Electronic Supplementary Information

Ruthenium Complexes Modified Carbon Nanodots for Lysosomal-Targeted One- and Two-Photon Imaging and Photodynamic Therapy

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Experimental section

1.1 TEM and AFM Measurement

The aqueous solutions of carbon nanodots (**CDs)** and nanohybrid **Ru1@CDs** were dropped onto a new 200-mesh copper grid or clipped mica and air-dried. The samples were analyzed using a Transmission Electron Microscopy (T12, FEI Tecnai G2 Spirit, Holland) or Bruker Multimode 8 AFM under ScanAsyst mode in air at room temperature.

1.2 Photophysical Properties of Ru(II) complex (Ru1) and nanohybrid Ru1@CDs

The concentration of **Ru1** was quantified by the strong absorption peak at 473 nm. Fluorescence spectra of **Ru1**, **CDs** and **Ru1@CDs** were measured in aerated disodium hydrogen phosphate/citric acid buffer solutions (pH 5.0 and 7.4).

1.3 pH-dependent $^1O_2$ Production Quantum Yields

The quantum yields for $^1O_2$ production (Φ_Δ) of the complexes under irradiation in aerated disodium hydrogen phosphate/citric acid buffer solutions (pH 5.0 and 7.4) were evaluated using a steady-state method with ABDA as the $^1O_2$ indicator[1] and $[Ru(bpy)_3]Cl_2$ as the standard (Φ_Δ = 0.18 in H_2O).[2] Briefly, air-equilibrated buffer solutions containing the tested **Ru1** or **Ru1@CDs** and ABDA (25 μM) were prepared in the dark and irradiated with a 450 nm LED light array. The absorption maxima of ABDA (378 and 380 nm at pH 5.0 and 7.4, respectively) were recorded every 20 s. The absorbance at 450 nm of the **Ru1** or **Ru1@CDs** and $[Ru(bpy)_3]Cl_2$ was kept at 0.15. The Φ_Δ of the **Ru1** and **Ru1@CDs** were calculated according to the following equation.

$$\emptyset_{\Delta(x)} = \emptyset_{\Delta(std)} \times \left(\frac{S_x}{S_{std}}\right) \times \left(\frac{F_{std}}{F_x}\right)$$

where subscripts x and std designate the sample and $[Ru(bpy)_3]Cl_2$, respectively, S stands for the slope of plot of the absorption maxima of ABDA against the irradiation time (s). F stands for the

absorption correction factor, which is given by $F = 1 - 10^{-OD}$ (OD represents the optical density of sample and $[Ru(bpy)_3]Cl_2$ at 450 nm).

1.4 Measurement of Two-Photon Absorption (TPA) Cross-section

The two-photon absorption spectra of the probes were determined over a broad spectral region by the typical two-photon induced luminescence method relative to Rhodamine B in methanol as the standard. Briefly, the two-photon excited fluorescence (TPEF) spectra were acquired with a nanosecond pulsed laser (OpoletteTM 355II; pulse width 100 fs; 80 MHz repetition rate; Spectra Physics Inc., USA). The two-photon induced fluorescence intensity was measured at 730-870 nm by using rhodamine B as the reference.[3] The intensities of TPEF of the reference and samples emitted at the same excitation wavelength were determined. The TPA cross sections were calculated according to the following equation.[4]

$$\delta_s = \delta_r \frac{\emptyset_r c_r I_s n_s}{\emptyset_s c_s I_r n_r}$$

Where I is the integrated fluorescence intensity, c is the concentration, n is the refractive index, Φ is the quantum yield, subscript 'r' stands for reference samples, and 's' stands for the samples.

1.5 Cell Lines and Culture Conditions

Human lung cancer (A549) and human normal liver cell (LO2) cells were obtained from Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Cells were maintained in RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium or DMEM (Dulbecco's modified Eagle's medium, Gibco BRL), which contained 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), and 100 U/mL penicillin (Gibco BRL). The cells were cultured in a humidified incubator, which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C.

1.6 Cellular Uptake

1.6.1 Confocal Microscopy. A549 cells were seeded in 35 mm culture dishes (Corning) for 24 h. Culture medium containing **Ru1** (40 μM) or **Ru1@CDs** (40 μM based on the concentration of **Ru1**) was added. After incubation for 2 h, 6 h, 12 h or 24 h in the dark, cells were washed with PBS three times and visualized by confocal microscopy immediately upon excitation at 405 nm.

1.6.2 Colocalization Assay. A549 cells were incubated with **Ru1** (20 μM) for 1.5 h, **Ru1@CDs** (20 μM based on the concentration of **Ru1**) or **CDs** (200 μg/mL) for 3.5 h. The cells were further co-incubated with LysoTracker® Deep Red (LTDR) (50 nM) or MitoTracker® Deep Red (MTDR) (150 nM) at 37 °C for another 30 min. Cells were washed three times with PBS and visualized by confocal microscopy immediately. The one-photon excitation wavelength is 405 nm. The two-photon excitation wavelengths for **Ru1**/**Ru1@CDs** and **CDs** are 810 nm. and 700 nm, respectively. The excitation wavelength of MTDR and LTDR is 633 nm. Emission was collected at 660 ± 20 nm (**Ru1**), 665 ± 20 nm (MTDR) and 668 ± 20 nm (LTDR).

1.6.3 Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Measurement. A549 or LO2 cells were seeded in 10 cm tissue culture dishes and incubated for 24 h. The medium was removed and replaced with fresh medium containing **Ru1** (2.5 μM) or equivalent **Ru1@CDs**. After 12 h incubation, the cells were washed with PBS, trypsinized and collected. The cells were counted, and digested with HNO_3 (65%, 0.2 mL) at room temperature for 24 h. The solution was then diluted to a final volume of 10 mL with Milli-Q water. The concentration of ruthenium was measured using the XSERIES 2 ICP-MS (Thermo Scientific, USA).

1.7 Cellular Uptake Mechanism Studies

A549 cells were pretreated with 20 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP), 50 μM chloroquine or 50 mM NH_4Cl for 1 h at 37 °C, and then incubated with **Ru1** (20 μM, 2 h) or **Ru1@CDs** (20 μM based on the concentration of **Ru1**, 4 h) at 37 °C. The cells were then washed twice with serum-free RPMI 1640 and visualized by confocal microscopy (LSM 710, Carl Zeiss, Germany). Emission was collected at 660 ± 20 nm upon excitation at 405 nm.

1.8 pH-dependent Emission in A549 Cells

A549 cells were incubated with **Ru1** (20 μM) or **Ru1@CDs** (20 μM based on the concentration of **Ru1**) for 6 h at 37 °C. The media was removed and the cells were then incubated with nigericin sodium salt solutions (20 μM) in disodium hydrogen phosphate/citric acid buffer solutions (pH 5.0 and 7.4) for 10 min. The cells were visualized immediately by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 660 ± 30 nm upon excitation at 405 or 810 nm. The emission intensity (n = 10 cells, excitation was 810 nm) was quantified using ImageJ software.

1.9 In Vitro Cytotoxicity Assay by One-Photon Photodynamics Therapy (OPPDT)

The cells were seeded in 96-well plates at 1×10^4/well and cultured for 24 h. Then the medium was replaced with medium containing different concentrations of **Ru1**, **CDs** or **Ru1@CDs**. After 24 h, the media was removed and fresh media was added. Then PDT groups were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). Then, 20 μL MTT (5 mg/mL) solution was added to each well. The plates were incubated in the dark for an additional 4 h. The media was carefully removed. DMSO was added (150 μL per well) and the plate was incubated at room temprature for 10 min with shaking. The absorbance at 595 nm was measured using a microplate reader (Infinite F200, Tecan, Switzerland). The cells treated under identical conditions in dark were kept as control groups. The

percentage of viability was calculated as the following formula: (viable cells)% = (OD of treated sample/OD of untreated sample) × 100%.

1.10 Hoechst 33342 Staining by OPPDT

A549 cells were seeded into 35 mm dishes (Corning) and incubated for 24 h. The cells were treated with **Ru1** (5 and 10 μM) or **Ru1@CDs** (5 and 10 μM based on the concentration of **Ru1**) for 24 h. Then the cells were washed twice with PBS and the samples were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). After incubated for 12 h, the cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. Then the cells were labelled with Hoechst 33342 (5 μg/mL in PBS) for 5 min and washed twice with PBS. The cells were imaged immediately with a confocal laser-scanning microscope with excitation at 405 nm and emission at 460 ± 20 nm.

1.11 Annexin V-FITC Staining by OPPDT

1.11.1 Confocal Microscopy. A549 cells were seeded into 35 mm dishes (Corning) and then treated with **Ru1** (5 and 10 μM) or **Ru1@CDs** (5 and 10 μM based on the concentration of **Ru1**) for 24 h. The cells were washed twice with PBS and the samples were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). After incubated for 12 h, the cells were stained using the annexin V-FITC apoptosis detection kit (Sigma Aldrich, USA) according to the manufacturer's recommendations. The samples were then analyzed immediately by confocal microscopy with excitation at 488 nm and emission at 530 ± 20 nm.

1.11.2 Flow Cytometry. A549 cells were cultured in six-well tissue culture plates for 24 h and then treated with **Ru1** (5 and 10 μM) or **Ru1@CDs** (5 and 10 μM based on the concentration of **Ru1**) for 24 h. Then the samples were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). After incubated for

12 h, the cells were harvested and stained using the annexin V apoptosis detection kit according to the manufacturer's recommendations. The samples were measured by flow cytometry with excitation at 488 nm and emission at 530 ± 20 nm. Data were analyzed by FlowJo software (Tree Star, USA). Ten thousand events were acquired for each sample.

1.12 Detection of Intracellular Reactive Oxygen Species (ROS)

1.12.1 Confocal Microscopy. A549 cells were seeded into 35 mm culture dishes and incubated for 24 h. The cells were treated with medium containing **Ru1** (2.5 and 5 μM) or **Ru1@CDs** (2.5 and 5 μM based on the concentration of **Ru1**) at 37 °C for 24 h in the dark. The cells were then washed twice with serum-free medium, and incubated with DCFH-DA (10 μM) for 15 min at 37 °C in the dark. The samples were washed twice with serum-free medium, and then irradiated with a 450 nm laser (20 mW/cm^2, 5 min). The samples were then analyzed immediately by confocal microscopy. Emission was collected at 530 ± 20 nm upon excitation at 488 nm.

1.12.2 Flow Cytometry. After treated with **Ru1** (2.5 and 5 μM) or **Ru1@CDs** (2.5 and 5 μM based on the concentration of **Ru1**) for 24 h, the cells were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). The cells were then harvested and incubated with DCFH-DA (10 μM) in serum-free medium for 15 min at 37 °C in the dark. After washed twice with serum-free DMEM, the samples were analyzed by flow cytometry with excitation at 488 nm and emission at 530 ± 15 nm. Mean fluorescence intensities (MFI) was analyzed using FlowJo 7.6 software (Tree Star, USA).

1.13 Detection of Intracellular ATP Levels and Caspase-3/7 Activity Assays

Measurement of adenosine triphosphate (ATP) content was carried out using the Cell Titer-Glo kit (Promega) was determined according to the manufacturer's instructions, and Caspase-3/7 activity was measured using Caspase-Glo® Assay kit (Promega, Madison, WI, USA) according to the

manufacturer's instructions. Briefly, cells were cultured in 96-well plates and treated with **Ru1** (5 and 10 μM) or **Ru1@CDs** (5 and 10 μM based on the concentrations of **Ru1**) for 24 h with or without light irradiation. For One-Photon PDT treatment, cells were irradiated with a 450 nm LED light array (20 mW/cm^2, 5 min). Relative luminescent units (RLU) were detected with a microplate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland). The results are averaged among 3 replicates, and have been normalized by the values obtained on untreated control cells. Error bars represent the standard deviation.

1.14 Detection of Cathepsin B Release by OPPDT

Cathepsin B activity was detected using the fluorogenic susbtrate Magic Red MR-$(RR)_2$ according to the manufacturer's instructions. Briefly, A549 cells seeded into 35 mm dishes (Corning) were treated with **Ru1** (2.5 and 5 μM) or **Ru1@CDs** (2.5 and 5 μM based on the concentrations of **Ru1**) for 24 h. For PDT treatment, cells were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). The cells were washed twice with PBS and then incubated with Magic Red MR-$(RR)_2$ at 37 °C for 1 h. After washed twice with PBS, the cells were visualized by confocal microscopy. Emission was collected at 630 ± 20 nm upon excitation at 543 nm.

1.15 Generation of 3D Multicellular Tumor Spheroids (MCTSs)

Generation and analysis of MCTSs: A number of 5000 diluted A549 cells were transferred to 1.5% agarose-coated transparent 96-well plates with 150 μL of culture media. The cells were then re-suspended in culture media and formed MCTSs aggregates approximately 400 μm in diameter after 3 days. The cell solution in the inlet was replaced with fresh cell culture media every two days to maintain the growing of MCTSs. After formation of the MCTSs, each MCTS in a 96-well plate was

imaged with a confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany) to record their color, integrity and diameter.

1.16 One- and Two-photon Luminescent Imaging on MCTSs

MCTSs of 400~500 μm diameter were treated with **Ru1** (10 μM) or **Ru1@CDs** (10 μM based on the concentration of **Ru1**) for 6 h and the DMSO volume was less than 0.1% (v/v). The images of spheroids were collected using a on a Zeiss LSM 710 confocal microscope (10× objective). The excitation wavelength of the laser was 810 nm. Emission was collected at 660 ± 20 nm.

1.17 Detection of Intracellular ROS Levels by Two-photon Laser

A549 cells were seeded into 35 mm culture dishes and incubated for 24 h. The cells were treated with medium containing **Ru1** (5 μM) or **Ru1@CDs** (5 μM based on the concentration of **Ru1**) at 37 ^{o}C for 24 h in the dark. The cells were then washed twice with serum-free medium, and incubated with DCFH-DA (10 μM) for 15 min at 37 °C in the dark. The samples were washed twice with serum-free medium, and then irradiated by an 810 nm laser (100 mW, 80 MHz, 100 fs). The samples were then analyzed immediately by confocal microscopy. Emission was collected at 530 ± 20 nm upon excitation at 488 nm.

1.18 Live/Dead Viability Assay by Two-photon Photodynamic Therapy (TPPDT)

Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, as determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM (3',6'-Di(O-acetyl)-4',5'-bis[N,N-bis(carboxymethyl)aminomethyl]fluorescein, tetraacetoxymethyl ester) to the intensely fluorescent calcein (λ_{ex} = 488 nm, λ_{em} = 520 ± 20 nm). The determination of cell viability is dependent on these physical and biochemical cell properties. After A549 cancer cells were treated with **Ru1** (10 μM) or **Ru1@CDs** (10 μM based on the concentrations of **Ru1**) for 12 h,

the cells (and their respective controls) were incubated with calcein AM (2 μM) solutions for 30 min and imaged directly using an inverted fluorescence microscope (Zeiss, Model Axio Observer D1, Germany). For TPPDT treatment, the cells were irradiated by an 810 nm laser irradiation (100 mW, 80 MHz, 100 fs, 20 s).

1.19 Cell Morphology Observation

A549 cells were seeded into 35 mm culture dishes and incubated for 24 h. The cells were treated with medium containing **Ru1** (5 μM) or **Ru1@CDs** (5 μM based on the concentration of **Ru1**) at 37 ^{o}C for 24 h in the dark. The cell morphology of A549 cells after TPPDT treatment (100 mW, 80 MHz, 100 fs) were imaged under inverted fluorescence microscope (Carl Zeiss, Göttingen, Germany) with a 63× objective.

1.20 Inhibition and Viability Test on MCTSs

A549 MCTSs were treated with the **Ru1** or **Ru1@CDs** at different concentrations, and incubated in the dark for 24 h. In parallel, the medium containing solvent was replaced with a solvent-free medium for the untreated MCTSs. Then, A549 MCTSs were subjected to two-photon irradiation (100 mW, 80 MHz, 100 fs) at 810 nm for 20 min and then were incubated in the dark for 48 h. The culture media were refreshed every day with the same concentration of the drug. The cell viability assay of MCTSs was performed using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells. Each MCTS was incubated with a 10 μL solution of calcein AM (2 mM) for 30 min at room temperature in the dark and imaged. The calcein AM can make the live MCTSs show marked green fluorescence and the dead MCTSs show weak or no fluorescence under a microscope.

1.21 Cytotoxicity Test on MCTSs

A549 MCTSs (diameter 400~500 μm) were treated by carefully replacing 50% of the medium with the drug-supplemented standard medium. In parallel, 50% of the solvent-containing medium was replaced by solvent-free medium for the untreated MCTSs. After incubated for 24 h in the dark, MCTSs were exposed to irradiation under the light (810 nm, 100 mW, 80 MHz, 100 fs) for 30 min. The MCTSs were then allowed to incubate for another 48 h. Three MCTSs were treated at indicated condition and the DMSO volume was less than 1 % (v/v). The cytotoxicity of the **Ru1** or **Ru1@CDs** toward the MCTSs was measured by the adenosine triphosphate (ATP) concentration with the Cell TiterGlo 3D Cell Viability kit (Promega).

1.22 In vivo toxicity and imaging of Zebrafish

The 1-day-old zebrafish embryos were incubated with **Ru1** (20 μM), **Ru1@CDs** (20 μM) in E3 media for 6 days at 28 ^{o}C. Every group had twenty four zebrafish embryos. The zebrafish were imaged by Zeiss LSM 710 NLO confocal microscope (10× objective).

1.23 Statistical Analysis

All biological experiments were performed at least twice with triplicates in each experiment. Representative results were depicted in this report and data were presented as means ± standard deviations (SD).

Supporting Figures and Tables

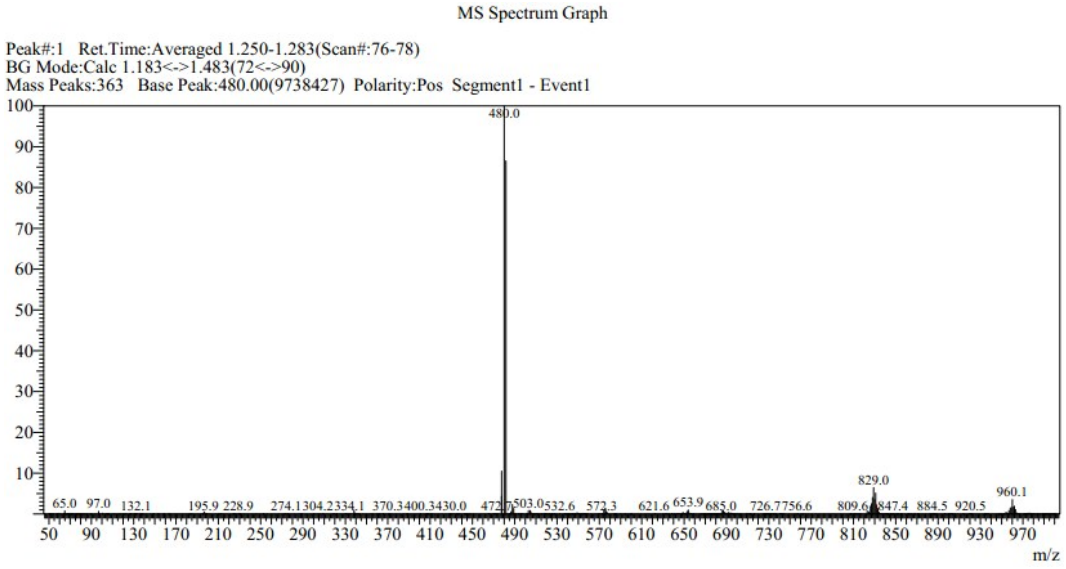

Figure S1 ESI-MS spectrum of **Ru1** in CH_3CN.

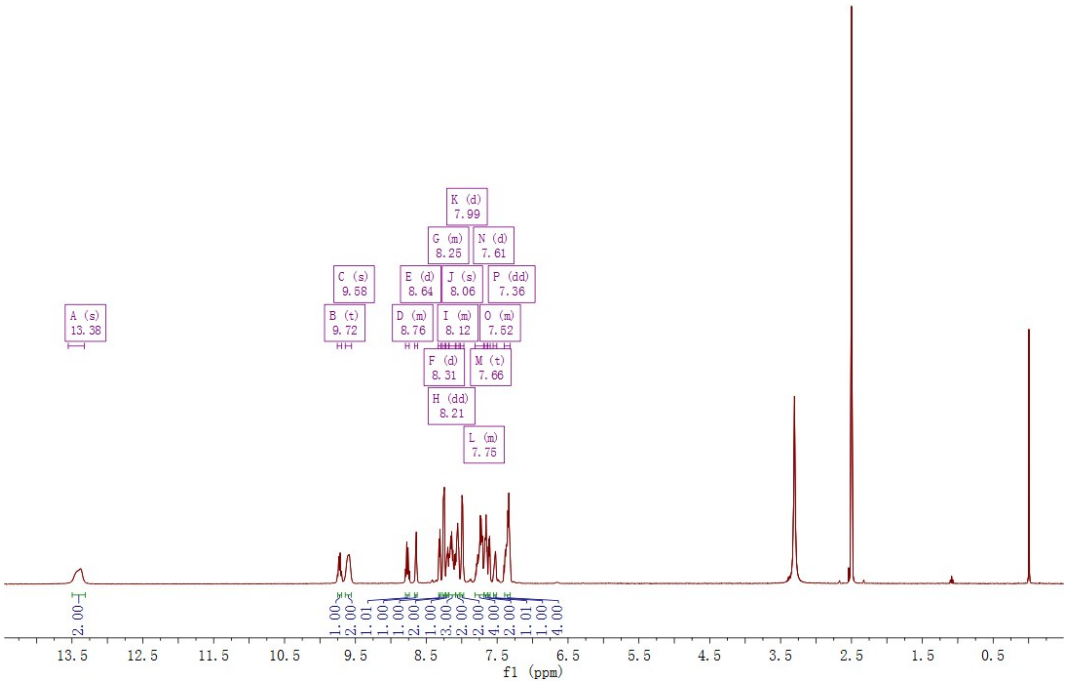

Figure S2 ^{1}H NMR of **Ru1** in DMSO-d_6.

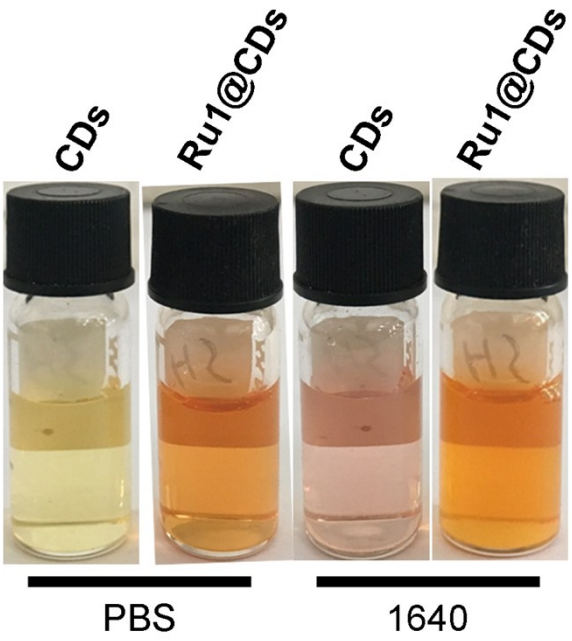

Figure S3 Stabilities of **CDs** and **Ru1@CDs**. **CDs** and **Ru1@CDs** was dispersed in PBS and cell culture media 1640 for a week.

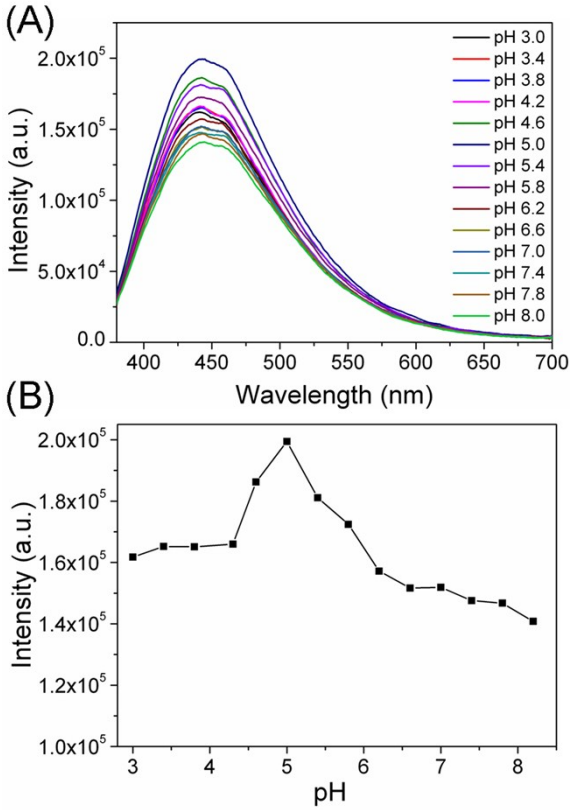

Figure S4 (A) pH-sensitive emission spectra of **CDs** (200 μg/mL, λ_{ex} = 360 nm) in disodium hydrogen phosphate/citric acid buffer solutions. (B) The fluorescence intensity of **CDs** at different pH.

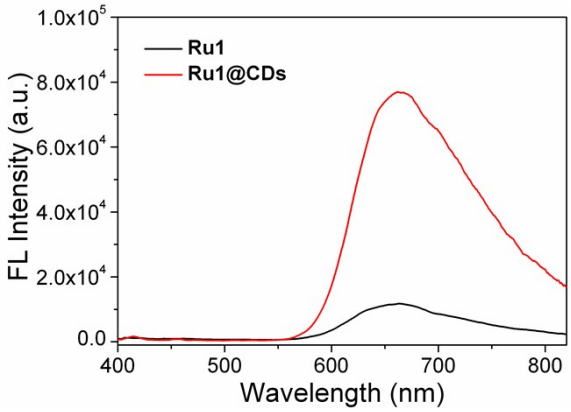

Figure S5 Fluorescence emission spectra of **Ru1** and **Ru1@CDs** (20 μM, λ_{ex} = 360 nm) in PBS.

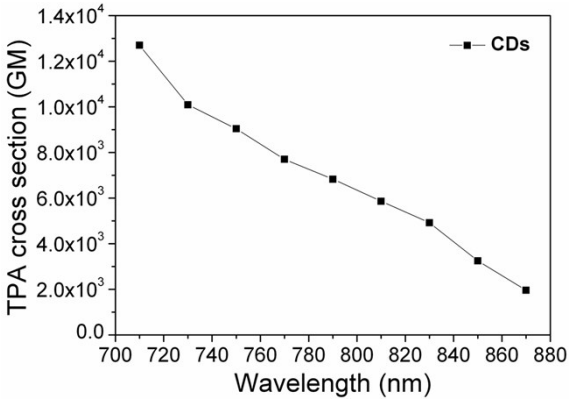

Figure S6 Two-photon absorption cross-sections of **CDs** at excitation wavelengths between 730 and 870 nm.

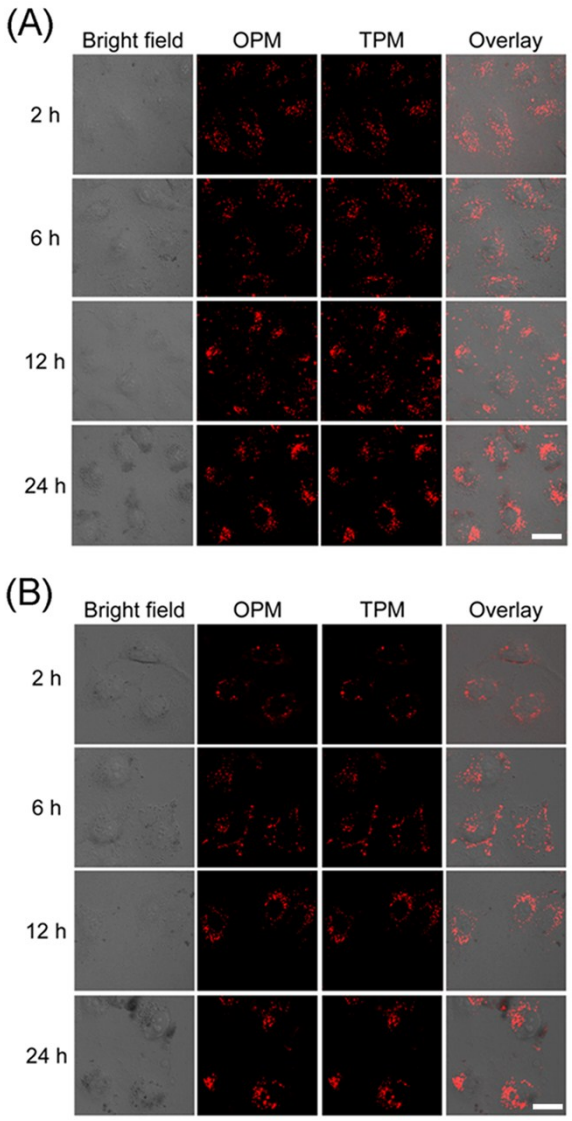

Figure S7 (A) Confocal fluorescence images of A549 after cells incubation with 40 μM **Ru1** in different time. (OPM: λ_{ex} = 405 nm, λ_{em} = 660 ± 20 nm; TPM: λ_{ex} = 810 nm, λ_{em} = 660 ± 20 nm). (B) CLSM images of A549 after cells incubation with 40 μM **Ru1@CDs** in different time. (OPM: λ_{ex} = 405 nm, λ_{em} = 660 ± 20 nm; TPM: λ_{ex} = 810 nm, λ_{em} = 660 ± 20 nm). Scale Bars : 10 μm

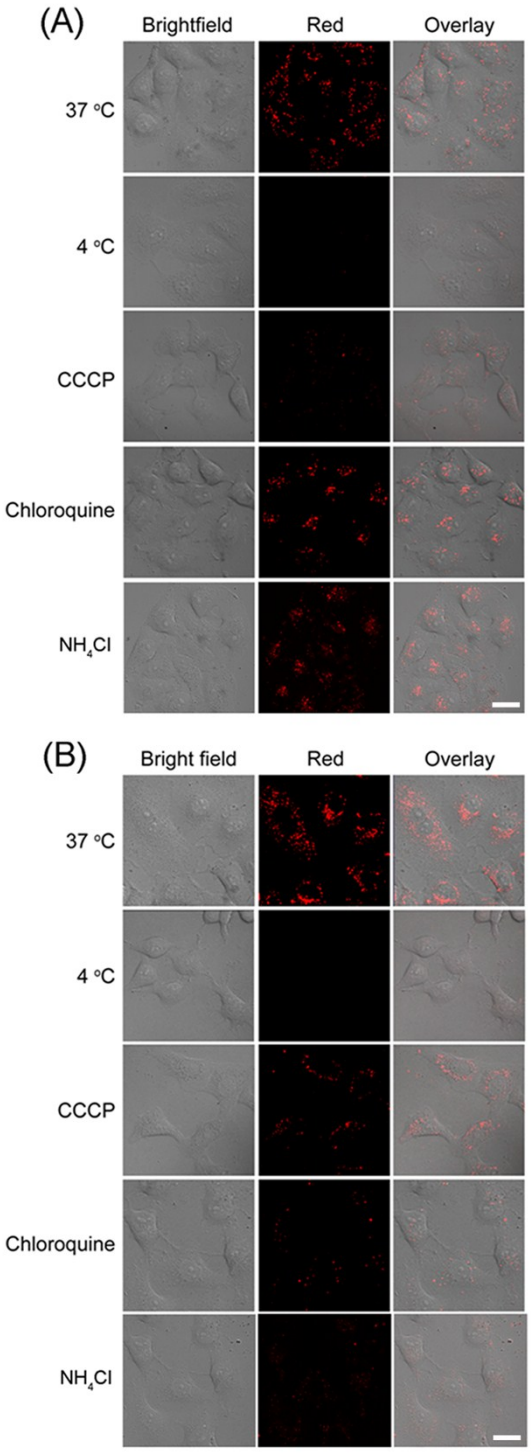

Figure S8 (A) Effect of incubation temperature (37 °C and 4 °C), metabolic inhibitor (CCCP, 30 μM) and chloroquine (50 μM) on cellular uptake of **Ru1** (20 μM, 2 h). (20 μM, 4 h). (B) Effect of incubation temperature (37 °C and 4 °C), metabolic inhibitor (CCCP, 30 μM), chloroquine (50 μM) and NH_4Cl (50 mM) on cellular uptake of **Ru1@CDs**. Scale bars: 10 μm.

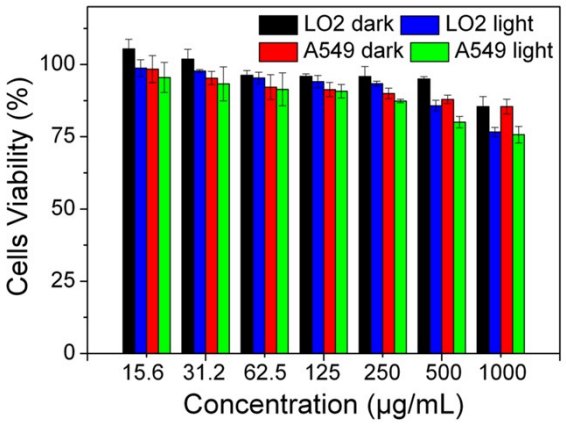

Figure S9 Cytotoxicity of the **CDs** in the absence and presence of 450 nm light towards A549 and LO2 cells.

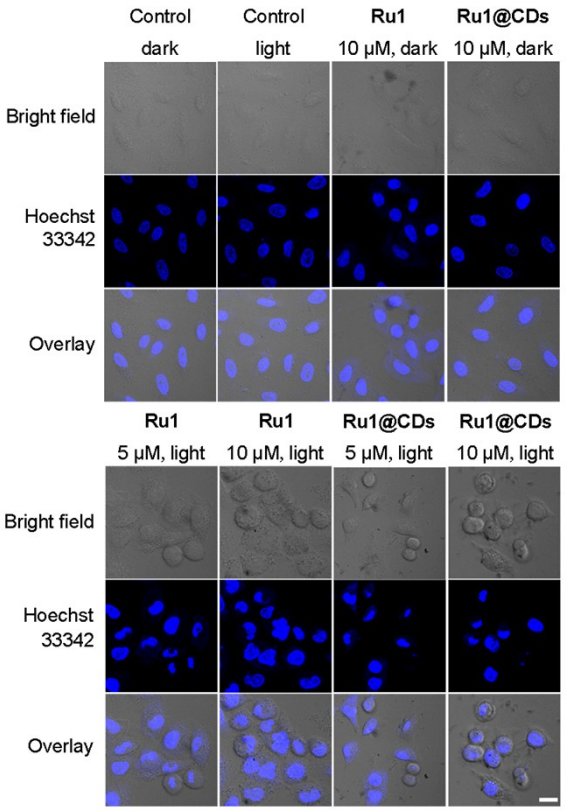

Figure S10 Hoechst stained A549 cells after treatment of **Ru1** and **Ru1@CDs** (5, 10 μM). The cells were irradiated with a 450 nm laser (20 mW/cm^2, 5 min). Scale bar: 10 μm.

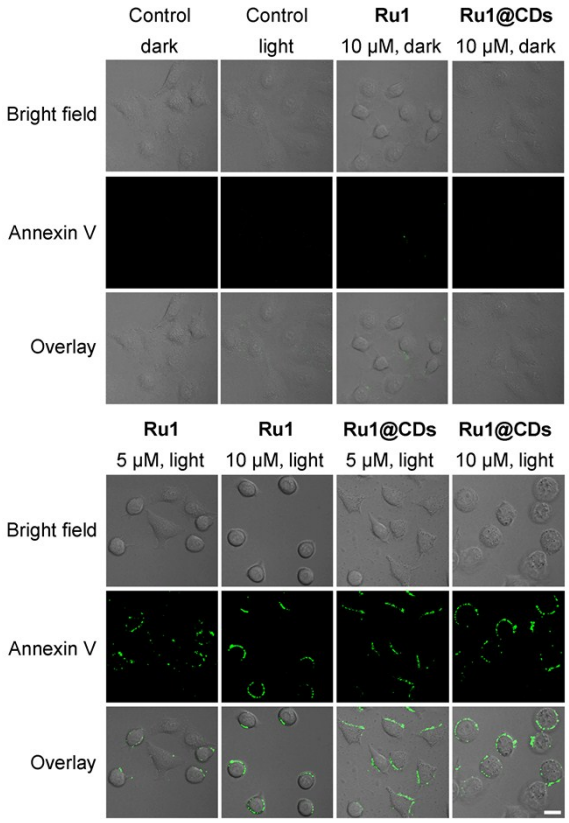

Figure S11 Representative confocal images of A549 cells treated with **Ru1** and **Ru1@CDs** (**Ru1** = 5, 10 μM) upon irradiation with a 450 nm laser (20 mW/cm^2, 5 min). Scale bar: 10 μm.

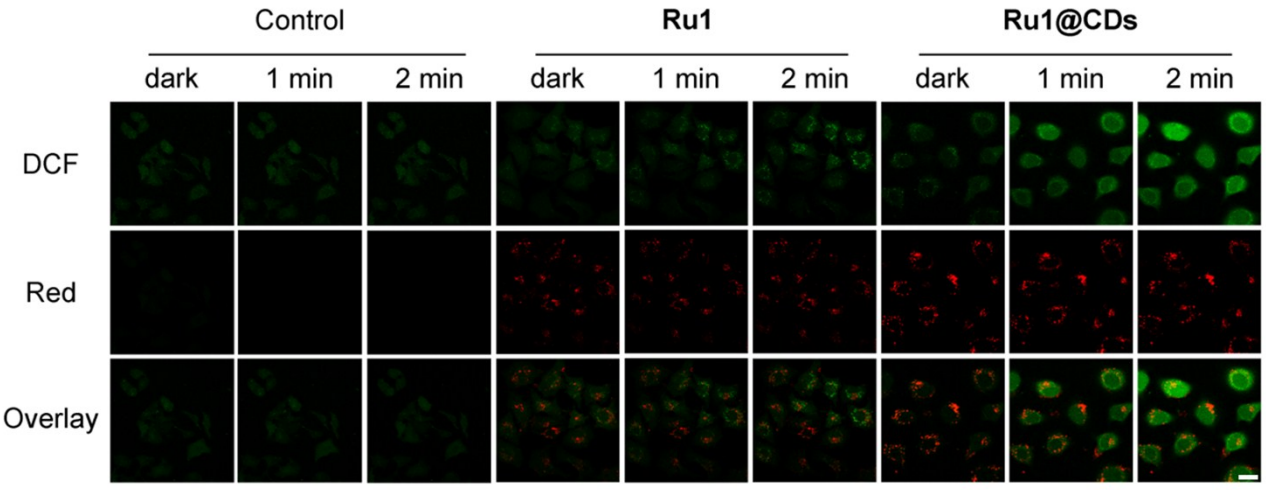

Figure S12 Confocal fluorescence images of cellular ROS levels detected by DCFH-DA A549 cells incubation with **Ru1** or **Ru1@CDs** for 24 h after which they were irradiated by 810 nm (100 mW, 0, 1, 2 min). Scale bar: 10 μm.

	Medium	λ_{ex}/nm	λ_{em}/nm	Φ_m[a]
Ru1	pH 5.0	470	665	0.075
	pH 7.4	470	658	0.020
Ru1@CDs	pH 5.0	470	664	0.085
	pH 7.4	470	656	0.022

Table S1 Quantum yields of luminescence at room temperature were calculated according to literature procedures. Solutions of $[Ru(bpy)_3]Cl_2$ were used as the standard, PBS (Φ_m = 0.028), Decay curves of compounds were recorded by an Edinburgh FLS 920 Spectrometer.

Supporting References

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