RAPTA-coordinated polydiacetylene self-assembly: A chameleon-like prodrug with a dual-lock strategy for real-time release monitoring of metallodrug

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Materials

The $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ precursors, 10,12-pentacosadiynoic acid (PCDA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide, N-Hydroxysuccinimide, 4-(Aminomethyl)pyridine, Ammonium hexafluorophosphate, and 1,3,5-Triaza-7-phosphaadamantane were obtained from Sigma-Aldrich (USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS) Dulbecco's Modified Eagle Medium (DMEM), Trypsin, Acridine orange (AO) and Ethidium Bromide (EtBr) was obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose, and antibiotics from Hi-Media Laboratories Ltd., Mumbai. The human gastric adenocarcinoma cell line - AGS was procured from the National Centre for Cell Sciences (NCCS), Pune, India. Ultrapure Milli-Q water was used in all experiments. Phosphate buffered saline (PBS) (pH 7.2) solution was prepared by the reported procedure. Commercial solvents were distilled and then used for the preparation of ligands and complexes.

Instrumentation

Microanalysis was carried out with a Vario EL elemental analyzer. UV-Vis spectroscopy was recorded on a Cary-60 UV-Vis spectrophotometer using cuvettes of 1 cm path length. ^{1}H NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer. High-resolution mass spectrometry was performed on a QTOF–HRMS equipped with an ESI source. Emission intensity measurements were carried out using an FLS-1000 Spectrofluorometer. 50W Blue LED lamp >420 nm was used for photo-irradiation. Particle size distribution and zeta potential measurements were recorded on nanoparticle SZ-100 (Horiba Scientific, UK). ICP-OES were taken to quantify the Ru content inside the cell using ICP-OES, Optima 5300 DV, Perkin Elmer. Confocal microscope images were taken using CLSM 700. Fluorescence images were taken using Leica DM6 fluorescence microscopy. HR-TEM images were taken using JEM-2100 Plus.

MTT assay

Before irradiation

Cell viability of RuPyPCDA, PyPCDA and RAPTA was determined using 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) assay. AGS- human gastric adenocarcinoma cells were procured from National Centre for Cell Science (NCCS), Pune. AGS-cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) culture media supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Invitrogen Corporation, CA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C until confluent. AGS cells were trypsinized and plated at a density of ~20, 000 cells/well in a 96-well plate and incubated at 37 °C in the CO_2 incubator. The cell viability % were determined using the MTT assay after 24 h incubation of different concentrations of compounds in the AGS-cell lines. The stock solutions of the compounds were prepared in a 10 mM PBS mixture immediately before dilution. Different concentrations of compound solution in µM were prepared by the dilution of the stock solution using culture media in triplicate. The MTT insoluble formazan was dissolved in DMSO and the MTT reduction was quantified by measuring the absorbance at 570 nm (Multiskan Spectrophotometer, USA). The obtained data

were plotted and fitted using GraphPad Prism software. The data were obtained for three biological replicates each and used to calculate the mean.

After irradiation

AGS-cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) culture media supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37˚C until confluent. AGS cells were trypsinized and plated at a density of ~20, 000 cells/well in a 96-well plate and incubated at 37 °C in the CO_2 incubator. The different concentration (25, 50, 75, 100, 125, and 150 µM) of RuPyPCDA assembly was incubated for 24 h at 37˚C and 5% CO_2. Then the cells were irradiated using a 50W blue light source ((≥420 nm) kept 5 cm apart from the cell-culture plate. After irradiation, the cell viability was measured using MTT assay as described above.

Cellular uptake of Ru compounds

Cell accumulation studies of Ruthenium complexes were conducted on AGS cells. Briefly, 3 x 10^6 cells were seeded on a Petri dish. The compound was added to 150 µM concentration after a 24-hour pre-incubation period in the drug-free medium at 37°C. A further 24-hour drug exposure period was then permitted. Then the cells were irradiated under blue light for 30 mins. After irradiation, the cells were counted, cleaned, trypsinized, and cell pellets were collected. After being digested to 200 µL of 72% v/v nitric acid at 80°C overnight (72%), each sample was diluted with milliQ water to get 1% v/v acid as the final concentration. ICP-OES was used to measure the concentration of Ru, and all experiments were performed in triplicate and the standard deviations were calculated. Similarly, the Ru content was also determined in a subcellular localization. Cell pellets were prepared as previously mentioned and a commercially available kit (Fraction PREP from BioVision) was used for this fractionation, following the manufacturer's instructions. Each resulting fraction was then digested with concentrated nitric acid (72% v/v) at 80°C overnight. After dilution with water to a final acid concentration of 1%, the amount of Ru in each fraction was measured using ICP-OES. All experiments were performed in triplicate and the standard deviations were calculated.

Mitochondrial membrane potential by JC-1 staining

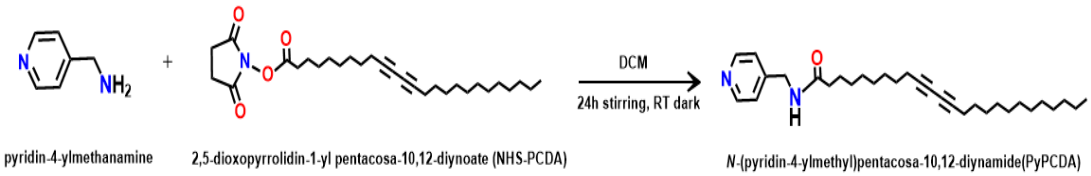
Culture cells on coverslips on appropriate 6-well cell culture plates at a density around 5 x 10^5 cells/ml overnight in an incubator (5% CO_2, 37°C). RuPyPCDA (150 µM) was incubated with cells. After 12 and 24 h of incubation, then 30 mins of blue light irradiation. The cell culture medium was replace with the same volume of JC-1, add 5 µl of JC-1 (200 µM) per 1 ml cell culture medium. Incubate the plates in an incubator (5% CO_2, 37°C) for 15-30 minutes. Immediately, the cells were visualized using a fluorescence microscope (Olympus, CKX-53, Japan), and the percentage of dead cells was quantified in at least three random microscopic fields.

Real-time monitoring using Confocal microscopy

20000 cells per well were seeded on a sterile coverslip in a 6-well plate and incubated for 24 h. After incubation, the cells were treated with a RuPyPCDA and incubated for 24 h. untreated cells were used as a control. The treated and control cells were washed with PBS after 24h

incubation. Then Imaging was done before and after irradiation of blue light using a confocal microscope with the appropriate filter.

Synthesis of [N-(pyridine-4-ylmethyl)pentacosa-10,12-diynamide] (PyPCDA).



N-Hydroxysuccinimide ester (NHS-PCDA) was synthesized by adopting previous literature.[1] NHS-PCDA (380 mg, 0.8 mmol) was dissolved in 5 mL of dichloromethane. To the organic solvent, 1 ml of pyridine-4-ylmethanamine was added. The mixture was stirred at ambient temperature for 12 h. After stirring, the product solution was extracted with water and dichloromethane in ratio 2:1. Then the organic layer was dried with magnesium sulfate and filtered, and the solvent was removed by evaporation. Then the product was purified by column chromatography using (Ethyl acetate/Hexane 1:3). Yield: 71%. Elemental analysis for [PyPCDA], calculated: C, 69.78; H, 4.89; N, 8.57, found: C, 69.81; H, 4.87; N, 8.42%. ESI-MS displays a peak at m/z 465.3839 for $C_{31}H_{48}N_2O$ $[M+H]^+$; calculated m/z: 464.7256. UV–Vis (conc., 500 µM) DCM, λ_{max} nm, (ε M^{-1} cm^{-1}): 232 (1780), 255 (1580). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.57–8.55 (m, 2H), 7.27-7.26 (d, 2H, J = 4 Hz), 7.20 (s, 1H), 4.48–4.46 (d, 2H, J = 8 Hz), 2.37–2.26 (m, 2H), 2.25–2.23 (m, 4H), 1.57–1.51 (m, 6H), 1.30-1.26 (m, 26H), 0.89–0.87 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 14.13, 19.22, 22.70, 24.79, 25.64, 28.28, 28.31, 28.37, 28.73, 28.77, 28.87, 29.06, 29.11, 29.22, 29.35, 29.49, 29.62, 29.64, 29.66, 31.93, 36.61, 42.32, 65.23, 65.31, 77.62, 77.66, 122.47,148.16, 149.46, 173.39 ppm.

Synthesis of [Ru(η^6-*p*-cymene)(PTA)(PyPCDA)(Cl)]PF_6 (RuPyPCDA).

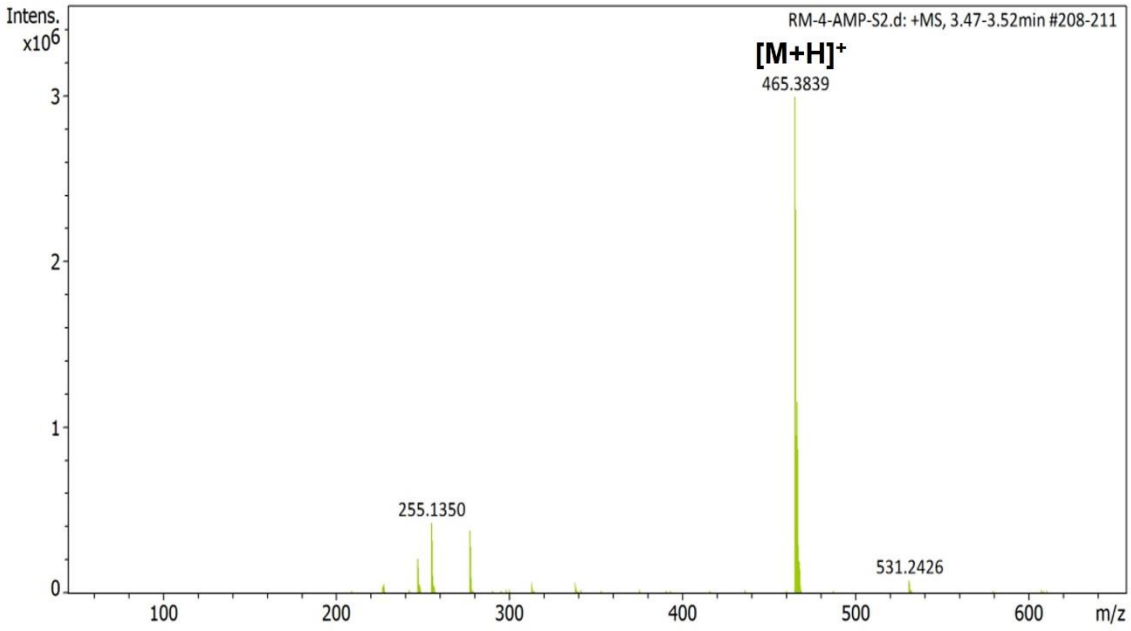
The desired ruthenium(II) arene complex was prepared in high yield in a single step (Scheme 1), in which [(η^6-*p*-cymene)$RuCl_2$]$_2$ (0.202 g, 0.3 mmol) and PyPCDA ligand 0.184 g 0.6 mmol) were dissolved in 25 mL of dichloromethane, and the reaction mixture was stirred for 24 h at room temperature under a dark and nitrogen atmosphere. A solution of NH_4PF_6 (0.110 g, 0.6 mmol) was added to the reaction mixture. The reaction mixture was stirred for 1 h and filtered to remove NH_4Cl and unreacted NH_4PF_6 through a Celite pad. Then, PTA (PTA = 1,3,5-triaza-7-phosphaadamantane; 0.094 g, 0.6 mmol) was finally added to the filtrate, which was stirred for 24 h at room temperature.[2] Then, the solvent was removed and the crude product was recrystallized using a DCM:MeOH solvent mixture (1:5 ratio) to get the orange-yellow-colored powder. Yield: 78%. Elemental analysis for [Ru(η^6-*p*-cymene)(PTA)(PyPCDA)(Cl)]PF_6, calculated: C, 69.78; H, 4.89; N, 8.57, found: C, 69.81; H, 4.87; N, 8.42%. ESI-MS displays a peak at m/z 892.4491 for $C_{47}H_{74}ClN_5OPRu$ $[M]^+$; calculated m/z: 892.6201, less abundant peaks at m/z 715.3747 for $[M\text{-}PTA]^+$ and 1052.4334 m/z for $[M+PF_6+H_2O]^+$. UV-Vis (conc., 300 µM) H_2O, λ_{max} nm, (ε M^{-1} cm^{-1}): 230 (6566), 245 (6600), 322 (2333), 445 (603). ^{1}H NMR (400 MHz, DMSO-d^6): δ 8.64–8.62 (d, 2H, J = 8 Hz), 8.31 (s, 1H), 7.38–7.37 (d, 2H, J = 4 Hz), 5.87–5.85 (d, 2H, J = 8 Hz), 5.83–5.82 (d, 2H, J = 4 Hz), 5.75 (s, 1H), 4.26 (s, 4H), 2.68–2.67 (m, 1H), 2.34–2.33 (m, 1H), 2.29–2.25 (m, 2H), 2.20–2.13 (m, 4H), 2.09 (s, 4H), 1.92 (s, 3H), 1.46–1.42 (m, 6H), 1.33-1.24 (m, 26H), 1.15–1.1 (m, 6H),0.87-0.84 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d^6) δ ppm: 14.43, 18.36, 18.74, 22.16, 22.57, 24.93, 28.15, 28.17, 28.63, 28.64, 28.78, 28.84, 28.97, 29.07, 29.17, 29.33, 29.41,

29.47, 31.77, 34.11, 49.17, 65.81, 71.13, 78.46, 85.88, 88.96, 124.95, 130.01, 131.12, 158.49, 174.95 ppm. ^{31}P NMR (162 MHz, (DMSO-d^6)): δ =-27.38 ppm (s, PTA), -144.20 ppm (sept, PF_6).

Preparation of RuPyPCDA Nano-assembly

1 mg/mL of RuPyPCDA complex in nanopure water was sonicated for 5 mins at room temperature and filtered using a 0.4 µm syringe filter to get a uniform size distribution. Then the self-assembly was irradiated under a UV light (254 nm, 400 µW/cm^2, Luzchem photo reactor) for 30 minutes to induce photopolymerization.[3,4] The color of RuPyPCDA solution was changed from yellow to pale green color and then the nanoformulation was stored in dark at 4^{o}C.

(a)



(b)

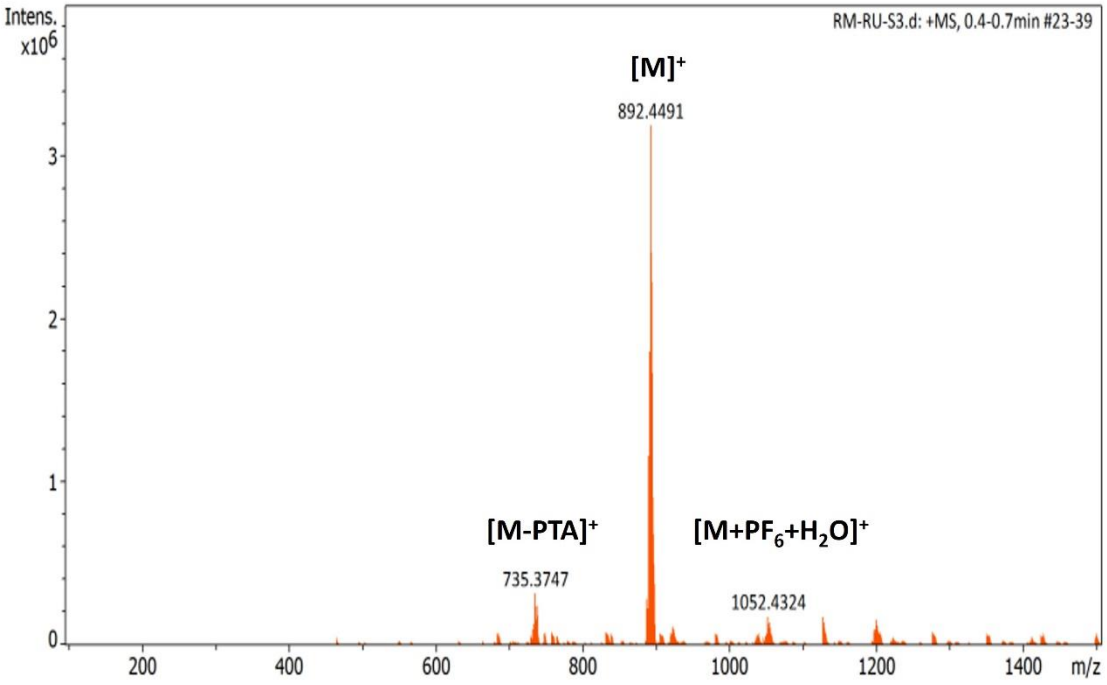


Figure S1: (a) ESI-MS of PyPCDA, and (b) ESI-MS of RuPyPCDA.

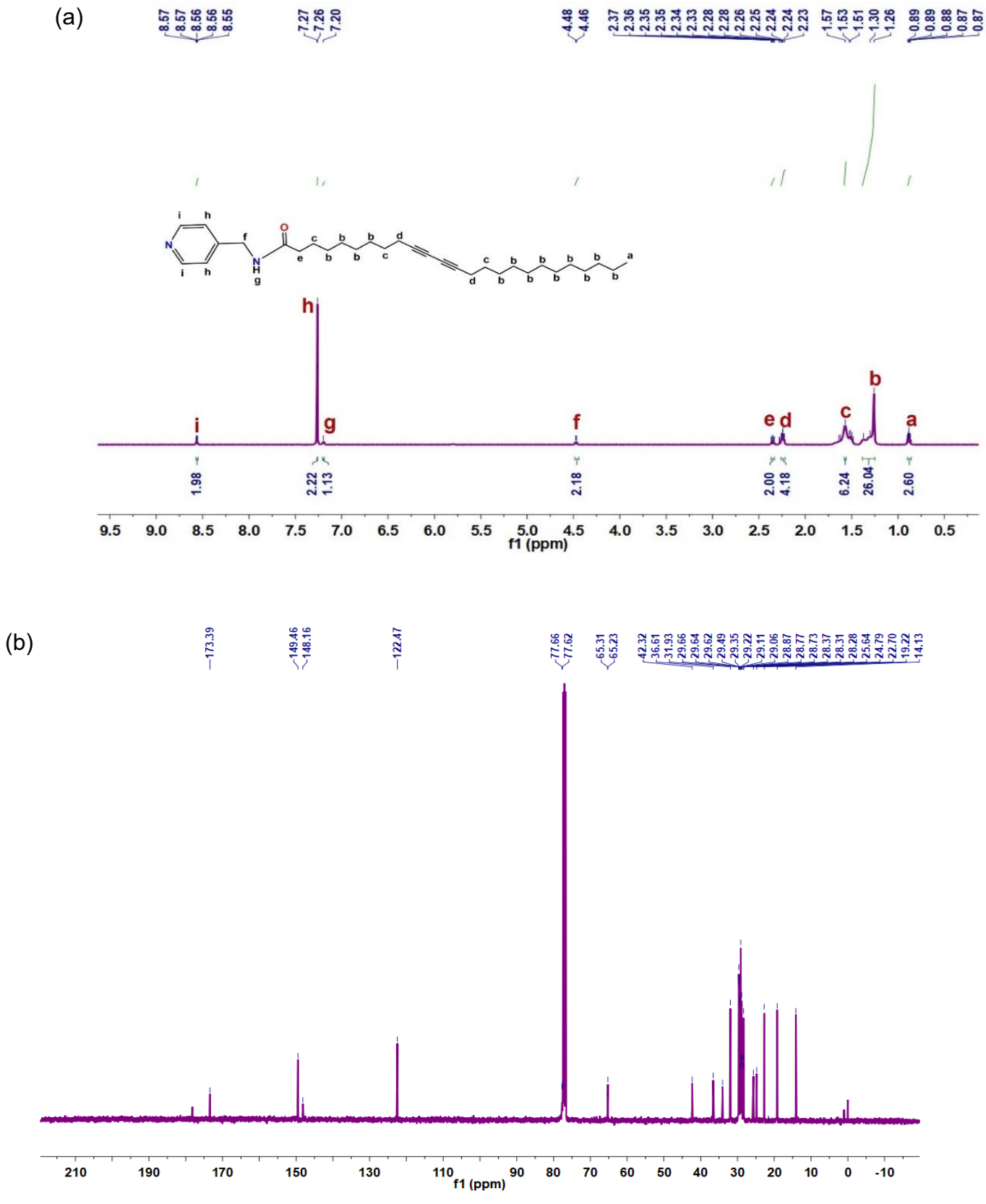
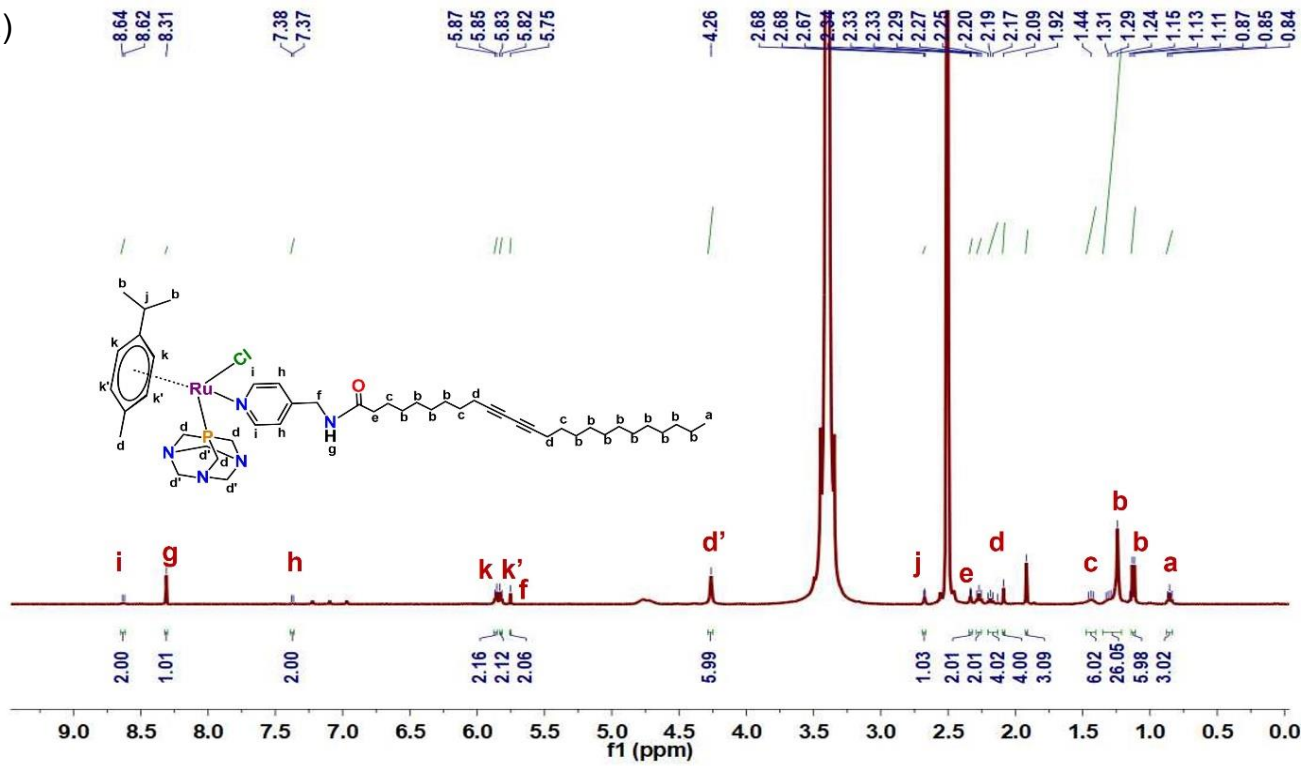
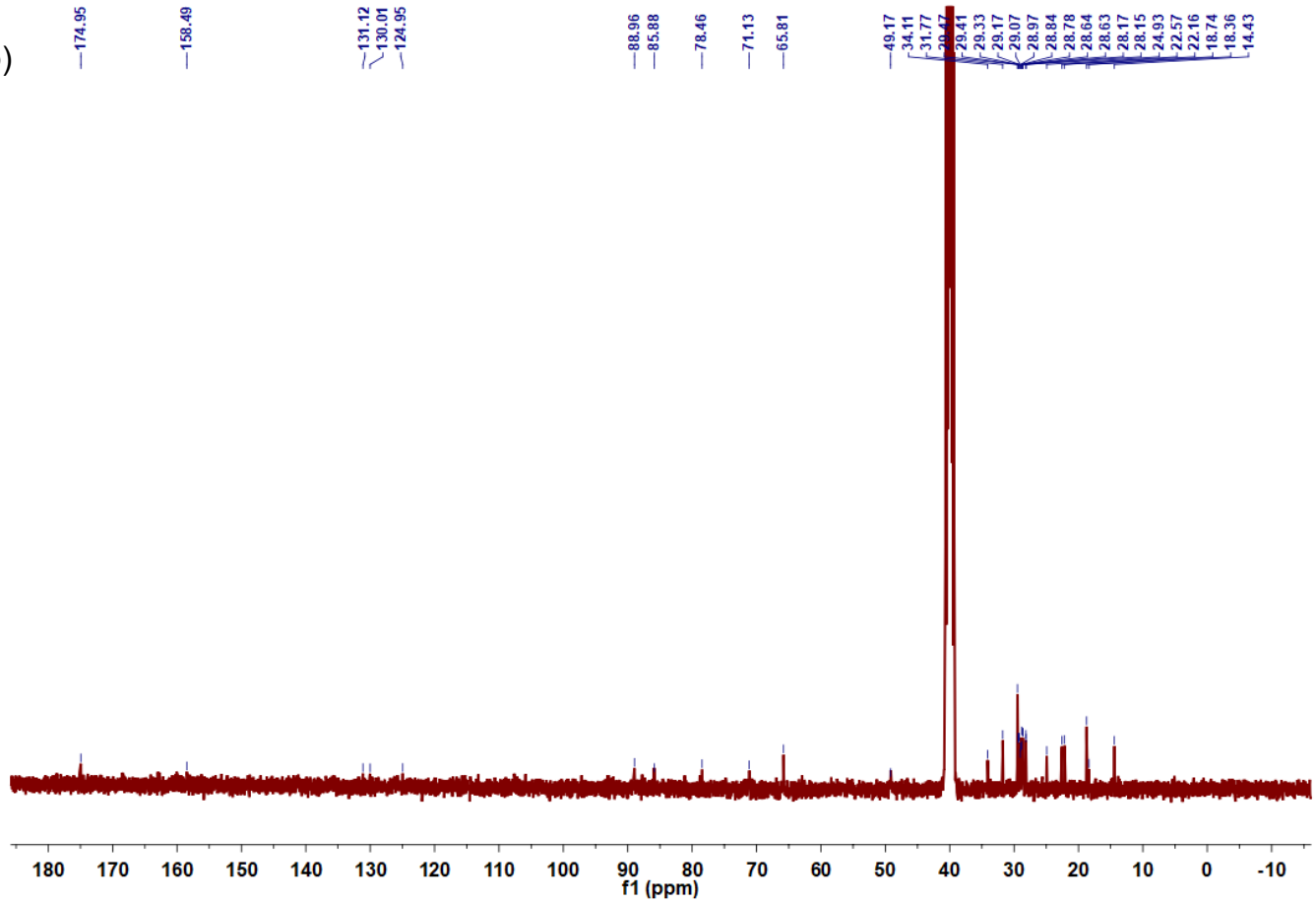


Figure S2: (a) ^{1}H NMR spectrum of PyPCDA in $CDCl_3$, and (b) ^{13}C NMR spectrum of PyPCDA in $CDCl_3$.

(a)


8.64
8.62
8.31
7.38
7.37
5.87
5.85
5.83
5.82
5.75
4.26
2.68
2.68
2.67
2.34
2.33
2.33
2.29
2.27
2.25
2.20
2.19
2.17
2.09
1.92
1.44
1.31
1.29
1.24
1.15
1.13
1.11
0.87
0.85
0.84
i
g
h
k k'
f
d'
j
e
d
c
b
b
a
2.00
1.01
2.00
2.16
2.12
2.06
5.99
1.03
2.01
2.01
4.02
4.00
3.09
6.02
26.05
5.98
3.02
f1 (ppm)

(b)


174.95
158.49
131.12
130.01
124.95
88.96
85.88
78.46
71.13
65.81
49.17
34.11
31.77
29.41
29.33
29.17
29.07
28.97
28.84
28.78
28.64
28.63
28.17
28.15
24.93
22.57
22.16
18.74
18.36
14.43
f1 (ppm)

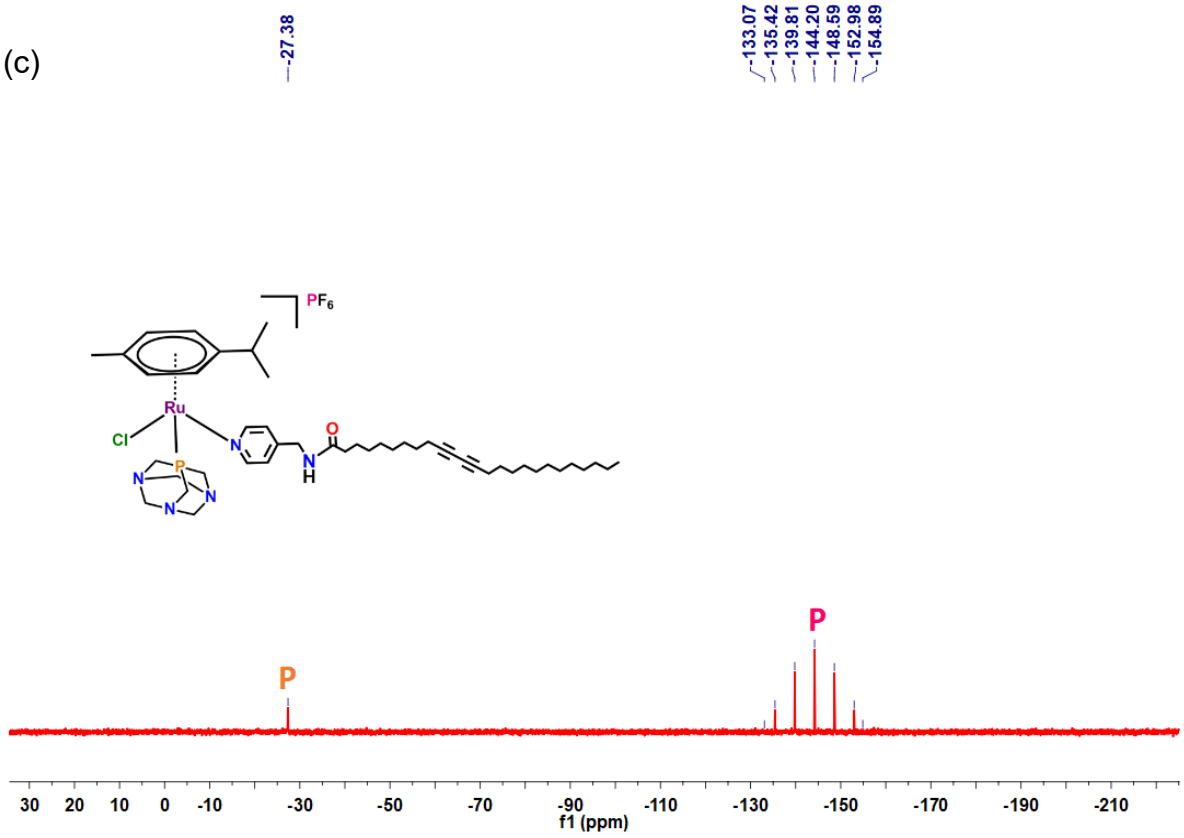


Figure S3: (a) ^{1}H NMR spectrum of RuPyPCDA in DMSO-d^{6}, (b) ^{13}C NMR spectrum of RuPyPCDA in DMSO-d^{6}, and (c) ^{31}P NMR spectrum of RuPyPCDA in DMSO-d^{6}.

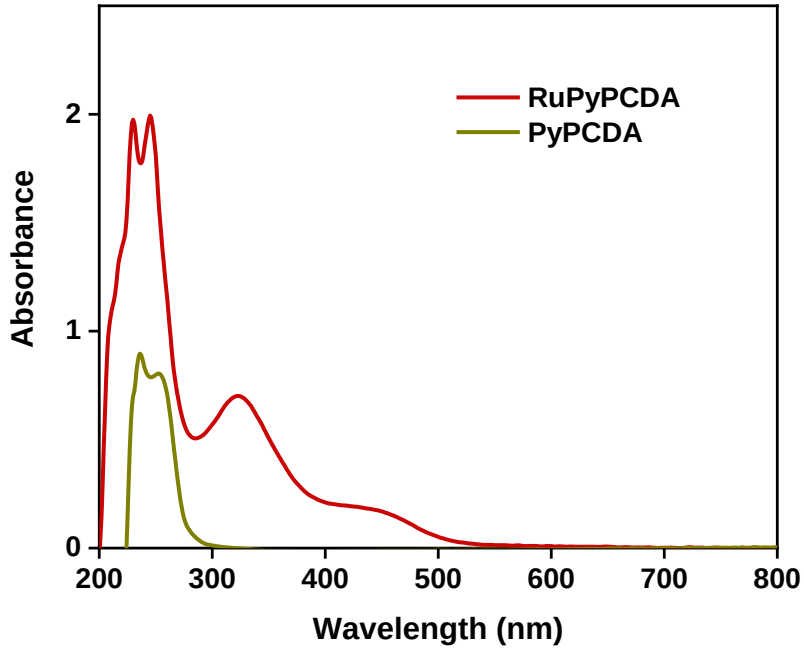


Figure S4: UV-Vis absorption spectra of PyPCDA in $CHCl_3$ and RuPyPCDA in H_2O.

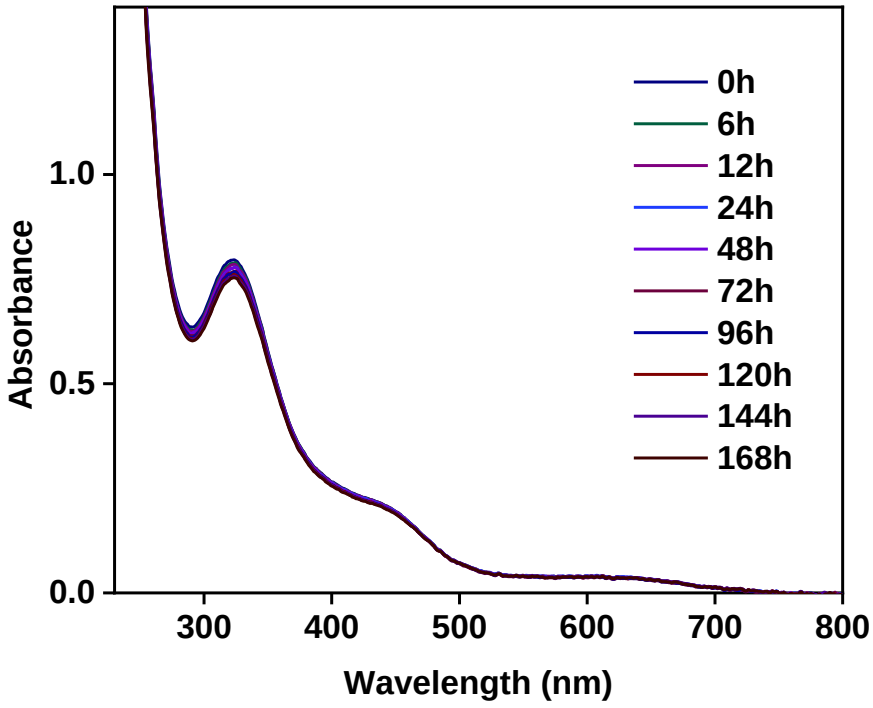


Figure S5: Stability profile of RuPyPCDA in H_2O at various time intervals up to 7 days.

Table S1. The particle size and charge of RuPyPCDA before and after blue light irradiation.

Sample	Particle size (nm)	Surface Charge (mV)	PDI
RuPyPCDA before irradiation	254.7	11.6	0.391
RuPyPCDA after irradiation	449	8.24	1.000

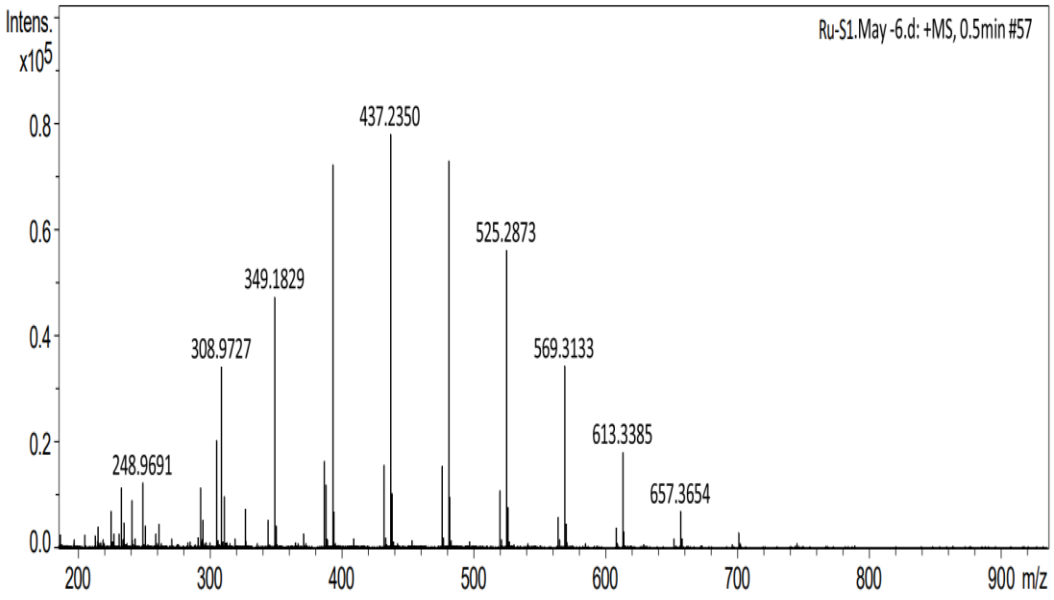


Figure S6: ESI-MS of RuPyPCDA after photo-release of 30 mins.

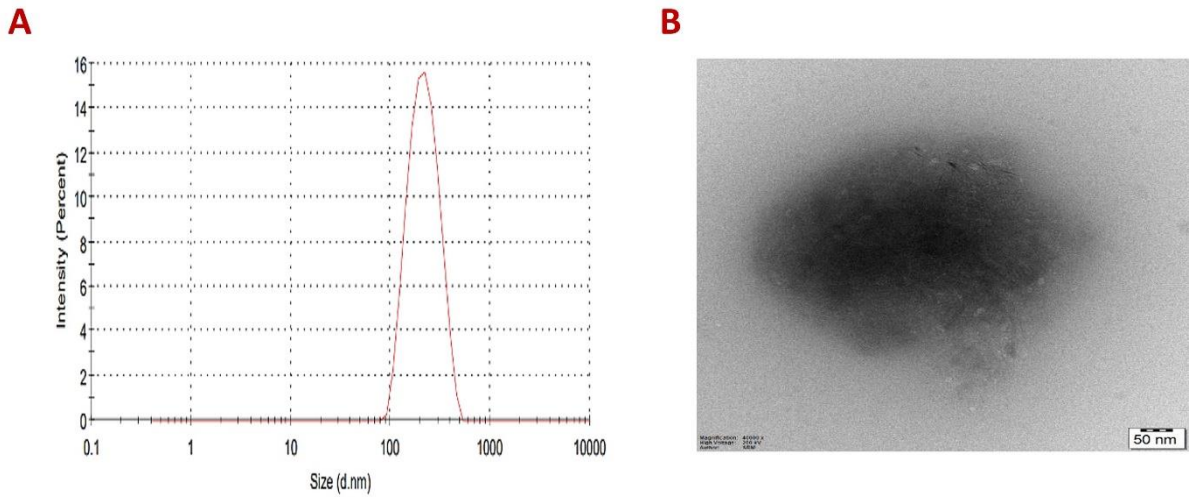


Figure S7: (A) Particle size of RuPyPCDA assembly and (B) TEM image of RuPyPCDA assembly after 30 mins of blue light irradiation.

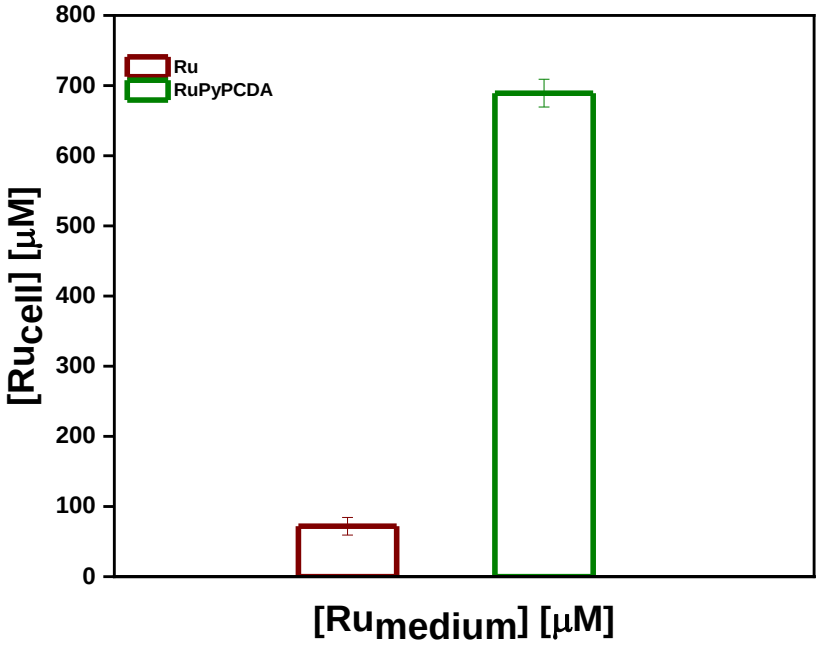


Figure S8: Cellular Uptake in AGS cells determined after 24 h of incubation with Ru and RuPyPCDA at equimolar of 150 μM concentration.

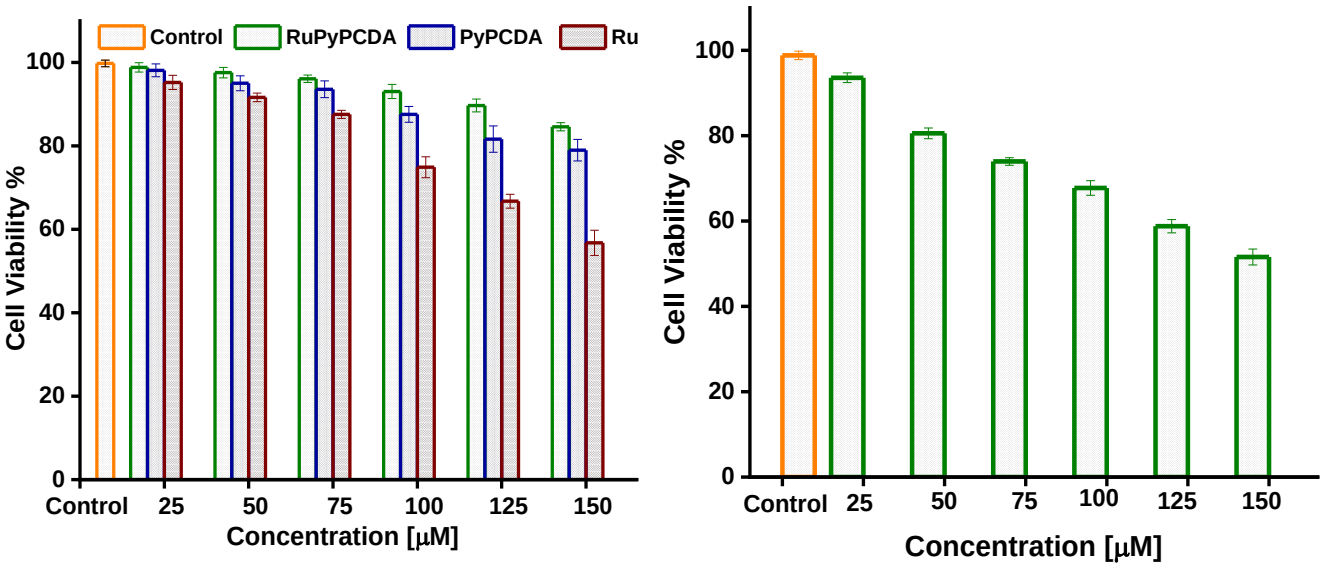


Figure S9: Cell viability tests of Ru, PyPCDA, and RuPyPCDA on the AGS cell line: (a) before blue light irradiation; (b) after blue light irradiation with different concentrations of RuPyPCDA. Values are presented as means ± standard deviations.

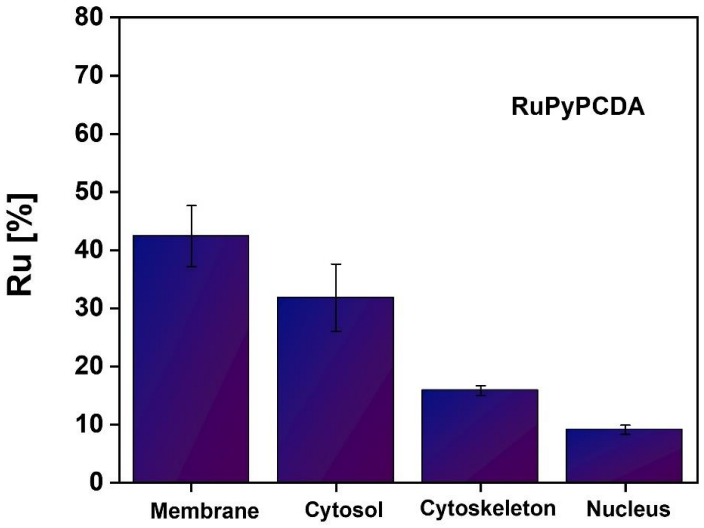


Figure S10: Ruthenium content of the cytosol, membrane, cytoskeleton, and nuclear fraction of AGS cells after 24h of exposure to the RuPyPCDA at 150 µM concentration.

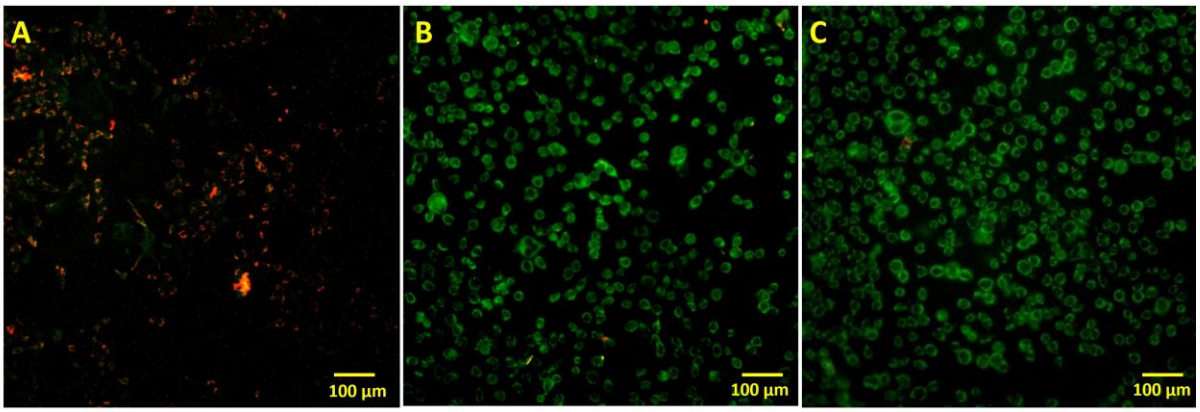


Figure S11: Images of JC-1 fluorescence intensity in AGS cells after exposure to RuPyPCDA at 150 µM with blue light irradiation. JC-1 fluorescence ratio as a marker of MMP loss. Scale bar 100 µm.

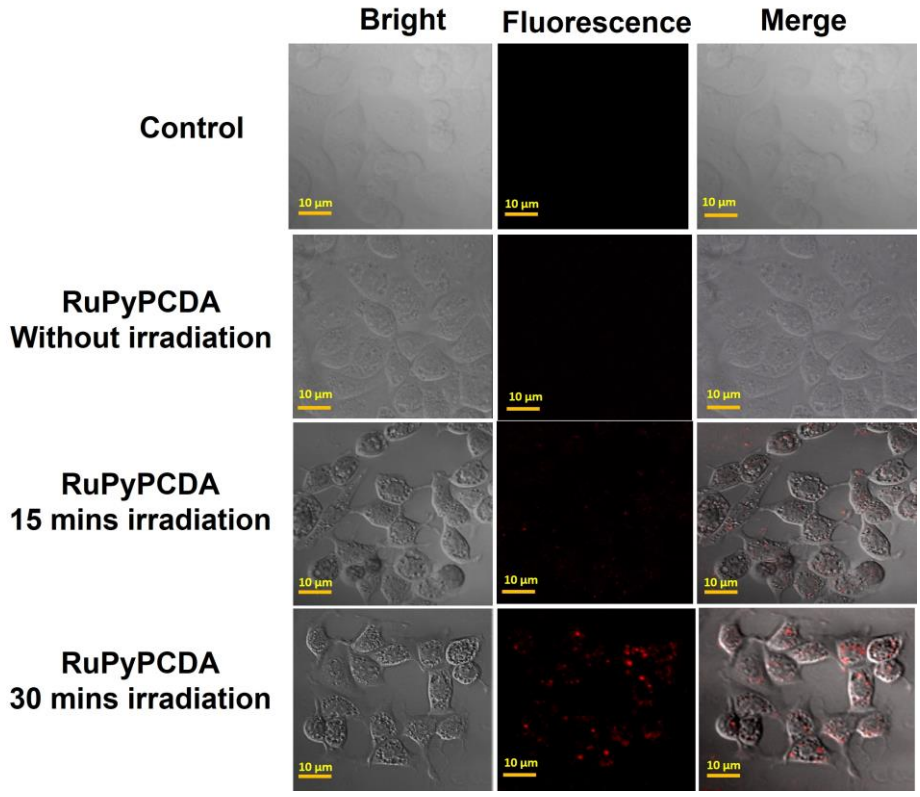


Figure S12: Confocal bright-field and fluorescence images (λ_{ex} = 555 nm) of AGS cells incubated with RuPyPCDA assembly before and after blue light irradiation. Scale bar 10 µm.

References

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2. R. Pettinari, A. Petrini, F. Marchetti, C. Di Nicola, R. Scopelliti, T. Riedel, L. D. Pittet, A. Galindo, and P. J. Dyson, P.J, *Chemistry Select* 2018, **3**(23), 6696-6700.
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4. C. Sumithaa, P. Gajda-Morszewski, W. Ishaniya, T. Khamrang, M. Velusamy, N. Bhuvanesh, M. Brindell, O. Mazuryk, and M. Ganeshpandian, *Dalton Transactions* 2024, **53**(3), 966-985.