Supporting Information

Design of Anticancer Organoruthenium Complex as Guest and Polydiacetylene-Coated Fluorogenic Nanocarrier as Host: Engineering Nanocarrier Using ene-yne Conjugation for Sustained Guest Release, Enhanced Anticancer Activity and Reduced Invivo Toxicity

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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 500 MHz NMR spectrometer. Mass spectrometry was performed on a Shimadzu LC MS-2020 spectrometer. Emission intensity measurements were carried out using an FLS-1000 Spectrofluorometer. Particle size distribution and zeta potential measurements were recorded on nanoparticle SZ-100 (Horiba scientific, UK). Fluorescence images were taken using Leica DM6 fluorescence microscopy. HR-TEM images were taken using JEM-2100 Plus. Powder X-Ray Diffractometer (XRD) images were taken using Bruker USA D8 Advance Davinci, BET analysis was taken using Quantachrome Instruments, Autosorb IQ series, TGA was measured in STA-2500 REGULUS-TGA-DSC Thermal analyzer, and FTIR spectra was analyzed using Shimadzu, IRtracer 100.

Single crystal X-ray diffraction (SC-XRD) analysis

A BRUKER Venture X-ray (kappa geometry) diffractometer was employed for crystal screening, unit cell determination, and data collection. The goniometer was controlled using the APEX3 software suite. The sample was optically centered with the aid of a video camera such that no translations were observed as the crystal was rotated through all positions. The X-ray radiation employed was generated from a Cu-Iμs X-ray tube (K_α = 1.5418Å with a potential of 50 kV and a current of 1.0mA). A Leica MZ 75 microscope was used to identify a suitable yellow block with very well-defined faces with dimensions (max, intermediate, and min) 0.067 x 0.042 x 0.032 mm^3 from a representative sample of crystals of the same habit. The crystal mounted on a nylon loop was then placed in a cold nitrogen stream (Oxford) maintained at 110 K. 45 data frames were taken at widths of 1°. These reflections were used to determine the unit cell. The unit cell was verified by examination of the *h k l* overlays on several frames of data. No super-cell or erroneous reflections were observed.

After careful examination of the unit cell, an extended data collection procedure (24 sets) was initiated using omega and phi scans. Integrated intensity information for each reflection was obtained by reduction of the data frames with the program APEX3. The integration method employed a three dimensional profiling algorithm and all data were corrected for Lorentz and polarization factors, as well as for crystal decay effects. Finally, the data was merged and scaled to produce a suitable data set. The absorption correction program

SADABS was employed to correct the data for absorption effects. Systematic reflection conditions and statistical tests of the data suggested the space group *P*21/*c*. A solution was obtained readily using XT/XS in APEX3. Hydrogen atoms were placed in idealized positions and were set riding on the respective parent atoms. All non-hydrogen atoms were refined with anisotropic thermal parameters. Elongated ellipsoids on the PF_6 suggested disorder and was successfully modeled between two positions with an occupancy ratio of 0.54:0.46. Appropriate restraints were added to keep the bond distances, angles, and thermal ellipsoids of the disordered group meaningful. The absence of additional symmetry or void were confirmed using PLATON (ADDSYM). The structure was refined (weighted least squares refinement on F^2) to convergence.

Lipophilicity

The lipophilicity of complexes was assessed by the "shake-flask" method in octanol-water phase partitions.[1] The complexes were dissolved in a mixture of water and n-octanol, followed by shaking for 24 hours. The mixture was allowed to settle over 30 minutes, and the resulting two phases were collected separately without cross-contamination of one solvent layer into another. The concentration of the complexes in each phase was determined by UV-Vis absorption spectroscopy at room temperature. The results are given as the mean values obtained from three independent experiments. The sample solution concentration was used to calculate log P. Partition coefficients for three complexes were calculated using the equation: log P = log[oct]/[aq].

Release Profile

The release profile of Ru(pyr) from AMSNs and PDA-AMSNs was carried out using the dialysis method.[2,3] 2 mL of Ru(pyr)@AMSNs and PDA-Ru(pyr)@AMSNs suspension were transferred into a dialysis bag (MWCO 12-14 kDa) and the bag was dipped in 50 mL of 1% DMSO/10 mM PBS (pH = 5.4 and 7.4) solution at 37 °C. The aliquot of the release medium (2 mL) was collected and replaced with an equal amount of the original PBS solution at different time intervals for six days. The amount of Ru(pyr) released from AMSNs and PDA-AMSNs was measured from the absorption intensity of the complex (λ_{max} = 345 nm) using a UV-Vis spectrophotometer for the plot of the percentage of complex released vs time. The experiments were performed in triplicate, and the results are presented as mean ± s.d.

Cytotoxicity

The evaluation of the cytotoxicity of the Ru complexes on HT-29 cells was conducted using an Alamar Blue assay.[4] The Alamar Blue test is based on the reduction of blue and non-fluorescent substrate (resazurin) to a pink and highly fluorescent product (resorufin) by the alive cells. Cells were seeded on a 96-well plate with a density of 3×10^4 cells per cm^2 one day before the experiments. Then, cells were incubated with various concentrations of the Ru compounds for 24 hours. All compounds were diluted in DMSO and then, added to the appropriate medium without FBS and L-glutamine to obtain the applied concentrations. The final DMSO concentration was kept constant at 0.1% (v/v). After the incubation, cells were washed with PBS and incubated in the resazurin sodium salt solution (50 μM) for 3 h. The cell viability was quantified at 605 nm using 560 nm excitation light (Tecan Infinite 200 microplate reader). Experiments were performed in triplicate and repeated at least three times to get the mean values ± standard deviation. The viability was calculated with respect to the untreated cells control. The IC_{50} values were determined using the Hill equation (OriginPro 2020 9.7)

$$y = y\_0 + ((y\_100 - y\_0)\ [[c]]\hat{}H)/([[[[IC]]\_50]]\hat{}H + [[c]]\hat{}H)$$

Cellular uptake of Ru compounds

Cellular uptake of the Ru complexes was determined by seeding HT-29 cells in 6-well plates with a density of 4×10^4 cells per cm^2 in a complete medium and cultured for 1 day.[5] Next, cells were incubated with non-toxic concentrations of the Ru complex (either 1/4 or 1/2 of IC_{50}) for 24 h dissolved in serum free medium. Subsequently, the incubated cells were washed with PBS, detached by trypsin treatment, counted, and centrifuged. The supernatant was removed, and cells were digested in concentrated nitric acid overnight at room temperature; for compounds encapsulated in silica the time was extended to 48 h and samples were kept at 60 °C followed by addition of concentrated fluoric acid and kept under the same conditions for another 48 h. The solutions were then diluted with Millipore water to a final nitric acid concentration of 1%. Samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS, NexION 2000C, Perkin Elmer). The results were calculated as the Ru concentration per cell. The experiments were repeated three times. Similarly, the content of Ru ions was measured in a subcellular fraction obtained as described in the following chapter. Further, the fluorescent images were obtained using an Olympus IX83 microscope equipped with a CellVivo chamber (λ_{ex} = 545 ± 25 nm, λ_{em} = 620 ± 30).

Subcellular fractionation

Subcellular fractionation was carried out by applying the subcellular protein fractionation kit for cultured cells (Thermo Fisher Scientific) according to the manufacturer's instructions.[6] Briefly, HT-29 cells were seeded in a 25 cm^2 flask in a complete medium and cultured for 24 h. Then, the medium was removed, and cells were treated with 1.5 μM of **Ru(pyr)** in serum-free medium for 24 h. After the incubation cells were washed with PBS, detached by trypsin treatment, and counted. 2 ml cells were collected for fractionation in 1.5 ml microcentrifuge tubes. The following fractions were obtained: CEB – cytoplasmic, MEB – membrane, NEB1 – soluble nuclear, NEB2 – chromatin-bound nuclear, and PEB – cytoskeletal. The experiments were performed in triplicate and each experiment was repeated three times to obtain mean values and standard deviation of the mean.

Evaluation of oxidative stress – in vitro studies

The cells were seeded into 96-well plate with the density of 3×10^4 cells per cm^2 in complete medium and cultured for 24 h. Then medium was removed and various concentration of the studied complexes were added for 24 h incubation. After the treatment, cells were washed with PBS and ROS probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, 20μM) was added to the culture for 30 min incubation. Then, the probe was removed, cells were washed with PBS, and fluorescence of the cells was quantified by a Tecan Infinite 200 plate reader. (λ_{ex} = 485 nm and λ_{em}= 535 nm). The experiment was performed in triplicates and repeated three times to get mean values and standard deviation of mean.

Antioxidant properties – in vitro studies

For evaluation of cellular antioxidant activity, HT-29 cells were seeded in a 96-well plate with a density of 3×10^4 cells per cm^2 in a complete medium and cultured for 1 day. Next, cells were incubated with non-toxic concentrations of the Ru complex (either 1/4 or 1/2 of IC_{50}) for 24 h dissolved in serum-free medium. Subsequently, the incubated cells were washed with PBS with Ca^{2+} and Mg^{2+} and ROS probe H_2DCF-DA (20μM) was added to the culture for 30 min incubation at 37 ^oC. The probe was then removed, cells were washed with PBS and the oxidant 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP, 300 μM) was added to the cells. The fluorescence of the cells was quantified by a Tecan Infinite 200 plate reader (λ_{ex} = 485 nm and λ_{em}= 535 nm) every 5 min for 1 hour. The experiments were performed in triplicate and repeated three times to obtain mean values and standard deviation of the mean.

Apoptosis inducing properties

The cell death was determined using Annexin V kit (Annex300F kit, BioRad). HT-29 cells were seeded in a 24-well plate with a density of 5×10^4 cells per cm^2 in a complete medium and cultured for 1 day. Next, cells were incubated with 3.0 μM Ru(pyr) complex dissolved in serum-free medium for 24 h. Subsequently, the incubated cells were washed with PBS and binding buffer. The cells were stained with Annexin V-FITC for 10 min in the dark and then, with propidine iodine (PI, 0.5 μM) for 5 min. Cells were analyzed by a flow cytometer BD Facs Versa (λ_{ex} = 488 nm, λ_{em}= 527 ± 16 nm, λ_{em}= 586 ± 21 nm). As a positive control, H_2O_2 (300 μM, 6 h incubation) was used. At the early stage of apoptosis, the relocation of phosphatidylserine (PS) from the inner side of the cellular membrane to the outer side takes place. Annexin V in the presence of Ca^{2+} ions selectively binds to PS. PI was used to assess cell necrosis.

Trypsin resistance assay

The impact of Ru compounds on cell susceptibility to detachment was assessed by checking their resistance to trypsin treatment. HT-29 cells were seeded in a 96-well plate with a density of 3×10^4 cells per cm^2 in a complete medium and cultured for 1 day. Then, the cells were incubated with various concentrations of Ru compounds (either 1/4 or 1/2 of IC_{50}) for 24 h. Subsequently, the cells were washed with PBS and kept in the buffer for 5 min. Then, 30 μL of 0.05% trypsin solution enriched with 0.53 mM EDTA was added to each well for 5 min of incubation at 37 °C, followed by washing with PBS and the addition of resazurin sodium solution to quantify adherent cells. The obtained results were normalized with appropriate wells without trypsin treatment to exclude the possible toxicity of the studied compounds and presented as a percentage of control cells. The experiments were carried out in triplicate, and each experiment was repeated five times to obtain the mean values and the standard error of the mean.

Cell cycle analysis

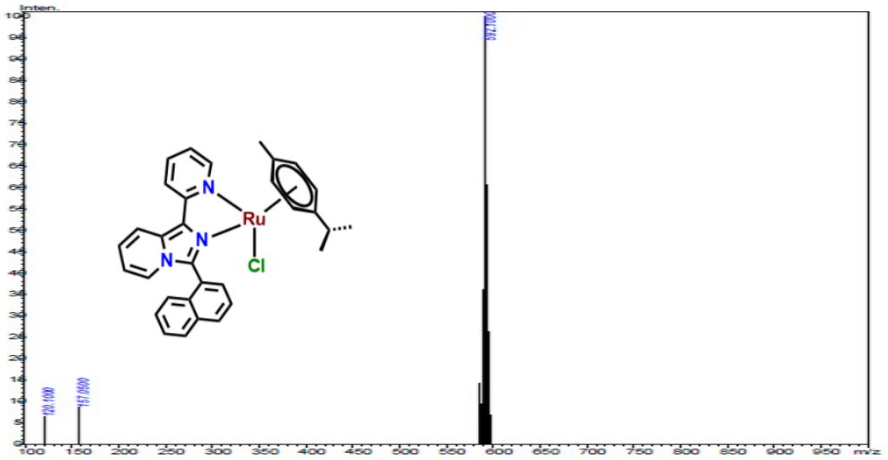
To assess the changes in cell cycle, PI test was used. Briefly, HT-29 cells were seeded in 6-well plates with a density of 4×10^4 cells per cm^2 in a complete medium and cultured for 1 day. Next, cells were incubated with various concentrations of Ru(pyr) complex (0.75 μM, 1.5 μM, 2.4 μM and 3.0 μM) dissolved in serum-free medium for 24 h. Subsequently, the incubated cells were washed with PBS, detached with trypsin and fixed using 70% methanol solution. Then the cells were stained with PI (100 μg/ml) for 30 min at 37 °C. After the incubation, the cells were washed and analyzed using BD Facs Versa flow cytometer (λ_{ex} = 488 nm and λ_{em}= 586 ± 21 nm).

Zebrafish Embryo Toxicity (ZET)

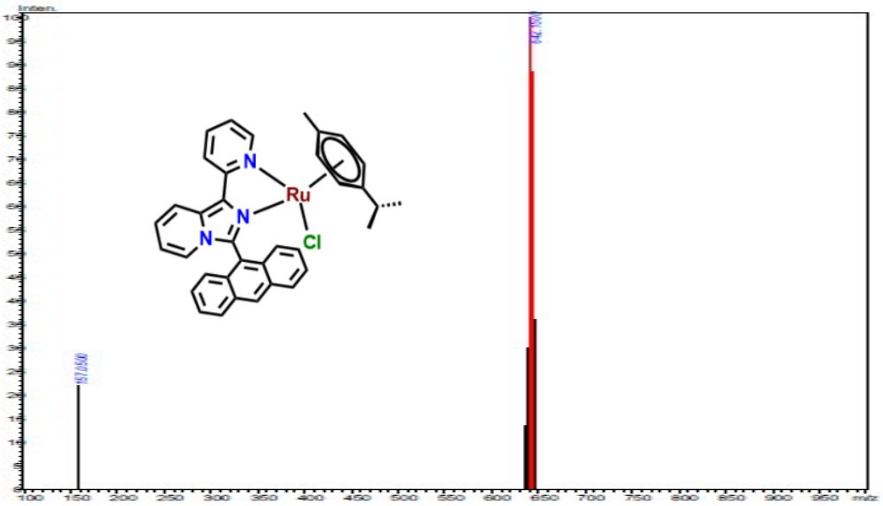
The embryos of wild-type Danio rerio (zebrafish) have been staged and nurtured at 27 ± 1 C as previously described and by following OECD 2013 guidelines.[7,8] The viable embryos were seeded in 24-well plates and exposed to five different concentrations (10, 25, 50, 100, and 200 μM) of Ru(pyr) and PDA-Ru(pyr)@AMSNs together with untreated control and vehicle control. The stock solution of the compounds was prepared in DMSO and then diluted using E3-medium but the final DMSO concentration was kept constant at 0.1% (v/v). The zebrafish embryo's mortality, malformations, and hatching rates were scrutinized under a stereo zoom microscope (Leica SAP0) up to 96 h with a time interval of 24h. Experiments were performed in triplicate to get the mean values ± standard deviation. The percentage of the hatching rate was calculated according to OECD guidelines as below.[9] The Lethal Concentration 50 (LC_{50}) was determined from the plot of the percentage of mortality vs. concentration using OriginPro 2020 9.7.

Hatching rate (%) = (Number of hatched larvae / Total number of embryo/larvae) x 100

(a)



(b)



(c)

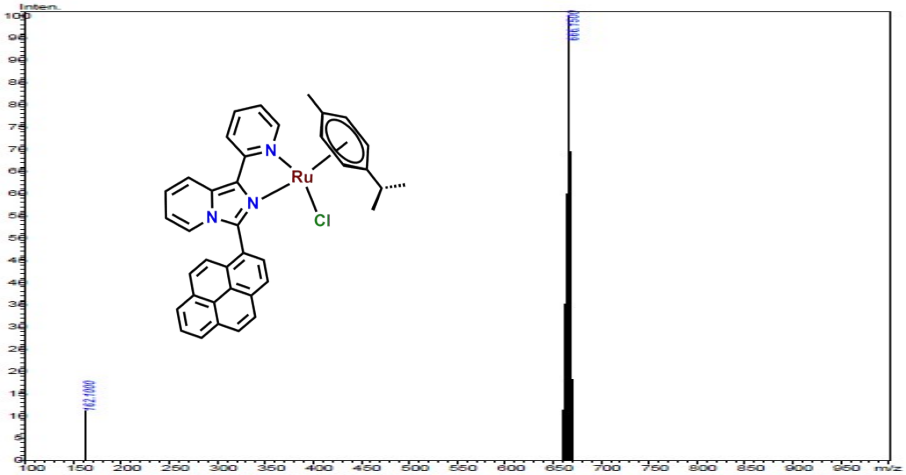


Figure S1: ESI-Mass spectra of (a) Ru(nap), (b) Ru(ant), and (c) Ru(pyr) in DCM/Methanol.

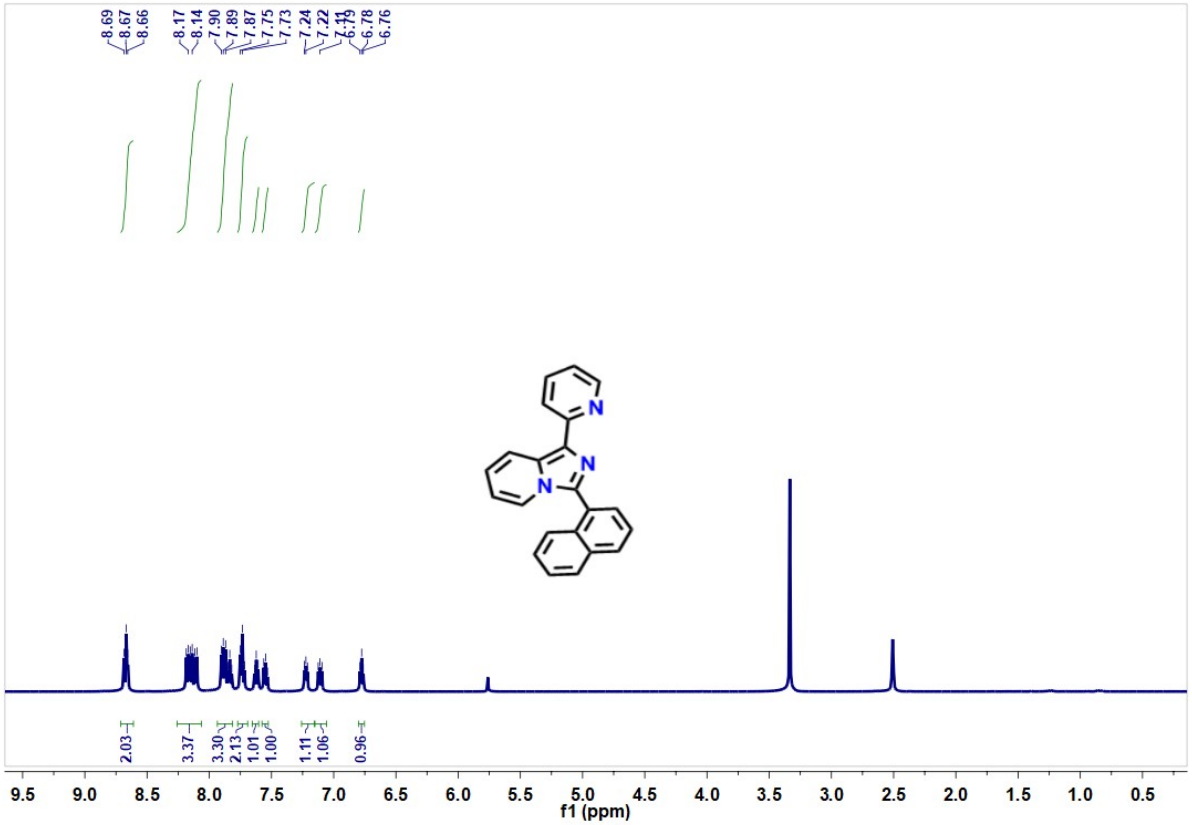


Figure S2. ^{1}H-NMR spectrum of L1 in $CDCl_3$.

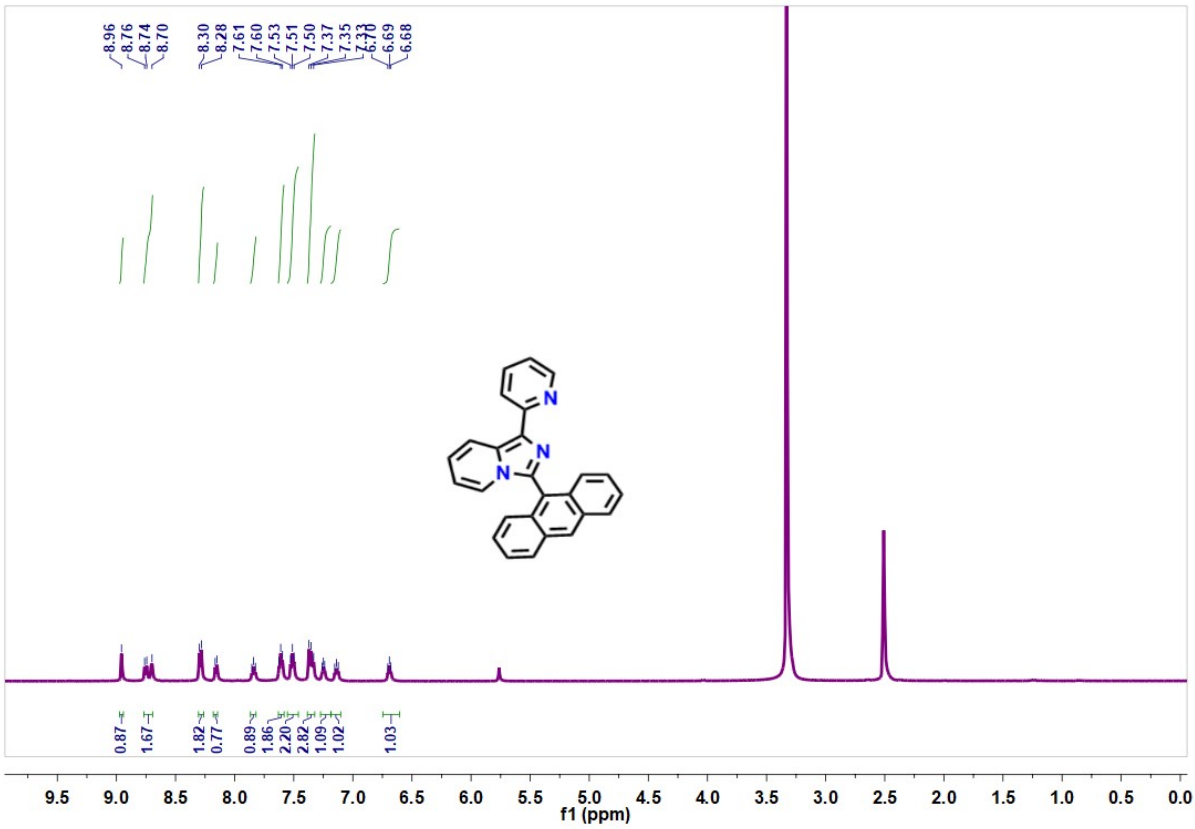


Figure S3. ^{1}H-NMR spectrum of L2 in $CDCl_3$.

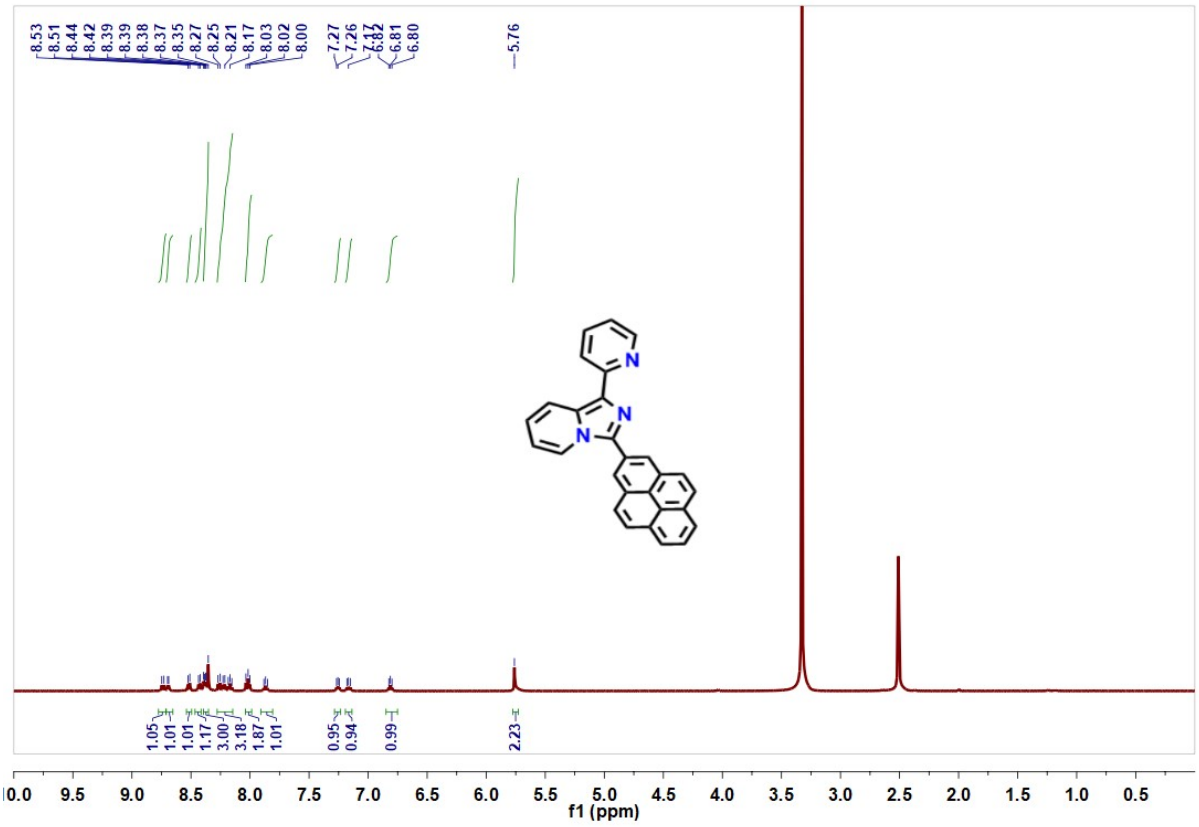


Figure S4. ^{1}H-NMR spectrum of L3 in $CDCl_3$.

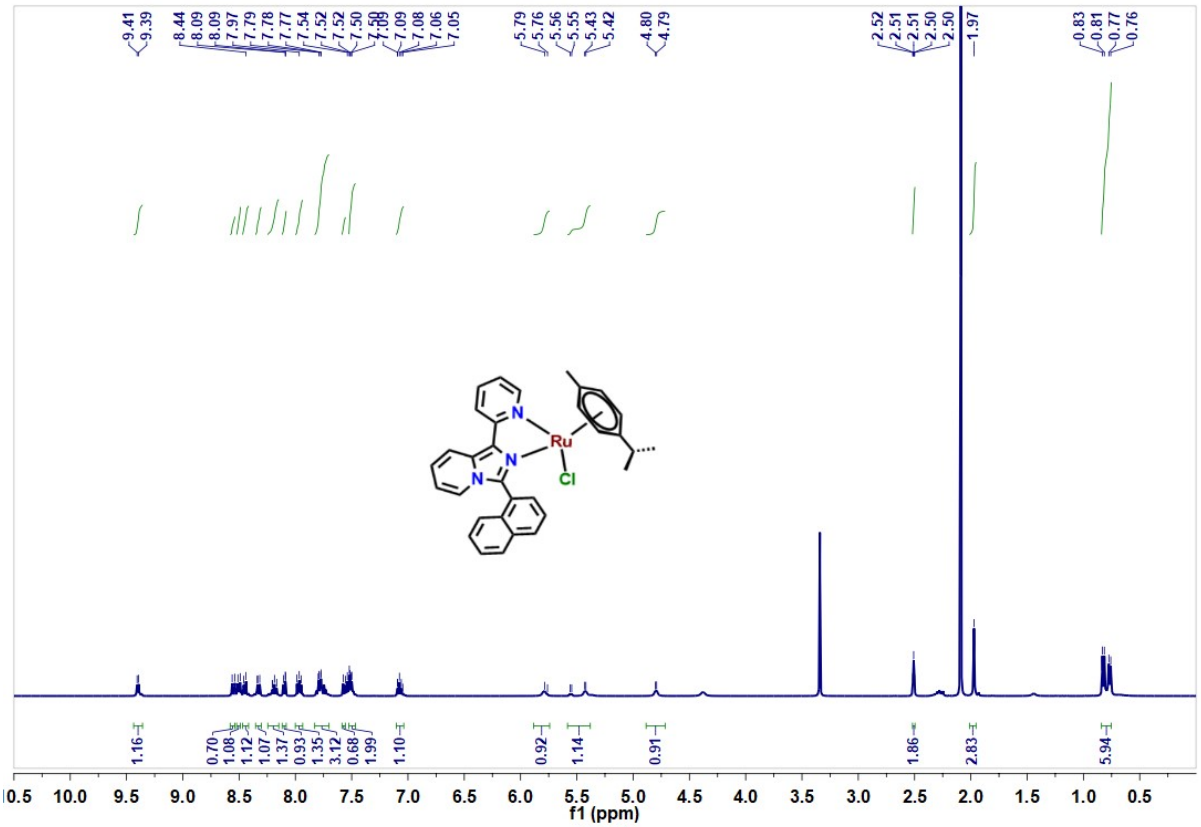


Figure S5. ^{1}H-NMR spectrum of **Ru(nap)** in DMSO-d^{6}.

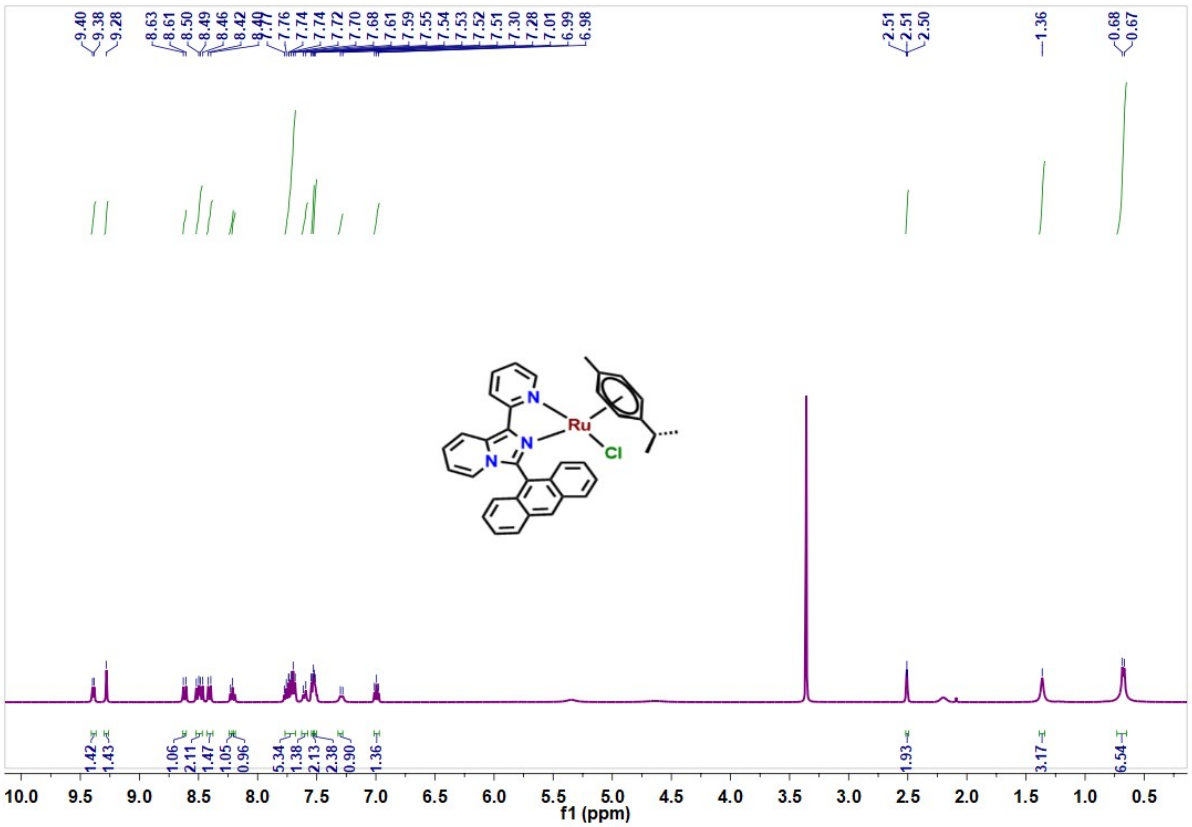


Figure S6. ^{1}H-NMR spectrum of **Ru(ant)** in DMSO-d^{6}.

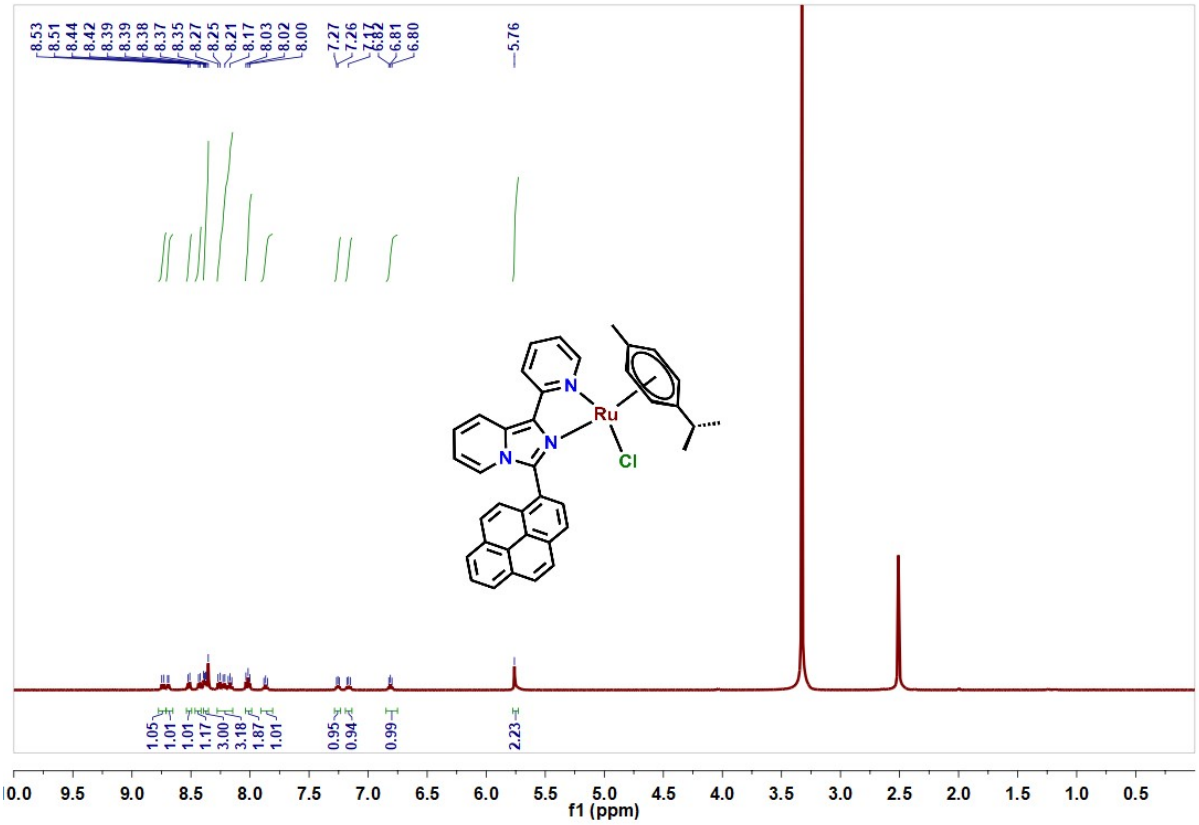


Figure S7. ^{1}H-NMR spectrum of **Ru(pyr)** in DMSO-d^{6}.

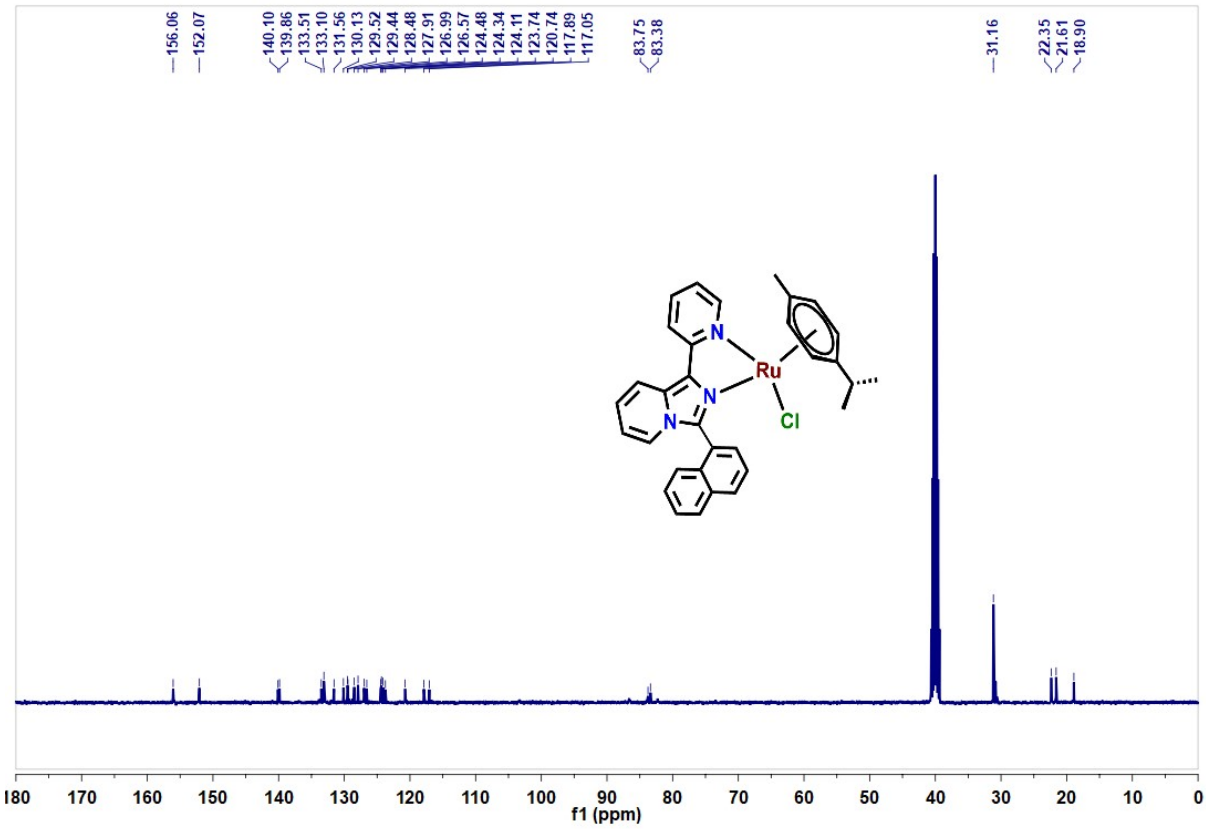


Figure S8. ^{13}C-NMR spectrum of **Ru(nap)** in DMSO-d^6.

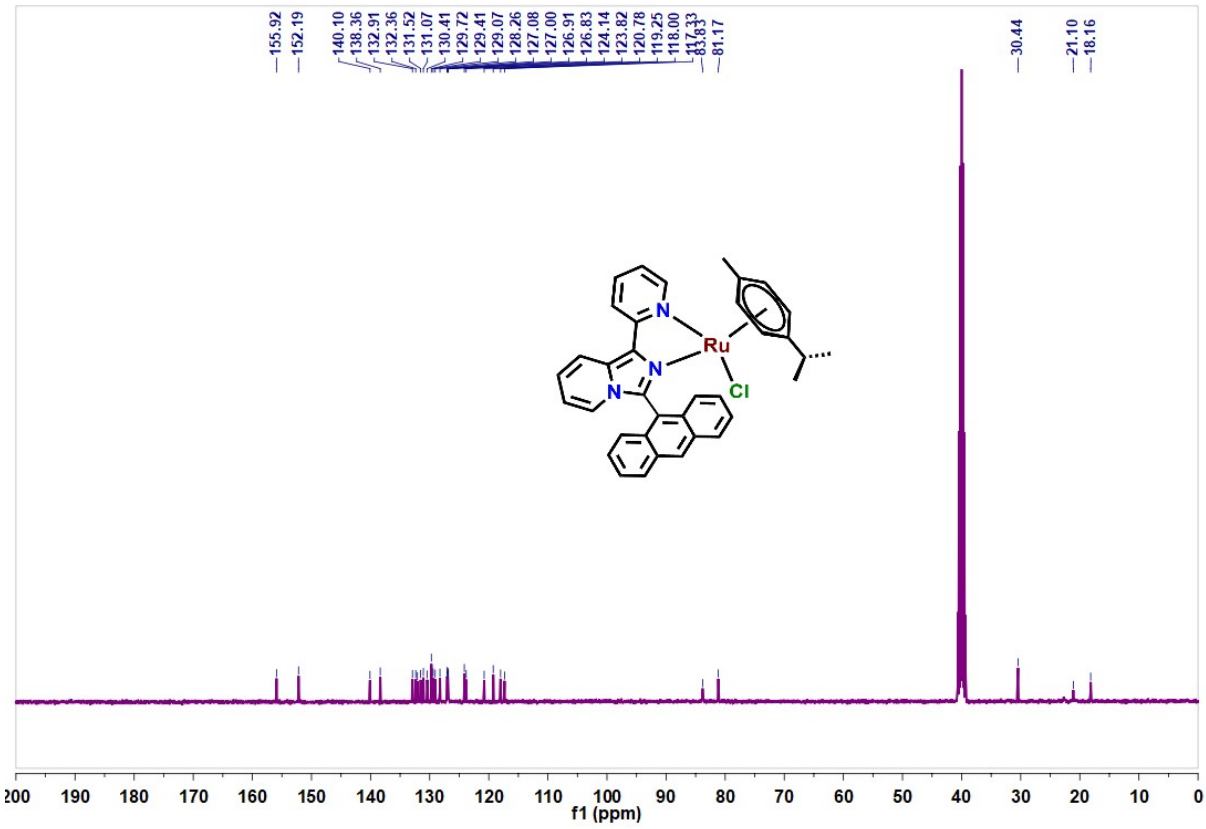


Figure S9. ^{13}C-NMR spectrum of **Ru(ant)** in DMSO-d^{6}.

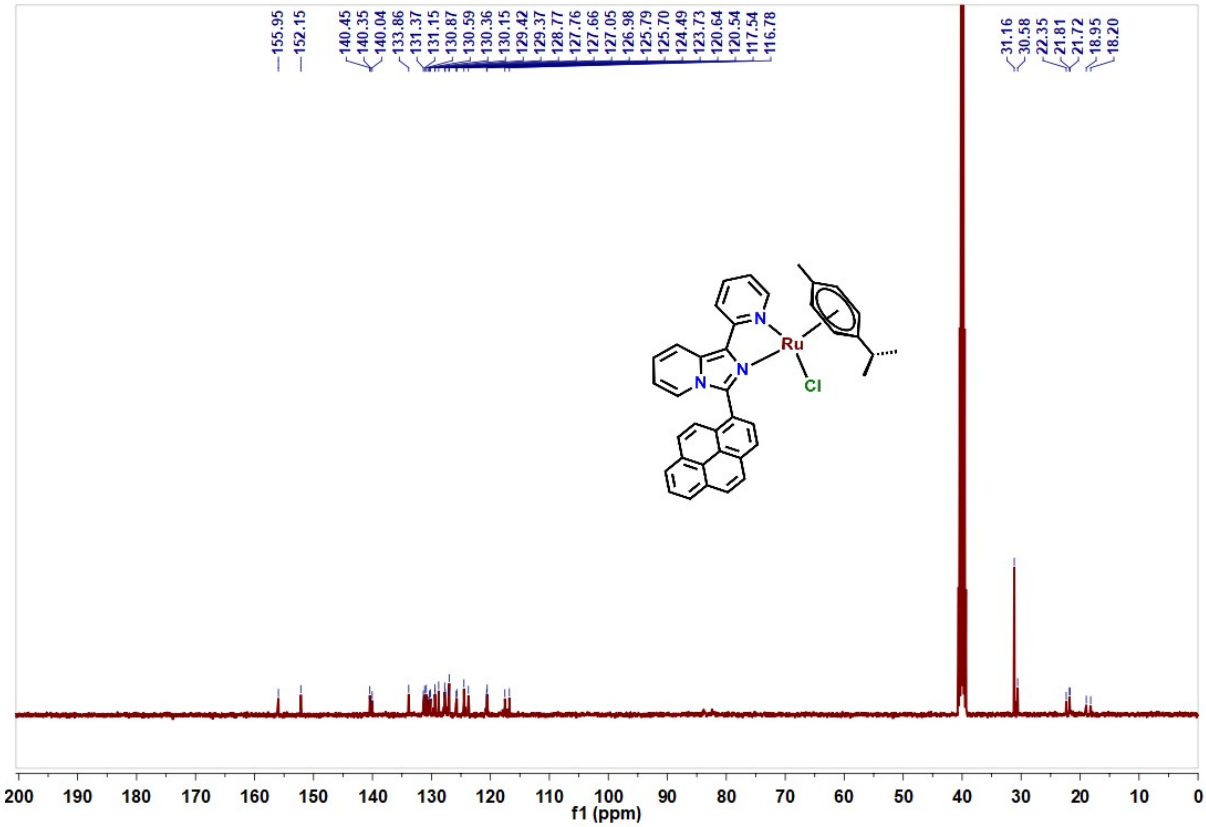


Figure S10. ^{13}C-NMR spectrum of **Ru(pyr)** in DMSO-d^{6}.

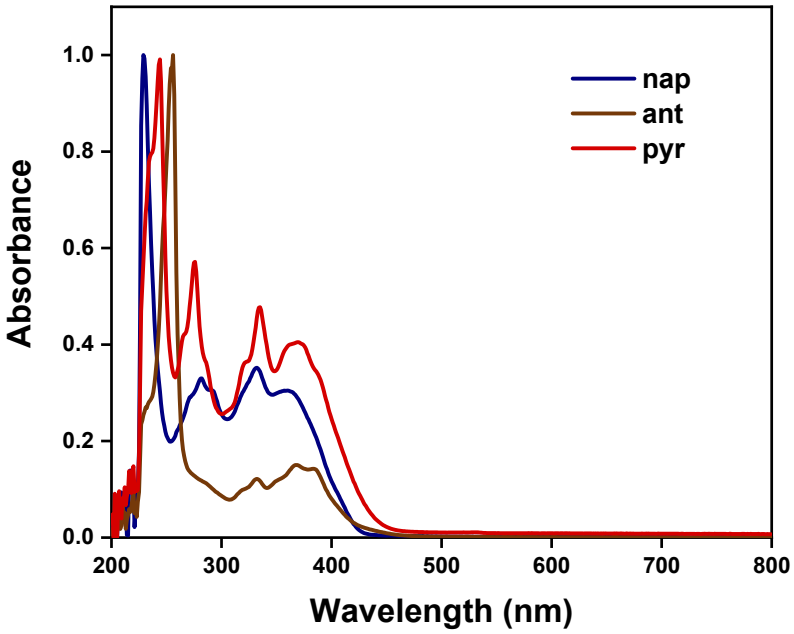


Figure S11. UV-Vis absorption spectra of nap, ant, and pyr in DCM/DMSO.

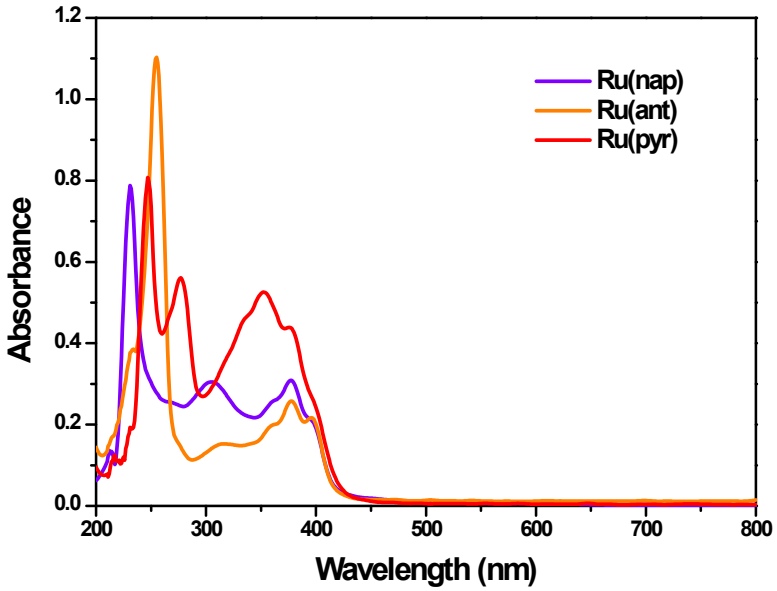


Figure S12: UV-Vis absorption spectra of Ru(nap), Ru(ant), and Ru(pyr) in DCM/DMSO.

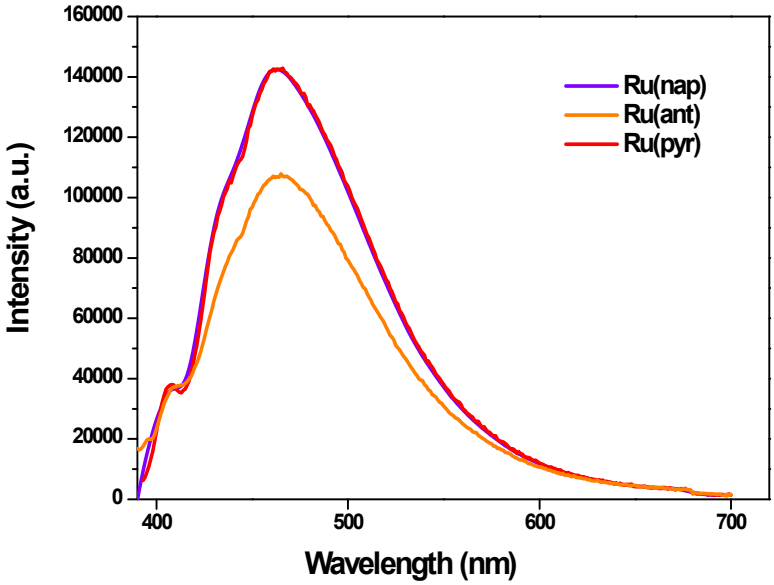


Figure S13. Emission spectra of Ru(nap) (λex=390 nm), Ru(ant) (λex= 365 nm), and Ru(pyr) (λex= 395 nm); (Conc., 13.3 μM of 0.1% (v/v) DMSO:DCM).

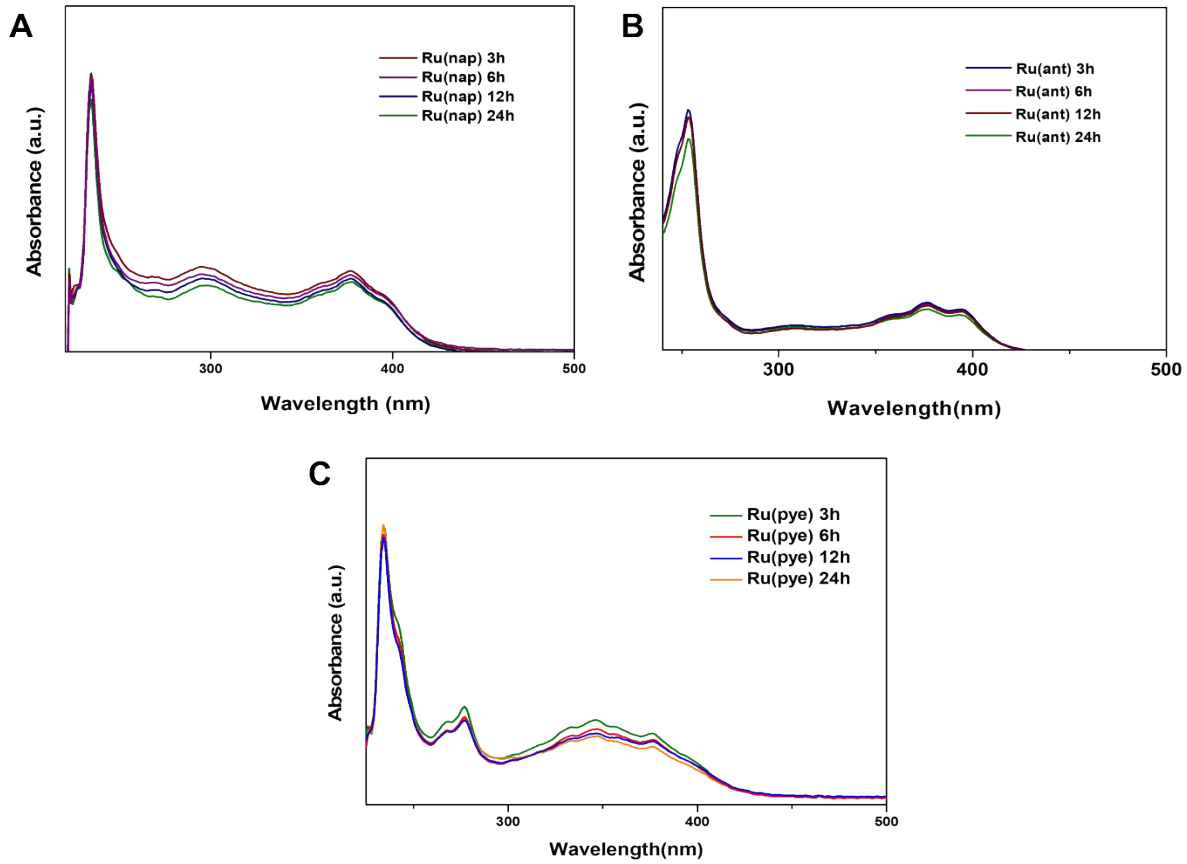


Figure S14: Stability studies of the (A) Ru(nap), (B) Ru(ant), and (C) Ru(pyr) in 1% DMSO-PBS solution at various time interval up to 24 h.

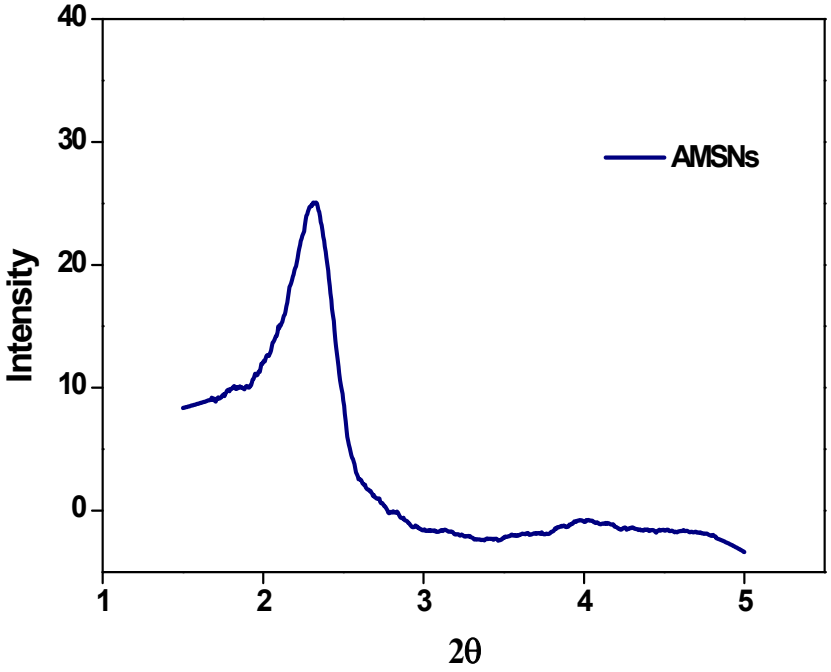


Figure S15: Powder XRD pattern of AMSNs.

Table S1: N_2 adsorption-desorption analysis of AMSNs, S_{BET} is the total surface area of AMSNs determined by Brunauer-Emmett-Teller (BET) method. Vp is the total pore volume and Dp is the pore diameter.

Sample	$S_{BET}(m^2/g)$	$Vp(cm^3/g)$	Dp(nm)
AMSNs	898.88	0.378	3.42

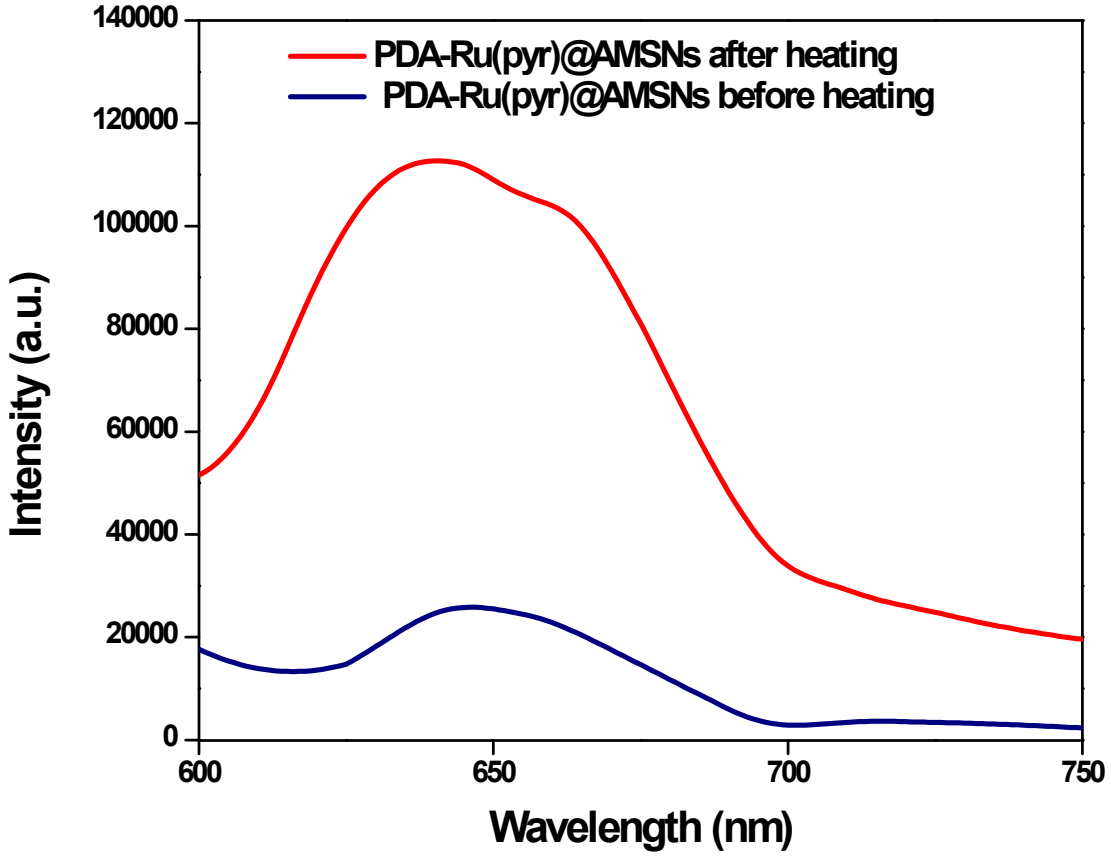


Figure S16: Emission spectra of PDA-Ru(pyr)@AMSNs before (λex= 600 nm) and after heating(λex= 564 nm).

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