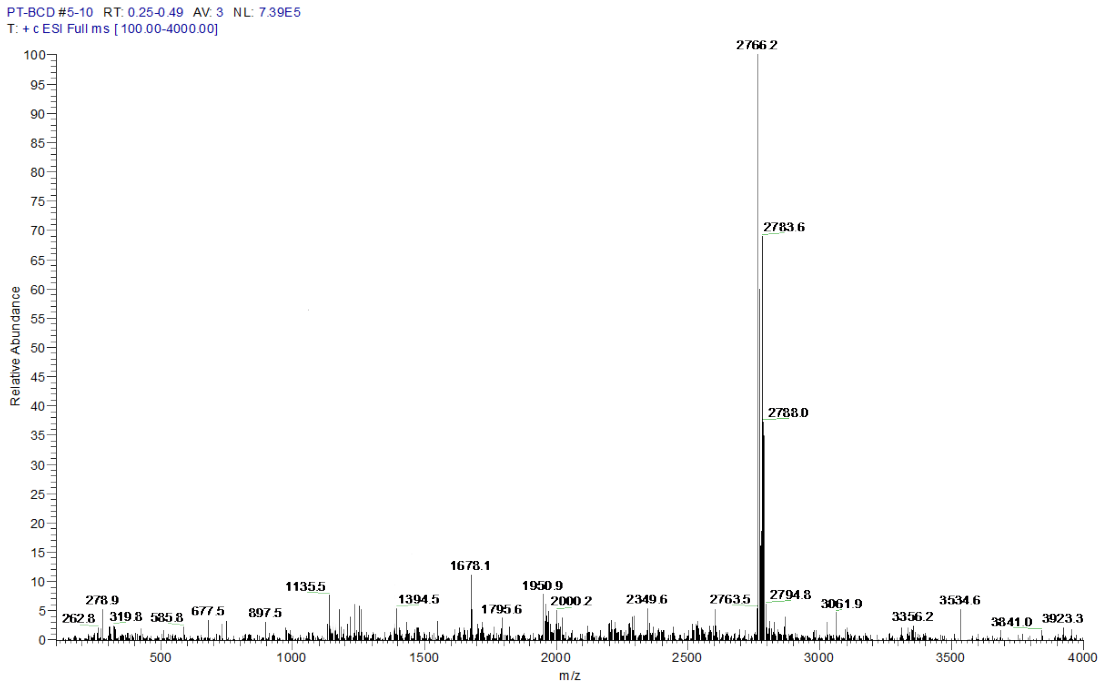


Fig. S1 ESI-MS spectrum of Pt-CD.

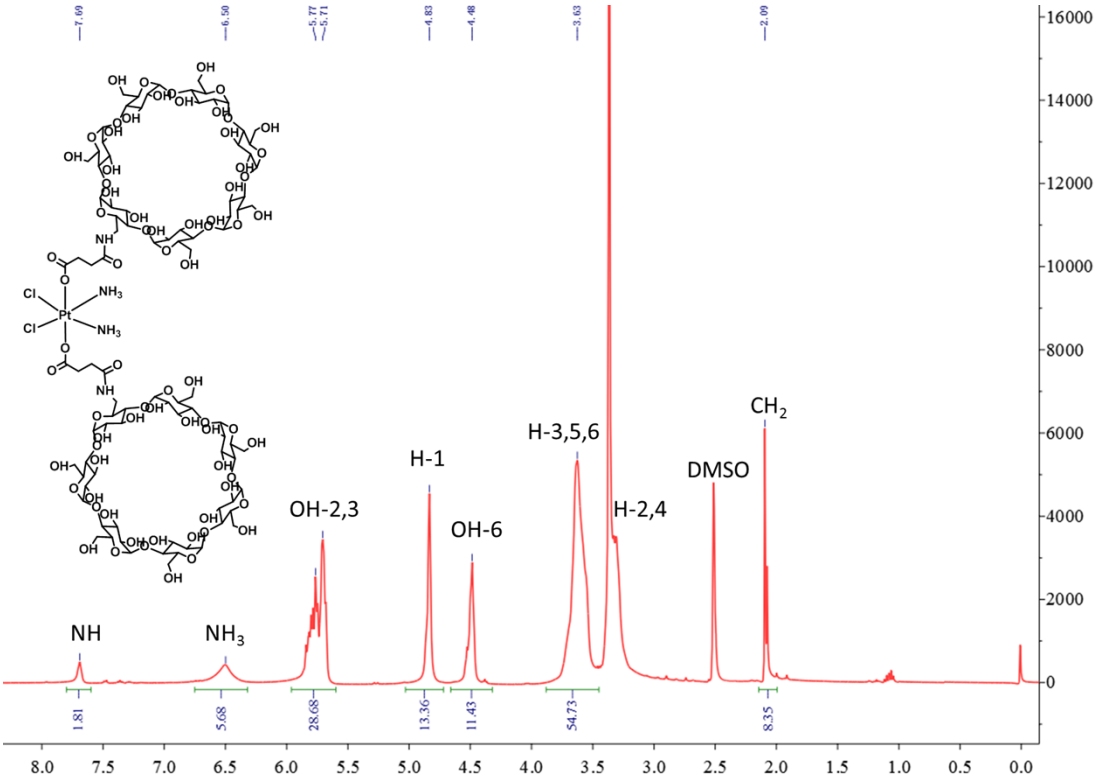


Fig. S2 ^{1}H NMR spectrum of Pt-CD.

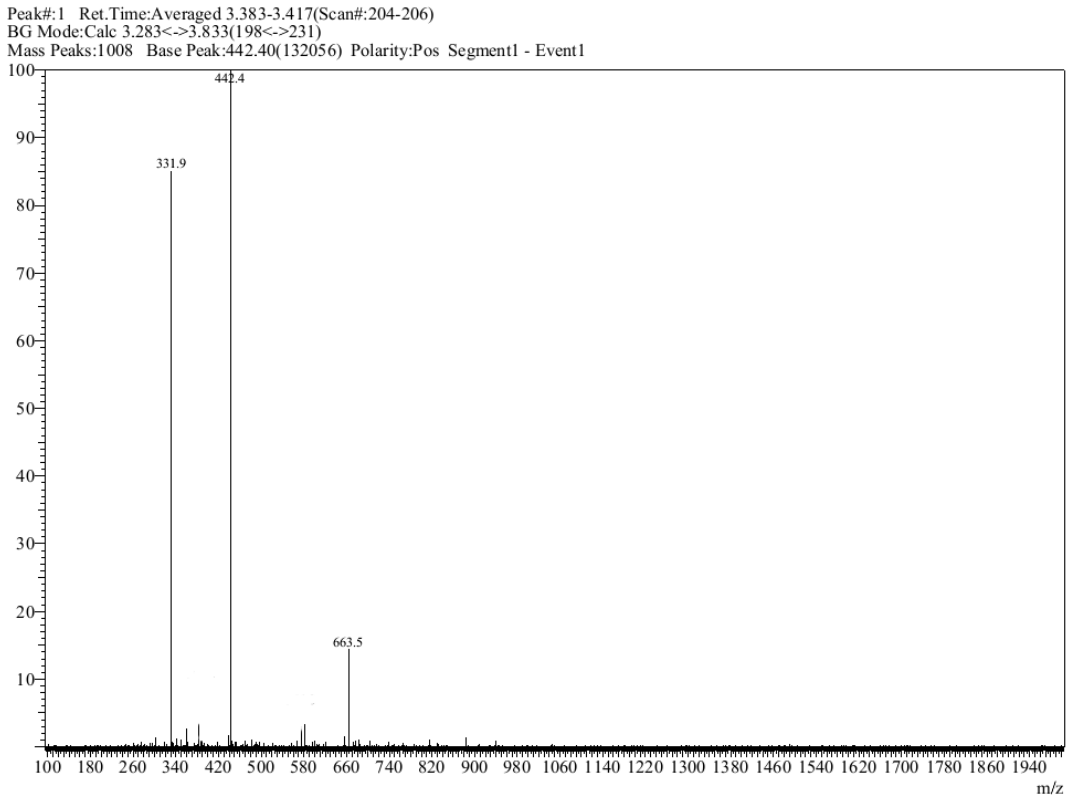


Fig. S3 ESI-MS spectrum of TPyP-Ad.

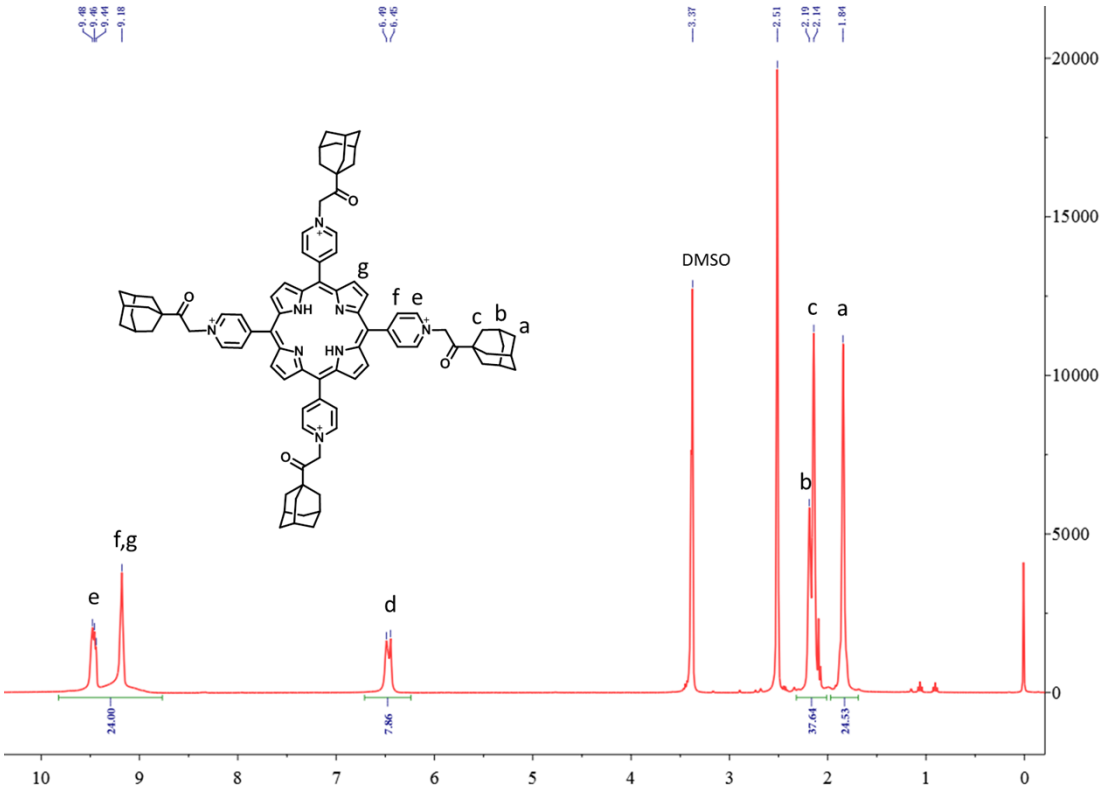


Fig. S4 ^{1}H NMR spectrum of TPyP-Ad.

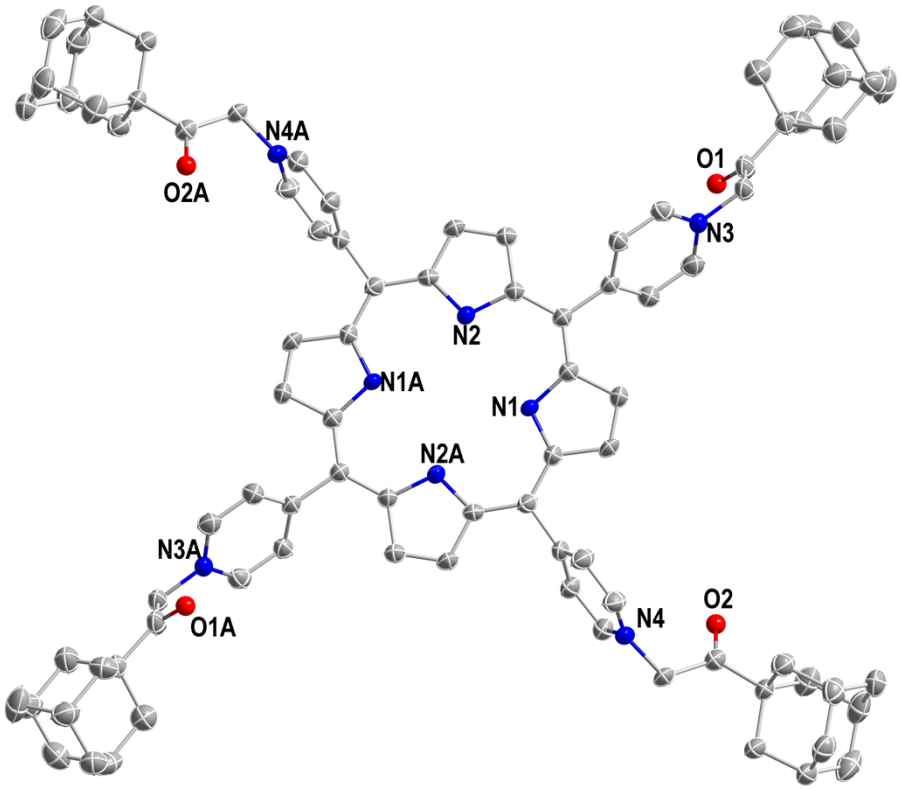


Fig. S5 X-ray crystal structure of TPyP-Ad. The hydrogen atoms and counter ions are omitted for clarity.

Table S1 Crystallographic data of TPyP-Ad.

Compound	**TPyP-Ad**•8CH_3OH•10H_2O
CDCC no.	1022409
Empirical formula	$C_{96}H_{144}Br_4N_8O_{22}$
Molecular weight	2081.83
Description	block, brown
Temperature (K)	150(2)
Crystal size (mm)	0.29×0.16×0.12
λ (Å)	0.71073
Crystal system	Monoclinic
Space group	P21/c
a (Å)	10.8583(4)
b (Å)	18.3496(9)
c (Å)	22.4335(9)
α (°)	90.00
β (°)	91.8340(10)
γ (°)	90.00
Volume, Å	4467.5(3)
Z	2
Absorption coefficient (mm^{-1})	1.883
F(000)	2184
θ range (deg)	3.03 –27.40
Completeness to θ_{max}	0.991
Density (calcd) (mg/m^{-3})	1.548
Reflections collected/unique	34173/10076
final R indices [I > 2σ(I)]	R1=0.0971; wR2=0.1938
R indices (all data)	R1=0. 0621; wR2=0.1695
GOF[b]	1.095

[a] $R1 = \sum \| F_0 | - | F_c \| / \sum | F_0 |, wR2 = \left\{ \sum \left[w\left(F_0^2 - F_c^2\right)^2 \right] / \sum \left[w\left(F_0^2\right)^2 \right] \right\}^{1/2}$ [b] $GOF = \left\{ \sum \left[w\left(F_0^2 - F_c^2\right)^2 / (n-p) \right] \right\}^{1/2}$

where *n* is the number of data and *p* is the number of parameters refined.

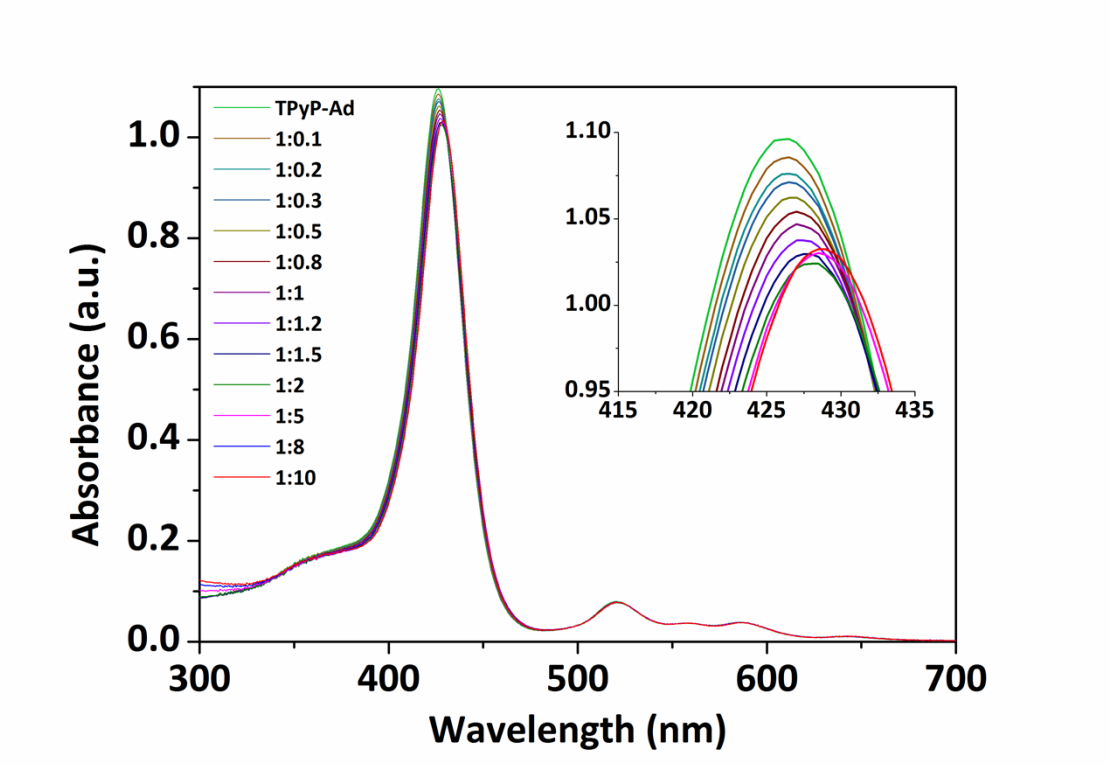


Fig. S6 Absorbance spectra of TPyP-Ad titrated with Pt-CD at different TPyP-Ad:Pt-CD ratios.

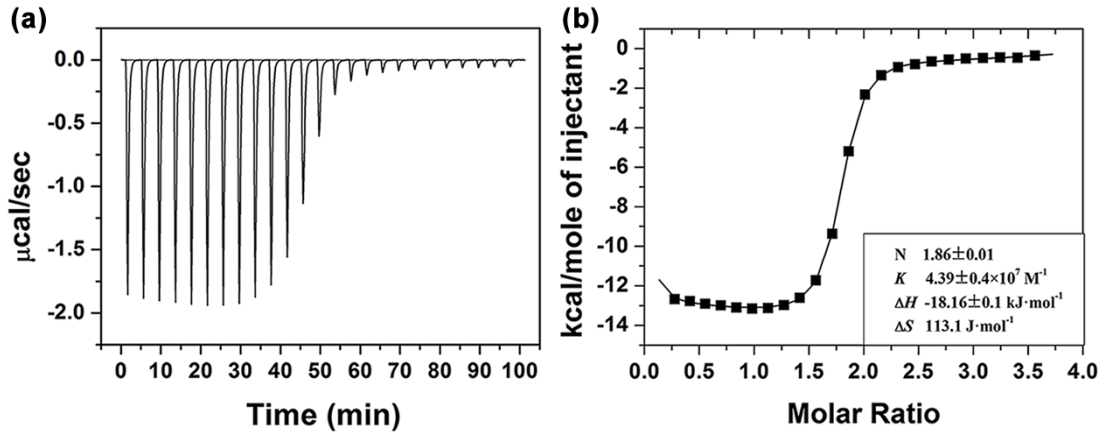


Fig. S7 (a) ITC calorimetric titration thermogram; (b) the integration of the heat signals of the microcalorimetric titration and the fitted curve.

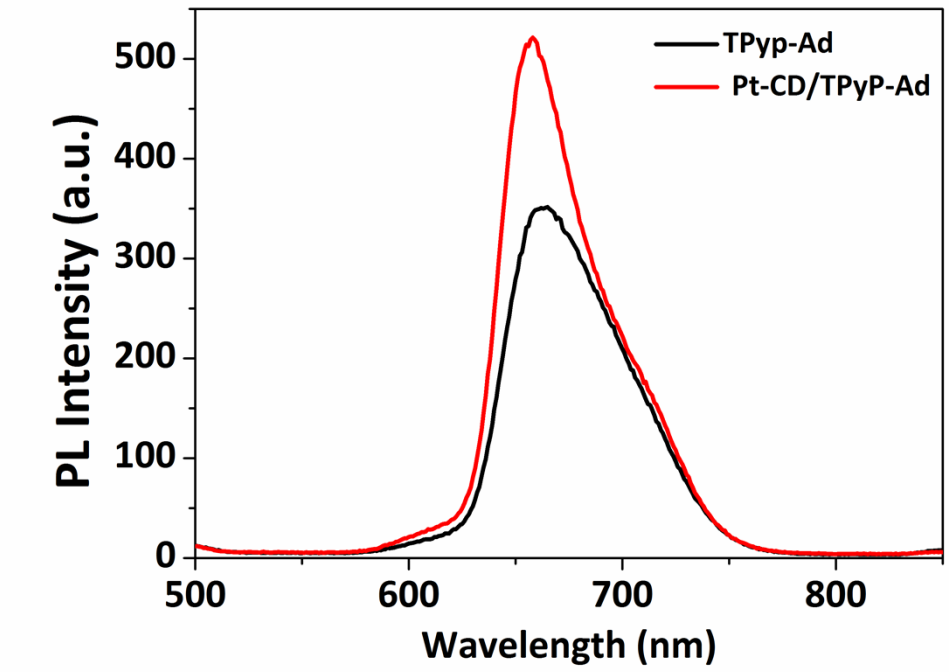


Fig. S8 Fluorescence spectra of TPyP-Ad and Pt-CD/TPyP-Ad.

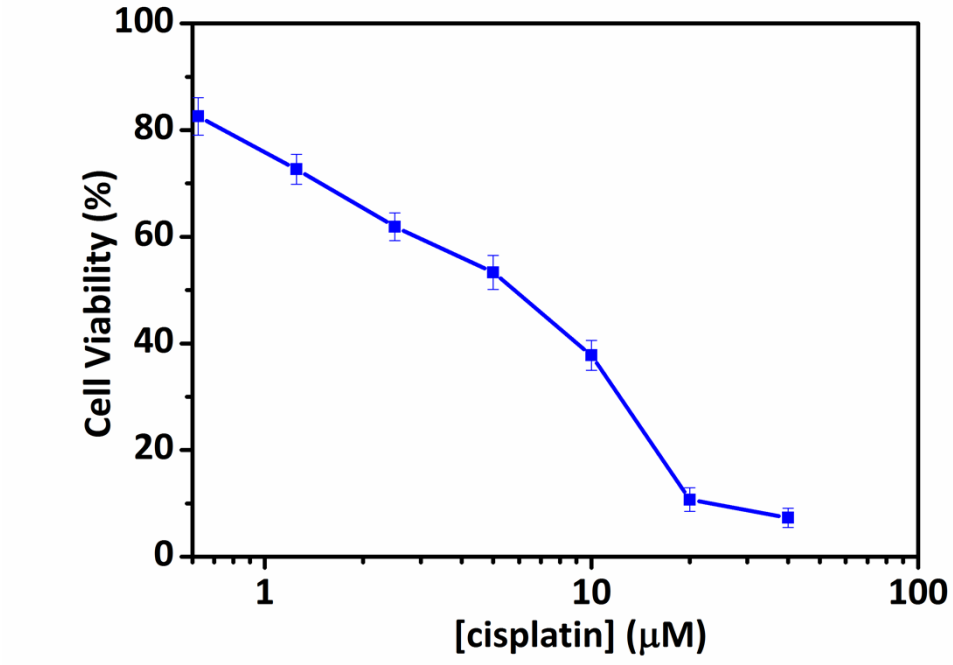


Fig. S9 Cytotoxicity profiles of cisplatin in A549 cells determined by MTT.

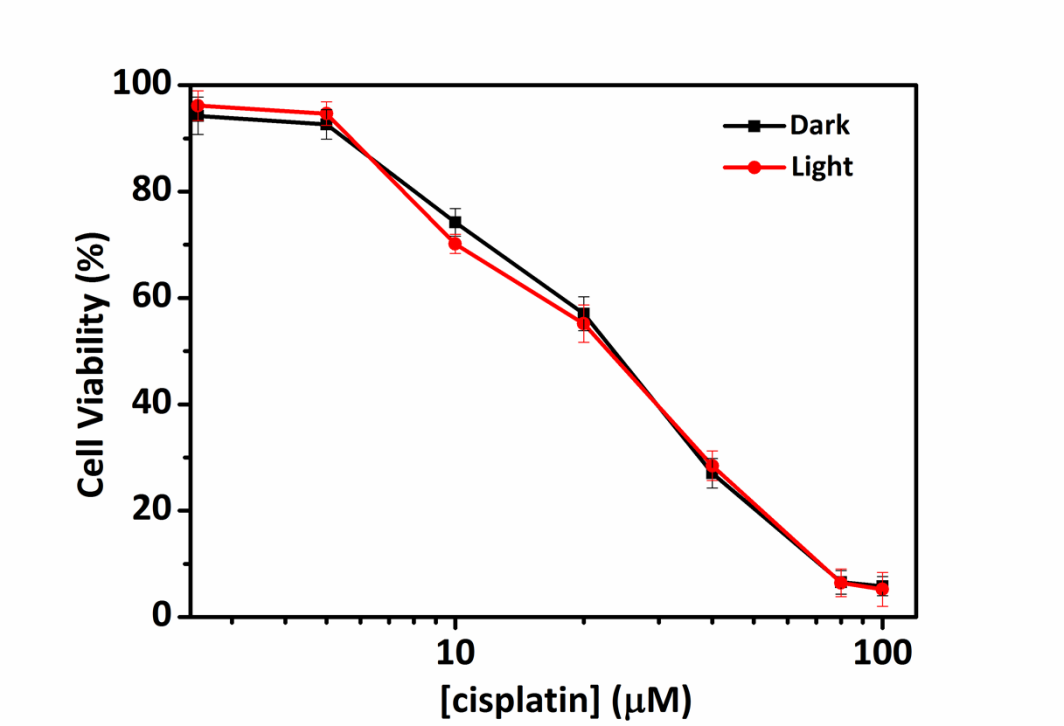


Fig. S10 Cytotoxicity profiles of cisplatin in A549R cells in the dark or with light irradiation determined by MTT. Light dose, 10 mW/cm^2, 2 min.

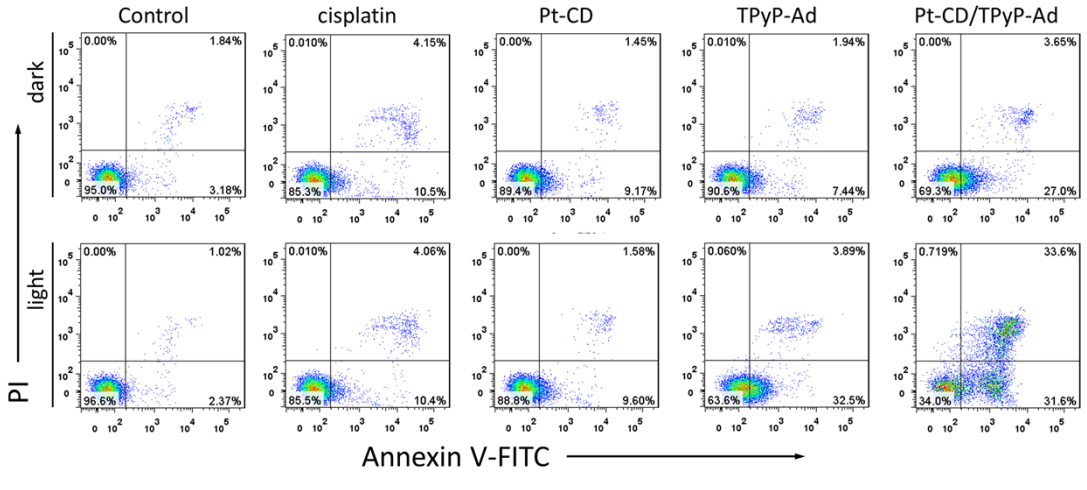


Fig. S11 Flow cytometric quantification of annexin-V and PI double staining A549R cells.

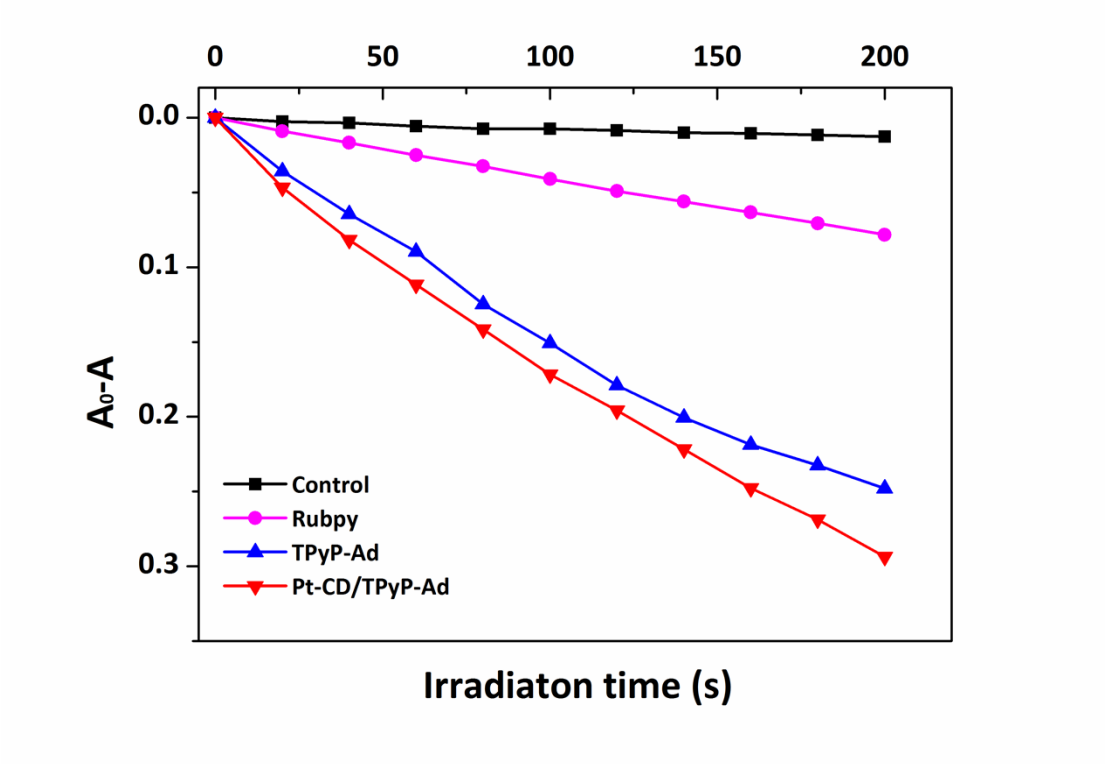


Fig. S12 Photooxidation of RNO under light irradiation.

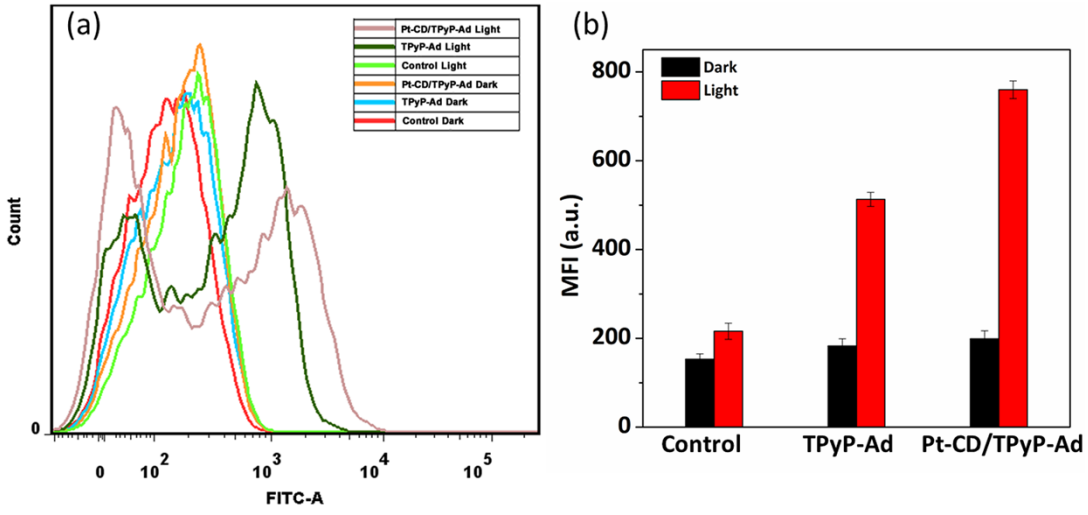


Fig. S13 Flow cytometric quantification of the cellular ROS levels by ROS probe DCFH-DA**.** A549R cells were treated with or without light irradiation after 24 h incubation with TPyP-Ad or Pt-bCD/TPyP-Ad. [TPyP-Ad]=2.5 μM. Light dose, 10 mW/cm^2, 2 min.