Polymeric Encapsulation of a Ruthenium(II) Polypyridyl Complex: From Synthesis to *in vivo* Studies against High-Grade Epithelial Ovarian Cancer

João P. M. António,[1,2] Albert Gandioso,[1] Fariba Nemati,[3] Nancy Soliman,[1,2] Robin Vinck,[1] Fan Sun,[4] Carine Robert,[2] Pierre Burcke[5], Didier Decaudin,[3] Christophe M. Thomas[2,*] and Gilles Gasser[1,*]

[1] Chimie ParisTech, PSL University, CNRS, Institute of Chemistry for Life and Health Sciences, Laboratory for Inorganic Chemical Biology, 75005 Paris, France

[2] Chimie ParisTech, PSL University, CNRS, Institut de Recherche de Chimie Paris, 75005 Paris, France

[3] Translational Research Department, Laboratory of Preclinical Investigation, PSL University, Institut Curie, 26 rue d'Ulm, Paris 75248, France

[4] Chimie ParisTech, PSL University, CNRS, Institut de Recherche de Chimie Paris, 75005 Paris, France

[5] Université de Paris, Institut de physique du globe de Paris, CNRS, F-75005 Paris, France

[6] Department of Medical Oncology, Institut Curie, 26 rue d'Ulm, Paris 75248, France

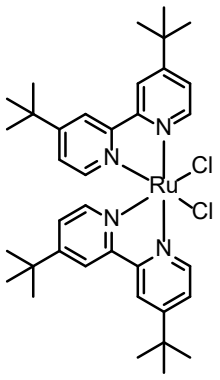
Supporting Information

General Remarks

NMR spectra were recorded on a Bruker Avance 400 MHz. ^{1}H and ^{13}C chemical shifts are reported in ppm versus $SiMe_4$ and were determined by reference to the residual solvent peaks for ^{1}H and ^{13}C NMR and to the chemical shift of TMS (0 ppm). Assignment of signals was made from multinuclear 1D (^{1}H, $^{13}C\{^{1}H\}$) and 2D (COSY, HMQC, HMBC) NMR experiments. All coupling constants are expressed in Hz and chemical shifts (δ) in ppm. Multiplicities are given as: s (singlet), d (doublet), dd (double doublet), dt (double triplet), t (triplet), td (triple triplet), tt (triple triplet), q (quartet), quint (quintuplet) and m (multiplet). Size exclusion chromatography (SEC) of polymers was performed in THF at 35 °C using an

Agilent 1260 Infinity Series GPC (ResiPore 3 µm, 300 x 7.5 mm, 1.0 mL/min, RI (PL-GPC 220) and Light scattering detectors). MALDI-TOF mass spectrometry analyses were recorded with a Axima Confidence spectrometer (Shimadzu), externally calibrated with PEG (M_n = 2000 g/mol). Elemental analysis was performed in a Flash 2000 CHNS-O analyzer (ThermoScientific, UK). The nanoparticle intensity-average diameters Dz and the polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Malvern ZetaSizer Nano ZS. RP-HPLC equipped with an Agilent Pursuit XRs 5C18 (Analytic: 100 Å, C18 5 µm 250 × 4.6 mm, Preparative: 100 Å, C18 5 µm 250 × 300 mm) Column was used to assess the hydrolytic release of **Ru** from S3 Ru-PLA nanoconjugates. The C18 reverse phase column was used with a flow rate of 1 mL.min-1 and UV absorption was measured at 350 nm. The runs were carried out with a linear gradient of A (CH_3CN) and B (distilled water): t=0–3 min, 20% A; t=7 min, 50 % A; t=20 min, 90 % A. The samples were filtered using a PureChem PP syringe filter (4mm, 0.2um) before HPLC injection. Reaction mixtures were analyzed by thin layer chromatography using Merck silica gel 60F254 aluminums plates and visualized by UV light or stained with potassium permanganate stain. Column chromatography was performed with silica gel Geduran® Si 60 (0.040-0.063 mm) purchased from Merck. All solvents were of analytical reagent grade and were purchased from VWR, Carlo Erba or Sigma-Aldrich. The water used in the reactions was freshly distilled prior to use. All reagents used were purchased from Fluorochem, Alfa Aesar, TCI or Sigma-Aldrich.

Synthesis and Characterization

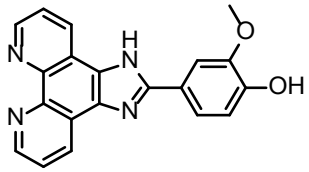


Ruthenium bis*(4,4'-*di-tert-butyl*-2,2'-*bipyridine) dichloride

$RuCl_3$ (1g) was dissolved in ethanol (25 mL) and refluxed for 3 h (solution turned green). Ethanol was evaporated and redissolved in DMSO (8 mL). The reaction mixture was stirred at reflux for 2 h. After cooling to room temperature, 50 mL of acetone were added and the mixture was stored at 4 ºC overnight. The precipitate was filtered, washed with cold acetone, and dried under vacuum to yield a yellow powder that was used in the following step without further purification.

$Ru(DMSO)_4Cl_2$ (343 mg, 1 equiv) was dissolved in DMF and tert-butyl-bipyridine (400 mg, 2.1 equiv) was added. Then LiCl (225 mg, 7.5 equiv) was added and the reaction was stirred at reflux for 8 h. The mixture was cooled down and left in the freezer overnight. The product was purified by column chromatography using CH_2Cl_2/MeOH 9:1 as eluent. The desired product was obtained as a deep purple solid (365 mg, 72.57 % yield).

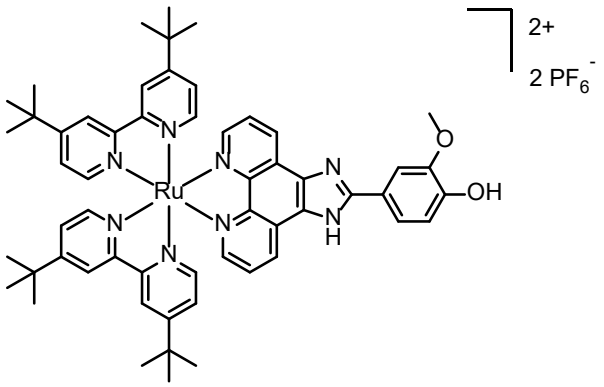
^{1}H-NMR (400 MHz, Chloroform-*d*) δ 10.10 (d, *J* = 5.9 Hz, 2H), 8.05 (d, *J* = 2.0 Hz, 2H), 7.92 (d, *J* = 2.0 Hz, 2H), 7.51 (dd, *J* = 5.7, 1.9 Hz, 2H), 7.49 – 7.43 (m, 2H), 6.92 (d, *J* = 3.8 Hz, 2H), 1.47 (s, 18H), 1.27 (s, 18H). Spectral data in accordance with previous reports.[1]



4-(1H-imidazo[4,5-f][1,10]phenanthrolin-2-yl)-2-methoxyphenol

1,10-phenanthroline-5,6-dione (570 mg, 1 equiv) and 4-(hydroxymethyl)benzaldehyde (578 mg, 1.4 equiv) were dissolved in acetic acid (10 mL) and sodium acetate (4.18 g, 20 equiv) was added. The mixture was refluxed for 2 h. After cooling, the resulting red solution was diluted with 50 mL of water and neutralized with NH_4OH, to form a yellow precipitate. The mixture was kept in the refrigerator overnight, followed by filtration and washing with water to obtain the pure yellow powder (882 mg, 95 % yield).

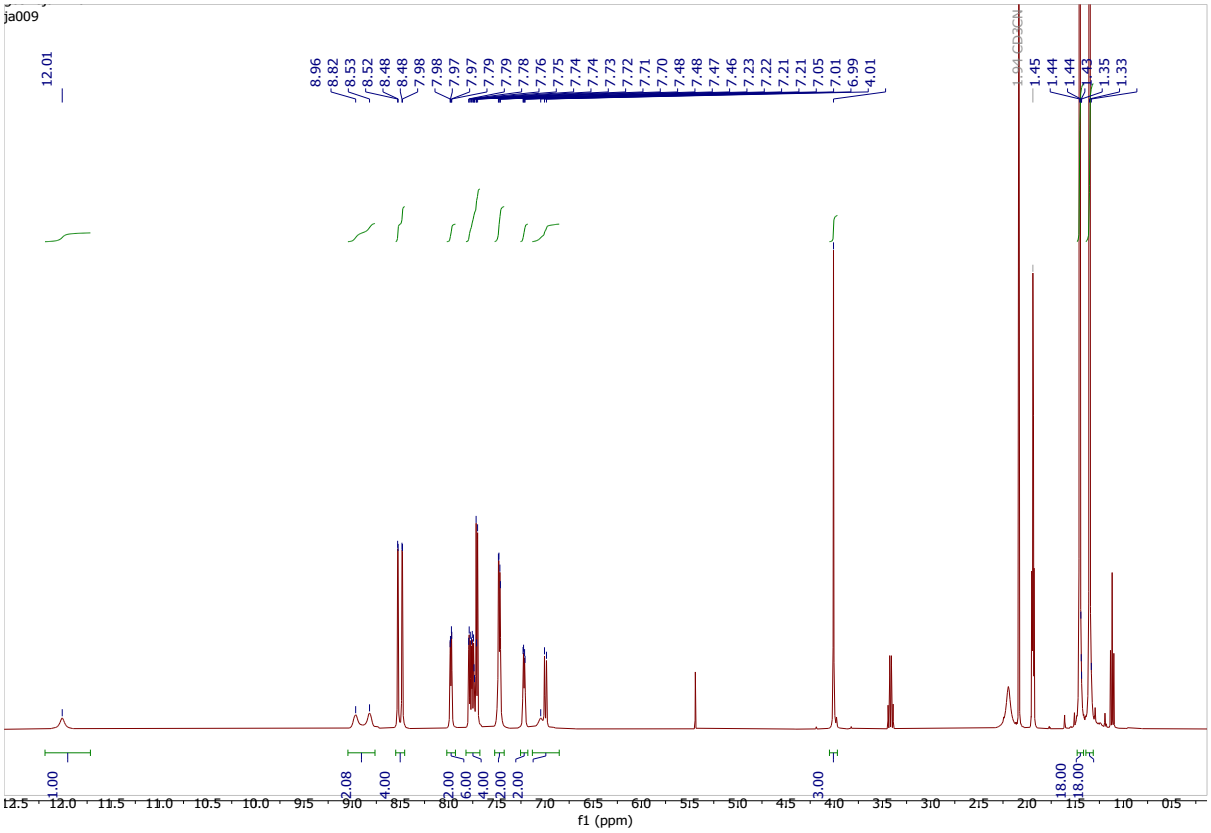
^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.01 (dd, *J* = 4.3, 1.7 Hz, 2H), 8.93 (dd, *J* = 8.2, 1.8 Hz, 2H), 7.87 (d, *J* = 2.0 Hz, 1H), 7.81 (dd, *J* = 8.2, 4.3 Hz, 2H), 7.76 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.99 (d, *J* = 8.2 Hz, 1H), 3.93 (s, 3H).

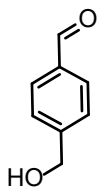


[Ru(4,4'-tert-butyl-2,2'-bipyridine)$_2$(2-methoxyphenol-1H-imidazo-1,10-phenanthroline)] (PF_6)$_2$

Ruthenium bis(4,4'-tert-butyl-2,2'-bipyridine) dichloride (565 mg, 1 equiv) and 4-(1H-imidazo[4,5-f][1,10]phenanthrolin-2-yl)-2-methoxyphenol (327 mg, 1.2 equiv) were dissolved in 50 ml of a water/ethanol 1:1 mixture and stirred at 90 ºC overnight. The resulting dark orange solution was evaporated under vacuum to remove the ethanol. A concentrated solution of NH_4PF_6 was added and an orange precipitate was formed, which was filtered and washed with cold water and diethyl ether. The product was purified by silica gel chromatography with acetonitrile/1 % KNO_3 aqueous solution 9:1. The pure product was obtained as an orange solid (927 mg, 91 % yield).

^{1}H-NMR (400 MHz, Acetonitrile-d_3) δ 8.89 (d, *J* = 57.6 Hz, 2H), 8.50 (dd, *J* = 18.0, 2.0 Hz, 4H), 7.98 (dd, *J* = 5.3, 1.3 Hz, 2H), 7.85 – 7.65 (m, 6H), 7.47 (dd, *J* = 6.0, 2.1 Hz, 4H), 7.22 (dd, *J* = 6.2, 2.0 Hz, 2H), 7.01 (m, 2H), 4.01 (s, 3H), 1.45 (s, 18H), 1.35 (s, 18H).



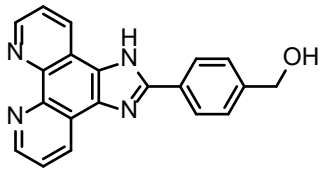


4-(hydroxymethyl)benzaldehyde

Terephthalaldehyde (1 g, 1 equiv) was dissolved in 12.5 mL of ethanol and 17.5 ml of THF added. The mixture was cooled with an ice bath and $NaBH_4$ (70 mg, 0.25 equiv) was added in small portions over

30 minutes. The reaction mixture was stirred for 6 h. Subsequently, the pH was adjusted to 5 with diluted HCl (the solution turned yellow) and extracted with ethyl acetate, dried with $NaSO_4$ and dried under vacuum. The product was purified by column chromatography using hexane/ethyl acetate (7:3 to 5:5) and a white solid was obtained (700 mg, 69 % yield).

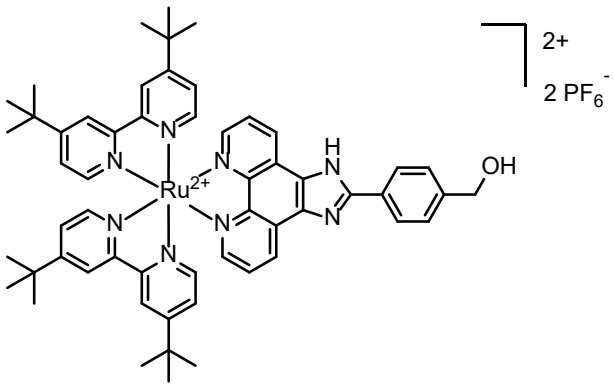
^{1}H-NMR (400 MHz, Chloroform-*d*) δ 9.88 (s, 1H), 7.77 (d, *J* = 8.2 Hz, 2H), 7.45 (d, *J* = 8.3 Hz, 2H), 4.72 (s, 2H). Spectral data in accordance with previous reports.[2]



(4-(1H-imidazo[4,5-f][1,10]phenanthrolin-2-yl)phenyl)methanol

1,10-Phenanthroline-5,6-dione (750 mg, 1 equiv) and 4-(hydroxymethyl)benzaldehyde (640 mg, 1.4 equiv) were dissolved in acetic acid (10 mL) and sodium acetate (5.18 g, 20 equiv) was added. The mixture was refluxed for 2 h. After cooling down, the resulting red solution was diluted with 50 mL of water and neutralized with NH_4OH, to form a yellow precipitate. The mixture was kept in the fridge overnight, followed by filtration and washing with water to obtain the pure yellow powder (788 mg, 72 % yield.

^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.02 (dd, *J* = 4.3, 1.8 Hz, 2H), 8.93 (dd, *J* = 8.1, 1.8 Hz, 2H), 8.26 (d, *J* = 8.0 Hz, 2H), 7.82 (dd, *J* = 8.1, 4.3 Hz, 2H), 7.54 (d, *J* = 8.0 Hz, 2H), 4.61 (s, 2H).



[Ru(4,4'-tert-butyl-2,2'-bipyridine)$_2$(4-hydroxymethyl-phenyl-1H-imidazo-1,10-phenanthroline)] (PF_6)$_2$

Ruthenium bis(4,4'-tert-butyl-2,2'-bipyridine) dichloride (500 mg, 1 equiv) and (4-(1H-imidazo[4,5-f][1,10]phenanthrolin-2-yl)phenyl)methanol (300 mg, 1.3 equiv) were dissolved in 50 ml of a water/ethanol 1:1 mixture and stirred at 90 ºC overnight. The resulting dark orange solution was

evaporated under vacuum to remove the ethanol. A concentrated solution of NH_4PF_6 was added and an orange precipitate was formed, which was filtered and washed with cold water and diethyl ether. The product was purified by silica gel chromatography with acetonitrile/1 % KNO_3 aqueous solution 9:1. The pure product was obtained as an orange solid (482 mg, 54 % yield).

^{1}H-NMR (400 MHz, Acetonitrile-d_3) δ 8.90 (d, *J* = 8.3 Hz, 2H), 8.53 (d, *J* = 2.0 Hz, 2H), 8.48 (d, *J* = 2.0 Hz, 2H), 8.18 (d, *J* = 8.4 Hz, 2H), 7.99 (dd, *J* = 5.2, 1.3 Hz, 2H), 7.77 (dd, *J* = 8.3, 5.3 Hz, 2H), 7.71 (dd, *J* = 6.0, 0.6 Hz, 2H), 7.56 (d, *J* = 8.7 Hz, 2H), 7.47 (dd, *J* = 6.1, 2.2 Hz, 4H), 7.22 (dd, *J* = 6.0, 2.0 Hz, 2H), 4.69 (s, 2H), 1.46 (s, 18H), 1.35 (s, 18H). **^{13}C-NMR** (101 MHz, CD_3CN) δ 163.56, 163.42, 158.04, 157.88, 153.57, 152.23, 152.04, 150.84, 146.78, 145.75, 130.88, 128.68, 128.13, 127.38, 127.27, 126.81, 125.68, 125.57, 122.50, 122.40, 118.36, 64.18, 36.37, 36.27, 30.54, 30.44. **HRMS:** m/z $[M+H]^+$ *Calculated* for $C_{56}H_{62}F_6N_8OPRu^+$ = 1109.3726; *Found* =1109.3728; m/z $[M+H]^{2+}$ *Calculated* for $C_{56}H_{62}N_8ORu^{2+}$ = 482.2040; *Found* = 482.2053. **Elemental Analysis**: *Calculated* for $C_{56}H_{62}F_{12}N_8OP_2Ru$ + $2H_2O$: C- 52.13; H- 5.16; N- 8.69; *Found*: C- 52.28; H- 5.08; N- 8.95

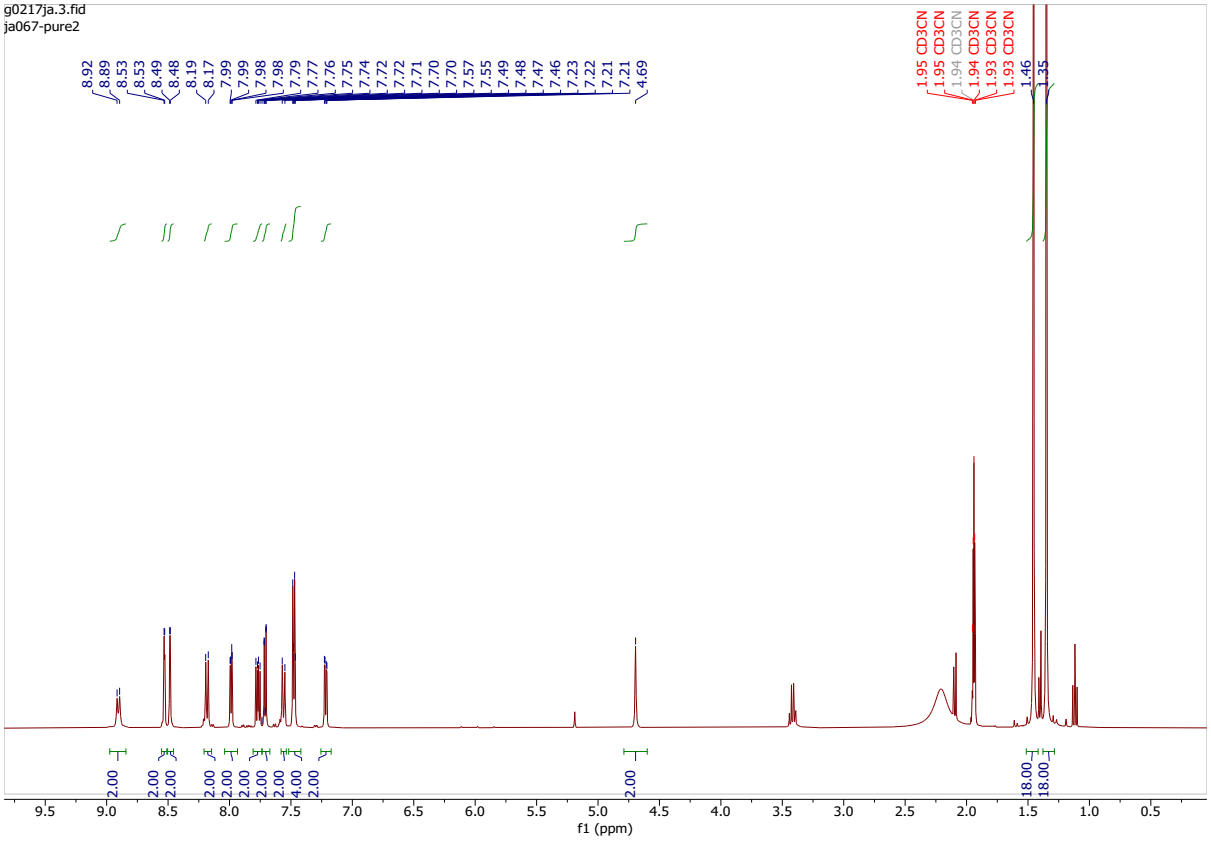


Figure S1. ^{1}H-NMR spectrum of **Ru** in CD_3CN.

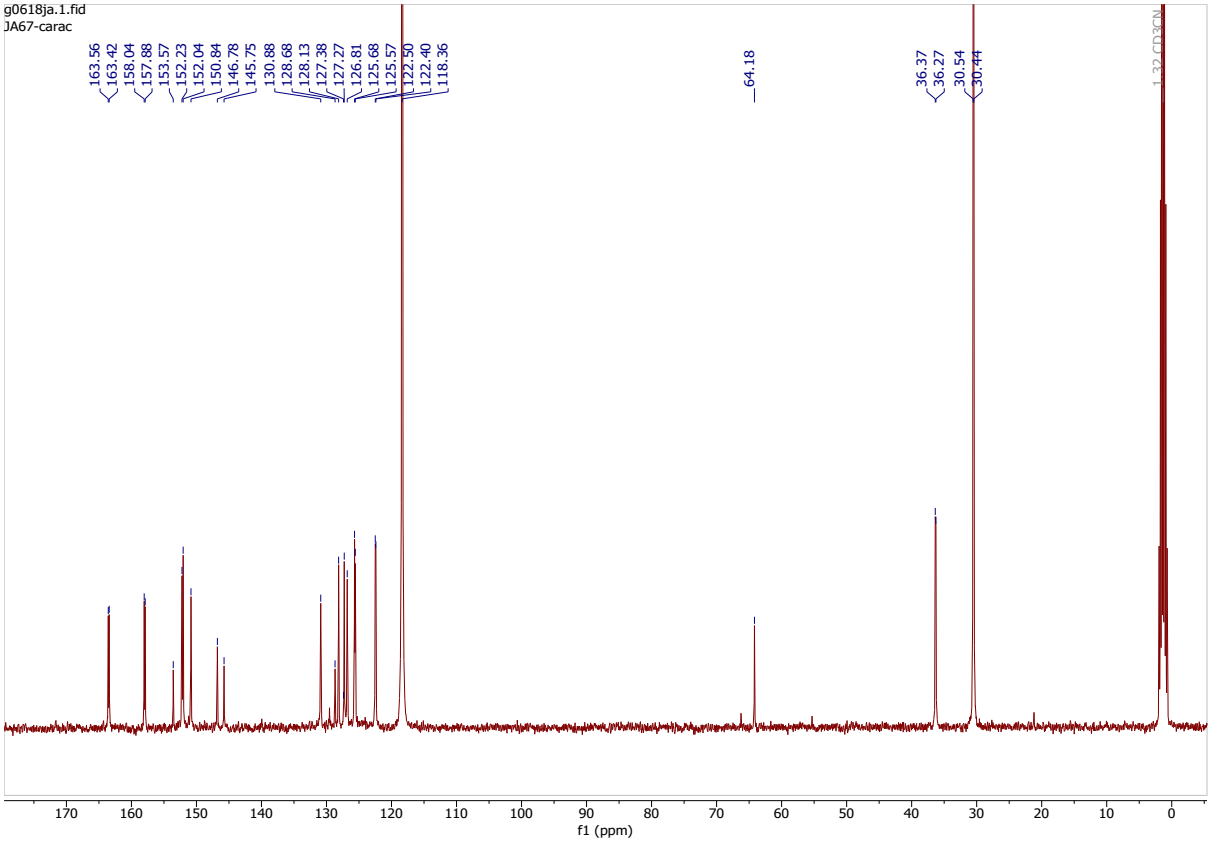


Figure S2. ^{13}C-NMR spectrum of **Ru** in CD_3CN.

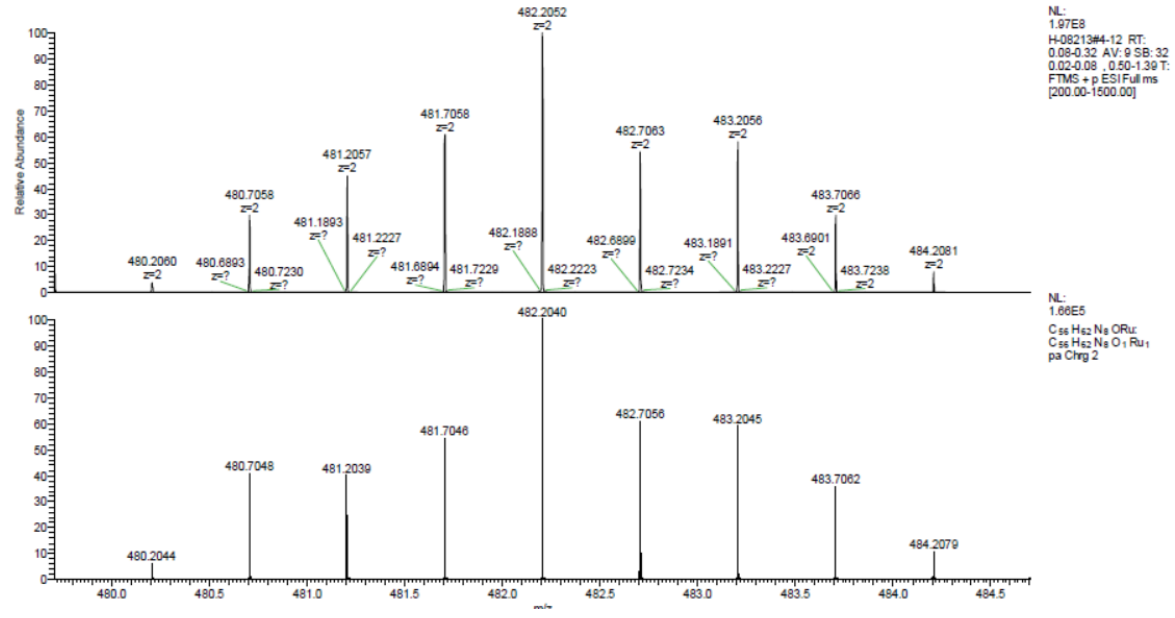


Figure S3. HRMS analysis of the theoretical (bottom) and experimental (top) isotopic pattern of **Ru**.

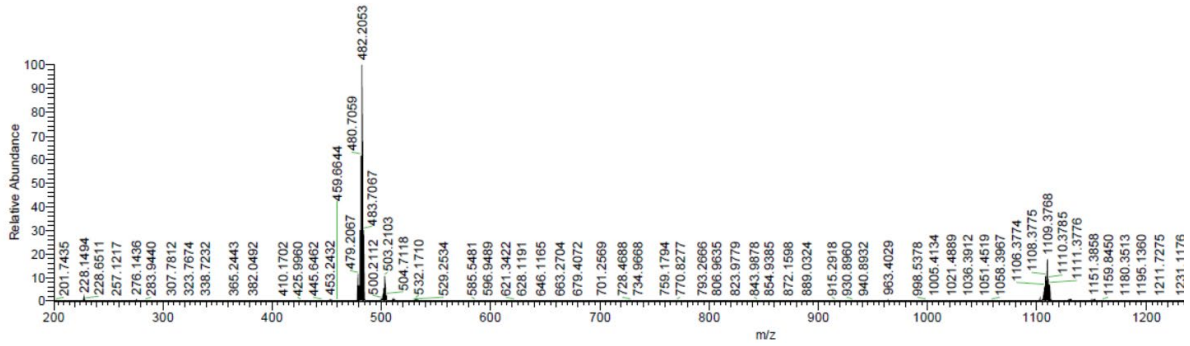


Figure S4. HRMS spectrum of **Ru**.

Stability in Plasma.

30 µL of a 10 mM solution of **Ru** in DMSO was diluted in 2 ml of human plasma + 0.5 mL of PBS buffer (pH 7.4) and incubated in a water bath at 37ºC (final concentration of ≈120 µM). Aliquots of 200 µL were removed at different time points and diluted in 400 µL of acetonitrile. The mixture was centrifuged and the supernatant was analyzed by HPLC (Figure S5).

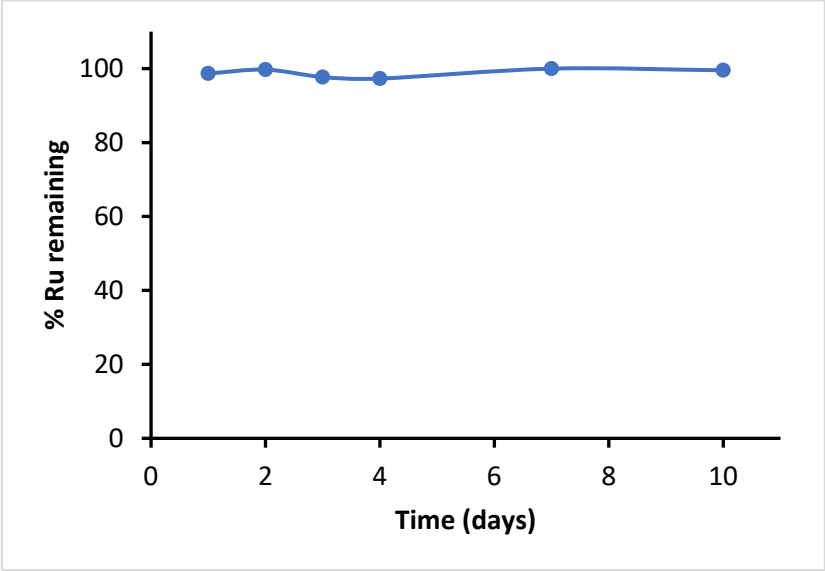


Figure S5. HPLC profile of the stability of **Ru** in plasma.

Polymerization

Standard ROP procedure

Solvents used were obtained from a Solvent Purification System and further dried with calcium hydride (CH_2Cl_2) or sodium/benzophenone (THF). L,D-Lactide was bought from Alfa Aesar and purified through two rounds of recrystallization in isopropanol and toluene, followed by sublimation and storage in the glovebox. $Ca[N(SiMe_3)_2]_2{\cdot}2THF$ was prepared as previously reported.[3] All polymerizations were performed in a glovebox with < 1 ppm of water and O_2.

In a typical polymerization procedure, 2 equiv. of **Ru** were dissolved in CH_2Cl_2 and added to $Ca[N(SiMe_3)_2]_2{\cdot}2THF$ (1 equiv) in THF. The CH_2Cl_2/THF proportion was 1:1 and the total volume of solvent was adjusted to achieve [Lactide] = 0.1 M. The reaction was stirred for 5 minutes and L,D-Lactide was added, followed by stirring at room temperature for 20 h. Reaction was quenched by contact with air, and the solvent was evaporated. The resulting orange solid was dissolved in CH_2Cl_2 and added dropwise to a 1:1 mixture of diethyl ether/pentane, to remove the unreacted monomer. The precipitate was filtered and washed with diethyl ether and pentane, to obtain the pure **Ru**-PLA.

^{1}H-NMR (400 MHz, Acetonitrile-d_3) δ 8.91 (m, 2H), 8.53 (d, *J* = 2.1 Hz, 2H), 8.49 (d, *J* = 2.1 Hz, 2H), 8.21 (m, 2H), 7.98 (d, *J* = 5.2 Hz, 2H), 7.76 (m, 2H), 7.71 (d, *J* = 6.0 Hz, 3H), 7.57 (m, 3H), 7.47 (dd, *J* = 6.1, 2.1 Hz, 5H), 7.27 – 7.14 (m, 2H), 5.27 (s, 2H), 5.23 – 5.03 (m, CH-PLA), 1.54-1.47 (m, CH_3-PLA), 1.46 (s, 18H), 1.35 (s, 18H).

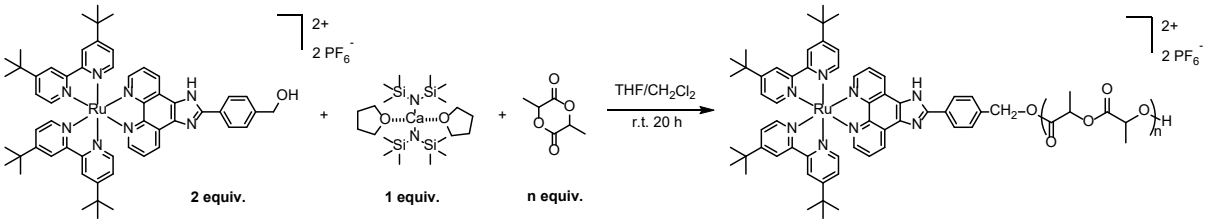


Figure S6. Reaction scheme of a typical ROP of lactide.

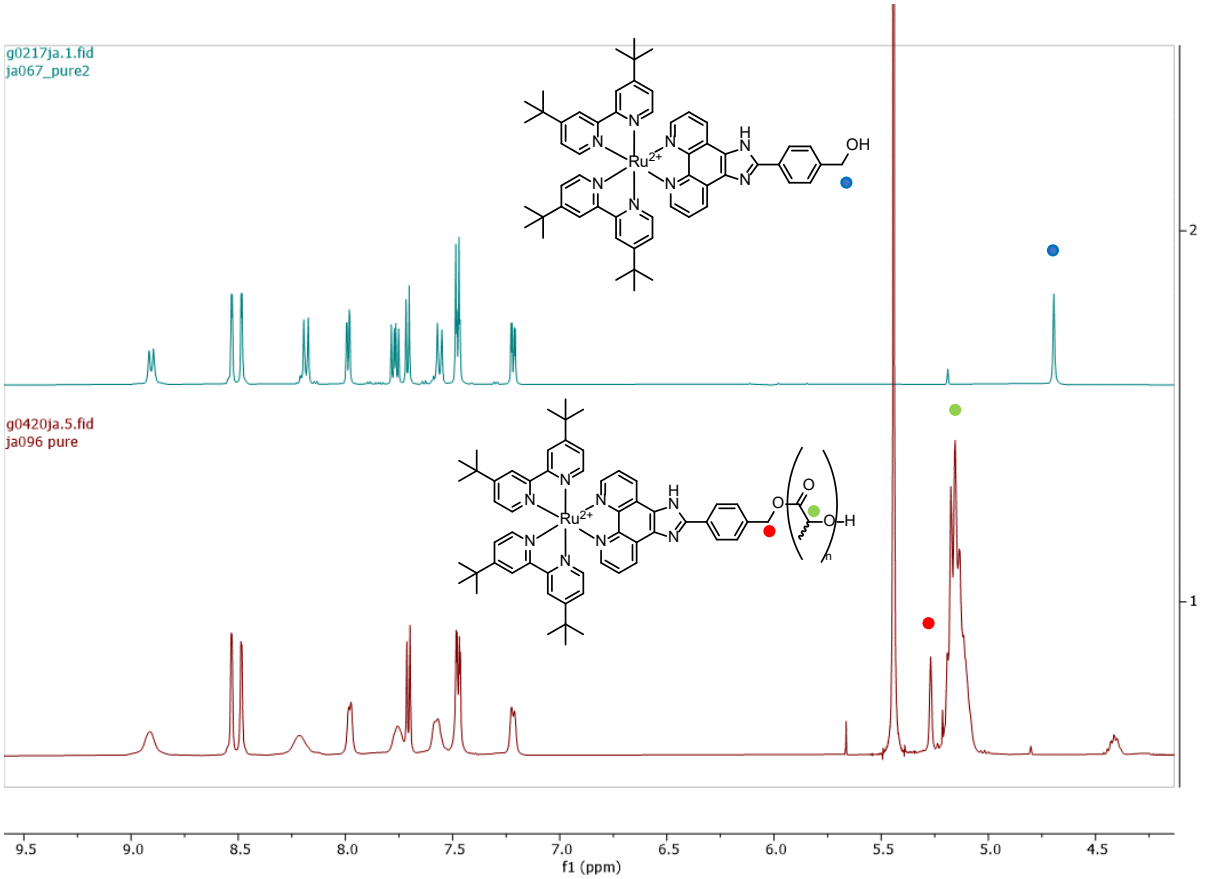
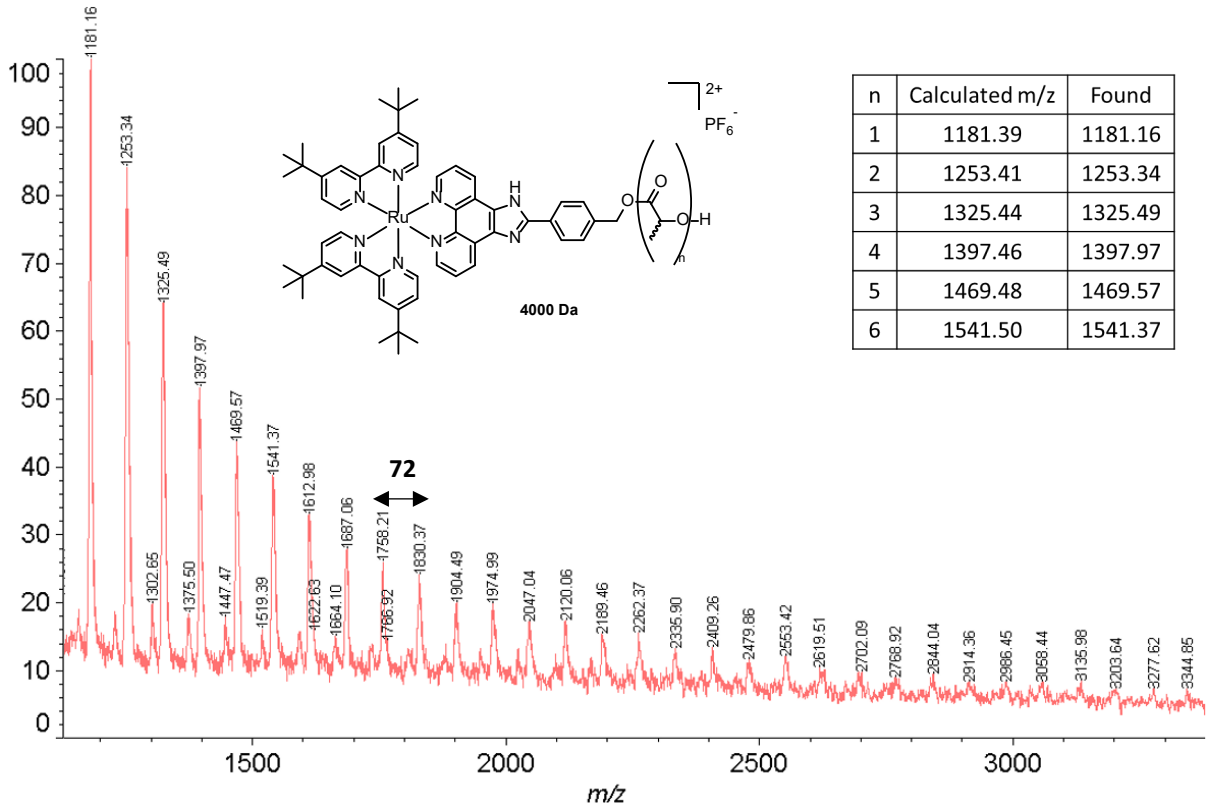


Figure S7. An overlay of ^{1}H-NMR spectra of **Ru** (top) and Ru-PLA (bottom). A shift in the benzylic CH_2 is observed from the **Ru** (blue dot) to the Ru-PLA (red dot).

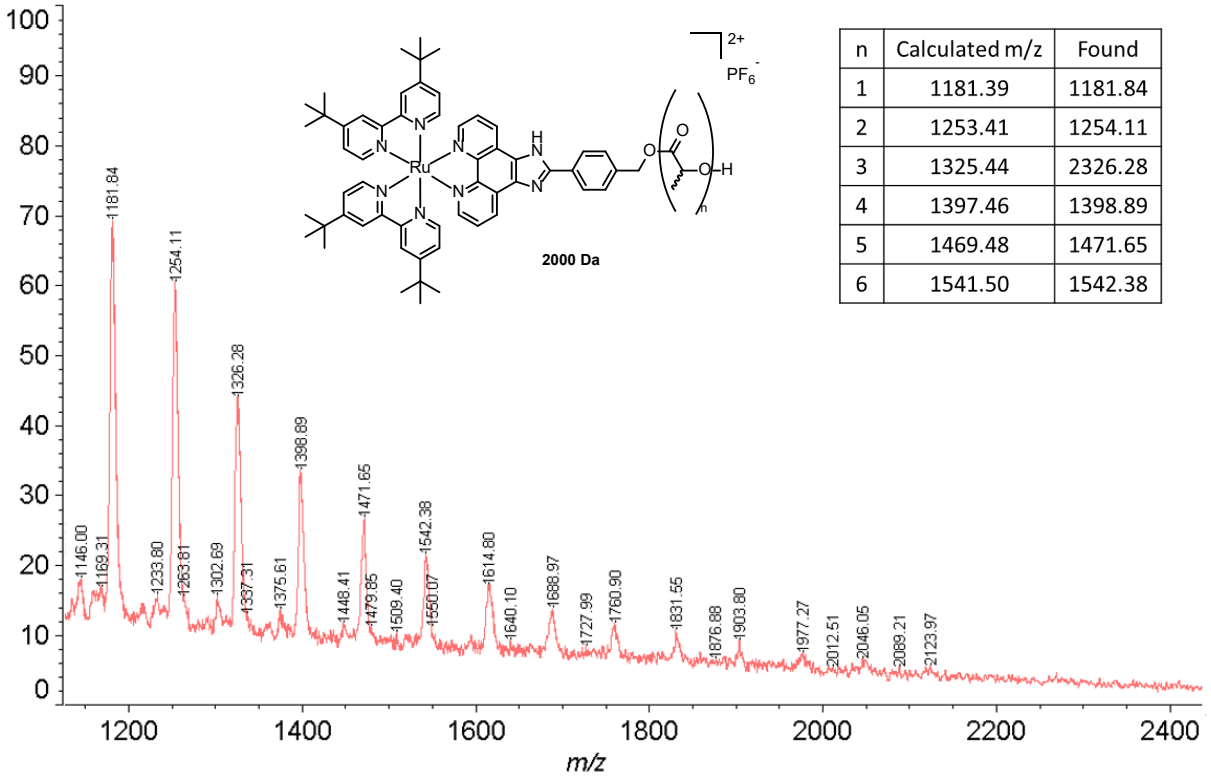
MALDI-TOF analysis

MALDI-TOF mass spectrometry analyses were recorded with a Axima Confidence spectrometer (Shimadzu), externally calibrated with PEG (M_n = 2000 g/mol). The samples were prepared with dithranol as matrix and LiCl as ionic adjuvant.



n	Calculated m/z	Found
1	1181.39	1181.16
2	1253.41	1253.34
3	1325.44	1325.49
4	1397.46	1397.97
5	1469.48	1469.57
6	1541.50	1541.37

Figure S8. MALDI-TOF spectrum of **P2** (4000 Da); Dithranol and LiCl were used in the preparation of the sample.



n	Calculated m/z	Found
1	1181.39	1181.84
2	1253.41	1254.11
3	1325.44	2326.28
4	1397.46	1398.89
5	1469.48	1471.65
6	1541.50	1542.38

Figure S9. MALDI-TOF spectrum of **P1** (2000 Da); Dithranol and LiCl were used in the preparation of the sample.

Size exclusion chromatography (SEC) analysis

Size exclusion chromatography of polymers was performed in THF at 35 °C using an Agilent 1260 Infinity Series GPC (ResiPore 3 µm, 300 x 7.5 mm, 1.0 mL/min, RI (PL-GPC 220) and Light scattering detectors). When using the RI detector, the number-average molecular masses (M_n) and polydispersity index ($Đ$) of the polymers were calculated with reference to a universal calibration vs. polystyrene standards. The M_n valuesobtained were corrected with the Mark-Houwink parameter for PLA (0.58).[4] The samples were prepared by dissolving 1 mg of polymer in 1 ml of HPLC grade THF (VWR).

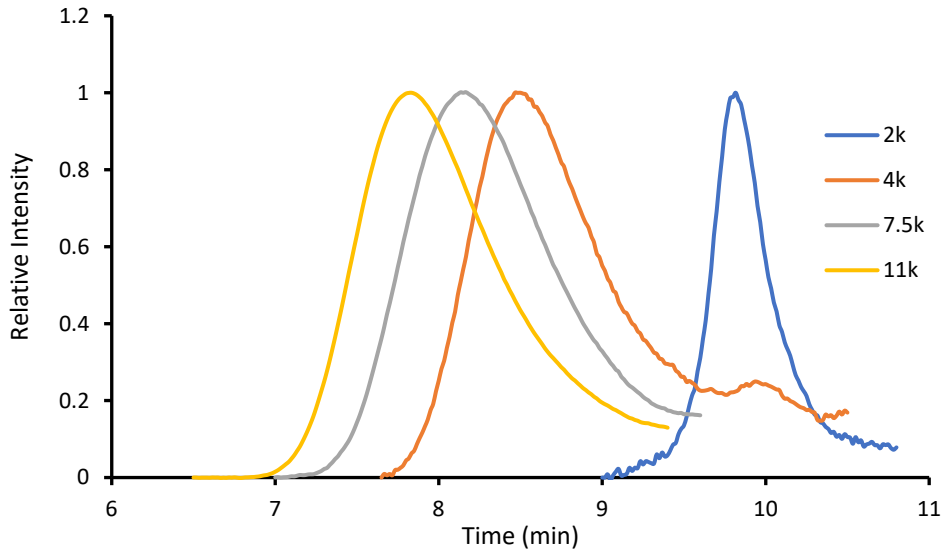


Figure S10. SEC-RI traces of **P1-P4**.

Nanoparticles Formulation

DLS Characterization

The nanoparticle intensity-average diameters Dz,the polydispersity index (PdI) and zeta potential were determined by dynamic light scattering (DLS) using a Malvern ZetaSizer Nano ZS (scattering angle = 173°) at a temperature of 25 °C with an equilibrium time of 120 s. All experiments were run in triplicate. The samples were diluted 10 times before analysis (final concentration ≈ 50 µM)

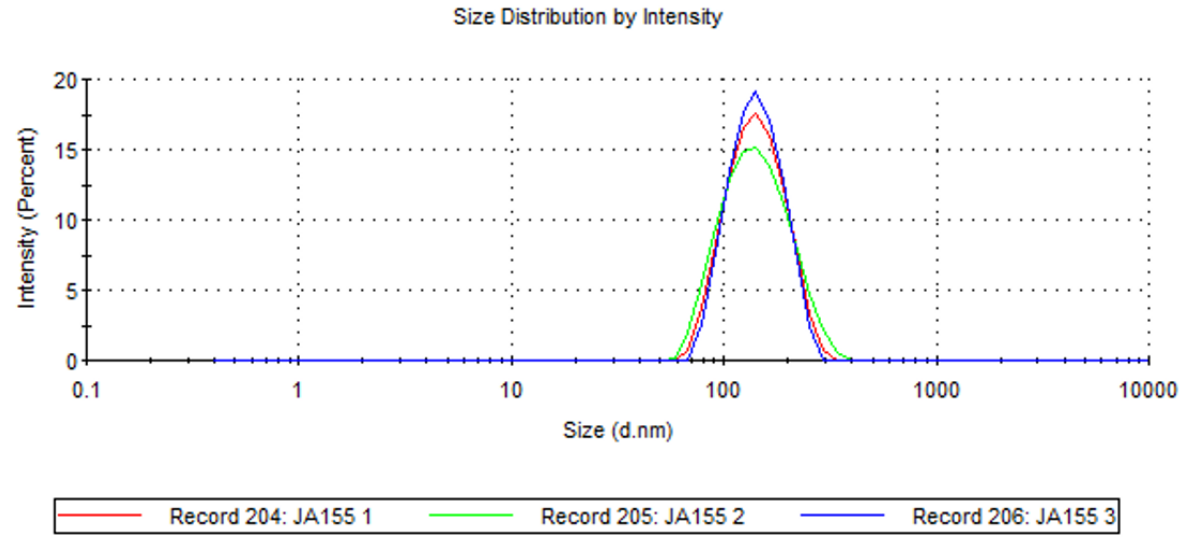


Figure S11. DLS analysis of the size distribution of **NP1**.

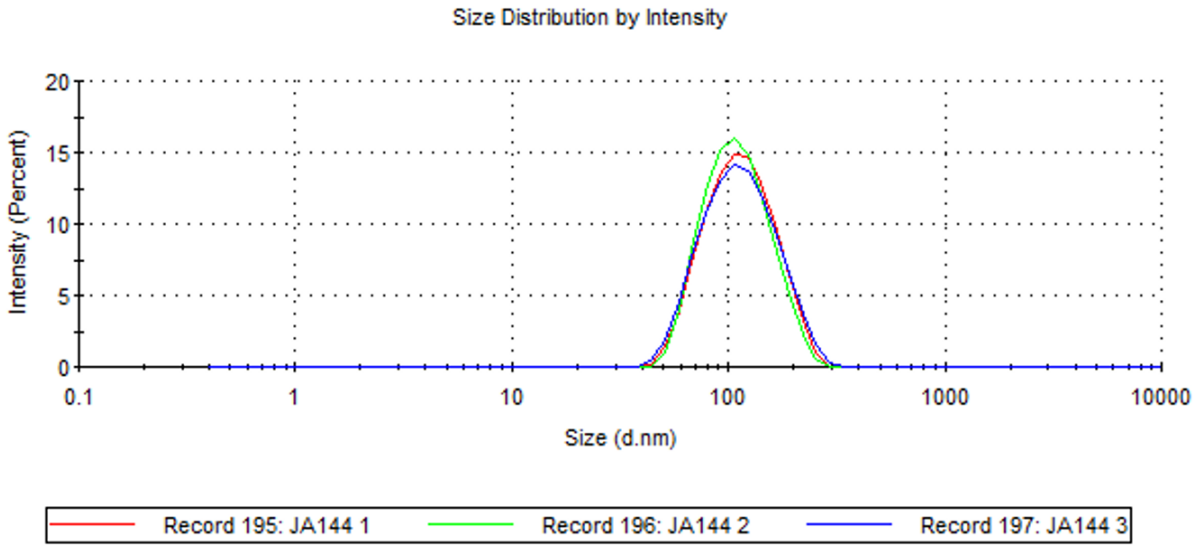


Figure S12. DLS analysis of the size distribution of **NP2**.

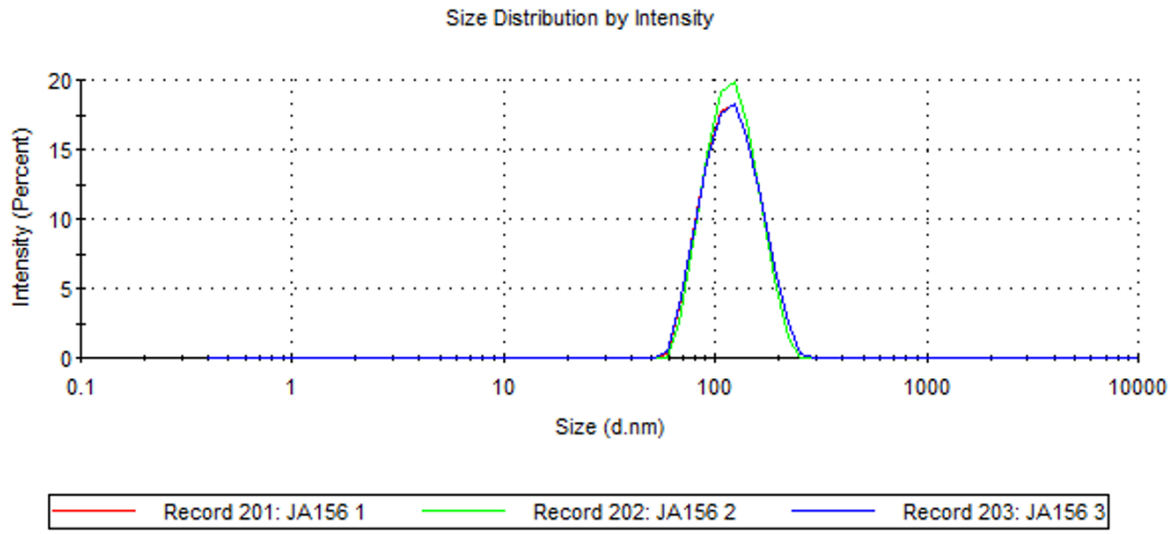


Figure S13. DLS analysis of the size distribution of **NP3**.

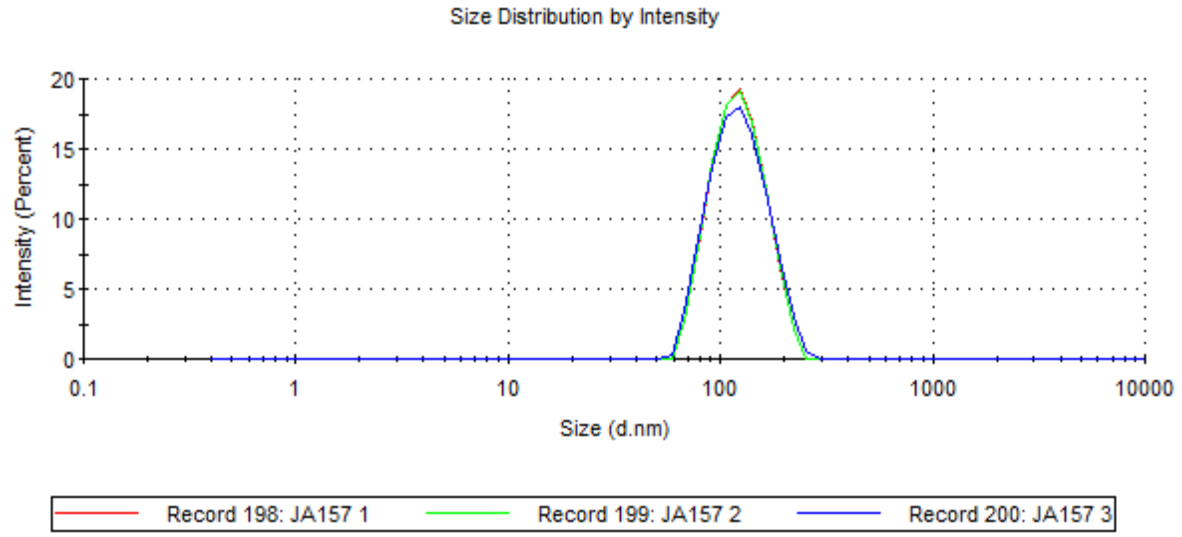


Figure S14. DLS analysis of the size distribution of **NP4**.

UV-Vis Characterization

The concentration of Ru in the nanoparticles was determined by ultraviolet-visible (UV-Vis) spectroscopy. Ru displays a characteristic absorption band with a maximum at 472 nm (Figure S15). Since the encapsulation in the nanoparticle does not affect its UV-Vis spectrum (Figure S16), a calibration curve was built to convert the observed absorbance into concentration (Figure S17).

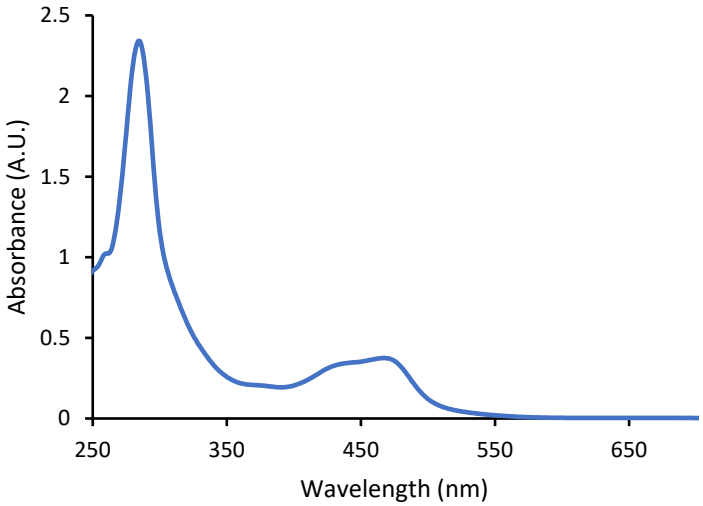


Figure S15. UV-Vis spectrum of **Ru** (20 µM).

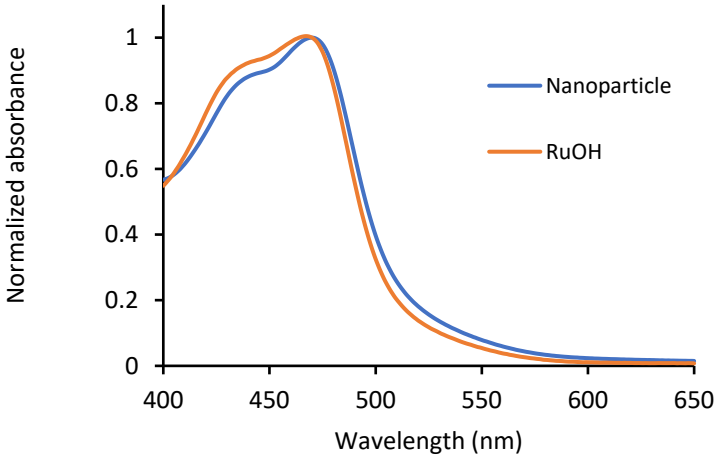


Figure S16. Overlay and normalization of the UV-Vis spectra of **Ru** and the nanoparticle.

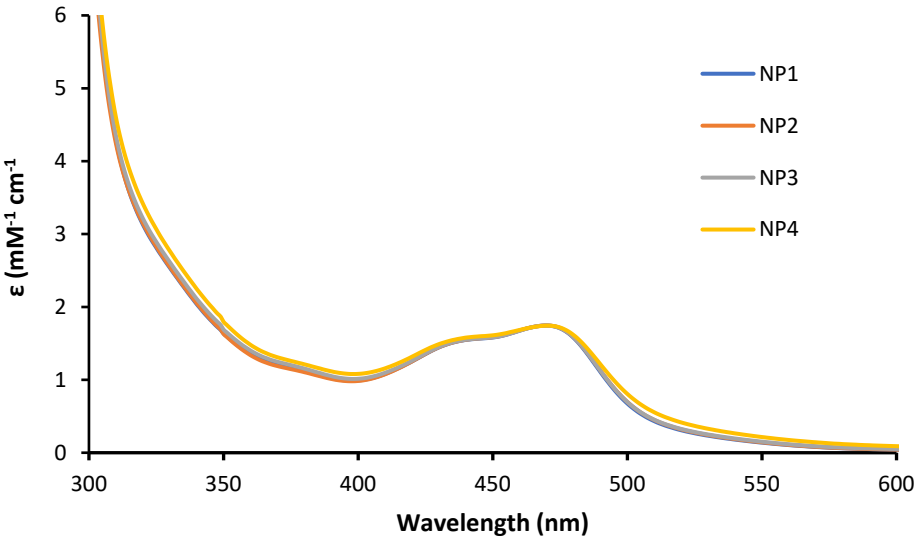


Figure S17. Overlay and normalization of the UV-Vis spectra of **NP1-NP4.**

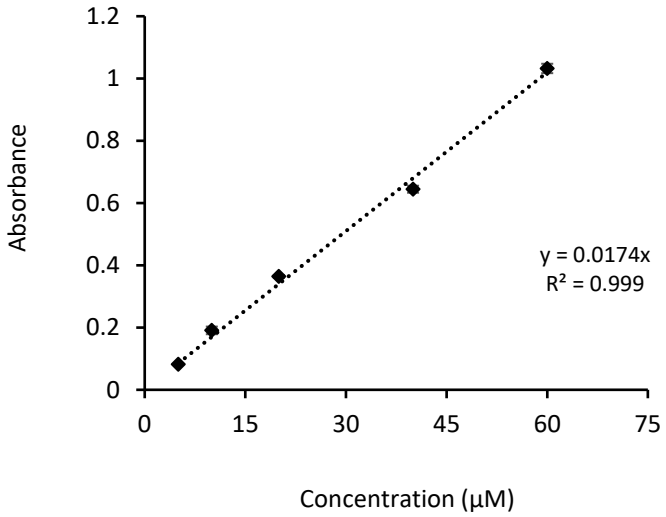


Figure S18. Calibration curve of Ru. Concentration range 5-60 µM. Representative data from three independent experiments is shown.

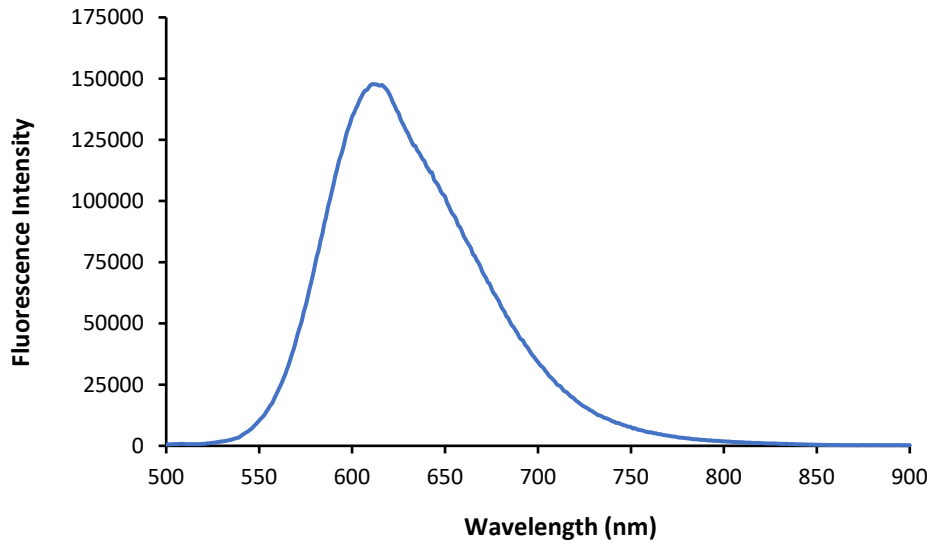


Figure S19. Emission spectrum of **Ru** (20uM) in ACN. Excitation at 472 nm. Average of 10 independent scans.

TEM characterization

A JEOL 2100Plus transmission electron microscope operated at 200kV was used to observe the morphology of the nanoparticles. A drop of the solution containing nanoparticles was placed and dried on a standard 3mm copper grid covered by a thin carbon film. A GATAN Rio16 high-resolution camera were employed to record the bright-field images of the nanoparticles. As shown in Figs. S20 and S21, the globular nanoparticles in sizes around 100 nm were observed in dark contrast on the transparent carbon film. The contrast results from both the thickness of nanoparticles and the presence of heavy Ru atoms that increase the interactions between the electron beam and the nanoparticles.

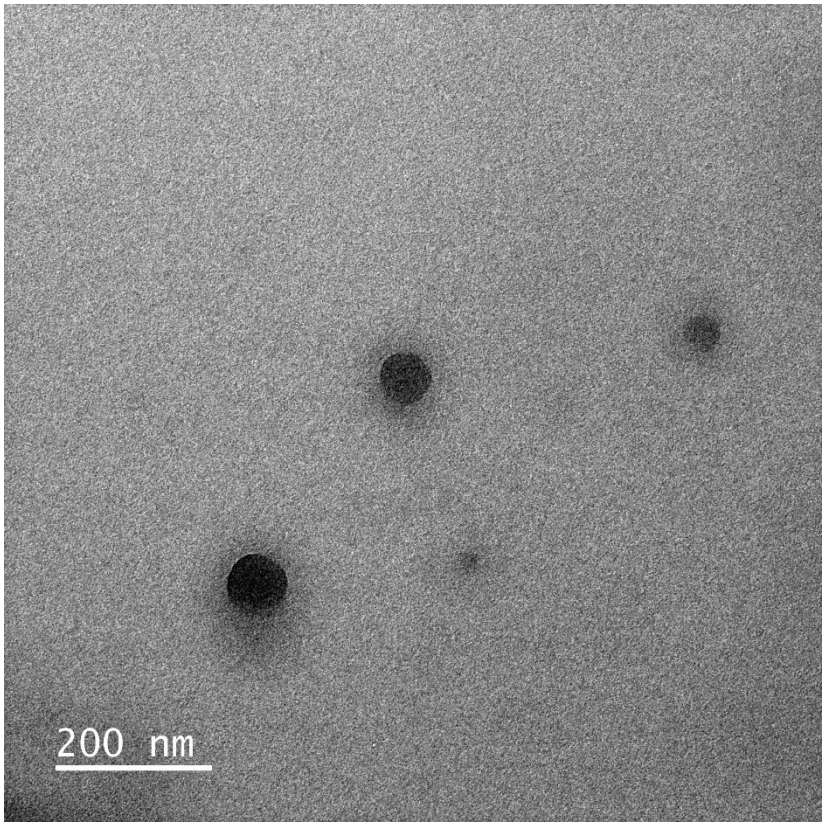


Figure S20. TEM image of three nanoparticles present in a **NP3** solution.

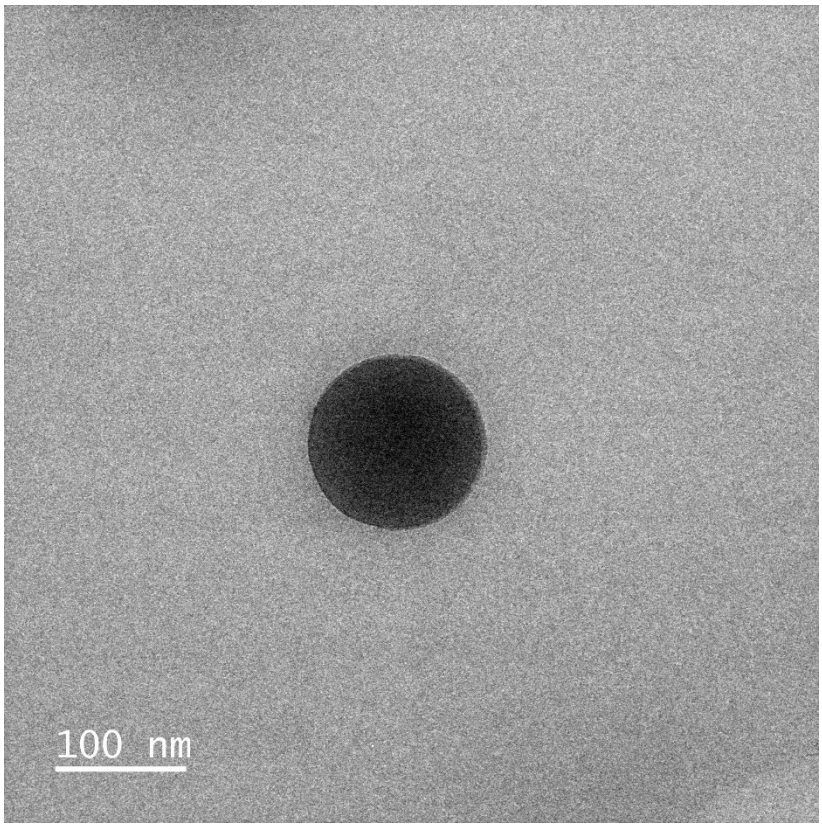


Figure S21. TEM image of a nanoparticle present in a **NP3** solution.

Release kinetics

The hydrolytic stability of the polymers was evaluated by reverse phase HPLC. 0.5 mL of the nanoparticle suspension were added to 4.5 mL of PBS (20 mM pH 7.4). The resulting PBS solution was divided into equal portions, added to five separate 1 mL Eppendorf tubes and incubated at 37 °C. At different time points, the corresponding Eppendorf tubes were taken out of the incubator and centrifuged at 10 000 g for 20 min. The supernatant was filtered and analyzed by HPLC. A calibration curve was built to convert the AUC into concentration. The resulting curves can be observed in Figure S22.

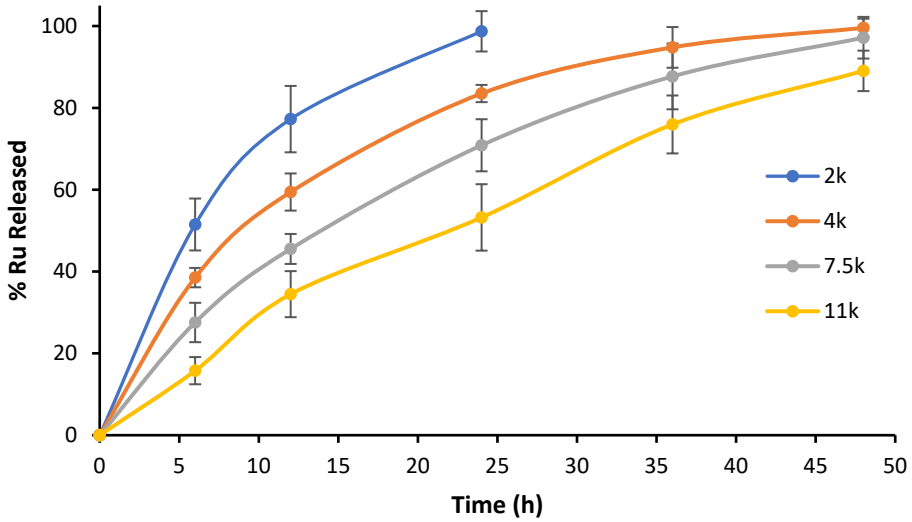


Figure S22. Profile of the hydrolytic stability of **P1-P4** in PBS pH 7.4, 37 ºC. Representative data from three independent experiments is shown.

Biological experiments

Cell Culture

The A2780 cell line was cultured in RPMI media (Gibco) supplemented with 10% fetal calf serum (Gibco) and 1% Penicillin-Streptomycin antibiotic (Gibco). The A2780 cisplatin-resistant cell line was cultured in RPMI media (Gibco) supplemented with 10% fetal calf serum (Gibco) and 1% Penicillin-Streptomycin antibiotic (Gibco). The resistance of A2780 cisplatin was maintained by cisplatin treatment (1 mm) for two weeks every month. Cells were used in the assays one week after the end of the treatment to avoid interfering with the results. The RPE-1 cell line was cultured in DMEM/F-12 media (Gibco)

supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin antibiotic (Gibco). Cell lines were maintained in a humidified atmosphere at 37 °C with 5% CO_2.

Cytotoxicity Assay Using a 2D Cellular Model

The cytotoxicity of the tested Ru complex and NPs 1-4 was assessed by a fluorometric cell viability assay using Resazurin (Acros Organics). Briefly, cells were seeded in triplicate in 96-well plates at a 4×10^3 cells/ well density in 100 µL. After 24 h, cells were treated with increasing concentrations of the ruthenium complex, NPs, and controls. Dilutions for Ru complex was prepared from 10 mM stock in DMSO was diluted to 100 - 0,01 µM with media. Regarding the NPs 1-4 according to the concentration of each NPs in 1% (w/v) Kolliphor P188 in MQ water were diluted to 100 - 0,01 µM with media. After 48 h or 72 h of incubation, the medium was removed and 100 µL of complete medium containing resazurin (0.2 mg/mL final concentration) was added. After 4 h of incubation at 37 °C, the fluorescence signal of the resorufin product was read (ex 540 nm, em 590 nm) in an Infinite 200 PRO Microplate Reader from TECAN. IC_{50} values were then calculated using GraphPad Prism software.

ICP-MS Cellular Uptake Studies

A2780 cells were seeded at a density of 1×10^6. The next day, cells were treated with 5 µM of the corresponding Ru and NPs 1-4 diluted in the cell culture medium from a stock solution of 10 mM dissolved previous in DMSO or in 1% (w/v) Kolliphor P188 in MQ water. After 12 h,, 24, 48, and 72 h cells were collected, counted, and stored at −80 °C. ICP-MS samples were prepared as follows: samples were digested using 70% nitric acid (0.5 mL, 70 °C, overnight) and then further diluted 1:100 (1% HCl solution in MQ water) analyzed using ICP-MS. All ICP-MS measurements were performed on a Agilent 7900 Quadrupole ICP-MS located at the Institut de Physique du Globe de Paris (France). The monitored isotopes are 99 and 101 Ru. Daily, before the analytical sequence, an indium internal standard was injected after inline mixing with the samples to correct for signal drift and matrix effects. A set of calibration standards was analyzed to confirm and model (through simple linear regression) the linear relationship between signal and concentration. The model was then used to convert measured sample counts to concentrations. The uncertainties were calculated using error propagation equations and considering the combination of standard deviation on the repeated consecutive signal acquisitions (n = 3), internal standard ratio and blank subtraction. The non-linear term (internal standard ratio) was linearized using a first-order Taylor series expansion to simplify error propagation. The amount of metal detected in the cell samples was transformed from ppb to µg of metal. Data were subsequently normalized to the number of cells and expressed as nanograms of metal/number of cells.

Fluorescence microscopy cellular uptake studies

The fluorescence microscopy cellular uptake studies of the tested Ru complex and NP 2 was assessed using Cytation 5 Cell Imaging Multi-Mode Reader (Biotek - Agilent). Briefly, cells were seeded in 6-well plates at a 25×10^4 cells/ well density in 3000 µL. After 24 h, cells were treated at 10 mM of the ruthenium complex and NP 2. After 4 h, 8 h, 12 h, and 24 h of incubation we visualize with the green filter of the Cytation 5 (496 nm, 525 nm) in live at 37 °C.

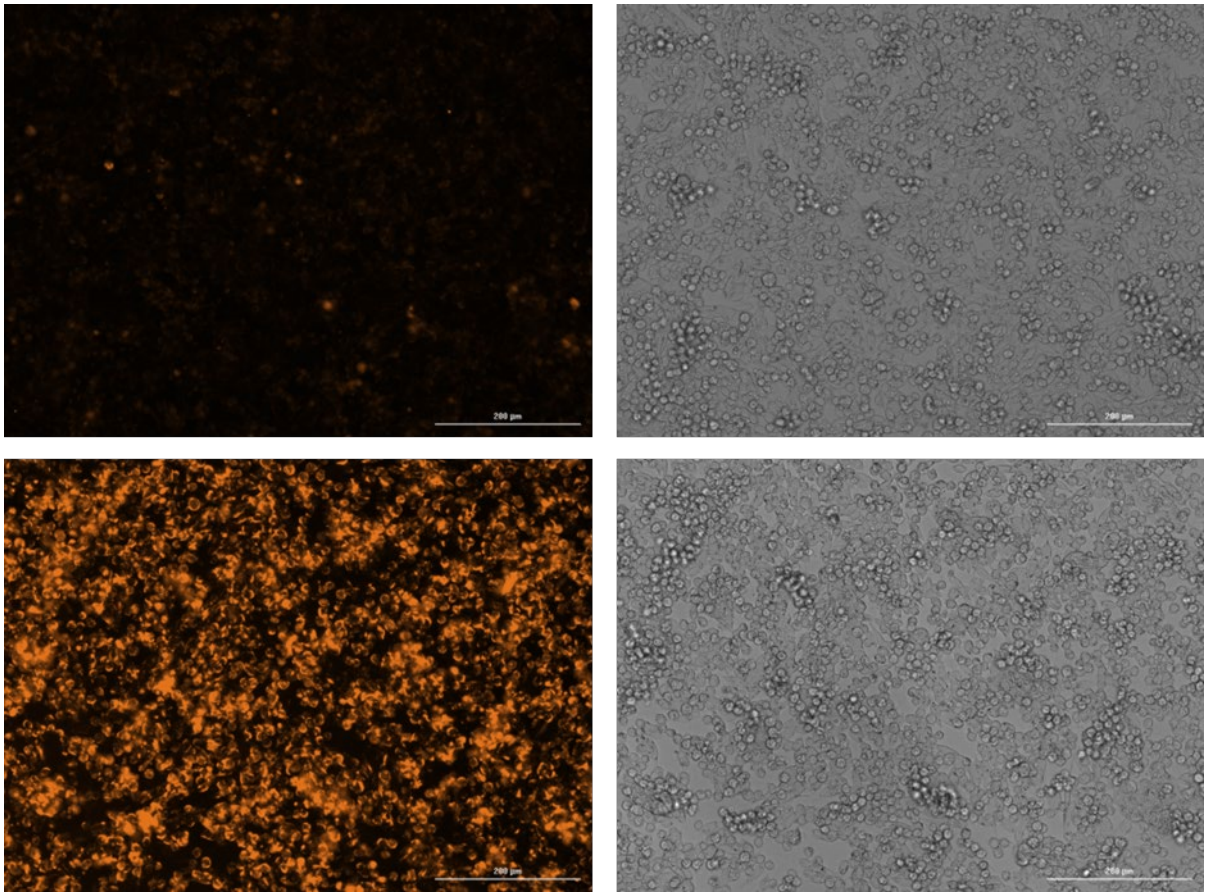
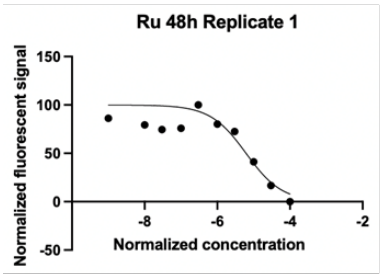
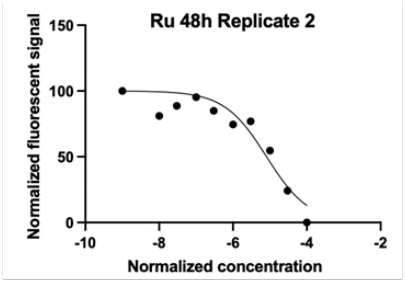
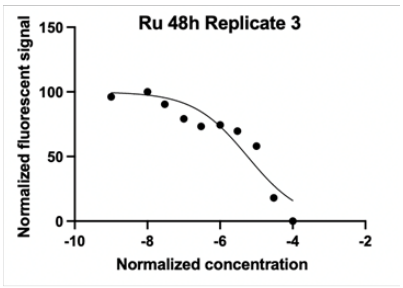
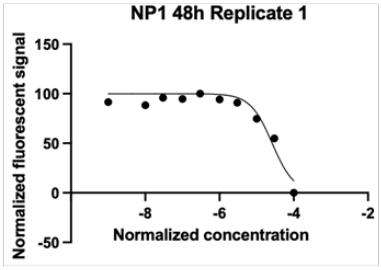


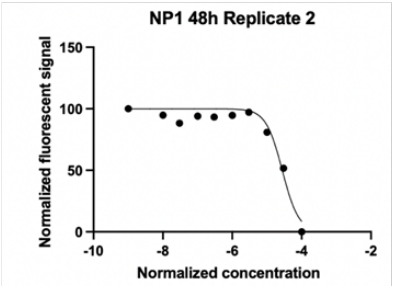
Figure S23. Cellular uptake of **Ru** (top) and **NP2** (bottom) by fluorescence microscopy. Single confocal planes of A2780 cells were incubated at 10 µM for 8 h at 37 °C.

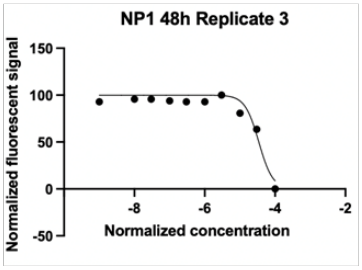

Ru 48h Replicate 1
Normalized fluorescent signal
Normalized concentration

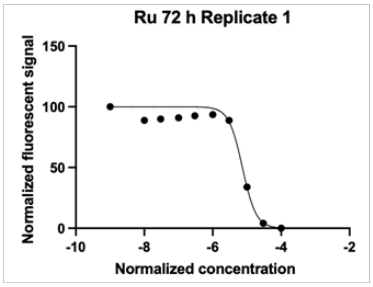

Ru 48h Replicate 2
Normalized fluorescent signal
Normalized concentration

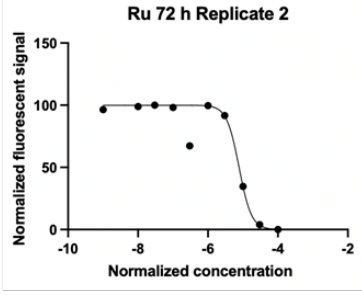

Ru 48h Replicate 3
Normalized fluorescent signal
Normalized concentration

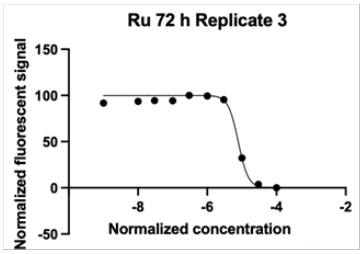

NP1 48h Replicate 1
Normalized fluorescent signal
Normalized concentration

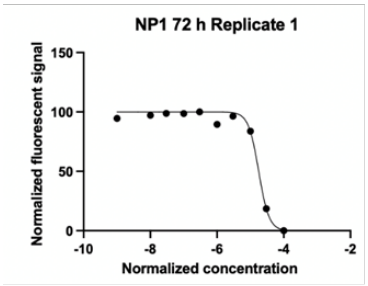

NP1 48h Replicate 2
Normalized fluorescent signal
Normalized concentration

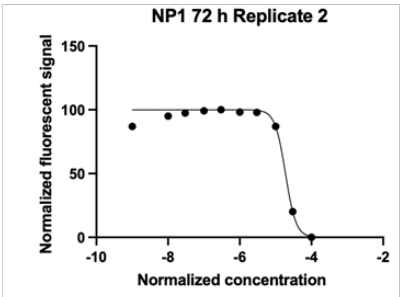

NP1 48h Replicate 3
Normalized fluorescent signal
Normalized concentration

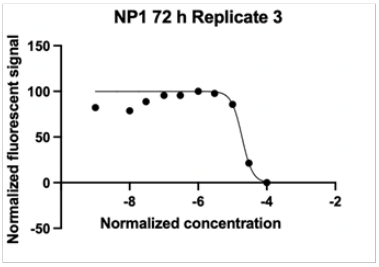

Ru 72 h Replicate 1
Normalized fluorescent signal
Normalized concentration

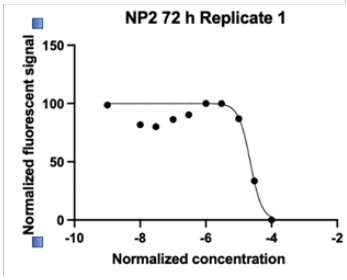

Ru 72 h Replicate 2
Normalized fluorescent signal
Normalized concentration

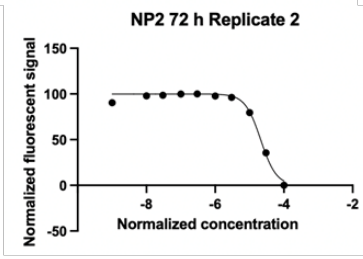

Ru 72 h Replicate 3
Normalized fluorescent signal
Normalized concentration

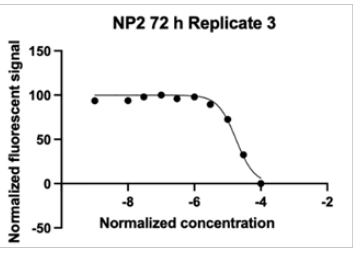

NP1 72 h Replicate 1
Normalized fluorescent signal
Normalized concentration


NP1 72 h Replicate 2
Normalized fluorescent signal
Normalized concentration


NP1 72 h Replicate 3
Normalized fluorescent signal
Normalized concentration


NP2 72 h Replicate 1
Normalized fluorescent signal
Normalized concentration


NP2 72 h Replicate 2
Normalized fluorescent signal
Normalized concentration


NP2 72 h Replicate 3
Normalized fluorescent signal
Normalized concentration

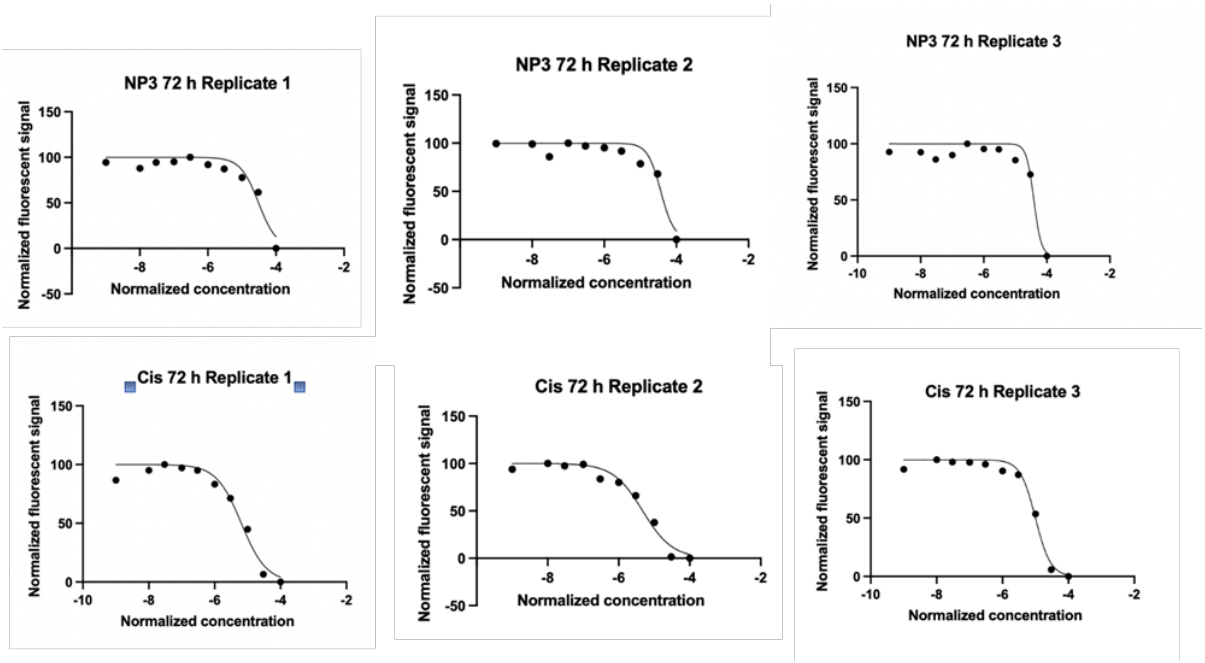
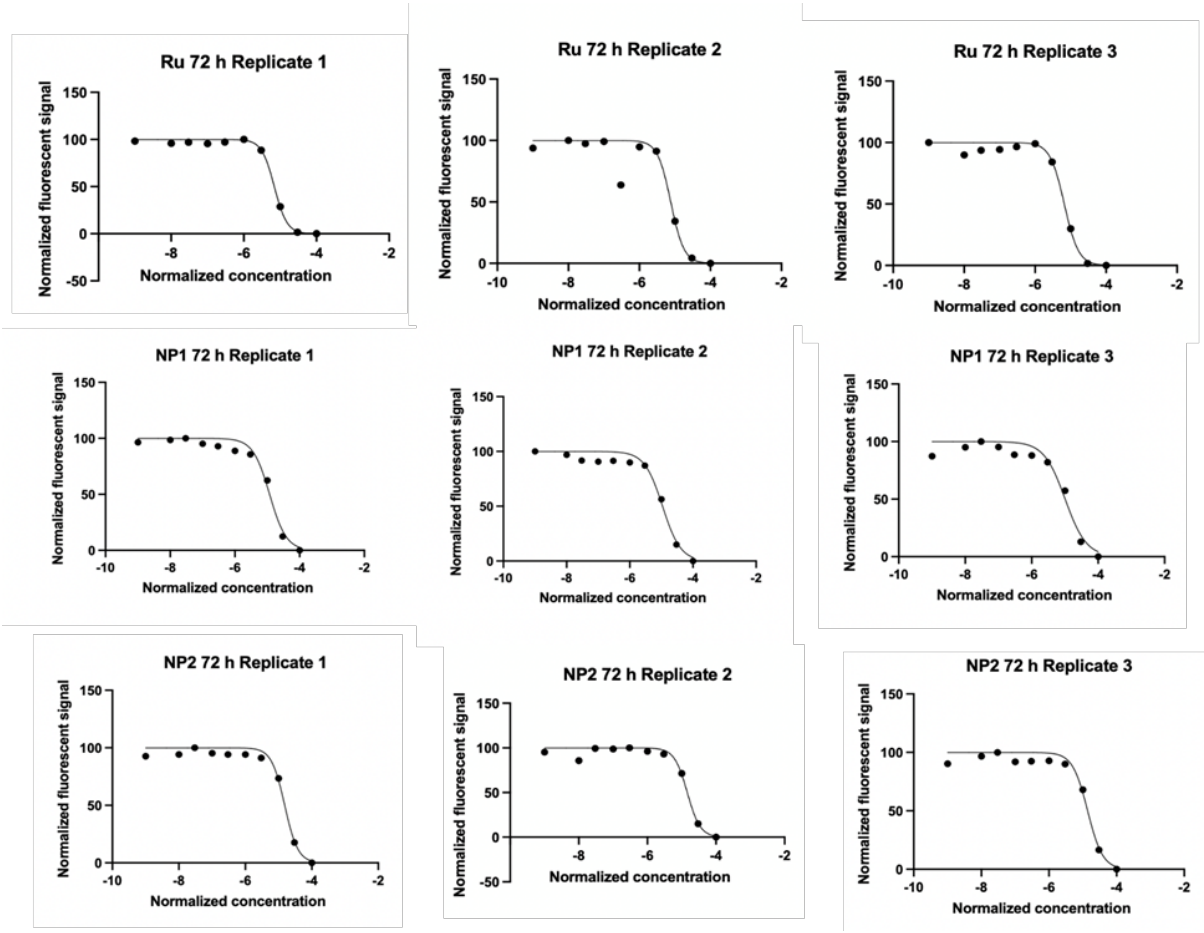


Figure S24. Fluorometric cell viability assay in A2780 cell line at 48h and 72h



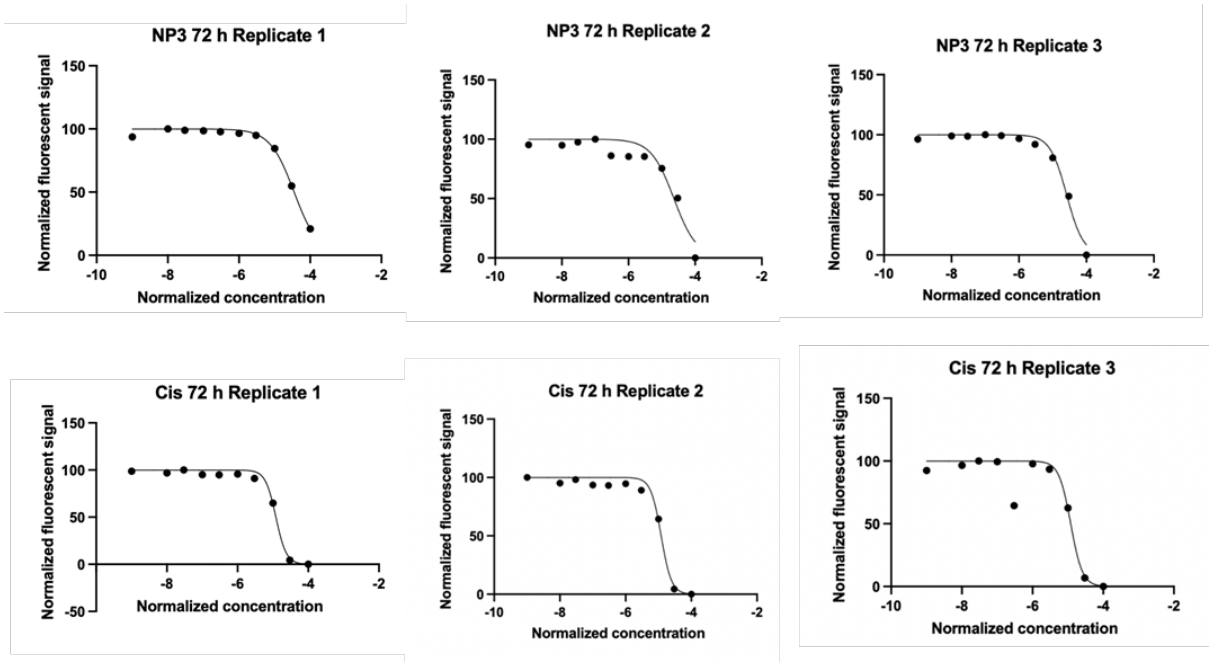


Figure S25. Fluorometric cell viability assay in A2780 Cisplatin resistant cell line at 72h.

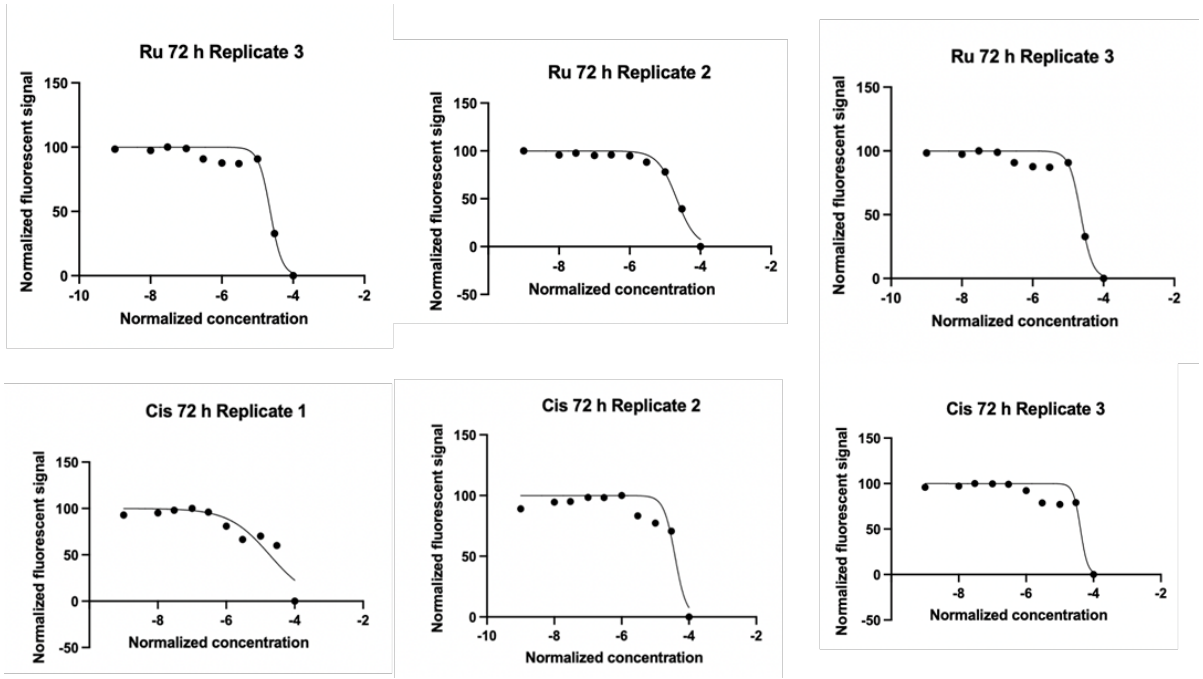


Figure S26. Fluorometric cell viability assay in RPE-1 cell line at 72 h.

In vivo Experiments

Components and treatment schedule

For *in vivo* studies, female swiss nude mice (Charles River, France) were used for tolerance and efficacy assessment. Ru alone was administered at 7.2 mg/kg. NPs were administered at 40 (39.5) and 36 mg/kg (35.9), the dose corresponding to a Ru dose of 6.6 and 7.2 mg/kg, respectively. All were administered weekly and intravenously.

Safety and Tolerability

For the tolerance experiment, mice were treated with NP, Ru and NP-Ru. The weight was measured 5 days for Week 1 and then 3 times/week. The weight of mice was then reported to the initial weight as relative tumor weight (RW).

Efficacy

For therapeutic efficacy, mice were xenografted with a tumor fragment of 20–40 mm^3 of OV54 serous adenocarcinoma ovarian cancer. Patient-Derived Xenografts (PDX) mice bearing tumors with a volume from 60 to 180 mm^3 were individually identified and randomly assigned to the control or treatment groups (8 to 10 mice per group). Tumor growth was evaluated by measuring twice a week of two perpendicular diameters of tumors with a caliper. The individual tumor volume was calculated as $V = a \times b^2/2$, a being the largest diameter, b the smallest. Tumor volumes were then reported to the initial volume as relative tumor volume (RTV). Means (and SD) of RTV in the same treatment group were calculated, and growth curves were established as a function of time. Additionally, to evaluate the overall response rate (ORR) to treatments observed in all models treated according to individual mouse variability, we decided to consider each mouse as a tumor-bearing entity. Hence, in all *in vivo* experiments, a relative tumor volume variation (RTVV) of each treated mouse was calculated from the following formula: [(RTVt/mRTVc)], where RTVt is the relative tumor volume of the treated mouse and mRTVc the median relative tumor volume of the corresponding control group at a time corresponding to the end of treatment. Then, for each treated mouse, we calculated [(RTVV)-1].

The experiment was stopped at the time of first ethical sacrifice. Mice were sacrificed 24 hours after the last treatment and, for 5 mice per group treated with NP, Ru and NP-Ru, tumor tissues, brain, lungs, liver, and kidney were collected and frozen. Tumor tissues were also fixed in formalin. Hence, between three and five tumors have been obtained from each group, according to the experimental design.

Statistical significance of observed differences between the individual RTVs corresponding to the treated mice and control groups was calculated using the two tailed Mann–Whitney test. Statistical significance of ORR between tested treatments was determined using a χ^2 test. Predictive markers have been defined using a Mann-Whitney test.

All *in vivo* experimental procedures were specifically approved by the Ethics Committee of the Institut Curie CEEA-IC #118 (Authorization APAFiS# 25870-2020060410487032-v1 given by the National Authority) in accordance with the international guidelines.

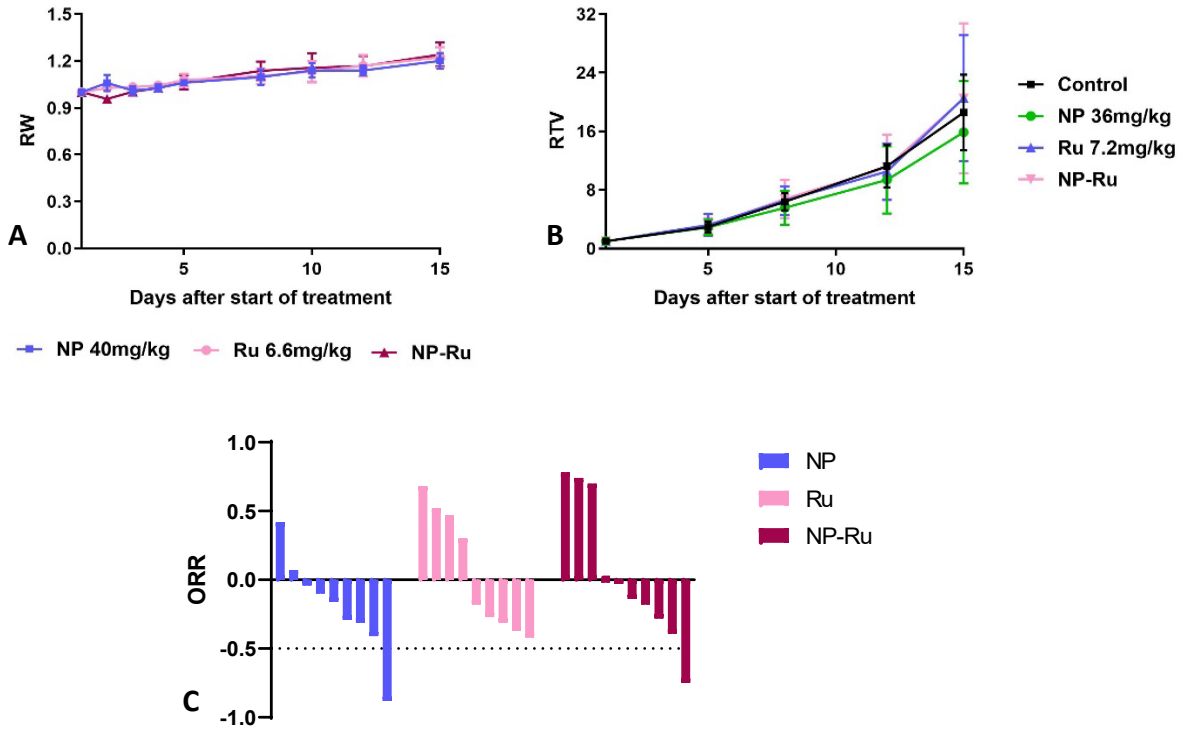


Figure S27. In vivo experiments: **A)** Evaluation of the toxicities of **NP**, **Ru**, and **NP-Ru**. **B)** Relative Tumor Volume (RTV) after **NP**, **Ru**, and **NP-Ru** administration. **C)** Overall Response Rate (ORR) after **NP**, **Ru**, and **NP-Ru** administration.

Biodistribution

ICP-MS on the biodistribution of organs samples were prepared as follows: isolated cellular fractions were lyophilized and digested using 2 mL of 70% nitric acid (80 °C, overnight). The samples were then further diluted (1:100 for all other samples) in Milli-Q water (containing a 1% HCl solution) and analysed using ICP-MS.

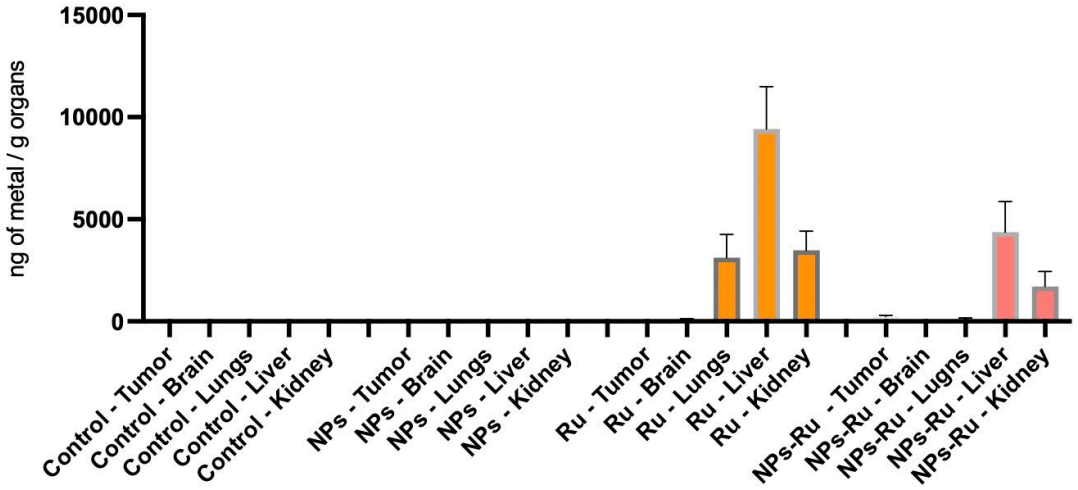


Figure S28. ICP-MS quantification of ruthenium content in various organs. Representative data from five independent experiments is shown.

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