Supplementary Information

High coatings of Ru(II) complexes on gold nanoparticles for single particle luminescence imaging in cells

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SECTION SA - SYNTHESIS AND CHARACTERIZATION

Figure S1. Schematic reaction scheme for the preparation of RuSH.

Synthesis of RuSAc: A solution of thioacetic acid (100 mg, 1.31 mmol) and ABCN (1,1'-azobiscyclohexane carbonitrile) (120 mg, 0.49 mmol) in dry THF (2 mL) was heated at 60 °C and degassed under nitrogen for 30 min. RuHex (105.9 mg, 0.100 mmol) was dissolved in THF (4 mL), and added dropwise to the solution. The double bond disappearance was monitored by proton NMR. The reaction was stirred at 60 °C followed by addition of one portion of ABCN (120 mg, 0.49 mmol) and thioacetic acid (100 mg, 1.31 mmol) after 15 hr, and another portion of ABCN (120 mg, 0.49 mmol) and thioacetic acid (100 mg, 1.31 mmol) after 24 hr. After a total of 40 hr stirring the reaction was pushed to completion and the ABCN was quenched with saturated NaHCO₃ solution (10 mL). The THF was removed in vacuo, and the residue was extracted in DCM/water, washed with brine, and dried over MgSO₄. The DCM was removed in vacuo and the solid was filtered under suction, and washed with a copious amount of hexane and diethyl ether to yield RuSAc (52.9 mg, 0.044 mmol, 43.8% yield) as a red solid.

1H NMR (400 MHz, CD₃CN, δ): 8.51 (d, 2H, J_Hd/Hi = 8.3 Hz, H_e/Hh), 8.50 (d, 2H, J_Hd/Hi = 8.3 Hz, H_e/Hh), 8.06-7.99 (m, 4H, H_d/Hi), 7.97 (d, 2H, J_H5 = 2.5 Hz, H3), 77.81 (d, 2H, J_Hc/Hj = 5.5 Hz, Hb/Hk), 7.71 (d, 2H, J_Hc/Hj = 5.5 Hz, Hb/Hk), 7.43-7.33 (m, 4H, H_c/Hj), 7.41 (d, 2H, J_H5 = 6.5 Hz, H6), 6.90 (dd, 2H, J_H6 = 6.5 Hz, J_H3 = 2.5 Hz, Hs), 4.18 (t, 4H, J_H8 = 6.5 Hz, H7), 2.85 (t, 4H, J_H11 = 7.3 Hz, H12), 2.27 (s, 6H, H13), 1.84-1.76 (m, 4H, Hs), 1.61-1.53 (m, 4H, H11), 1.52 – 1.38 (m, 8H, H9/H10).

13C NMR (100 MHz, CDCl₃, δ): 153.0 (C6), 152.7/152.5 (Cb/Ck), 138.3 (Cd/Ci), 128.5 (Cq/Cj), 125.1 (Cp/Ch), 115.1 (C3), 112.3 (C3), 70.6 (Cj), 30.8 (C13), 30.2 (C11), 29.4 (C12), 29.1 (C8), 28.9/25.8 (Cq/C10).

1464, 1444, 1396, 1339, 1278, 1222, 1120. UV-VIS (MeCN) $\lambda_{\text{max}}$ in nm (log $\varepsilon$): 462 (4.08), 436 (sh), 289 (4.79), 228. Emission (MeCN) $\lambda_{\text{max}}$ in nm: 645.

Synthesis of RuSH: NH$_4$OH (aq) solution (50 μl, 30 % wt) was added to RuSAc (100 μl, 1.52 mM in MeCN, 152 nmol) and shaken for ten min in a sealed vial to give RuSH in situ, directly prior to titration into the colloid. TOF LD-MS(+): m/z 829.7 [M - 2PF$_6$]$^+$. 

Figure S2. Emission spectra monitoring the effect of Zonyl 7950 addition (0-112 mg L$^{-1}$) to a solution of RuSAc (0.16 μM RuSAc in 1 % CH$_3$CN / H$_2$O). $\lambda_{\text{exc}}$ = 450 nm.

Nanoparticle Synthesis and Characterization

Au$_{13}$NP Synthesis according to Grabar et al.$^1$ UV-VIS (H$_2$O) $\lambda_{\text{max}}$ [nm]: 520 (SPR), 260. Diameter = 13± 1 nm (DLS number distribution). Zeta potential = -40 ± 5 mV (2 nM in deionised water).

Au$_{100}$ The method of Ziegler and Eychmüller$^2$ was followed with minor modifications. UV-VIS (H$_2$O) $\lambda_{\text{max}}$ [nm] = 566 (SPR). Diameter = 98 ± 27 nm (DLS number distribution). Zeta potential = -33 ± 3 mV (4 pM in deionised water).

Z●AuNP$_{13}$ and Z●AuNP$_{100}$ Zonyl® 7950 (purchased from Sigma Aldrich), CH$_3$CH$_2$CCOOC$_2$H$_4$(CF$_2$)$_n$F, MW ca. 500, 1 μl, 1.15 g mL$^{-1}$) was added to AuNP$_{13}$ (1 mL, 9 nM), or AuNP$_{100}$ (1 mL, 40 pM), to give fluorosurfactant coated nanoparticles. For their characterisation the 13 nm coated particles were centrifuged twice at 12,000 g for 15 min, and the 100 nm particles at 12,000 g for 90 s (twice), and the supernatant was decanted from each pellet to remove unbound material. Z●AuNP$_{13}$, with a zeta potential of - 61 ± 4 mV (2 nM in deionised water), a diameter of 20 ± 2 nm (DLS number distribution) and XPS$^3$ data: Au 4f$_{7/2}$ = 84.31 eV (62.5%), 85.27 eV (37.5%). Z●AuNP$_{100}$, with a zeta potential of - 47 ± 3 mV (4 pM in deionised water) and diameter of 92 ± 21 nm (DLS number distribution).


3We acknowledge M. Walker, Science City Photoemission Facility, University of Warwick for the XPS data.
RuS•AuNP13 and RuS•AuNP100 A solution of RuSH (20 μl, 3.5 mM) added to Z•AuNP13 (1 mL, 9 nM) or Z•AuNP100 (1 mL, 40 pM), was stirred for 20 min. The Z•AuNP13 and Z•AuNP100 were prepared as above and used without centrifugation. The 13 nm coated particles were centrifuged at 12,000 g for 15 min, and the 100 nm particles at 12,000 g for 90 s, and the supernatant was decanted from each pellet to remove unbound material. The nanoparticles were re-suspended in deionised water (1 mL), and the procedure was repeated twice.

RuS•AuNP13: UV-VIS (H2O) λmax [nm] = 522 (SPR). Diameter = 18 ± 5 nm (DLS number distribution), zeta potential = -48 ± 2 mV (2 nM in deionised water). Emission (H2O, λexc = 450 nm) λmax [nm] = 640. XPS data: Au 4f7/2 = 84.15 eV (91%), 84.54 eV (9%).

RuS•AuNP100: UV-VIS (H2O) λmax [nm] = 567 (SPR). Diameter = 76 ± 22 nm (DLS number distribution), 100 nm (mean diameter by NanoSight Particle Tracking), zeta potential = -35 ± 3 mV (4 pM in deionised water). Emission (H2O, λexc = 450 nm) λmax [nm] = 640.

Calculating the concentration and complex coverage of the gold nanoparticles

Mass of HAuCl4 (49 % Au by assay) = 0.09889g
Mass (Au) = 0.49 x 0.09889 = 0.04846 g, moles (Au) = 0.04846 / 197 = 2.4597 x 10^{-4} moles.
[Au] = 2.4597 x 10^{-4} moles / 0.275 dm^3 = 8.944 x 10^{-4} M.

Number of Au atoms per 13 nm nanoparticle = (6.5 x 10^{-9})^3 / (140 x 10^{-12})^3 = 106,802 (assuming that the particles are spherical and estimated atomic radius of gold = 140 pm).

Concentration of AuNP13 = 8.944 x 10^{-4} M / 100,082 = 8.94 nM ~ 9 nM.

During the first growth step, 6 mL of the AuNP13 seeds are diluted in 80 mL. Assuming no new seeds are formed, the concentration of the AuNP25 formed = 0.7 nM.

In the second growth step 9 mL of the AuNP25 seeds are diluted to 80 mL. Assuming no new seeds are formed, the concentration of the AuNP50 formed = 80 pM.

In the final growth step, 40 mL of the AuNP50 are diluted to 80 mL, rendering AuNP100 at 40 pM.

The coverage of RuS in RuS•AuNP13
ICPOES results give a Ru:Au atomic ratio of 1:63.
From the calculation of the number of Au atoms per 13 nm particle, 100,082 Au atoms per nanoparticle are estimated which leads to 100,082/63 = 1590 RuS complexes per nanoparticle.

Surface area of 13 nm RuS•AuNP13 = 4π (6.5 x 10^{-9})^2 = 5.3 x 10^{-16} m^2 (assuming particles are spherical). Surface area per complex = 5.3 x 10^{-16} / 1590 = 3.33 x 10^{-19} m^2 (0.57 nm x 0.57 nm).

If a diameter of RuS•AuNP13 is estimated to 17 nm (based on the large error involved in the measurement), the calculation of the number of Au atoms per 17 nm particle will lead to (8.5 x 10^{-9})^3 / (140 x 10^{-12})^3 = 223806 atoms per nanoparticle which leads to 223806/63 = 3552 RuS complexes per nanoparticle. Surface area will then be estimated = 4π (8.5 x 10^{-9})^2 = 9 x 10^{-16} m^2 (assuming particles are spherical). Surface area per complex = 9 x 10^{-16} / 3552 = 2.5 x 10^{-19} m^2 (0.5 nm x 0.5 nm).

The coverage of RuS in AuNP100
The concentration of RuS left on the nanoparticles was estimated to 4.3 μM, from absorbance values of the supernatant at 460 nm. Ratio Ru complexes : Au100NP = 4.3 μM / 40 pM = 107,500.

Surface area of Au100NP = = 4π (50 x 10^{-9})^2 = 3.14 x 10^{-14} m^2 (assuming particles are spherical). Surface area per complex = 3.14 x 10^{-14} / 107,500 = 2.92 x 10^{-19} m^2. (0.54 nm x 0.54 nm).

The value is reported as 10^5 to include the error in the calculation of the concentration of the seed particles Au13NP which is result from the measurements of their diameter.
Figure S3. Normalized UV-Vis absorption spectra of gold nanoparticles after each growth step. AuNP13 seeds (bold solid line), AuNP25 following first growth step (solid line), AuNP50 following two growth steps (bold dashed line) and AuNP100 after three growth steps (dashed line).

Figure S4. DLS intensity distributions (a) and (b), and number distributions (c) and (d). In (a) and (c): RuS●AuNP13 (bold full line), Z●AuNP13 (dashed line) and AuNP13 (full line). In (b) and (d) RuS●AuNP100 (bold full line), Z●AuNP100 (dashed line) and AuNP100 (full line). (e) NanoSight nanoparticle tracking analysis of Ru●AuNP100 showing relative number distribution of size (bold full line), with a mean size of 100 nm, and a mode diameter of 79 nm. The cumulative number distribution is displayed (dashed line).
Figure S5. Transmission electron micrographs of Ru•AuNP13, (a) and Ru•AuNP100 (b) showing monodisperse nanoparticles of (a) 17 ± 5 nm and (b) 108 ± 15 nm.

Photophysical Characterization of RuS•AuNP13 and RuS•AuNP100

![Graphs showing photophysical characterization](image)

Figure S6. UV-Vis absorption spectra (solid line), excitation spectra, $\lambda_{em} = 630$ nm (dashed line), and luminescence spectra, $\lambda_{exc} = 450$ nm (bold solid line) of 2 nM RuS•AuNP13 (top) and 10 pM RuS•AuNP100 (bottom).

Figure S7. Effect of the nanoparticle to the lifetime of RuSH (0.3 μM, 1 % CH$_3$CN in H$_2$O) by monitoring the luminescence at 630 nm upon addition of Z•AuNP13 (0-850 pM). $\lambda_{exc} = 450$ nm. An estimation of RuS/NP is given.
SECTION SB - IMAGING IN CELLS

Cell Culture:
The human alveolar adenocarcinoma A549 cell line (line 86012804, HPA) was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), penicillin (100 µg mL\(^{-1}\)), streptomycin (100 µg mL\(^{-1}\)) L-glutamine (100 µg mL\(^{-1}\)), hereafter called complete media. Cells were cultured in 20 mL of complete media in vented T\(_{75}\) flasks at 37°C in 5 % CO\(_2\) and cells were routinely sub cultured using a 1–4 dilution using a standard trypsin-EDTA protocol. For microscopy, cells were grown in complete media on sterile glass coverslips for a minimum of 24 hours until 60 % cell confluent. Prior to dosing with nanoparticles cells were washed in PBS and the cell media was replaced with 2.7 mL fresh media. RuS•AuNP\(_{13}\) (300 µL, 9 nM) or RuS•AuNP\(_{100}\) (300 µL, 40 pM) were added to the cell media for the required incubation time of 24 hours. For the final 30 minutes Hoechst 33258 (3 µl, 2.5 µg mL\(^{-1}\)) was added to each well. Cell media was removed and the cells washed twice in PBS followed by fixation with methanol (3 mL) for 5 minutes followed by two further washes with PBS. Coverslips were removed and mounted on a droplet of Hydromount media (national diagnostics) on glass slides, sealed with clear nail varnish and stored for 24 hours at 4°C before imaging.

Confocal Microscopy

Pulse-Chase with A549 cells dosed with RuS•AuNP\(_{13}\)

Figure S8. Pulse-chase confocal images of the distribution of RuS•AuNP\(_{13}\) in A549 cells following incubation for 24 hr (pulse), (a) immediately after the 24 hr treatment, (b) 1 day, (c) 2 days, (d) 3 days post-treatment. Nuclear Hoechst staining, blue channel, \(\lambda_{\text{exc}} = 405\) nm, \(\lambda_{\text{em}} = 410-455\) nm and ruthenium luminescence, red channel, \(\lambda_{\text{exc}} = 453\) nm, \(\lambda_{\text{em}} = 555-800\) nm. Scale bar 10 µm; acquisition time 70 s per image.
Figure S9. Confocal images of distribution of RuS•AuNP13 in A549 cells following incubation for 24 hr. (a) white light transmission image, (b) reflection image $\lambda_{\text{exc}} = 633$ nm, $\lambda_{\text{em}} = 623-643$ nm, (c) ruthenium luminescence, red channel, $\lambda_{\text{exc}} = 453$ nm, $\lambda_{\text{em}} = 555-800$ nm, (d) nuclear Hoechst staining, blue channel, $\lambda_{\text{exc}} = 405$ nm, $\lambda_{\text{em}} = 410-455$ nm, (e) overlay of blue channel and transmission image, (f) overlay of blue channel and reflection image, (g) overlay of red channel; Acquisition time: 2 min 15 s.
Figure S10. (a) Confocal image sequence from 3D z-stack of live cells with RuS•AuNP13 and nuclear Hoechst staining, with enlargement of highlighted slice shown in (b – left). (b – right) orthogonal view of the central z-stack slice (xz plane below, yz plane-right) illustrating ruthenium fluorescence not from within the same z-plane as the nuclear Hoechst luminescence (arrow).
Additional Images of A549 cells dosed with RuS•AuNP100

Figure S11. Images of distribution of single RuS•AuNP100 nanoparticles in cells monitored by confocal luminescence and reflection microscopy. (a) nuclear Hoechst staining, blue channel, $\lambda_{\text{exc}} = 405\ \text{nm}$, $\lambda_{\text{em}} = 410-455\ \text{nm}$ (b) ruthenium luminescence, red channel, $\lambda_{\text{exc}} = 453\ \text{nm}$, $\lambda_{\text{em}} = 555-800\ \text{nm}$ (c) overlay of blue and red channels, (d) reflection image $\lambda_{\text{exc}} = 488\ \text{nm}$, $\lambda_{\text{em}} = 478-498\ \text{nm}$, (e) white light image and (f) overlay of blue channel and white light image. White scale bar is 20 $\mu\text{m}$. 
Figure S12. (a) Confocal image sequence from 3D z-stack of single cell with RuS•AuNP100 and nuclear Hoechst staining, with enlargement of highlighted slice shown in (b – left). (c – right) orthogonal view of the central z-stack slice (xz plane below, yz plane-right) illustrating ruthenium fluorescence interspersed within the same z-plane as the nuclear Hoechst luminescence (arrow).
Theoretical and Experimental PSF Analysis of single RuS•AuNP100 in A549 cell

Figure S13. a) theoretical 100 nm gold nanoparticle modelled as a section of a sinusoidal wave representing a simple model of the nanoparticle sphere, b) Gaussian model of the measured point-spread function from confocal luminescence measurements, c) result of the convolution of the measured point-spread function from confocal luminescence measurements with a simple model of the nanoparticle sphere, d) reflection microscopy image (left) and confocal luminescence image (right) indicating chosen nanoparticle (inset scale-bar 1 µm), e) and f) comparison of the result of the convolution of the measured point-spread function with the simple model of the nanoparticle sphere (solid line) and the actual measured intensity profile of a nanoparticle in the reflection and confocal luminescence image luminescence images respectively.
**Figure S14.** MTT Assay showing percentage cell viability of A549 cells after 24 hour incubation with AuNP (hashed) and RuS•AuNP (grey) for the 13 nm particles (left) and the 100 nm particles (right). Dosing was performed with 0.2-4.5 nM 13 nm particles and 0.8-20 pM 100 nm nanoparticles (equating to 2 – 50 % dilutions of the media with water). The equivalent dosing with deionised water blanks (white) are presented on each graph.

**Figure S15.** AK Assay showing percentage cell viability of A549 cells after 24 hour incubation with AuNP100 (hashed) and RuS•AuNP100 (grey), dosing with 0.8-20 pM nanoparticles (equating to 2 – 50 % dilutions of the media with water) and the equivalent dosing with deionised water blanks (white).

Viability was measured in four independent experiments; each experiment consisted of duplicate plates with duplicate measurements at each concentration. The error bars for each data point represents the mean ± 1 standard deviation (SD) of these measurements. Data outside of 1 SD of the mean of these sixteen readings were classified as outliers and disregarded.
Confocal microscopy studies of the cells dosed with RuS•AuNP13 correspond to a concentration of 0.9 nM and those dosed with RuS•AuNP100 correspond to a concentration of 4 pM RuS•AuNP100, indicated by the arrows. All treatments resulted in a concentration-dependent decrease in the ability of cells to reduce MTT, a measure of mitochondrial activity (P < 0.001), as assessed by a 1-way ANOVA. However, when treatment with particles were compared to the matched water blank there was no statistically significant difference between either AuNP or RuS•AuNP 13 and 100 nm particles and the appropriate water blank, with the exception of RuS•AuNP100 at 4 pM and 13.6 pM, as assessed by a Bonferroni corrected t-test (P < 0.05). Even when correcting for multiple comparisons the t-test is a non-conservative prone to false positives and the lack of a consistent concentration-dependent difference between the water blanks and the RuS•AuNP100 particles suggests that this is not biologically significant.

Furthermore there was no statistically significant concentration dependent release of adenylate kinase (AK assay), which a measure of cytoplasmic membrane integrity at any of the treatments investigated (1-way ANOVA).

**MTT Assay:** Cells were seeded in a 96-well plate at density of 8000 cells per well. Following incubation for 24 hours at 37 °C in 5 % CO2, removal of media and a PBS wash, particles or water in complete media at the required concentrations were added for a further 24 hour incubation. The media was removed (and retained for the adenylate kinase assay – see below) and the cells washed twice with PBS followed by incubation in 10 µL MTT (5 mg mL⁻¹ stock in PBS) in 100 µl per well complete media. After 3 hours incubation the supernatant was discarded and DMSO (200 µl per well) added to dissolve the resultant formazan crystals. Absorbance against a DMSO blank was immediately read in a microplate reader at 590 nm. Percentage cell viability was expressed relative to the untreated cells in complete media.

**Adenylate Kinase (AK) Release Assay:** The Adenylate kinase assay was performed using the commercially available bioluminescent ToxiLight™ kit (Lonza Ltd., Wokingham, UK). AK detection reagent (100 µl) was added to each well of a 96-well white luminescence plate (Greiner Bio-One Ltd, UK). Upon reaching room temperature 20 µl aliquots of the test supernatant was transferred to each well and samples incubated for 5 min. Bioluminescence was then immediately measured with a microplate reader (Bio-tek Instruments Inc., Kineticale software for Windows). The assay was performed on duplicate plates with duplicate measurements for each concentration tested. Percentage viability was expressed relative to 100 % lysed cells (positive controls), using Biosoft N25-9 detergent.

**Flow Cytometry:**
Cells were cultured in 6-well plates to approximately 60 % confluence before treatment with RuS•AuNP100 (4 pM) for 24 hours. Cells were washed in PBS, detached with trypsin-EDTA solution (3 min, 37°C), and pelleted by centrifugation. The supernatant was removed and cell pellets re-suspended in PBS and transferred to flow cytometry tubes for analysis (FACScalibur, BD Biosciences, USA). Forward scatter, side scatter and red fluorescence (λex=488 nm with light collected through a 670 nm band pass filter) of 50,000 cells were collected and Weasel freeware software was used for data analysis:

(http://www.wehi.edu.au/faculty/advanced_research_technologies/flow_cytometry/weasel_for_flow_cytometry_data_analysis/)
Figure S16. Cellular uptake of **RuS•AuNP100** as assessed by flow cytometry. The scatter plots represent red nanoparticle fluorescence ($\lambda_{exc} = 488$ nm with light collected through a 670 nm band pass filter) and forward scatter (y-axis, a measure of cell size).

**Forward Scatter vs. Side Scatter:**

*Figure S17.* FACS median side scatter data from control (undosed) A549 cells (left, dotted line in graph on right) and cells dosed with **RuS•AuNP100** (middle and solid line in graph on right). An increase in the median side scatter, from 273.8 to 361.9 is indicative of increased scattering from the dosed cells, over and above the expected cell granularity, induced by the presence of the gold nanoparticles.