Electronic Supplementary Information

Rational Design of Polymeric Core Shell Ratiometric Oxygen-Sensing Nanostructures

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Materials, Reagents, and Instruments for Chemical Synthesis

Synthesis of $[Ru(bpy)_2\text{-phen-Ar-COOH}]^{2+}$

The $[Ru(bpy)_2\text{-phen-Ar-COOH}]^{2+}$ oxygen probe was synthesised and prepared as described previously. [1]

Synthesis of BODIPY reference

The BODIPY reference probe was synthesised as described previously.[2]

Synthesis of poly(styrene) nano-structures

Materials.

All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. γ-Benzyl-L-glutamate and ε-benzyloxycarbonyl-L-Lysine were supplied by Bachem. Anhydrous dimethyl formamide (DMF) was used directly from the bottle under an inert and dry atmosphere. N3-fluorenylmethoxycarbonyl-L-lysine (Fmoc-L-lys) and ε-benzyloxycarbonyl-L-Lysine (ZLL) NCA was synthesized following a literature procedure. [3] Butyl phthalimidomethyl trithiocarbonate (RAFT CTA 1) and the PS homopolymer (PS TTC) were synthesized according to a literature procedure by Moad *et. al.* [4] The preparation of amine end-functional polystyrene ($PS_{38}\text{-}NH_2$) is described in a previous publication. [5]

Chain extension of PS-NH_2 *via* the ROP of NCAs.

PS_{38}-$PZLL_{64}$-$PFLL_5$: Chain extension of the PS-NH_2 macroinitiator was done according to a method described in previous work. [6] The NCA monomer of ε-benzyloxycarbonyl-L-Lysine (1.23 g, 4.02 mmol) was dissolved in 12 ml anhydrous DMF in a Schlenk tube. The solution was degassed *via* three successive freeze-pump-thaw cycles and kept under vacuum. The reaction flask was immersed in a 0 °C water bath and a solution of PS-NH_2 (224.5 mg, 0.057 mmol, $[M]_0/[I]_0 = 70$) in 1 mL anhydrous DMF was injected through a rubber septum with a syringe. The reaction was left to stir for several days until the ZLL NCA had been completely consumed as monitored by ATR FTIR spectroscopy. The polypeptide was further chain extended *via* the introduction of Fmoc-L-lysine NCA (0.113 g, 0.29 mmol, $[M]_0/[I]_0 = 5$) dissolved in 3 mL DMF. After full monomer conversion monitored via ATR FTIR spectroscopy the polymer was precipitated into an excess of diethyl ether, filtered and dried under vacuum. Isolated yield: 0.89 g

Selective Fmoc deprotection

PS_{38}-$PZLL_{64}$-PLL_5: The hybrid copolymer PS_{38}-$PZLL_{64}$-$PFLL_5$ (0.8 g) was dissolved in 8 ml DMF. To this 2 ml of piperidine was added. The solution was stirred for 2 h at room temperature. The resulting polymer was purified *via* dialysis against methanol for 16 hours and then DDI water for 24 hours using Spectra/Por dialysis membranes (MWCO, 3.5 kDa). The product was lyophilized and isolated as a white powder. Isolated yield: 0.72 g.

Attachment of Ru complex

PS_{38}-$PZLL_{68}$-$(Ru\text{-}L)_2$: A general procedure for 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)/*N*-hydroxysulfosuccinimide (Sulfo-NHS) coupling was employed to attach the ruthenium ligand to the lysine residues of the hybrid copolypeptide. The Ruthenium ligand (Ru-L) (38 mg, 0.054 mmol, 2 eq w.r.t lysine repeat units), EDC (11 mg, 0.054 mmol, 1.2 eq w.r.t. the Ru-L) and sulfo-NHS (2.5 mg, 0.027 mmol, 0.5 eq w.r.t. EDC) were dissolved in 3 mL DMSO and stirred for 20 minutes. The latter was added to a solution of PS_{38}-$PZLL_{64}$-PLL_5 (0.61 g, 0.027 mmol) in 5 mL DMSO. The reaction mixture was stirred overnight. The resulting product was purified *via* dialysis against DDI water using Spectra/Por dialysis membranes (MWCO, 3.5 kDa) for 72 h at room temperature to remove any unbound Ruthenium ligand. The product was subsequently lyophilized and isolated as an orange polymer. Isolated yield: 0.52 g.

PZLL deprotection

PS_{38}-PLL_{68}-(Ru-L)$_2$: A general procedure was used for the deprotection of the PZLL pendant groups: PS_{38}-$PZLL_{68}$-(Ru-L) (0.44 g) was dissolved in trifluoroacetic acid (12 mL) and a minimal amount of THF to improve solubility. A solution of HBr (33 wt. % in acetic acid) (1.3 mL, 6-fold excess with respect to ε-benzyloxycarbonyl-L-Lysine repeat units)) was added slowly to the reaction at 0 ºC. After 4 h, the solution was added to 200 mL diethyl ether and the precipitate washed several times with diethyl ether. The product was dialyzed against DDI water using Spectra/Por dialysis membranes (MWCO, 3.5 kDa) for 72 h at room temperature. The product was lyophilized and isolated as an orange powder. Isolated yield: 0.21 g.

Preparation of BODIPY nanoparticles *via* a mini-emulsion polymerization.

The mini-emulsion polymerization of a 95/5 (v/v) solution of styrene/divinyl benzene (DVB) and BODIPY were carried out in a 25 mL two-neck reactor equipped with a reflux condenser, nitrogen inlet and magnetic stirrer. A typical reaction proceeded as follows: PS_{38}-PLL_{68}-(Ru-L)$_2$ (80 mg) was added to the reactor under an inert atmosphere and dissolved in 9 mL degassed distilled water. A styrene/DVB monomer solution (0.80 g) was deoxygenated separately for 20 min by bubbling nitrogen through it. The BODIPY dye (0.3 mg) was dissolved in this solution and injected into the reactor. The reaction mixture was left under heavy stir using a T 10 basic ULTRA-TURRAX® homogenizer for 5 min, while being kept on an ice bath. The reaction flask was transferred to a heated oil bath (70 °C) and a deoxygenated initiator solution (10 mg of potassium persulfate in 1 mL of water) was injected to start the polymerization. The reaction was left to proceed for 4 hours after which the the resulting latex was dialyzed against DDI for 48 hours using Spectra/Por dialysis membranes (MWCO, 3.5 kDa). The latex was characterized using DLS and FESEM.

Oxygen Calibration Studies

To measure the oxygen sensitivity, RuBODIPYNP solutions in PBS were purged with nitrogen for 10 minutes at room temperature. The oxygen concentration was measured using a PreSense Oxygen Probe. The particles were slowly re-aerated to give a range of oxygen concentrations (measured in μmol/L). The emission spectra and lifetimes were measured to

construct an oxygen calibration curve for the particles. The emission spectra were collected using a Varian Cary Fluorimeter, with an excitation and emission slit width of 10 nm. The lifetimes were collected using time correlated single photon counting (TCSPC).

RuBODIPYNP preparation for cell uptake

Cetyltrimethylammonium bromide (CTAB) from Sigma Aldrich, was used to induce a cationic surface charge on the particles for cell uptake. 100 μM CTAB solution was added to the RuBOIPYNP and left to sonicate overnight.

RuBODIPYNP uptake

CHO and HeLa cells were seeded at $1.5\ \tilde{A}—\ 10^5$ cells in 2 mL media on 35 mm glass-bottom culture dishes. Cells were grown for 48 h at 37 °C at 5 % CO_2. The growth media was removed, and replaced with 2 mL Lebovitz media for 2 h. 44μg/mL of RuBODIPYNP in Lebovitz media were added to the cells and incubated for 5 minutes in the absence of light at 37 °C and 5 % CO_2. The RuBODIPYNP/media solution was removed and replaced with Lebovitz media. Cells were left to incubate for 2, 4, and 6 h at 37 °C in the dark. Cells were washed twice with PBS supplemented with 1.1 mM $MgCl_2$ and 0.9 mM $CaCl_2$ and imaged immediately. Cells were imaged using a Leica TSP DMi8 confocal microscope, using a 100 X oil immersion objective lens. The nanoparticles were excited using a 470 nm white light laser. The BODIPY emission was collected using a band pass 505-550 nm filter, and the ruthenium emission was collected using a long pass 605 nm filter. DRAQ 7 (Biostatus, UK), which stains the nucleus of dead cells, was added to the culture dish (1:100 final concentration) to monitor cell viability. DRAQ 7 was excited using a 633 nm white light laser and emission was collected using a long pass 650 nm filter.

Phosphorescent lifetime imaging microscopy (PLIM) of RuBODIPYNP

CHO cells were seeded at 1.5 x 10^5 cells in 2 mL media on 35 mm glass-bottom culture dishes. Cells were grown for 48 h at 37 °C at 5 % CO_2. The growth media was removed, and replaced with 2 mL Lebovitz media for 2 h. 44μg/mL of RuBODIPYNP in Lebovitx media were added to CHO cells and incubated for 5 minutes in the absence of light at 37 °C and 5

% CO_2. The nanoparticles/media solution was removed and replaced with Lebovitz media. Cells were left to incubate for 4 h at 37 °C in the dark. CHO cells were washed twice with PBS supplemented with 1.1 mM $MgCl_2$ and 0.9 mM $CaCl_2$. Live PLIM images were acquired using MicroTime System attached to a Leica TSP Dmi8 confocal microscope using a 100X oil immersion objective. Each PLIM image was acquired for 20 minutes at 512 x 512 resolution. A 405 nm laser was used to excite the sample. The BODIPY emission was collected using a 515-580 nm band pass filter at 8 ps resolution, and the $[Ru(bpy)_2$-Phen-Ar-COOH$]^{2+}$ was collected using a 570-700 nm band pass filter at 256 ps resolution. The data was analysed using the PicoQuant Symphotime Software. Lifetimes were fit to a mono exponential decay until an X^2 value of 0.9-1.1 was achieved. The average lifetime was taken from 2 separate experiments.

Cytotoxicity assay

CHO cells were seeded in a 96-well plate in 100 µL media at 1 x 10^4 cells per well for 24 h at 37 °C with 5 % CO_2. RuBODIPYNP were added to give final concentrations of 1, 2, 5, 10, 25, and 50 % v/v in CHO media. The cells were incubated for 5 minutes. The particles were removed and replaced with CHO media and left to incubate in the absence of light for 24 h at 37 °C at 5 % CO_2. In both cases, 10 µL of Resazurin (Alamar Blue) reagent was added to each well and incubated for a further 7 h in the absence of light at 37 °C. The resazurin was converted to resorufin in viable cells and its absorbance was measured at 570 nm, with background measured at 600 nm using a Tecan 96-plate reader.

Polymer Analysis

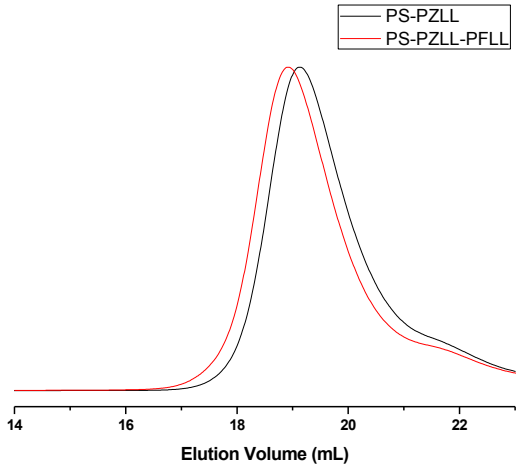


Figure S1 GPC traces of diblock copolymer PS-PZLL and tri-block copolymer PS-PZLL-PFLL **2**.

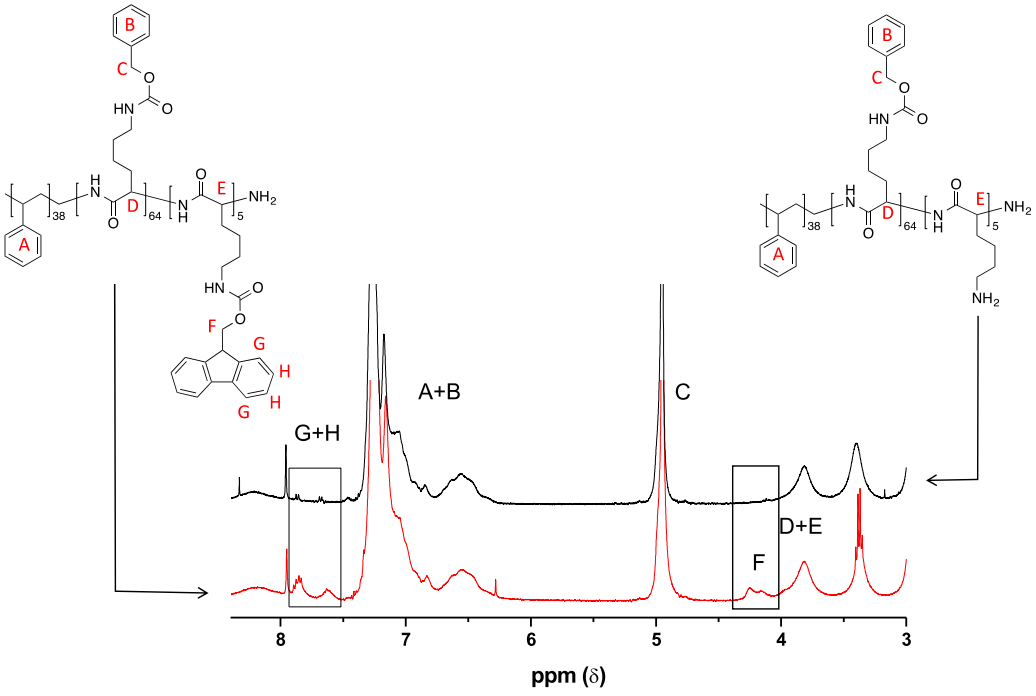


Figure S2 Sections of ^{1}H-NMR spectra of PS_{38}-$PZLL_{64}$-$PFLL_5$ and PS_{38}-$PZLL_{64}$-PLL_5 demonstrating successful deprotection by removal of the Fmoc group (black boxes).

Dynamic Light Scattering from Core-Shell particles RuBODIPYNP

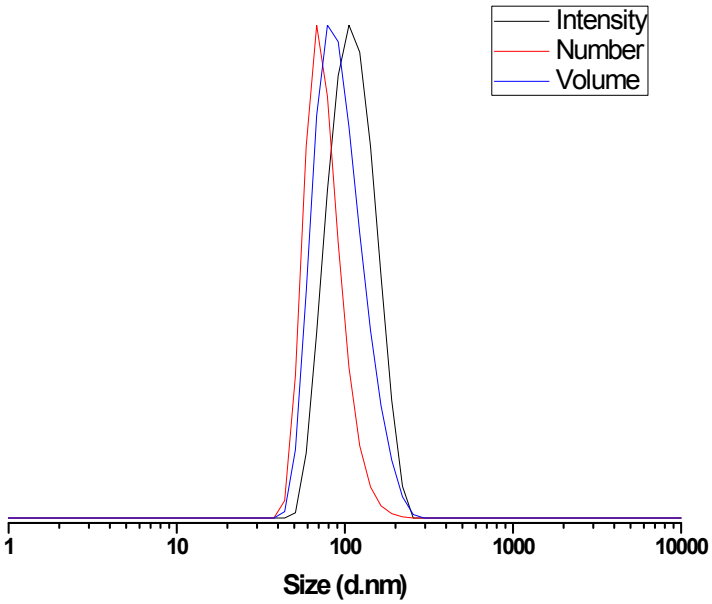


Figure S3 Dynamic Light Scattering (DLS) of core shell RuBODIPY-NP

Emission Spectra of RuBODIPYNP

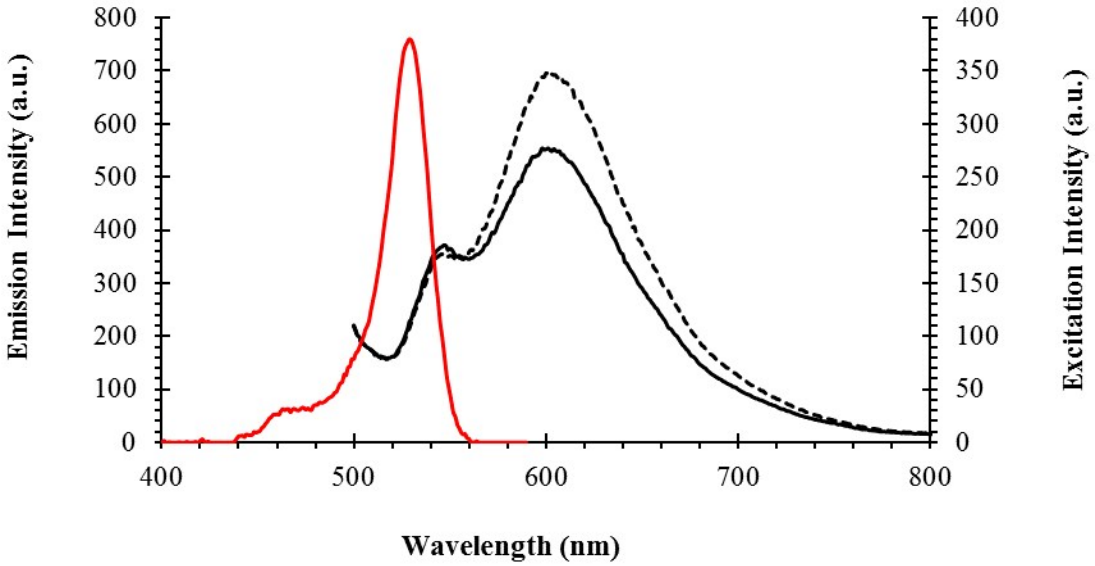


Figure S4 Excitation (—) and emission spectra of RuBODIPYNP when aerated (—) and deaerated (---) in PBS solution. Solution was degassed with nitrogen for 10 minutes. The complex was excited at 458 nm at a slit width of 10 nm.

TCSPC Lifetime analysis of RuBODIPYNP particles

Representative lifetime plots for the particles in PBS solution (pH 7.4)

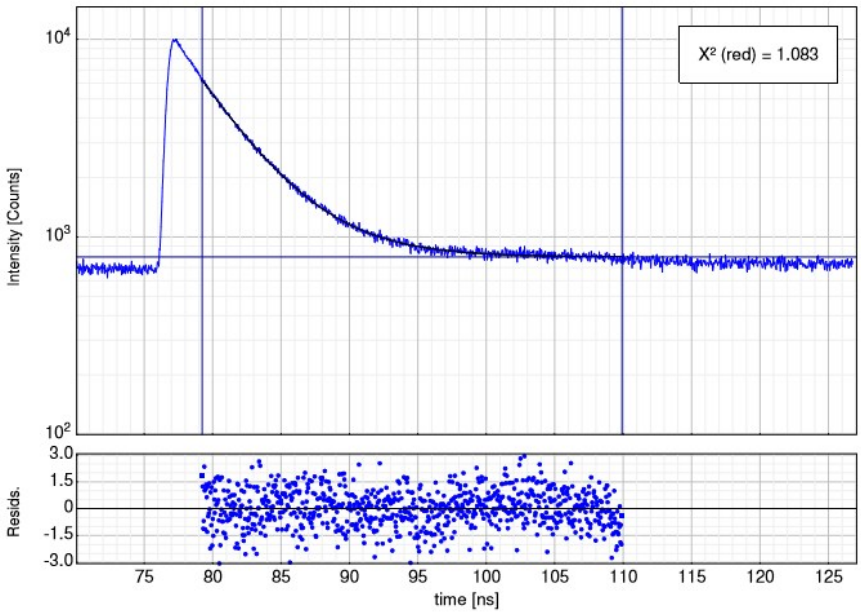


Figure S5 TCSPC lifetime decay of the BODIPY core component, aerated, in PBS solution (pH 7.4)

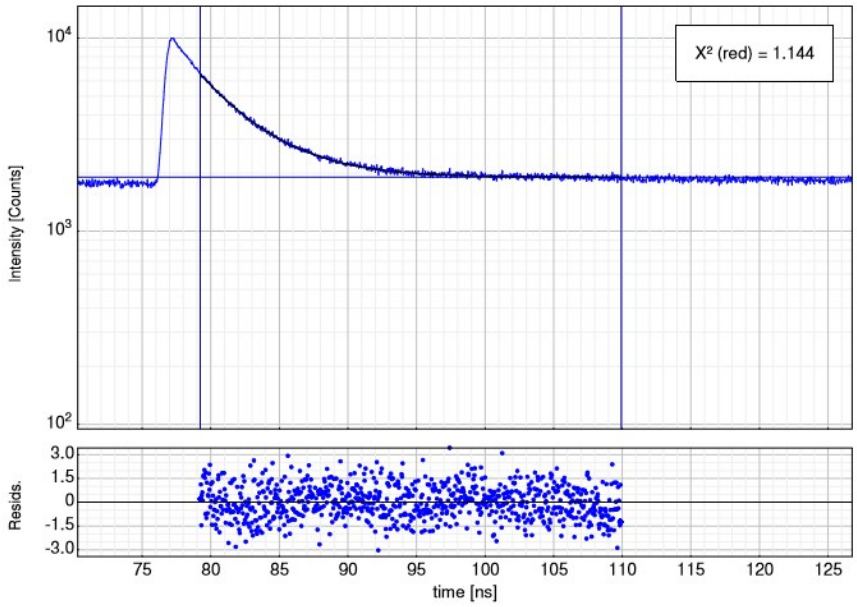


Figure S6 TCSPC lifetime decay of the BODIPY core component, de-aerated with nitrogen, in PBS solution (pH 7.4).

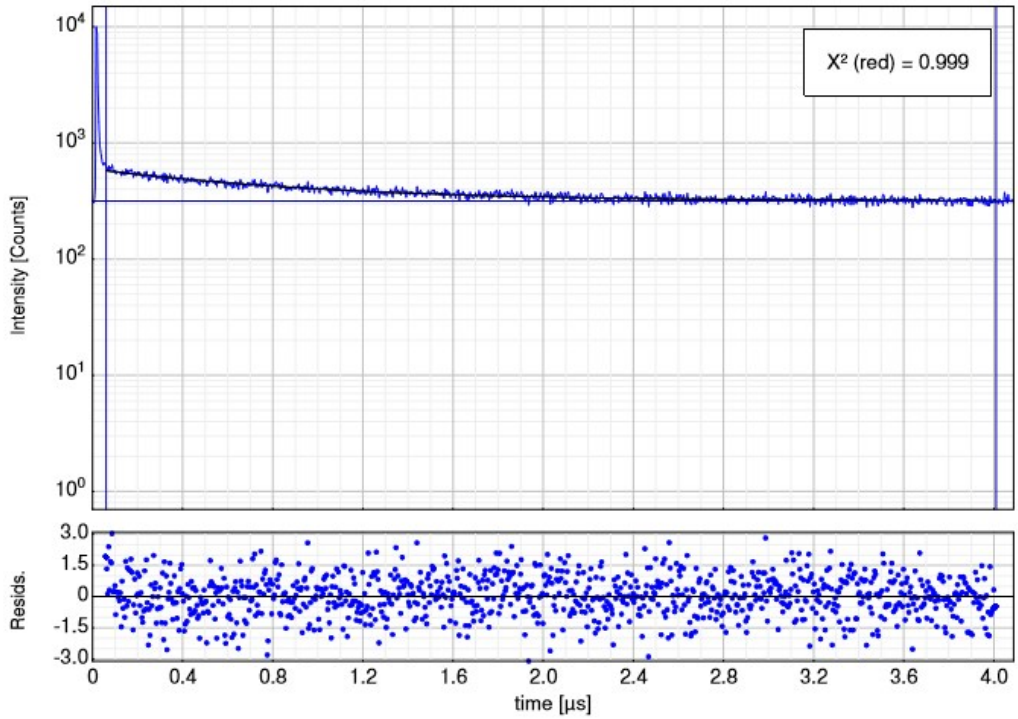


Figure S7 TCSPC lifetime decay of $[Ru(bpy)_2\text{-phen-Ar-COOH}]^{2+}$ component, aerated, in PBS solution (pH 7.4).

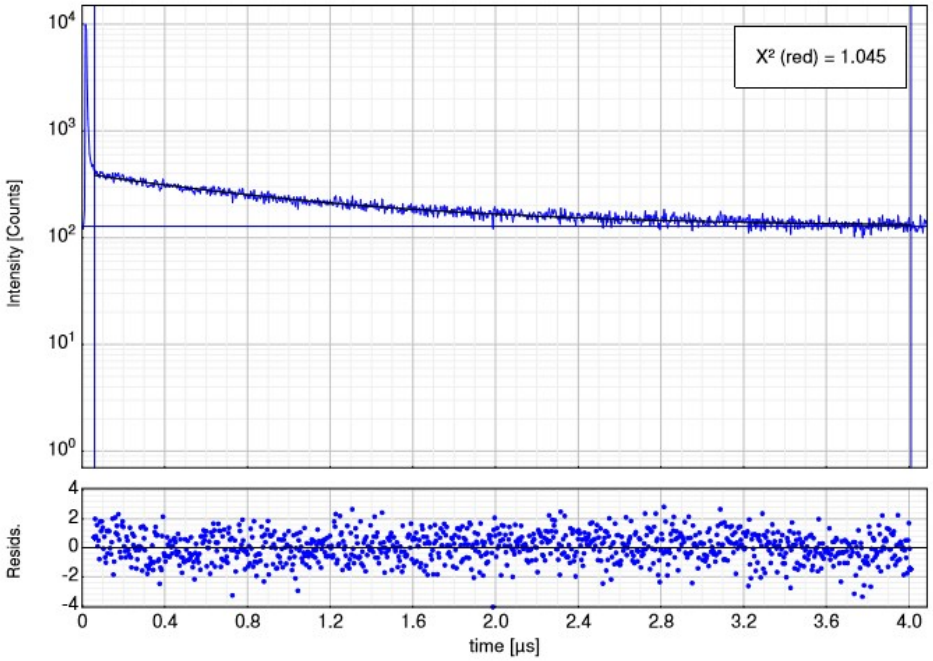


Figure S8 TCSPC lifetime decay of $[Ru(bpy)_2\text{-phen-Ar-COOH}]^{2+}$ component, de-aerated with nitrogen, in PBS solution (pH 7.4).

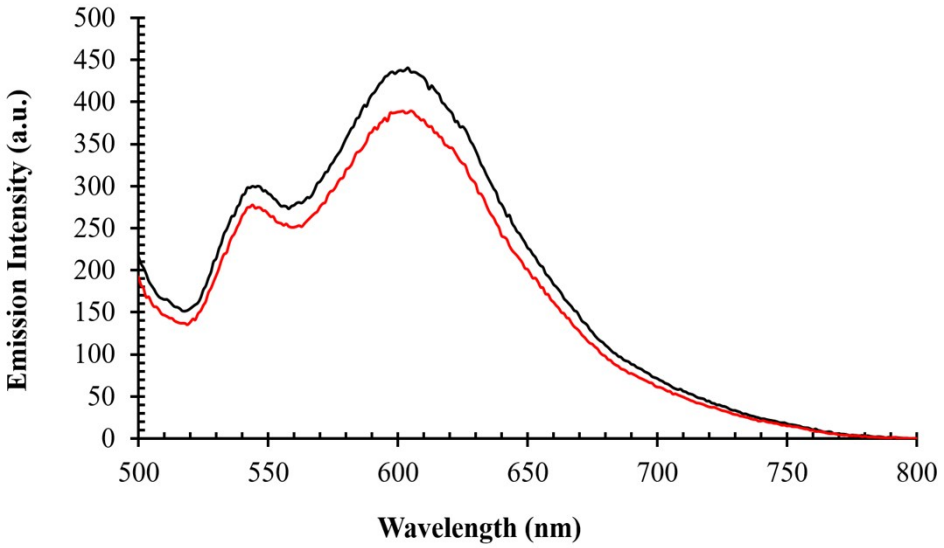
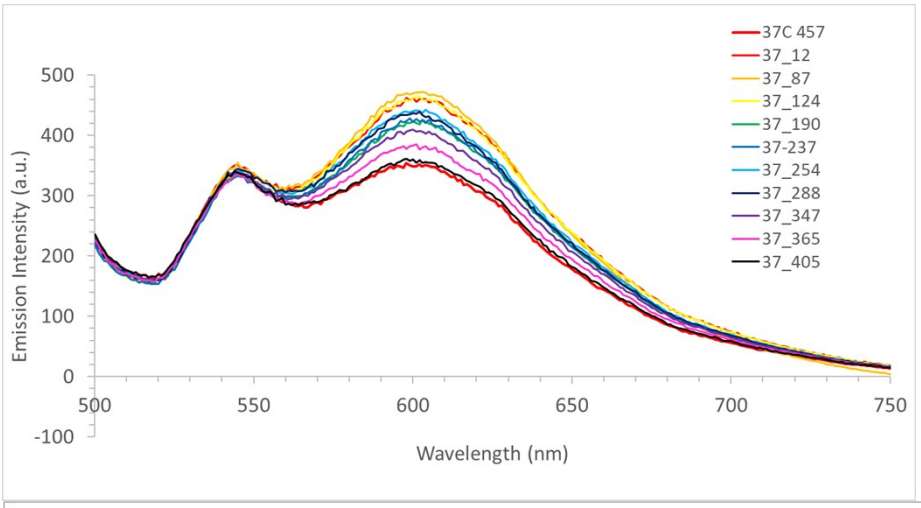
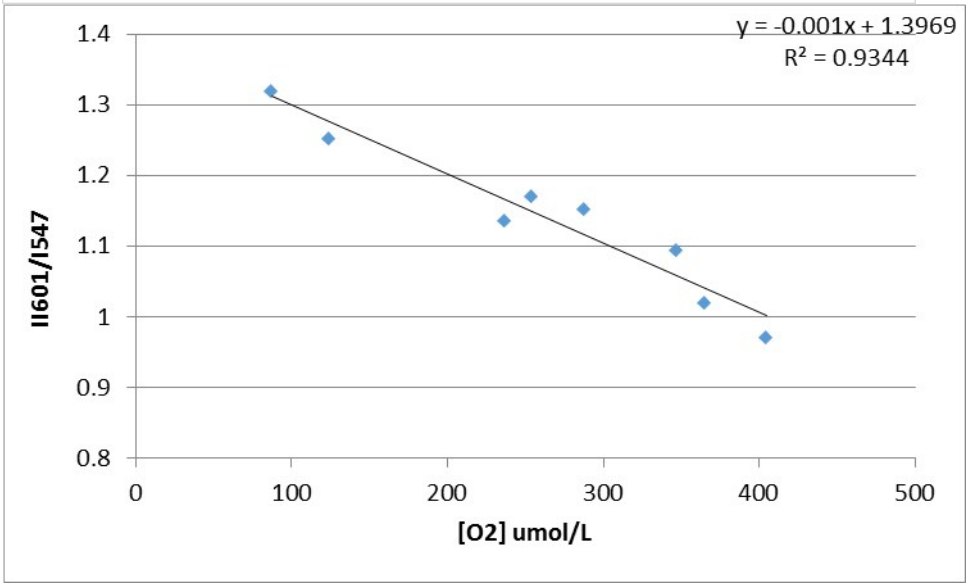


Figure S9 Emission spectra of RuBODIPYNP (–) and following 1 month (–) suspension in PBS solution when excited at 458 nm, slit width 10 nm.





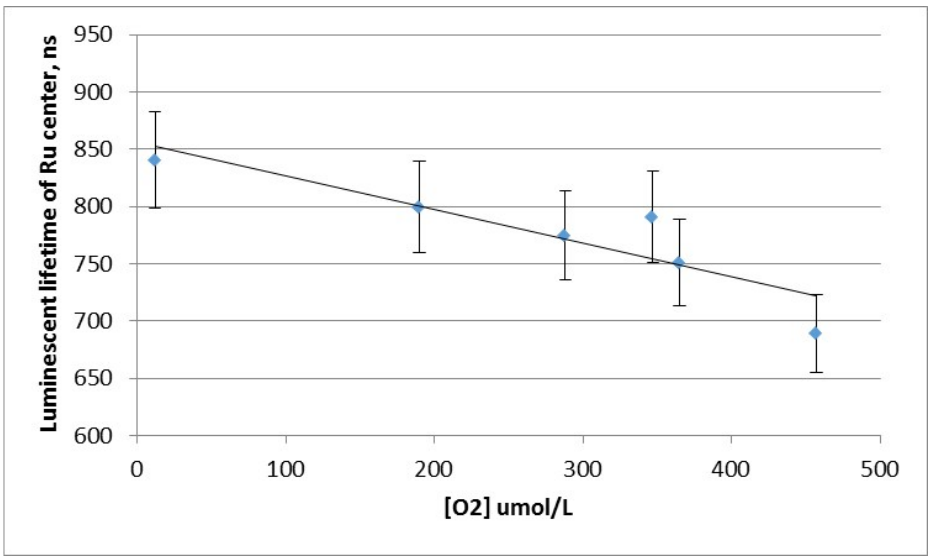


Figure S10 Top**,** Emission spectra of RuBODIPYNP in PBS at 37°C solution as a function of O_2 concentration when excited at 458 nm, slit width 10 nm. middle ratiometric response to

O_2, Bottom, lifetime of Ru center at 37oC as a function of O2 concentration in PBS buffer. n= 2

Spectra of RuBODIPYNP at pH 3.8

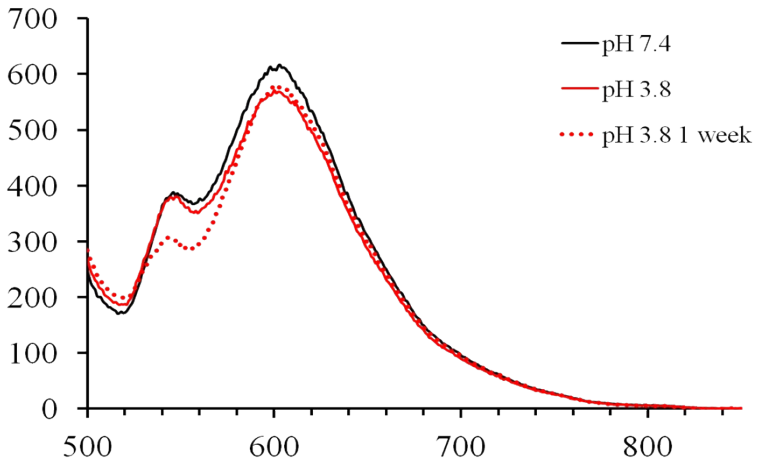


Figure S10 Emission spectra of RuBODIPYNP in PBS whose pH was adjusted to pH 3.8 using 0.1 M HCl, and compared to pH 7.4. The particles were left in aqueous solution at pH 3.8 for 1 week to assess any changes to the emission.

Spectra of RuBODIPYNP in 0.1 mM CTAB solution

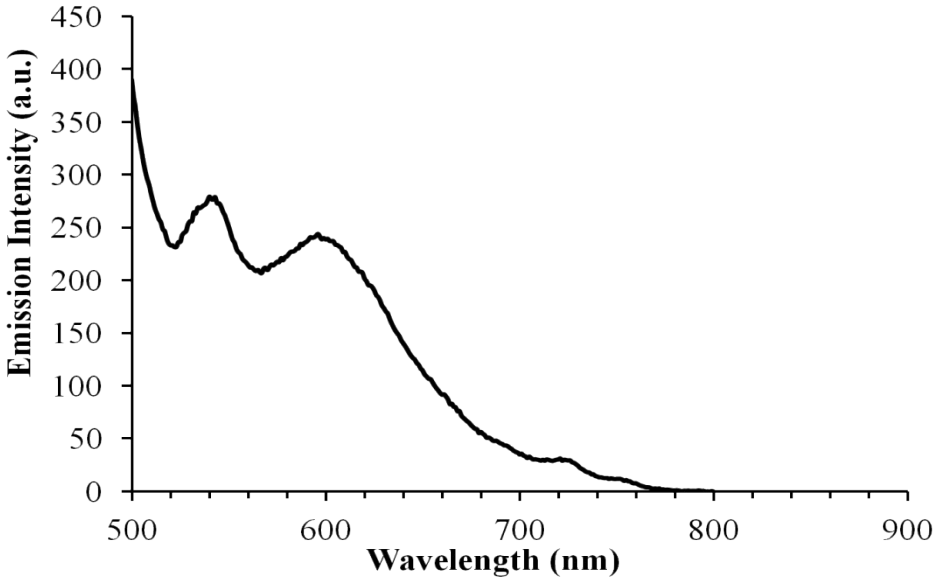


Figure S11 Emission spectrum of RuBODIPYNP in 0.1 mM CTAB/PBS. Solution had been incubated overnight.

Cell Studies with RuBODIPYNP particles

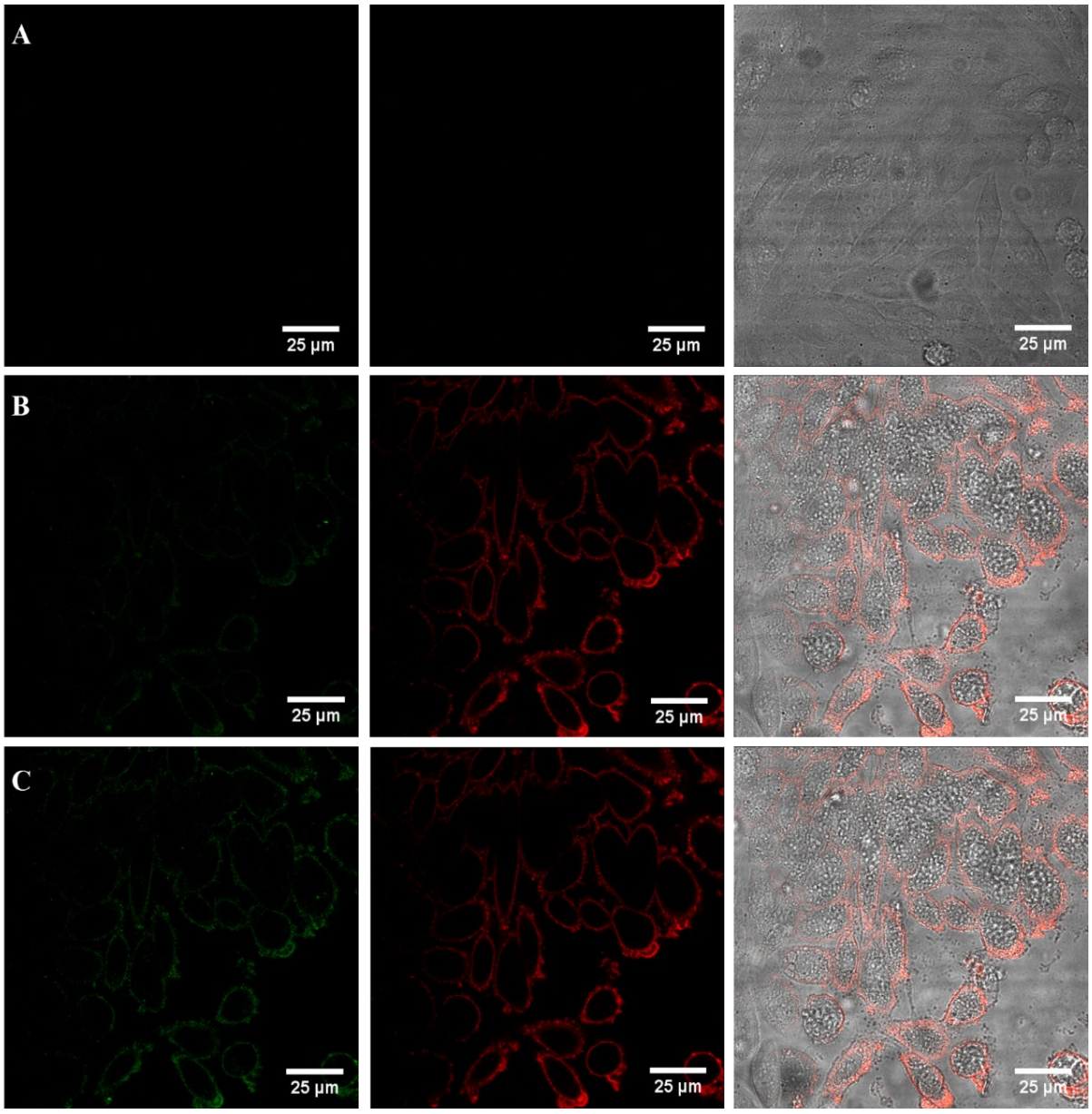


Figure S12 Live cell confocal imaging of RuBODIPYNP pre-treated with 100 µM CTAB prior to exposure to CHO cell membrane. 44µg/mL particles/media added to live CHO cells and imaged after 1 minute of addition (A), and 2 minutes of addition (B). Image of particles after they had been removed after 5 minutes NP exposure and washed and re-introduced to fresh supplemented PBS (C).

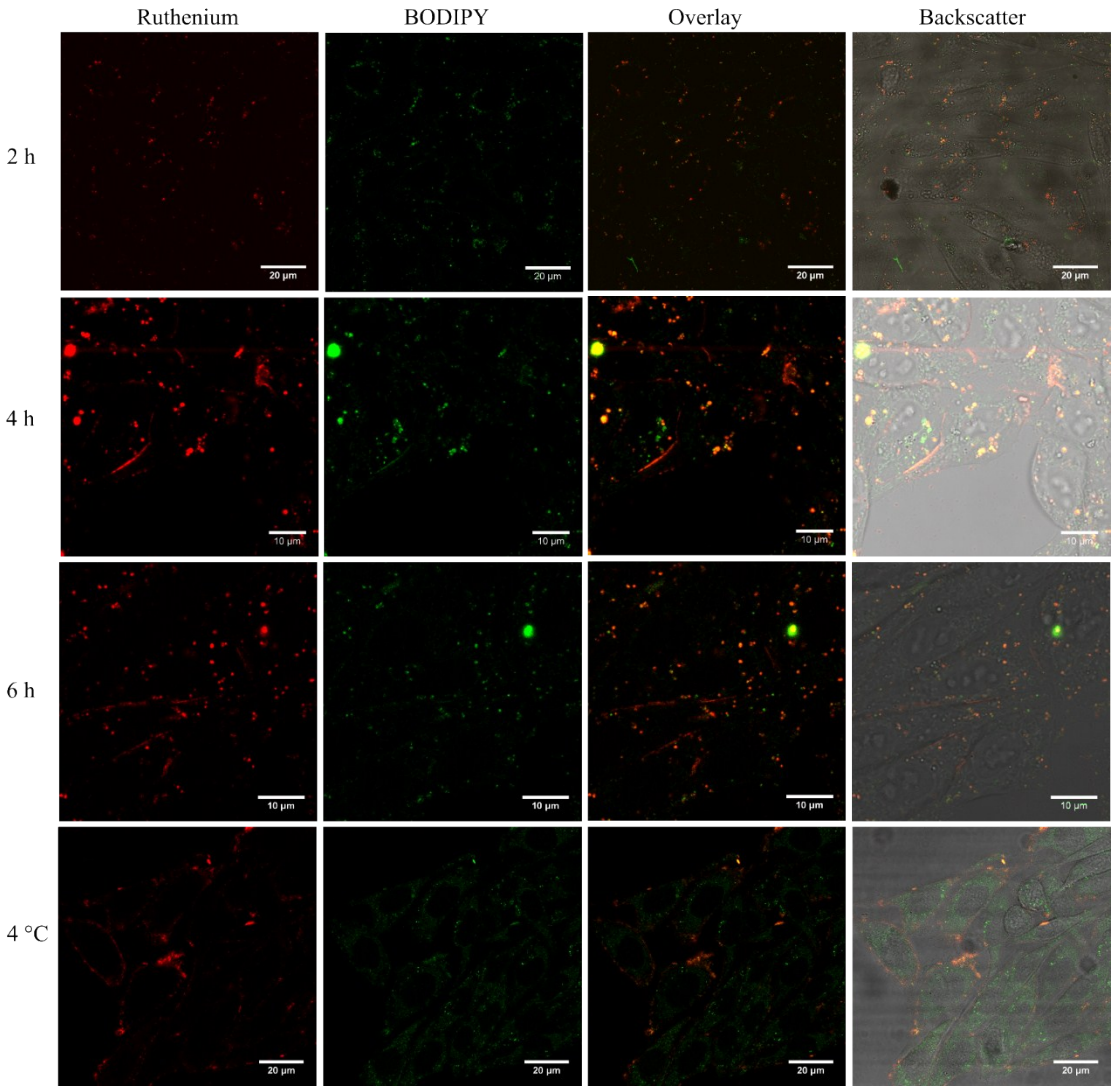


Figure S13 Confocal images of live CHO cells incubated with 44 µg/mL RuBODIPYNP in Lebovitz media for 2 h, 4 h, and 6 h at 37 °C. CHO cells were incubated for 4 h at 4 °C to assess mode of uptake. The particles were excited using a 458 nm white light laser. The BODIPY emission was collected between 505 and 550 nm, and the ruthenium emission was collected using a long pass 605 nm filter.

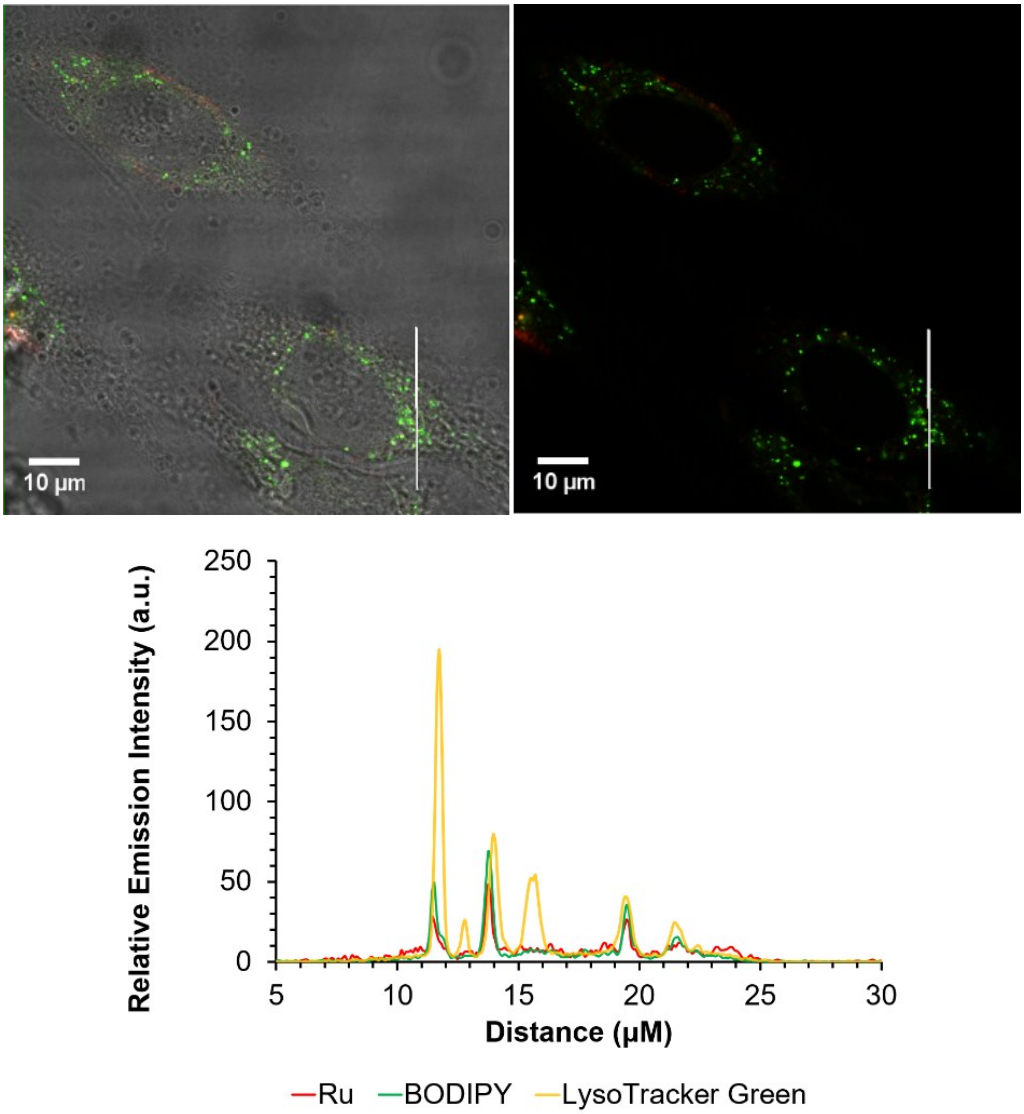


Figure S14 Co-localisation of RuBODIPYNP in CHO cells across the cytoplasm, and the corresponding profile demonstrates the ruthenium (—) and BODIPY (—) channels of the particles, LysoTracker Green (100 nM) in yellow (—).

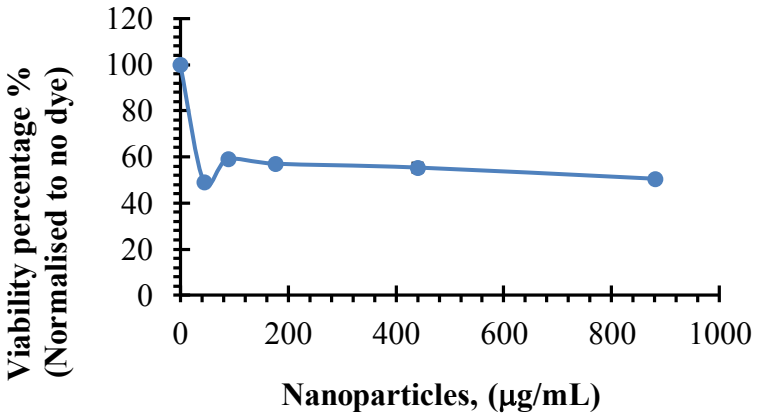


Figure S15 Cytotoxicity of RuBODIPYNP. Particles were added to CHO cells to yield final % v/v concentrations of particles to media and incubated for 24 h in the dark at 37 ºC at 5 % CO_2. CHO viability was assessed using the Resazurin (Alamar Blue) assay (n=3).

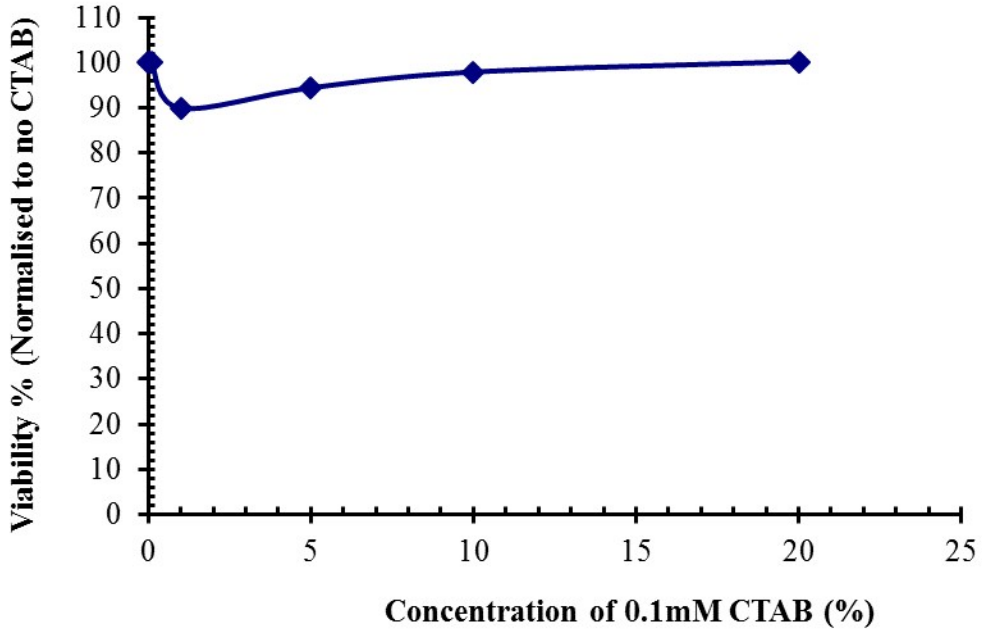


Figure S16 Cytotoxicity control of 0.1mM CTAB solution. The solution was added to CHO cells to yield final % v/v concnetraions of 0.1mM nCTAB to media. The cells were incubated for 24 h in the dark at 37 ºC at 5 % CO_2. CHO viability was assessed using the Resazurin (Alamar Blue) assay.

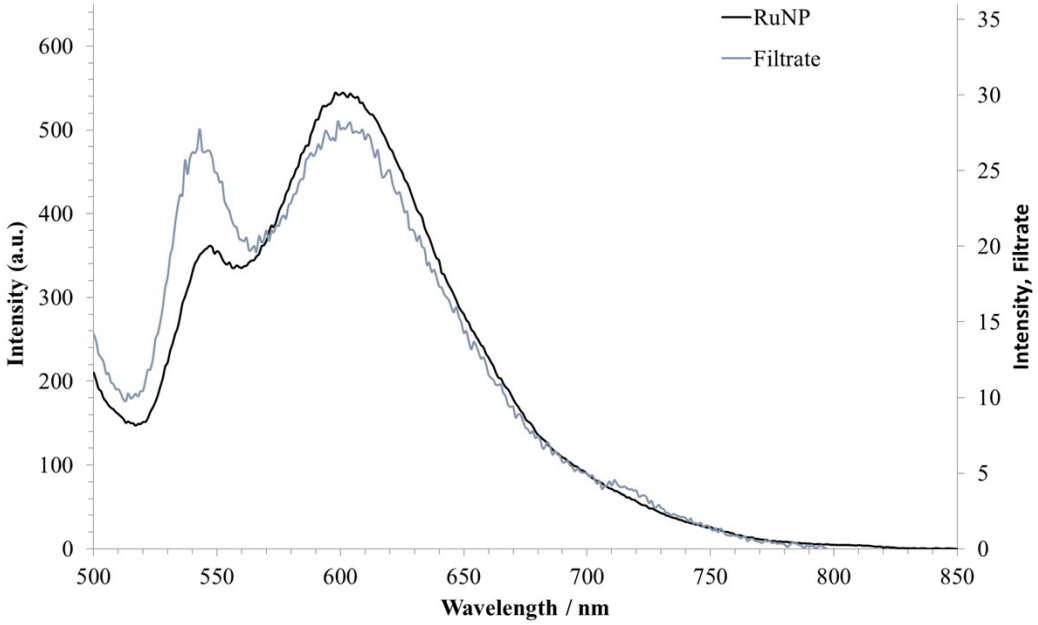


Figure S17 Emission spectra of a 1 month old suspension of a RuBODIPYNP in PBS buffer(–) and from the liquor remaining after centrifugation of the particle suspension 5500 rpm for 5 minutes, (–). It is important to note that the BODIPY derivative does not emit in aqueous solvent and so its presence in the liquor, which is PBS buffer, indicates that centrifugation has not removed all of the particles from the filtrate.

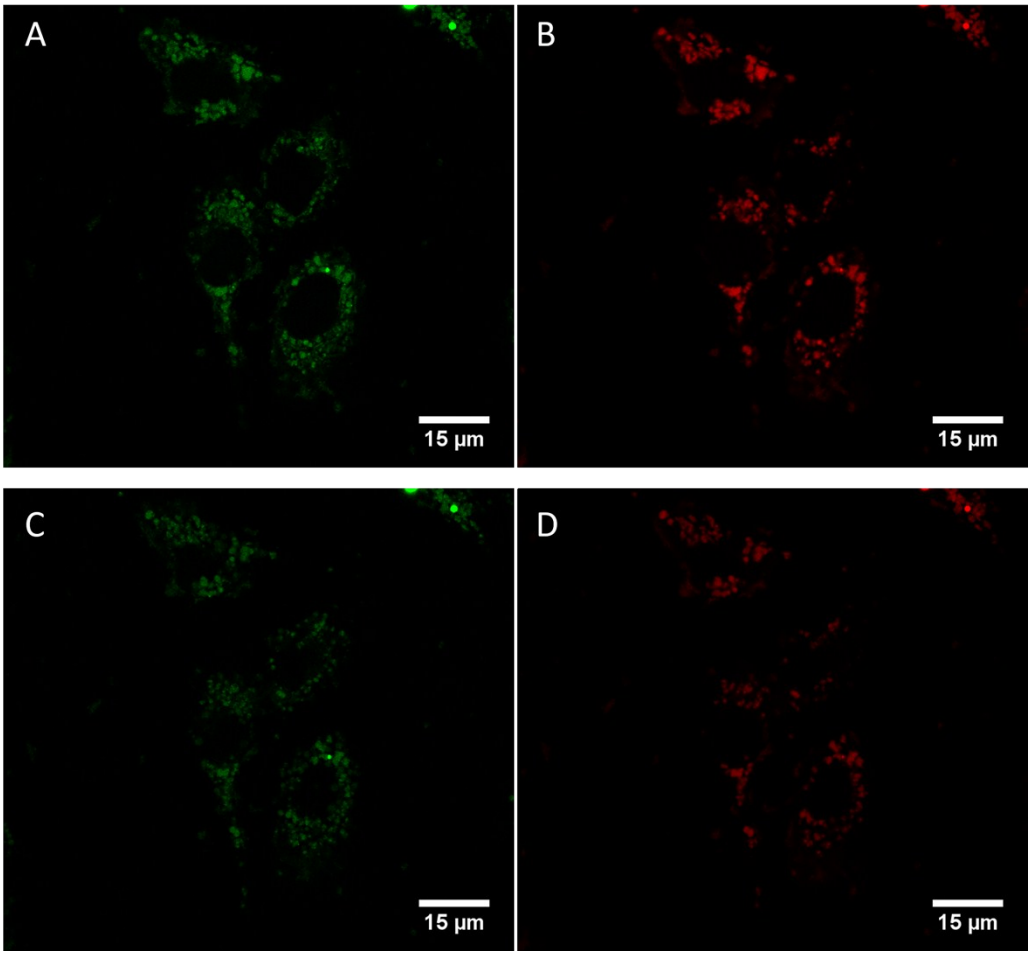


Figure S18 Confocal images of RuNP in HeLa cells showing the BODIPY channel in green (A), and Ru channel in red (B). The cells were imaged every 2 minutes, and a final image at T60 minutes shows there was no evidence of photobleaching in both the BODIPY (C) and Ru (D) channels. The particles were excited using a 458 nm white light laser. The BODIPY emission was collected between 505 and 550 nm, and the ruthenium emission was collected using a long pass 605 nm filter.

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