Supplementary information

for

Biocompatible Ruthenium Polypyridyl Complexes as Efficient Radiosensitizer

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1.Experimental section

1.1 Materials

4,4'-dimethyl-2,2'-bipyridine, 4,7-diphenyl-1,10-phenanthroline, $RuCl_3$, Sodium perchlorate ($NaClO_4$), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 1,6-diaminohexan, di-tert-butyl decarbonate (Diboc) and methoxy PEG acetic acid ($PEG_{5000}COOH$) were purchased from Shanghai Aladin Reagent Company, 4'-methyl-2,2'-bipyridine-4-carboxylic acid (bpyCOOH)[1], [Ru $(dip)_2Cl_2$][2] and N-(Aminohexyl)-4'-methyl-2,2'-bipyridine-4-carboxamide·HCl ($bpyC_6NH_2 \cdot HCl$)[3] were synthesized according to reported literatures. Dulbecco's modified Eagle's medium (DMEM) medium and fetal bovine serum (FBS) were bought from Gibco® Thermo Fisher Scientific Inc. Thiazolyl blue tetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Mito-tracker Green (Mitochondria) and Hoechst 33342 were purchased from Sigma-Aldrich. The water used in cellular experiments was ultrapure, supplied by a Milli-Q water purification system from Millipore. All the chemicals and solvents were analytically pure.

1.2 Cell lines

Human melanoma A375 cells, human cervical epithelial carcinoma (Hela), human hepatoma (HepG2 and 97L), human normal liver cells (L02 and HL-7702), CHEM-5 human glial cells, H9C2 cardiac muscle and WI38 lung epithelial fibroblast cells were purchased from American Type Culture Collection (ATCC, Manassas, Virginia).

1.3 Synthesis of $[Ru(dip)_2bpyC_6NH_2] (ClO_4)_2$ (Ru-CNH_2)

A solution of [Ru $(dip)_2Cl_2$] (81.64 mg, 0.11 mmol), $bpyC_6NH_2 \cdot HCl$ (41.87 mg, 0.12 mmol) in 20ml ethylene glycol monoethyl ether was heated to reflux for 6 h. The reaction solution turned from initial purple to red with refluxing, eventually to a bright red transparent liquid. After cooled to room temperature, the solution was added to 30 ml Saturated sodium perchlorate solution and the precipitation was filtered. The residue dissolved in minimal methanol was dropped diethyl ether and the precipitation was filtered to obtain crude product. The crude product was purified by neutral alumina column chromatography with dichloromethane / methanol (10:1, v/v) as eluent. The principal red band was collected and the Solvent was removed to produce a red solid.

1.4 Synthesis of $[Ru(dip)_2bpyC_6PEG_{5000}OCH_3] (ClO_4)_2$ (Ru-PEG)

A solution of PEG_{500}0COOH (0.5 g, 0.1 mmol), EDC (21.09 mg, 0.11 mmol) and NHS

(12.66 mg, 0.11 mmol) in 100 ml dichloromethane was stirred under room temperature for 30 min, and Ru-CNH2 (127.7 mg, 0.1 mmol) dissolved in 20 ml dichloromethane was added and he mixture solution was keep at 40℃ for 12 h. After cooled to room temperature, the solution was washed with Saturated sodium carbonate solution (100 ml) and water (100 ml) for three times, the solvents was removed under vacuum. The crude product was purified by neutral alumina column chromatography with dichloromethane / methanol (10:1, v/v) as eluent. The orange solid obtained by slow evaporation of the solution and dried in vacuo.

1.5 Preparation of nanostructures of Ru-PEG (Ru-Nano).

Ru-PEG (10.0 mg) dissolved in acetonitrile (500 μl) was dropwised in to a vial with 10 ml PBS under mildly stirring. The solution mixture was stirred at room temperature overnight, and the volatile solvent was removed under reduced pressure. The supernatant was dialyzed against PBS solution using semipermeable minidialysis tubes (molecular weight cut-off = 7000 Da; GE Healthcare) for 2 days. The nanoparticles were used for following experiments after purified and ruthenium content was determined by inductively coupled plasma mass spectrometry (ICP-MS) analysis.

1.6 hemocompatibility of Ru-CNH2 and Ru-Nano

Different concentrations of Ru-CNH_2 and Ru-Nano were pre-mixed with heparinized RBCs and then incubated for different time at 37 ℃. Thereafter, RBCs were centrifuged to collect the supernatant and the absorbance was measured for the collected supernatant by spectrophotometry at 540 nm and hemolysis was determined based on eqn (1).

$$\text{Hemolysis}(\%) = (A_{\text{sample}} - A_{\text{NC}})/(A_{\text{PC}} - A_{\text{NC}}) \times 100\% \quad (1)$$

where A_{sample} is the absorbance of the sample at 540 nm, A_{NC} is the absorbance of the negative control at 540 nm, and A_{PC} is the absorbance of the positive control at 540 nm. The collected cells were then investigated further for agglutination, and each sample was placed on a glass slide, under a cover slip and observed using a phase contrast microscope (Life Technologies, EVOS FL auto).

1.7 MTT assay

The cell viability of HepG2, A375, 97L, HeLa, WI38 HL-7702, L02, Chem-5, H9C2 cells after treatment with different concentrations of Ru-CNH_2 and Ru-Nano for 72 h was measured by MTT assay[4].

1.8 Mitotracker & Hoechst staining

Mitotracker & Hoechst staining assay was applied to visualize distribution of Ru-Nano in HepG2 cells at a concentration of 10 μM as our reported methods[4].

1.9 Ru-Nano and Radiation treatment

The HepG2 cells were initially cultured for 24 h and then treated with various doses of Ru-Nano for 6 h. Afterward, the cells were irradiated by X-ray (0, 2, and 4 Gy) followed by incubation at 37.0 °C for 72 h.

1.10 Clonogenic assay

The HepG2 cells were seeded on six-well plates at 2000 cells per ml (2 ml) and were incubated for 24 h. After treatment for 6 h with different concentrations of the Ru-Nano, the cells were exposed to different X-ray dosages and incubated at 37 °C for 7 days. The cells were fixed with 4.0% paraformaldehyde (vol/vol) for 10 min and stained with 0.5% crystal violet (wt/vol) for 20 min. The survival fraction of the clones was used to evaluate the effects of different treatments.

1.11 Determination of intracellular ROS overproduction

The effects of the Ru-Nano and radiation on intracellular ROS generation in the HepG2 cells were examined using the 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence probe. Briefly, the HepG2 cells (2×10^5 cells per ml) were treated with the Ru-Nano for 6 h. Then, the cells were treated with X-ray (2 Gy) and incubated with DCFH-DA at 37 °C for 30 min. The intracellular reactive oxygen species (ROS) level was measured as the fluorescence intensity of DCFH-DA (excitation and emission wavelengths of 458 and 525 nm, respectively). Fluorescence images were acquired from the HepG2 cells to examine whether the combined radiotherapy induced variations in ROS. The cell-free model was the same as that described above except that the cultured HepG2 cells were replaced by PBS.

1.12 Change of mitochondrial morphology

The HepG2 cells were cultured on 2 cm glass-bottom dishes for 24 h. After treatment with the Ru-Nano (10 μM) for 6 h and the cells were irradiated with X-ray (2 Gy). After incubation for 12 h, the cell monolayer was rinsed with ice-cold PBS 3 times. The cell mitochondria and nuclei were stained with 100 nM Mito-tracker for 2 h and 1 μg ml^{-1} H33342 for 15 min. Mitochondrial morphology changes were observed by fluorescence microscopy (EVOSFL auto, Life Technologies, 100 ×).

1.13 Flow cytometric analysis

The cell cycle distribution and cell apoptosis after the treatment of Ru-Nano and X-ray were analyzed by flow cytometric analysis as previously reported[4].

References:

1. B. M. PEEK, G. T. ROSS, S. W. EDWARDS, G. J. MEYER, T. J. Meyer and B. W. ERICKSON, *Int. J. Peptide Protein Res.*, 1991, **38**, 114-123.
2. R. Caspar, C. Cordier, J. B. Waern, C. Guyard-Duhayon, M. Gruselle, P. Le Floch and H. Amouri, *Inorg. Chem.*, 2006, **45**, 4071-4078.
3. H. Szelke, H. Wadepohl, M. Abu-Youssef and R. Krämer, *Eur. J. Inorg. Chem.*, 2009, **2009**, 251-260.
4. Y. Tian, Y. Huang, P. Gao and T. Chen, *Chem. Commun.*, 2018, **54**, 9394-9397.

2. Results and Figures

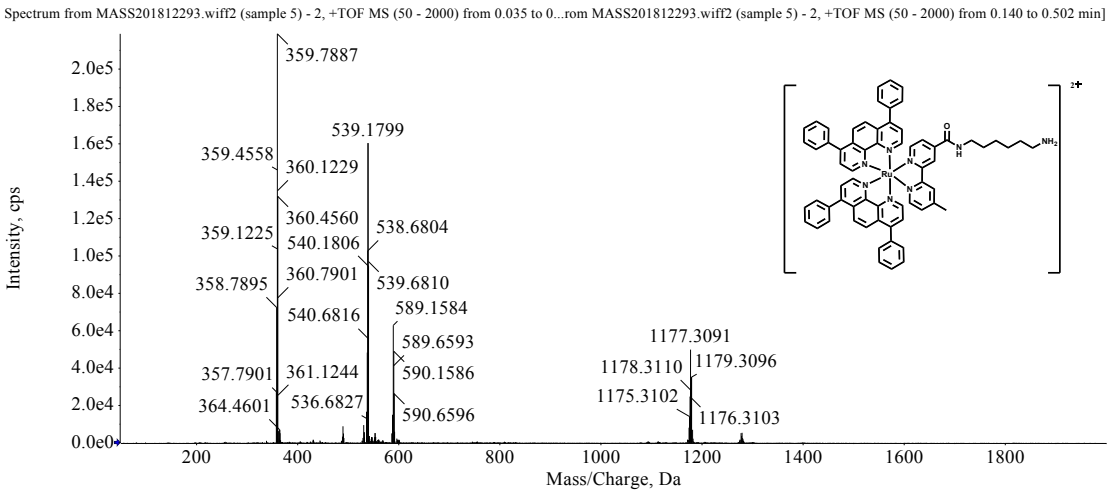


Figure S1. The ESI-MS spectra of Ru-CNH_2 in methanol.

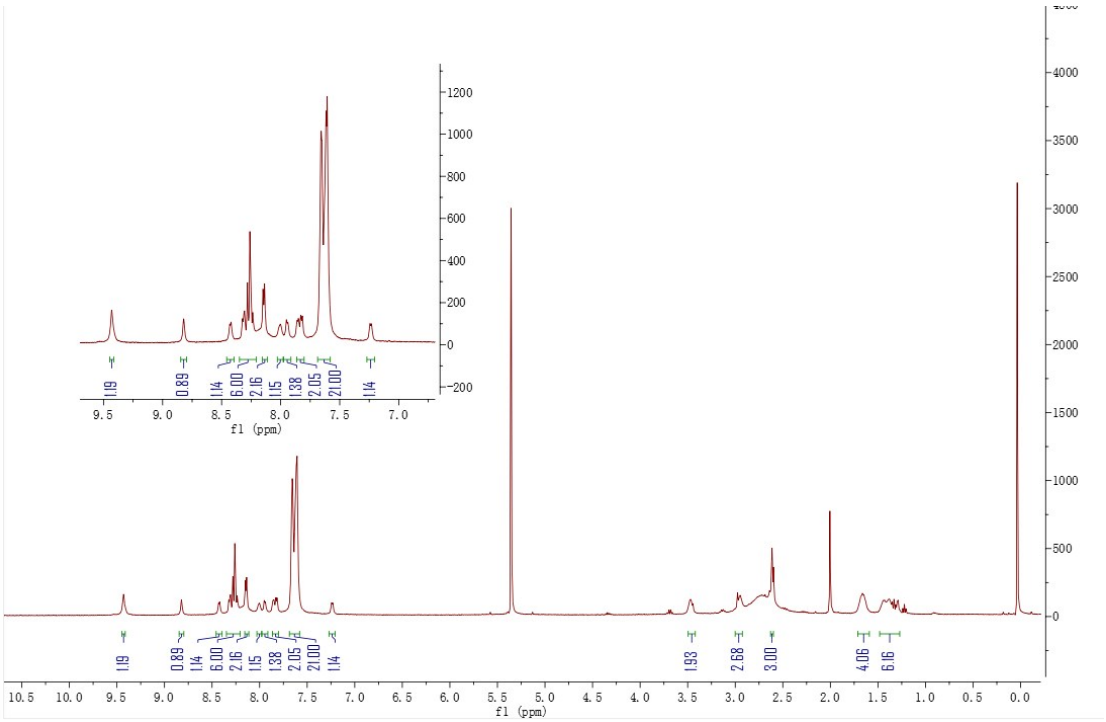


Figure S2. The 1H NMR spectra of Ru-CNH_2 in CD_2Cl_2.

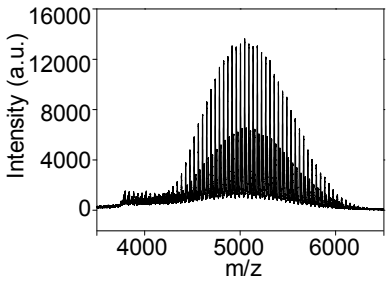


Figure S3. MALDI-TOF MS of H3CO–PEG_{5000}–COOH, using α-cyano-4 hydroxycinnamic acid with sodium trifluoroacetate as the matrix.

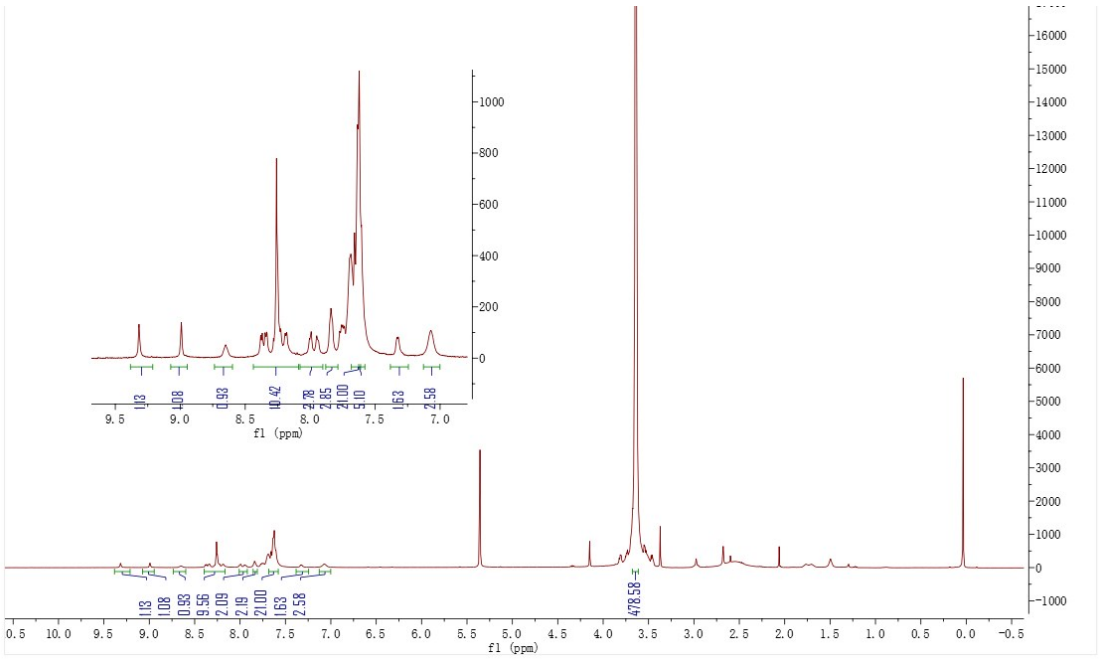


Figure S4. ^{1}H NMR spectra of $Ru(dip)_2bpyC_6PEG_{5000}OCH_3$ in CD_2Cl_2

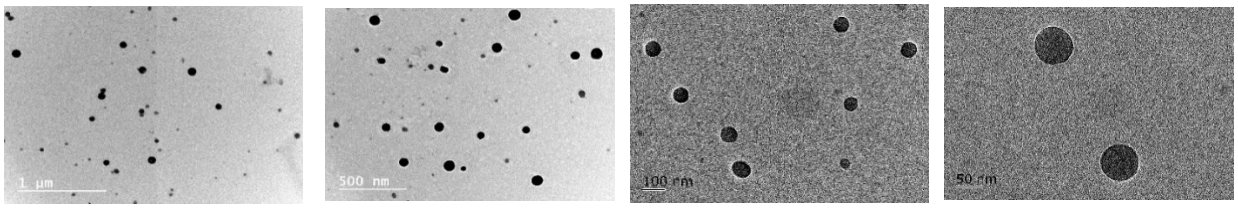


Figure S5. TEM images of Ru-Nano

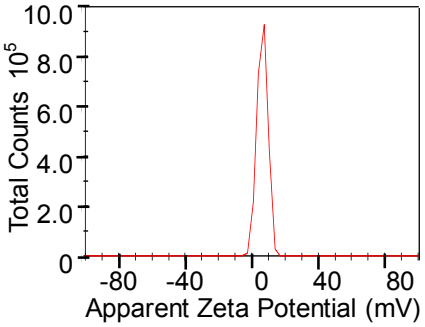


Figure S6. zeta potential of Ru-Nano in aqueous solution

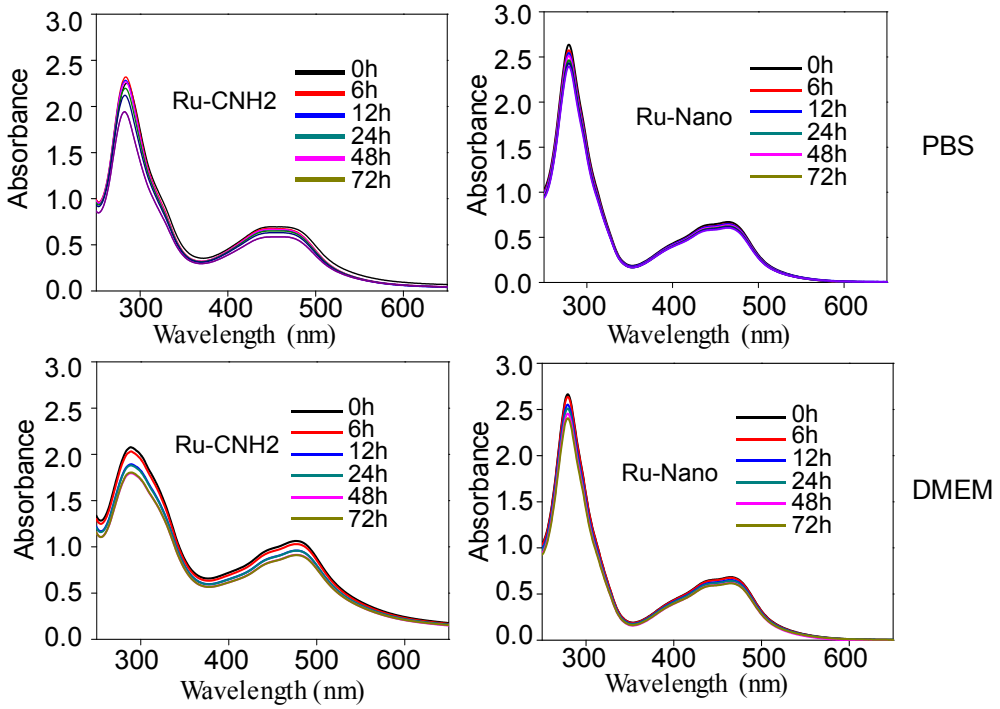


Figure S7. UV-vis spectra of Ru-Nano and $RuCNH_2$ in PBS solution and DMEM solution.

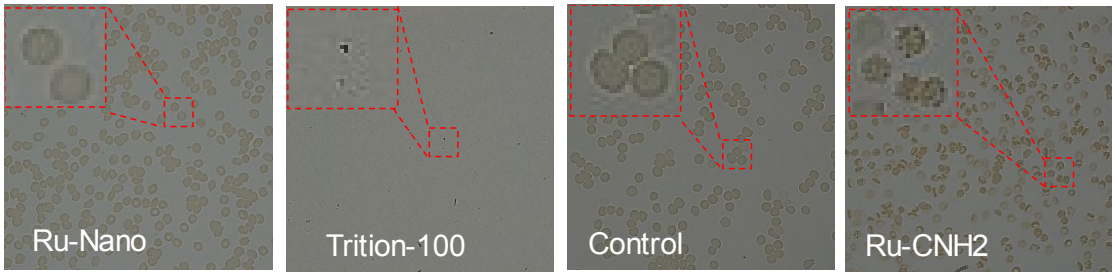


Figure S8 Light images of Red Blood Cells (5 μM) with different treatment for 4 h.

Table S1. Growth inhibition of the Ru-Nano and Ru-CNH_2

Complex		IC_{50} (μM)									SI[a]	SI[b]
		Cancer cells						Normal cells				
	logP	WI38	H9C2	97L	HepG2	A375	HeLa	L02	Chem-5	HL-7702		
Ru-Nano	-0.22	27.46	13.59	12.8	9.34	12.82	17.9	11.41	10.8	17.7	1.22	1.90
Ru-CNH_2	0.41	20.35	6.41	15.7	13.62	25.97	30.5	7.30	3.68	3.80	0.54	0.28

SI[a] (Safe Index) = IC_{50} (L02)/ IC_{50} (HepG2), SI[b] (Safe Index) = IC_{50} (HL-7702)/ IC_{50} (HepG2)

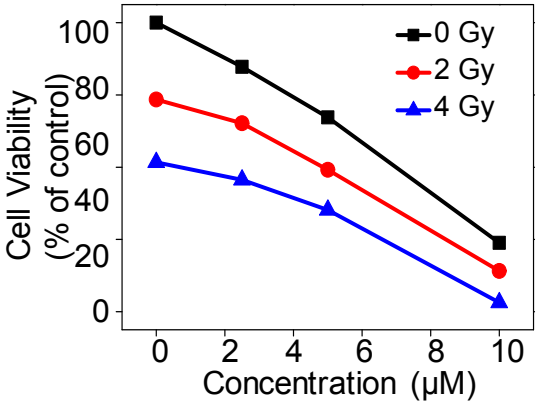


Figure S9. Cell Viability under the co-treatment of Ru-Nano with different concentrations and X-ray radiation with different doses.

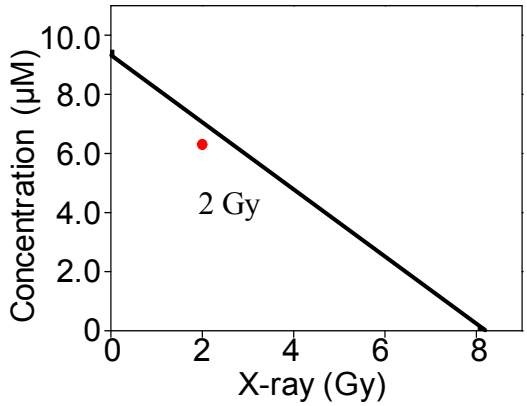
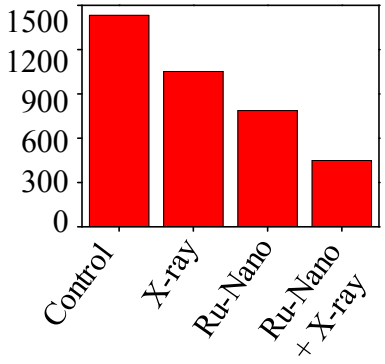


Figure S10. Isobologram analysis of the synergistic antiproliferative effect of the combined application of X-ray and Ru-Nano.



	Migration Rate (%)	
	X-ray	Non-X-ray
Control	34.1	48.7
Ru-CNH_2	11.7	23.7
Ru-Nano	6.25	11.1

Figure S11 Quantitative analysis of the (a) colony formation experiment, (b) wounding healing assay.

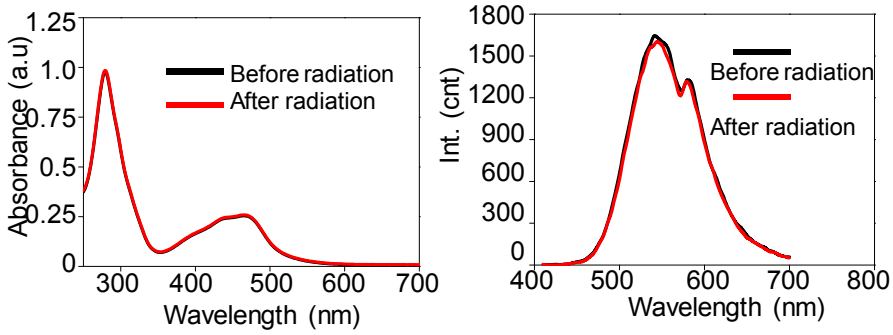


Figure S12. (a) UV-vis and (b) fluorescence spectra of the nanostructures before (black) and after (red) 2 Gy X-ray radiation.