Supporting Information

Mononuclear Ruthenium (II) Theranostic Complexes that Function as Broad-spectrum Antimicrobials in Therapeutically Resistant Pathogens Through Interaction With DNA.

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S1. Chemistry Methods

S1a. $[Ru(N\text{-}N)_2(DPQ)][PF_6]_2$

Four compounds were synthesised by the following general procedure. $[Ru(N\text{-}N)_2Cl_2]$[1] and DPQ[2] were suspended in a 1:1 solution of EtOH:H_2O. The suspension was refluxed for 12 hours under argon, cooled to room temperature and filtered. NH_4PF_6 was added to form a brown hexafluorophosphate salt.

S1a(1). $[\{Ru(1,10\text{-phenanthroline})\}_2(DPQ)][PF_6]_2$

$[Ru(Phen)_2Cl_2]$ (1.01 g, 1.90 mmol), DPQ (0.495 g, 2.36 mmol) and EtOH:H_2O (50 mL). Mass = 0.910 g (0.946 mmol, 49.8 %). MS (TOF MS LD+) m/z (%): 671 (100) $[M\text{-}2PF_6]^{2+}$, 817 (50) $[M\text{-}PF_6]^+$. ^{1}HNMR (DMSO-d^6) δ (splitting integration): 7.52 (2H, dd), 7.63 (2H, dd), 7.85 (2H, dd), 7.94 (4H, dd), 8.30 (4H, q), 8.37 (2H, dd), 8.51 (2H, dd), 8.62 (2H, dd), 8.73 (2H, dd).

S1a(2). $[Ru(5\text{-methyl-}1,10\text{-phenanthroline})_2(DPQ)][PF_6]_2$

$[Ru(5MP)_2Cl_2]$ (1.00g, 1.78 mmol), DPQ (0.500 g, 2.38 mmol), and EtOH:H_2O (50 mL). Mass = 1.05 g (1.06 mmol, 59.6 %). MS(TOF MS LD+) m/z (%): 350 (85) $[M\text{-}2PF_6]^{2+}$, 846 (25) $[M\text{-}PF_6]^+$. ^{1}HNMR (DMSO-d^6) δ (splitting integration): 2.51 (6H, s), 7.22 (6H, ddd), 7.89 (4H, m), 8.21 (2H, t), 8.28 (2H, d) 8.64 (2H, d), 8.77 (2H, dd), 8.90 (2H, d).

S1a(3). $[Ru(2,9\text{-dimethyl-}1,10\text{-phenanthroline})_2(DPQ)][PF_6]_2$

$[Ru(DMP)_2Cl_2]$ (1.03 g, 1.75 mmol), DPQ (0.495 g, 2.36 mmol), EtOH:H_2O (50 mL). Mass = 0.95 g (0.933 mmol, 53.5%). MS(TOF MS LD+) m/z (%): 364 (100) $[M\text{-}2PF_6]^{2+}$, 874 (85) $[M\text{-}PF_6]^+$. ^{1}HNMR (DMSO-d^6) δ (splitting integration): 2.58 (6H, s), 7.28 (2H, d), 7.46 (2H, d), 7.81 (2H, d), 7.93 (2H, t), 8.16 (2H, d), 8.30 (4H, dd), 8.39 (2H, d), 8.72 (2H, d).

S1a(4). $[Ru(3,4,7,8\text{-tetramethyl-}1,10\text{-phenanthroline})(DPQ)][PF_6]_2$

$[Ru(TMP)_2Cl_2]$ (1.01 g, 1.57 mmol), DPQ (0.495 g, 2.36 mmol) and EtOH:H_2O (50 mL). Mass = 0.861 g (0.801 mmol, 51 %). MS(TOF MS LD+) m/z (%): 784 (51) $[M\text{-}2PF_6]^{2+}$, 929 (100) $[M\text{-}PF_6]^+$. ^{1}HNMR (DMSO-d^6) δ (splitting integration): 2.23 (6H, s), 2.39 (6H, s), 2.79 (6H, s), 2.85 (6H, s), 7.46 (2H, dd), 7.85 (2H, d), 7.65 (2H, s), 7.95 (2H, s), 8.41 (4H, d), 8.48 (2H, d).

S1b. $[Ru(N\text{-}N)_2(tpphz)][PF_6]_2$

Four compounds were synthesised by the following general procedure. 5,6-diamino-1,10-phenanthroline was dissolved in hot methanol, this was added to a boiling solution of $[Ru(N\text{-}N)_2DPQ][PF_6]_2$ in acetonitrile. The reaction mixture was heated to reflux at 80 °C for 6 hours. The solution was cooled to room temperature and filtered. NH_4PF_6 was added to form a red hexafluorophosphate salt. The crude product was washed with water, ethanol and diethyl ether. It was then purified on a grade I alumina column with acetonitrile/water/KNO_3. The red band was collected, the solvent removed under reduced pressure and the red solid dried *in vacuo*.

S1b(1). $[\{Ru(1,10\text{-phenanthroline})\}_2(tpphz)][PF_6]_2$

5,6-diamino-1,10-phenanthroline (100 mg, 0.48 mmol), hot methanol (20 mL), $[Ru(Phen)_2DPQ][PF_6]_2$ (650 mg, 0.71 mmol), acetonitrile (30 mL). Mass = 0.251 g (0.221 mmol, 31.1%). , ^{1}HNMR (CD_3CN-d^6) δ (splitting integration): 7.76 (8H, m), 8.19 (4H, dd), 8.34 (4H, dd), 8.68 (4H, t), 8.80 (2H, d), 9.43 (4H, dd). MS; m/z (%): 991.4 $[M\text{-}PF_6]^+$ (100); 845.4 $[M\text{-}2PF_6]^+$ (35); 423.2 $[M\text{-}2PF_6]^{2+}$ (25). $C_{48}H28N_{10}[^{102}Ru]^{2+}$ Calculated 423.0765. Found 423.0783. Elemental analysis calculated for $C_{48}H_{28}F12N_{10}P_2Ru.5H_2O$: C, 50.76; H, 2.48; N, 12.33. Found C, 50.86; H, 2.28; N, 12.20.

S1b(2). $[Ru(5\text{-methyl-}1,10\text{-phenanthroline})_2(tpphz)][PF_6]_2$

5,6-diamino-1,10-phenanthroline (88.2 mg, 0.42 mmol), hot methanol (17 mL), $[Ru(5MP)_2DPQ][PF_6]_2$ (600 mg, 0.61 mmol), acetonitrile (30 mL). Mass = 0.298 g (0.185 mmol, 28%). ^{1}HNMR (CD_3CN-d^6) δ (splitting integration): 2.58 (6H, s), 7.86 (8H, m), 8.28 (2H, s), 8.39 (6H, dd), 8.55 (2H, s), 8.71 (4H, dd), 8.92 (4H, d). MS; m/z (%): 437 $[M–2(PF_6)]^{2+}$. $C_{50}H_{32}N_{10}[^{102}Ru]^{2+}$ Calculated 437.0922. Found 437.094. Elemental analysis calculated for $C_{50}H_{32}F_{12}N_{10}P_2Ru.4H_2O$: C, 51.60; H, 2.77; N, 12.03. Found C, 51.40; H, 3.01; N, 11.71.

S1b(3). $[Ru(2,9\text{-dimethyl-}1,10\text{-phenanthroline})_2(tpphz)][PF_6]_2$

5,6-diamino-1,10-phenanthroline (100 mg, 0.48 mmol), hot methanol (20 mL), $[Ru(DMP)_2DPQ][PF_6]_2$ (685 mg, 0.67 mmol), acetonitrile (35 mL). Mass = 0.221 g (0.256 mmol, 38%). ^{1}HNMR (CD_3CN-d^6) δ (splitting integration): 2.21 (12H, s), 7.30 (4H, d), 7.82 (4H, d), 7.94 (4H, d), 8.08 (4H, d), 8.23 (4H, d), 8.62 (4H, d). MS; m/z (%): 1047 $[M–(PF_6)]^+$, 451 $[M–2(PF_6)]^{2+}$. $C_{52}H_{36}N_{10}[^{102}Ru]^{2+}$ Calculated 451.1101. Found 451.1098. Elemental analysis calculated for $C_{52}H_{36}F_{12}N10P_2Ru.5.5H_2O$: C, 52.40; H, 3.04; N, 11.75. Found C, 52.43, H, 3.08; N, 11.81.

S1b(4). $[Ru(3,4,7,8\text{-tetramethyl-}1,10\text{-phenanthroline})(tpphz)][PF_6]_2$

5,6-diamino-1,10-phenanthroline (88.2 mg, 0.42 mmol), hot methanol (17 mL), $[Ru(TMP)_2DPQ][PF_6]_2$ (606 mg, 0.56 mmol), acetonitrile (30 mL). Mass = 0.272 g (0.389 mmol, 45%), ^{1}HNMR (CD_3CN-d^6) δ (splitting integration): 2.29 (6H, s), 2.32 (6H, s), 2.80 (6H, s), 2.86 (6H, s), 7.71 – 7.78 (4H, m), 7.84 (4H, s,), 8.15 (4H, d), 9.30 (4H, d), 9.59 (4H, d). MS; m/z (%): 479 $[M–2(PF_6)]^{2+}$. Accurate mass analysis: $C_{56}H_{44}N_{10}[^{102}Ru]^{2+}$ Calculated 479.1391. Found 479.1405. Elemental analysis calculated for $C_{56}H_{44}F_{12}N_{10}P_2Ru.4H_2O$: C, 53.89; H, 3.55; N, 11.22. Found C, 53.80; H, 3.56; N, 11.30.

S1c. $[Ru(N\text{-}N)_2(dppz)][PF_6]_2$

[Ru(N-N)2Cl2], dppz and LiCl were boiled under reflux in ethanol/water (3:1) for 5 hours. The solution was cooled, and the solvent removed by reduced pressure. The residue was dissolved in ethanol (20 mL) and filtered. The product was precipitated by addition of aqueous NH4PF6 and collected by filtration. The crude product was chromatographed on grade l alumina with acetonitrile/toluene (1:1). The product was concentrated by rotary evaporation and recrystalised using a diethyl ether precipitated.

S1c(1). $[Ru(phen)_2(dppz)][PF_6]_2$

Compound was synthesised from an already established procedure.[3]

S1c(2). $[Ru(TMP)_2(dppz)][PF_6]_2$

$[Ru(TMP)_2Cl_2]$ (1.00 g, 1.55 mmol), dppz (1.05 g, 3.71 mmol), LiCl (0.40 g), water/ethanol 3:1 (100 mL). Mass of product 0.92 g (0.80 mmol, 52 %), ^{1}HNMR (CD_3CN-d^6) δ (splitting integration): ^{1}H NMR (CD_2Cl_2) δ (splitting integration): 9.60 (dd, 2H), 8.53 (m, 4H), 8.42 (d, 2H), 8.16 (s, 2H), 8.00 (d, 2H), 7.89 (dd, 2H), 7.76 (m, 4H), 2.14 (dd, 24H). MS; m/z (%): 428 $[M\text{-}2(PF_6)]^{2+}$. Accurate mass analysis: $C_{50}H_{42}N_8[^{102}Ru]^{2+}$ Calculated 428.1311. Found 428.1323. Elemental analysis calculated for $C_{50}H_{42}F_{12}N_8P_2Ru.5H_2O$: C, 52.41; H, 3.69; N, 9.78. Found C, 52.40; H, 3.50; N, 9.80.

S1d. $[\{Ru(3,4,7,8\text{-tetramethyl-}1,10\text{-phenanthroline})_2\}_2(tpphz)][PF_6]_4$

$[Ru(TMP)_2Cl_2]$ (1.12 g, 1.73 mmol) and tpphz (0.260 g, 0.68 mmol), water/ethanol (80 mL). Mass = 1.22 g (0.58 mmol, 85.7 % yield). MS; m/z (%): 911 (10) $[M\text{-}2(PF_6)]^{2+}$, 559 (100) $[M\text{-}3(PF_6)]^{3+}$. ^{1}HNMR (MeCN-d^6) δ (splitting integration): 2.1 (s, 48 H), 7.8 (s, 4H), 7.9 (t, 8H), 8.2 (dd, 4H), 8.4 (s, 8H), 9.9 (dd, 4H). ^{1}HNMR (Acetone-d^6) δ (splitting integration): 2.1 (dt, 48H), 8.0 (m, 4H), 8.1 (s, 4H), 8.2 (s, 4H), 8.52 (d, 4H), 8.6 (s, 8 H) , 10.1 (d, 4H). Elemental analysis $[\{Ru(3, 4, 7, 8\text{-Tetramethyl-}1,10\text{-phenanthroline})_2\}_2(tpphz)]$ $(PF_6)_4.5.5H_2O$, $C_{88}H_{87}N_{14}O_{5.5}Ru_2P_4F_{24}$ Calculated: C; 47.93, H; 3.97, N: 8.89. Found C; 47.92, H; 3.83, N; 8.82. Accurate mass analysis: $C_{88}H_{76}N_{14}[^{102}Ru]_2^{4+}$ Calculated 383.1111. Found 383.1112.

S1e. Anion metathesis

The hexafluorophosphate salt of each complex was dissolved in the minimum volume of acetone and a saturated solution of ammonium chloride in acetone added. The resultant precipitated chloride salt was collected by filtration, washed with cold acetone and dried in vacuo.

S1f. Photochemistry

S1f(1). Absorption spectra

An emission quartz cuvette was loaded with varying concentrations of the $[Ru(TMP)_2(tpphz)]$ chloride salt dissolved in water (0-20 μM). Each reading was baseline corrected. The absorption spectra at 200-800 nm were obtained with a Cary 500 Scan UV-vis-NIR Spectrophotomer, set to double beam mode (spectral band width = 2 nm), with a medium scan speed of 600 nm min^{-1}.

S1f(2). Molar extinction coefficients

Molar extinction coefficients were calculated using the Beer-Lambert law. They were calculated from a plot of measured absorbance against sample concentration.

$A = \varepsilon cl$ *Equation 5.1: Beer-Lambert Law*

S1f(3). Excitation and emission spectra

A fluorescence quartz cuvette was loaded with varying concentration of the $[Ru(TMP)_2(tpphz)]^{2+}$ chloride salt dissolved in water (0-20 μM). The excitation and emission spectra were recorded using a Fluoromax-3 Spectrophotometer, slit width 5 nm, scan speed 100 nm min^{-1}. The emission spectra were taken at the 424 nm peak seen in the absorption spectra.

S1g. DNA binding studies

S1g(1). DNA preparation

A tris aqueous buffer was produced (25 mM NaCl, 5 mM Tris, pH = 7). CT-DNA was dissolved in tris buffer. Sonication (2 × 15 minutes) was used to break down the CT-DNA into an average of 150-200 base pair fragments. The sample purity was determined by UV-vis spectroscopy with A260/A290 > 1.9 which indicates a protein free sample. The concentration of the CT-DNA samples was determined by UV-vis spectroscopy ($\varepsilon_{260\,nm}$ = 13200 mol L^{-1} cm^{-1}).

S1g(2). Luminescence titration

A tris aqueous buffer was produced (25 mM NaCl, 5 mM Tris, pH = 7) and a 5 μM solution of the $[Ru(TMP)_2(tpphz)]$ was dissolved into the buffer. This buffer solution was loaded into a quartz cuvette and equilibrated at 25 °C for 30 minutes. The emission spectra were recorded. A 200 μM stock solution of CT-DNA was made up and 10 μL was added to the buffer solution and mixed with a pipette. The emission spectrum was recorded showing an enhancement in emission and this was repeated until the emission became constant. The emission intensity for each addition of DNA was calculated using the Flouoromax-3 Spectrophotometer software Origin.

S1h. Determination of LogP

Relative lipophilicities or partition coefficients of 1^{4+} - 4^{4+} across l-octanol and H_2O ($\log P_{oct/wat}$) were determined via the shake-flask method.[5] Aqueous stocks of 1^{4+} - 4^{4+} were prepared at 200 and 300 μg mL^{-1} were added to equal volumes of 1-octanol that had been pre-saturated with H_2O for 36 h. After incubation with shaking at 37 °C for 24 h, the 1-octanol and aqueous phases were recovered, and the relative distribution of each compound was determined by UV-vis absorbance spectroscopy at 430 – 455 nm.

S2. Microbiology and Microscopy Methods

S2a. Bacterial strain information and general growth procedures

Microbiological studies were conducted with: (1) wild-type K-12 derivative laboratory strain *Escherichia coli* MG1655; (2) CTX-M-15 type extended spectrum β-lactamase (EBSL)-producing clinical isolate *E. coli* EC958 (ST131)[4] (3) *Enterococcus faecalis* vancomycin-resistant V583 clinical isolate (ATCC 700802):[5] (4) wild-type laboratory strain *Staphylococcus aureus* SH1000; (5) a clinical isolate strain of *Pseudomonas auerginosa* PA2017 (University of Surrey) and (6) a clinical isolate strain of *Acetinobacter baumannii* AB184. Bacteria were routinely grown under aseptic, aerobic conditions in autoclave-sterilised culture medium at 37 °C. Lysogeny Broth (LB) (Formedium), Mueller-Hinton II (MH-II) (Sigma-Aldrich) and Brain-Heart Infusion (BHI) (Sigma-Aldrich) were prepared as per manufacturers' instructions. Glucose defined minimal medium (GDMM) was prepared by dissolution of 4 g L^{-1} K_2PO_4, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl, 10 mg L^{-1} $CaCl_2$ and 2.6 g L^{-1} K_2SO_4 in deionized H_2O with supplementation of 10 mL L^{-1} Trace Elements solution. The pH was adjusted to 7.4 by the addition of NaOH. GDMM was sterilised by autoclaving and then further supplemented with 1 mM $MgCl_2$ and 20 mM glucose prior to growth studies. Trace elements contained 5 g L^{-1} ethylenediaminetetraacetic acid (EDTA), 0.5 g L^{-1} $Fe(III)Cl_3.6H_2O$, 50 mg L^{-1} ZnO, 10 mg L^{-1} $CuCl_2.2H_2O$, 10 mg L^{-1} $CoNO_3.6H_2O$, 10 mg L^{-1} H_3BO_3, 0.12 mg L^{-1} $(NH_4)_2MoO_4$ and 17 mg L^{-1} Na_2O_4Se.

Prior to experiments, bacterial starter cultures were prepared by inoculating LB (*E. coli*) or BHI (*E. faecalis*) with a single colony of bacteria and then grown for overnight at 37 °C with shaking for 16 – 18 h. For *E. coli* EC958 and *E. faecalis* V583, starter cultures were additionally supplemented with 50 μg mL^{-1} ampicillin or 10 μg mL^{-1} vancomycin. Starter cultures were washed once and resuspended in the appropriate growth medium for each experiment. For short-term storage, bacterial stocks were maintained on nutrient agar plates at 4 °C for 2 – 3 weeks. For long-term storage, strains were stored as cell suspensions in 30 % (v/v) LB 70 % (w/v) glycerol at – 70 °C.

S2b. Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

MICs and MBCs of $\mathbf{1}^{4+}$ - $\mathbf{4}^{4+}$ and ampicillin (comparison) were determined via the standard broth-dilution method in 96-well microtitre plates in either MH-II, as recommended by European Committee of Antimicrobial Susceptibility Testing (EUCAST) or in GDMM. For *E. faecalis*, both media were further supplemented with 10 % (v/v) BHI to facilitate growth. The MIC of each compound was evaluated using 2-fold increasing concentrations of each compound between 2 - 512 μg mL^{-1} against a bacterial inoculum of 10^7 – 10^9 colony forming units per mL (CFU mL^{-1}), corresponding to an optical density at 600 nm (OD_{600}) of 0.05 – 0.075. Plates were incubated at 37 °C for 20 h. After this time, the level of turbidity in each well was used to determine the extent bacterial cell growth in the presence of the compounds. The minimal concentration of compound that did not permit bacterial growth was determined to be the MIC. For MBC determination, 10 μL samples of each well were then transferred to nutrient agar plates and further incubated at 37 °C. The lowest concentration of compound in which no CFU were observed after plating was determined to be the MBC. MIC/MBCs were determined from 3 independent biological repeats.

S2c. Time-kill assays

E. coli MG1655 and EC958 strains were grown on GDMM to early exponential phase, diluted to approximately 10^8-10^9 CFU mL^{-1} and 4^{4+} was added at 0 – 25.6 μM (0 - 8 x MIC). Immediately prior to mono-TMP addition, and at 1-6 h thereafter, culture growth (turbidity at OD_{600}) and viability (CFU mL^{-1}) were measured. Cell viability was determined via the standard viable counts method, which is based on the ability of a single viable bacterial cell to form a colony when grown on agar plates. 10-fold serial dilutions of cell culture samples were prepared in sterile PBS and then 3 x 10 μL of each dilution was spotted onto nutrient agar plates. Plates were then incubated overnight at 37 °C to permit colony growth and cell viability was determined as the average number of counted CFU mL^{-1}. Time-kill assays were performed as three independent biological repeats.

S2d. Single stain microscopy

S2d(1). Preparing the culture

An overnight culture of bacteria (EC958) was grown in GDMM. The cells were diluted to OD_{600}-0.05 and grown to OD-0.3-0.4 in GDMM (50 mL). 1 mL of culture was harvested, and the compound added at MIC concentration. 1 mL cultures were then harvested at 10, 30 and 60 minutes. The bacteria was pelleted (centrifuge, 14,000 RPM, 90 seconds), and supernatant removed. The pellet was suspended in fixant (16% paraformaldehyde, 500 μL) and Milli-Q water (500 μL), and placed on a rotary wheel at room temperature for 30 minutes. Samples were frozen as a pellet.

S2d(1). Preparing the slides

Coverslips were sonicated in 1M KOH for 15 minutes, then coated in polylysine solution for 30 minutes. The pellets were suspended in PBS, and 5 μL was dried onto the middle of the coverslip using nitrogen gas. Coverslips were washed with distilled water and mounted onto slides using a SlowFade Gold Antifade Mountant. Slides were imaged using the structured illumination (SIM) microscope. Imaging was done using the 1514 immersion oil, and mol_probes microscope setting. A phase contrast image was taken (DIC) and the 405 nm laser used to excite the compound, luminescent images were collected in the A568 channel. OMX SI reconstruction was performed on images.

S2e. Uptake of $\mathbf{4}^{4+}$ by *E. coli* and determination of cellular ruthenium and iron levels

Uptake and cellular accumulation of $\mathbf{4}^{4+}$ by *E. coli* EC958 was determined by measuring bacterial cell metal content by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) as follows. *E. coli* EC958 cultures were grown to mid-

exponential phase in LB broth, washed in PBS and then resuspended in PBS +/- 20 mM glucose to approximately 10^8-10^9 CFU mL^{-1}. 4^{4+} was added to cells at MIC level (1.6 μM) and then 20 mL samples of culture were harvested at 5, 10, 20 and 60 min after 4^{4+} addition. Samples of non-4^{4+} treated cells were also taken for comparison. Samples were centrifuged at 5,000 x *g* for 20 min at 4 °C to obtain cell pellets and the supernatant containing unbound extracellular 4^{4+} was discarded. The resultant cell pellets were then washed twice in 0.5 % (v/v) Aristar nitric acid to remove loosely bound residual 4^{4+}. To prepare cell material for ICP-AES, cell pellets were resuspended in 0.5 mL Aristar nitric acid (69 % (w/v) and then placed in a sonicator bath for 30 min to completely dissolve cells. The resulting digest was then diluted to a final volume of 5 mL with diluted nitric acid and then samples were analysed on a Spectro CirosCCD (Spectro Analysis) Inductively-Coupled Plasma-Atomic Emission Spectrophotometer. Levels of Ru and Fe in the samples were determined by a calibration curve using multi-element standard solutions containing 0.1, 0.2, 5 and 10 mg L^{-1} Ru and Fe.

S2e. Membrane damage ATP release assays

Loss of *E. coli* membrane integrity following 4^{4+} exposure was determined by leakage of ATP, an intracellular marker, from *E. coli* cells.[6] Levels of extracellular ATP of *E. coli* grown with or without 4^{4+} were determined as follows. *E. coli* EC958 cells were grown to mid-exponential phase in GDMM and then 4^{4+} (0 – 1.6 μM) was added to cultures. Immediately prior to, and at time-intervals after 4^{4+} addition, samples of cell culture were harvested by centrifugation at 15, 000 x *g* for 5 min at 4 °C to remove cells. Supernatants were recovered and retained at – 20 °C for ATP analysis. ATP analysis was conducted using the bioluminescence-based Molecular Probes' ATP Determination kit (Invitrogen, Fisher Scientific). The levels of ATP in super-natants were derived via a standard curve of ATP standards from 1 nM - 1 μM. Luminescence measurements of ATP standards and culture supernatants were measured in duplicate on a Lumat[3] Luminometer (Berthhold Technologies, UK).

S2f. Galleria Mellonella toxicity screen

TruLarv™ Galleria Mellonella were used for this study to ensure they were reared without antibiotics and were all a similar weight. For each compound concentration 7 Galleria were used, and for the control 15 Galleria were used. Insects were injected on the initial day with 10 μL of the correct concentration stock solution of 4^{4+} or water (control) into their left pro-leg. Once injected Galleria were stored in a petri dish containing filter paper and incubated at 37.5 ºC. Three analysis tests were conducted at 0, 24, 48, 72, 96 and 120 hours. Activity scores were recorded: 0-no movement, 1-corrects itself, 2-movement on stimulation, 3-movement without stimulation. Live/dead scores recorded to produce percentage survival curves. Melanisation was scored on a scale of 0-4: 0 – completely black, 1 – black spots, 2- tail/line black and 4 – none. Cocoon formation was not observed in this case. At the end of the toxicity screen Galleria were disposed of in a humane manner.[7]

S2g. Baclight™ membrane potential assay

BacLight Membrane Polarization Assay. Membrane polarization was studied using the Mol Probes BacLight polarization kit. Overnight cultures of EC958 were grown as previously described. Cells were grown to the exponential phase (OD: 0.4) in GDMM at 37.5 °C. One solution of bacteria was treated with 4^{2+} (3.2 μM) and incubated for 60 min at 37.5 °C. The other solution was the untreated control. OD_{600} was measured, and cells were diluted in PBS to a final concentration of 10^6 CFU/mL. Four flow cytometry tubes were prepared to sample. A,B: 1 mL of the cells treated with 1^{2+} and the unstained control were incubated with 10 μL of DiOC2(3) (3 mM) for 30 min in the dark in separate flow cytometry tubes. The membrane polarization positive control tube was prepared with 10 μL of carbonyl cyanide 3- chlorophenylhydrazone (CCCP) (500 μM) 10 μL of DiOC 819 2(3) (3mM); the cells were incubated for 30 min in the dark before reading. In addition, a fourth control was prepared in the absence of DiOC2(3) on 4^{2+} stained cells. Readings were taken on an LSR II flowcytometer, and data were analyzed using the FlowJo software.

S2h. Dual-stain microscopy

S2h(1). NHS-ester co-stain

Initial pellets were grown, treated with 1^{4+} and fixed as before. After fixing the sample was washed with PBS. Cells were resuspended in PBS (250 μL), and NHS-ester 405 was added (50 μg/mL). Samples were incubated on a rotary wheel at room temperature for 5 min. Cells were pelleted (14 000 rpm, 90 s) and washed with PBS (3 washes). Cell suspensions were mounted as described previously. Laser 405 nm, emission filter DAPI. OMX SI reconstruction, and OMX SI image registration was performed on the images, and the images were processed and analyzed using the FIJI ImageJ software.

S2h(2). DAPI co-stain

Initial pellets were grown and fixed as before. After fixing the sample was washed with PBS. Cells were suspended in 300 µL of DAPI (300 nM) in PBS. Samples were incubated on a rotary wheel at room temperature for 5 minutes. Cells were pelleted (14,000 RPM, 90 seconds) and washed with PBS (3 washes). Cell suspensions were mounted as previous. Laser 405 nm, emission filter DAPI. OMX SI reconstruction and OMX SI image registration was performed on the images, and the images were processed and analysed using the FIJI Image J software.

S2i. Transmission electron microscopy

Cell pellets up until fixation were prepared in the same way as the single-stain super-resolution microscopy samples. The cells were fixed using 3% glutaraldehyde. Cells were dehydrated using a series of ethanol washes (70−100% ethanol) and TEM samples sectioned in Araldite resin by microtome. Samples were examined on a FEI Tecnai instrument operating at 80 kV equipped with a Gatan 1 K CCD camera. Images were processed and analyzed using FIJI ImageJ software.

S2j. Hyvolution Stimulated Emission Depletion (STED) microscopy

HyVolution and STED Microscopy. Initial pellets were grown, fixed and mounted as previously discussed. Imaging was done on a commercial LEICA SP8 3X gSTED SMD confocal microscope (Leica Microsystems, Manheim, Germany), with capability of also performing high-resolution microscopy via Hyvolution. The STED nanoscope is equipped with 3 depletion lines and it is also equipped with a 3D STED additional vortex to obtain higher spatially resolved images in XY and Z (enhanced axial super-resolution). The excitation laser beam consisted of a pulsed (80 MHz) supercontinuum white light laser (WLL). For a cleaner emission the excitation lines had a cleanup notch filter (NF) in the optical pathway. Images were taken using the pulsed White Light Laser (WLL) line excitation at 475 nm and recording the emission between 600 and 650 nm. The pulsed STED images were taken with a pulsed 775 nm depletion Laser, again in every case the respective NF were in place. The objective employed was a Leica 100×/1.4 NA oil objective. The pinhole was set at one Airy unit. The gated HyD detectors were set with the gated option on and the temporal gated selected was from 2 to 6.5 ns. For the 3D STED images, the depletion lasers were split in two, the second vortex was set at 65%. For the Hyvolution mode, the pinhole was set at 0.5 Airy units. Every image was acquired in the Leica HyD photon counting detectors. The Hyvolution technology, developed by Leica Microsystems with the deconvolution Huygens software company (SVI, Netherlands), employs a reduced pinhole size at the detection pathway for the image acquisition. The image is later deconvoluted using the Huygens software package. The deconvolution software uses the raw data information from the Leica acquisition files for an optimal deconvolution, for 3D objects we employed a theoretical PSF, as it automatically counteracts the possible aberrations caused for in-depth imaging. The single plane images were deconvolved with a theoretical calculated PSF provided by the software and by an experimentally calculated PSF from fluorescent beads; in both cases no apparent difference was observed. Image analysis and deconvolution of STED images resolved the localization and distribution of the organometallic dye at the bacteria. As expected the images had low intensity counts; to maximize signal-to-noise we deconvolved the images using a commercially available software (Huygens package software, SVI, (Netherlands). To quantify the background level of noise we used either an automated quantification provided by the software or a manual by means of computing the averaged background intensity from regions outside the cell. We obtained better results with the manual process. For the deconvolution we used 40 iterations, a signal-to-noise ratio of 15, and the classical maximum likelihood estimation method provided by the software. The quantification of the resolution obtained at specific position on the bacteria was done employing the full width at half-maximum (fwhm) method. The intensity line profiles over a distance drawn at the bacteria were represented using OriginPro (OriginLab Corporation, Northampton, MA), and the calculation was done by fitting the peaks to a Gaussian function. The same software package was used for data plotting and analysis. The data was represented in form of normalized distribution population. The figures have been developed employing the free open source software Inkscape. The surface rendered images were obtained by Huygens Professional, LAX software (Leica SP8) to generate the surface rendered 3D STED images, and the orthogonal views; the rest of the images have been post processed with Fiji (ImageJ; NIH).

S3. Supplementary data

S3a. UV-Vis absorption data

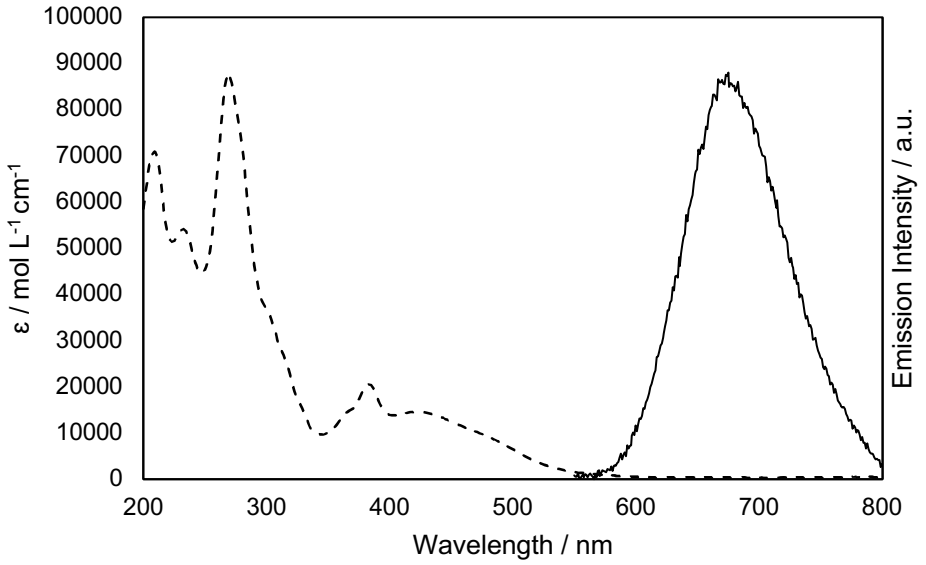


Figure 1. *Absorption (- - - -) (Cary300 spectrophotometer) and emission (——) (Fluoromax3 fluorimeter) spectra for compound 4^{2+} (0-20 μM) between 200-800 nm, collected in water at 37.5°C.*

Complex	Solvent	Λ_{max}/nm	ε/$M^{-1}cm^{-1}$	Transition
$\mathbf{1}^{2+}$	Water	424	14606	MLCT
$\mathbf{1}^{2+}$	Acetonitrile	420	13510	MLCT

Figure 2. *UV-Vis spectroscopy data showing the molar extinction coefficient and absorption maxima 1^{4+} in water and MeCN. Conducted on a Cary 300 UV/Vis spectrophotometer at 27.5 °C.*

S3b. Emission data

Complex	Solvent	Λ_{max}/nm (MeCN)	Λ_{max}/nm (water)
$\mathbf{1}^{2+}$	Water	670	650

Figure 3. *Luminescent emission data showing the emission maxima for 1^{4+} in water and MeCN. Conducted on a Fluoromax 3 fluorimeter at 27.5 °C.*

S3c. DNA binding studies

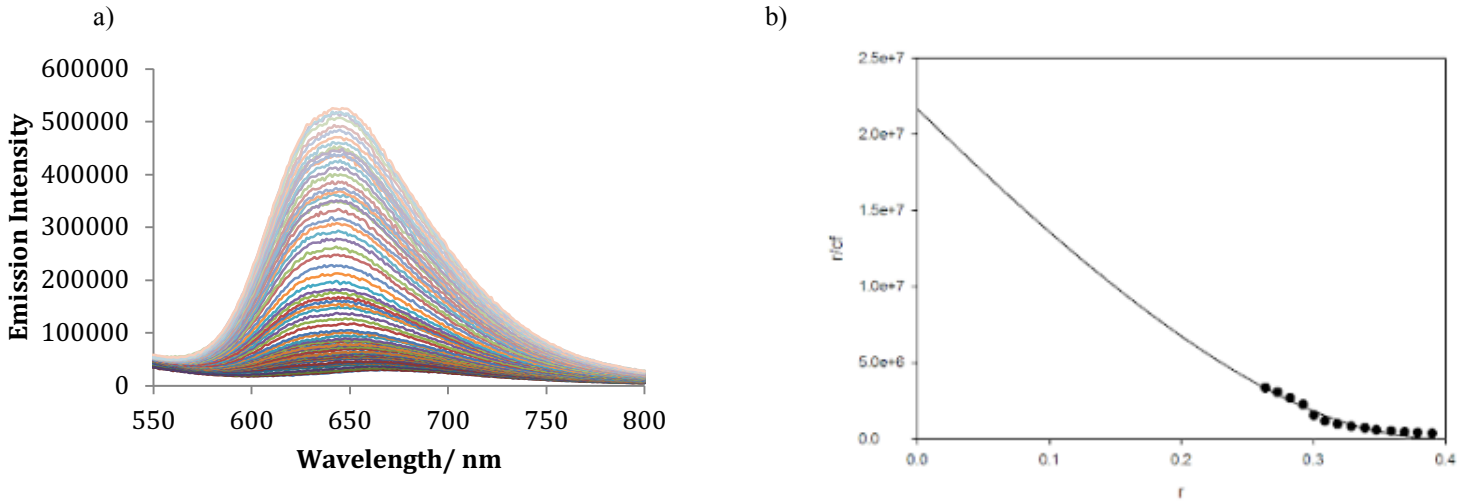


Figure 4. *DNA binding studies data for [{Ru(3,4,7,8-tetramethyl-1,10-pheananthroline)$_2$}$_2$(tpphz)][Cl]$_4$, collected using Calf-thymus DNA on a Fluoromax 3 fluorimeter at 27.5 °C in Tris Buffer 5mM. a) the emission spectra collected at increasing concentrations of DNA. b) McGhee Von Hippel plot to determine K_b – 2.4 x 10^6 M^{-1}*

Complex	I_b/I_f	K_b/M^{-1}	n
TMP	10	2.4×10^6	0.92 ± 0.02

Figure 5. *DNA binding constant K_b (M^{-1}) and site size n for compounds 1^{2+} collected using Calf-thymus DNA on a Fluoromax 3 fluorimeter at 27.5 °C in Tris Buffer 5mM.*

S3d. Comparison of LogP and MIC

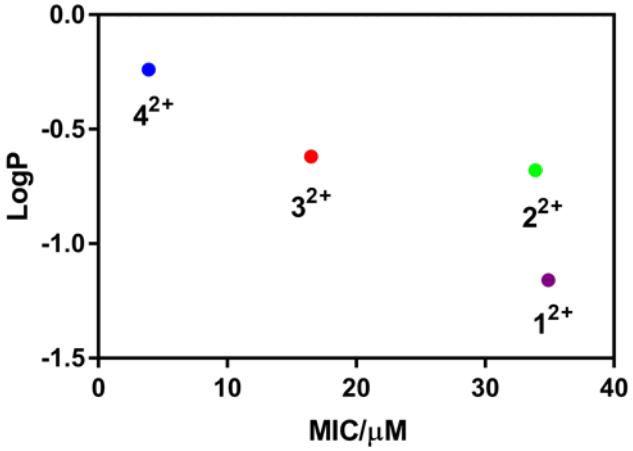


Figure 6. *A comparison of the LogP values determined and the activity (MIC) of each complex against EC958 (E. coli), to show activity increases with relative increase in lipophilicity. LogP data was collected using the shake flask method.*

S3e. Uptake experiments accessory data

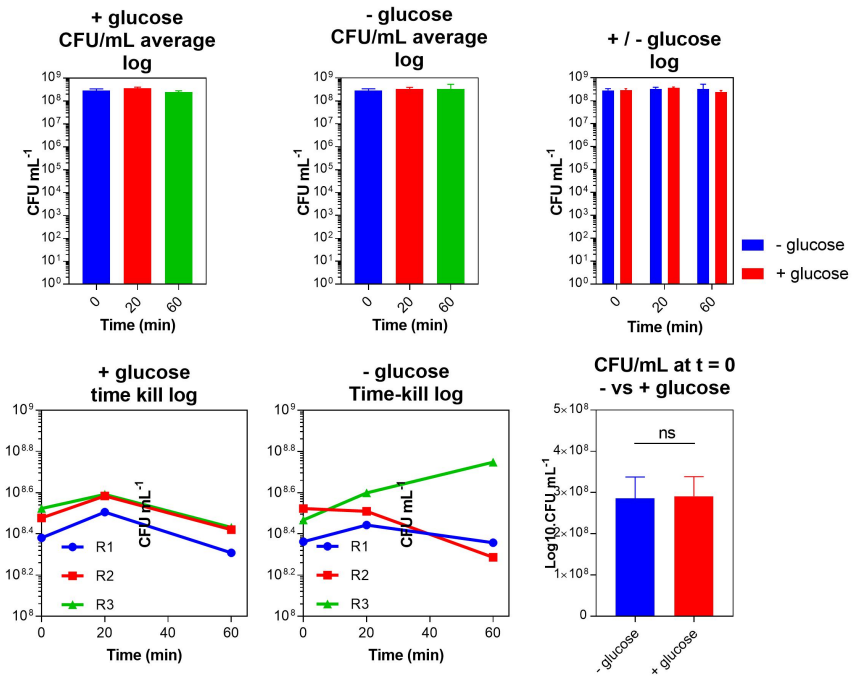
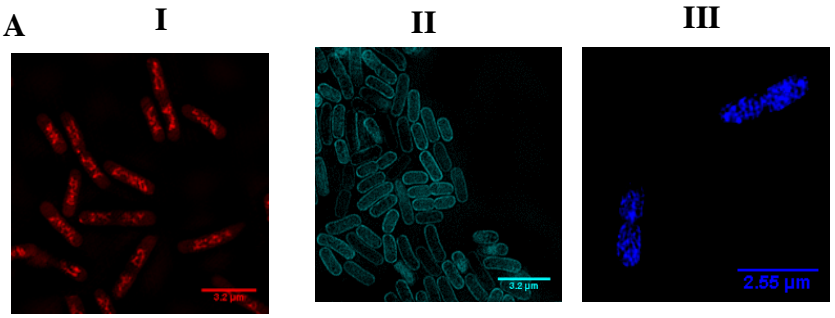
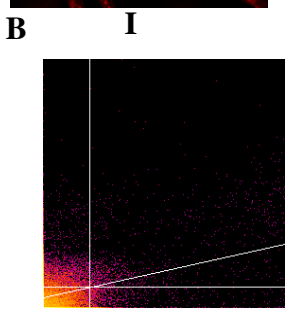


Figure 7. *Top: CFU/mL average at each uptake time-point in the presence and absence of glucose with a comparison (right). EC958 cells were diluted in PBS and plated on Nutrient Agar: incubated at 37.5°C overnight. Bottom: Time-kill assay in the absence and presence of glucose over 60 minutes. EC958 cells were grown in GDMM with incubation at 37.5°C.*

S3f. NHS-ester and DAPI co-staining experiments





Co-stain	Pearson's coefficient	Correlation
DAPI	0.695 (P = 1.00)	Strong
NHS-ester	0.055 (P = 0.95)	None

Figure 8. *(A) Localization of* $\mathbf{4}^{4+}$ *(i), NHS-ester 405 (ii) and DAPI (iii) in E. coli EC958 cells visualized through SIM 60 min. (i) excitation 450 nm, A568 filter; (ii) excitation 405 nm, DAPI filter; (iii) excitation 405 nm, DAPI filter. (B) Colocalisation threshold* $\mathbf{4}^{2+}$ *and DAPI (i) and Pearson's colocalisation constant for* $\mathbf{1}^{4+}$ *and DAPI (ii). After treatment with 3.6 µM* $\mathbf{4}^{4+}$*, cells were washed with PBS before fixing with paraformaldehyde (16%).*

S3g. ATP release assay accessory data

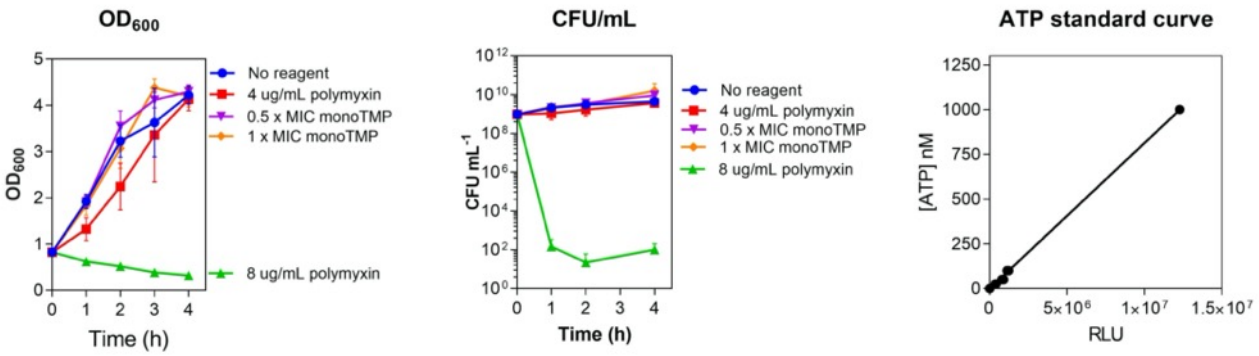


Figure 9 . *Left: OD_{600} readings, centre: CFU/mL readings, over 4 hours for cells treated with polmyxin (4, 8 µg/mL), monoTMP (0.5, 1 MIC) and no reagent. EC958 incubated at 37.5 °C in GDMM, for CFU counts cells were diluted in PBS, plated onto Nutrient Agar and incubated overnight at 37.5 °C. Right ATP standard curve from ATP (1 nM - 1 µM) standards, luminescence read on a Lumat3 Luminometer (Berthhold Technologies, UK).*

S3h. BacLight membrane potential assay

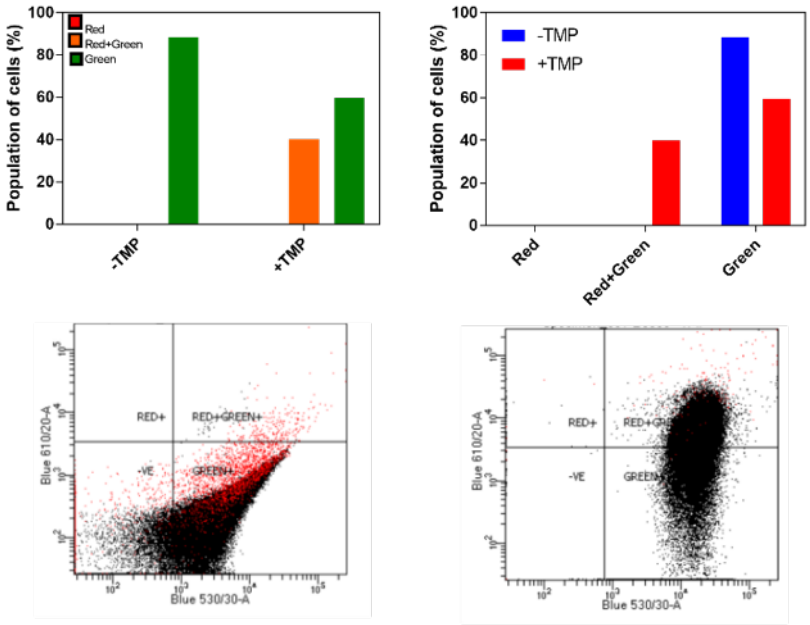


Figure 10. *A) Detection of membrane potential in E. coli EC958 cells. Percentage population of cells (%) containing red/red+green/green fluorescence are given. Cells were incubated with 30 µM of DiOC2(3) for 30 minutes in the presence or absence of 3.9 µM of* ***4***$^{2+}$ *(B) The analysis of the flow cytometry data using red and green fluorescence parameters. The red-versus-green fluorescence dot plots were collected with log amplification.*

S3i. Cellular uptake of $\mathbf{4}^{2+}$ by *E. coli* EC958 cells

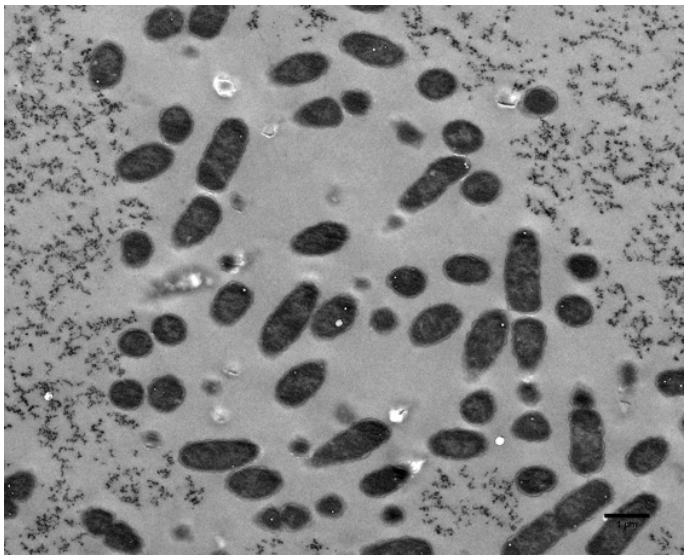


Figure 11. *TEM image showing* $\mathbf{4}^{2+}$ *cellular contents leaked from the cell.* Regions around the cells stained with $\mathbf{4}^{2+}$ show distinctively less compound stained intracellular contents.

S3j. Transmission electron microscopy (TEM)

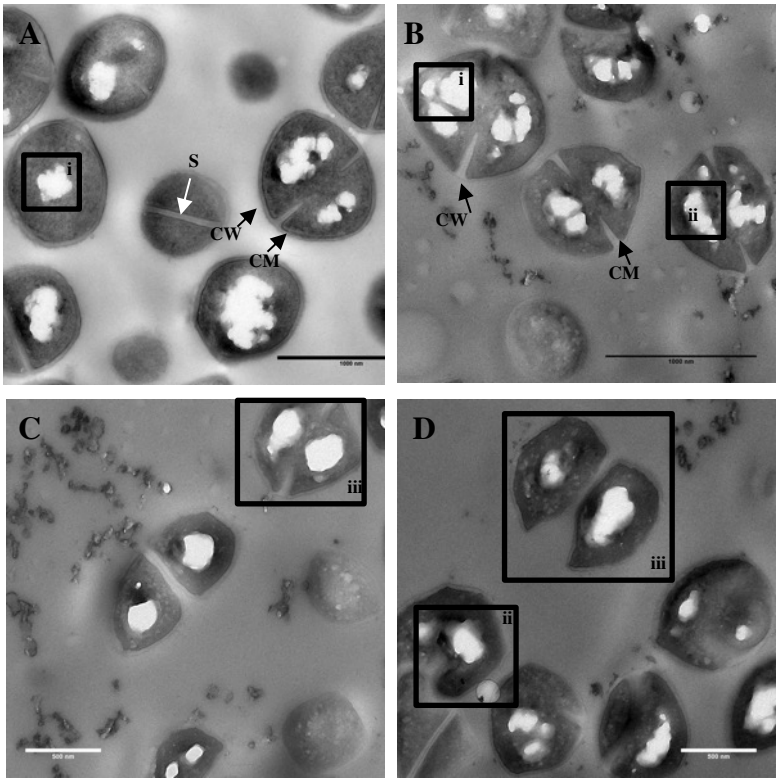


Figure 12. *Transmission electron microscopy –TEM – imaging. Localisation of* $\mathbf{4}^{2+}$ *in S. aurues SH1000 cells at 0 (A), 10 min (B), 60 min (C) and 120 min (D) following treatment with MIC concentrations of* $\mathbf{4}^{2+}$*, directly using the complex as a contrast agent to show accumulation of* $\mathbf{4}^{2+}$ *in the cytoplasm, inner-membrane and cellular DNA. Condensed DNA (i),* $\mathbf{4}^{2+}$ *localisation around condensed DNA (ii), and osmotic morphological changes (iii) are identified. The cell septum (S), cell wall (CW) and cell membrane (CM) are labelled. Cells were fixed with glutaraldehyde and the control was stained with osmium tetroxide, uranyl and led acetate.*

S3k. *Galleria* mellonella percentage melanisation and activity scores

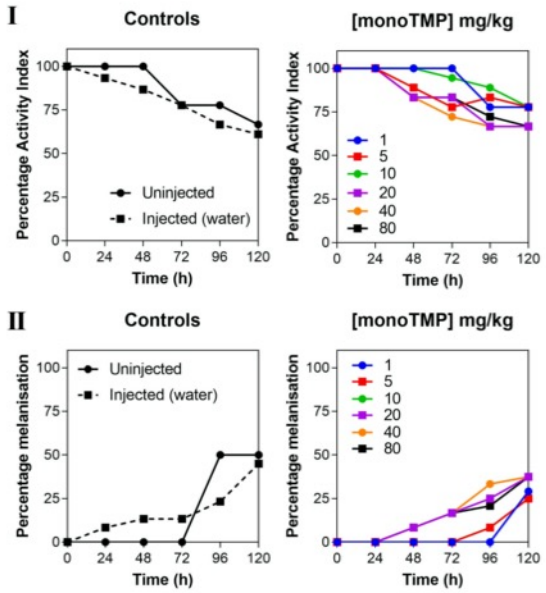


Figure 13. *Galleria Mellonella percentage melanisation and activity. Determination of* $\mathbf{4}^{2+}$ *toxicity in the insect model Galleria Mellonella. Larvae were injected with 10 µL of water (control), or* $\mathbf{4}^{2+}$ *(0-80 mg/kg) The larvae were incubated at 37.5 °C and live/dead scores were conducted at 120 hours.*

S3l. *S. aureus* SH1000 uptake experiment

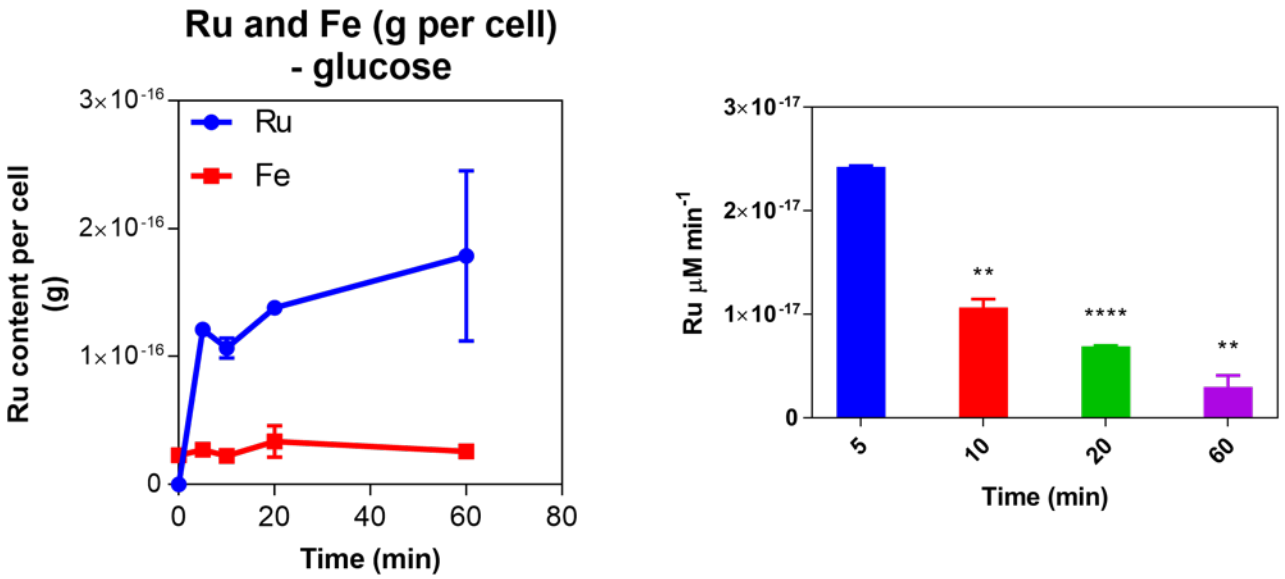


Figure 14. Accumul*ation experiments. ICP-AES data for the uptake of ruthenium by S. aureus SH1000 in the absence of glucose after exposure to* $\mathbf{4}^{2+}$ *(left). Ru (blue) and Fe (red) levels per cell are expressed as metal (g) per cell. Fe levels were calculated as a control. Conditions: concentration of* $\mathbf{4}^{2+}$ *= 7.8 µM. Cells were washed with 0.5%(v/v) nitric acid to remove unbound complex. Error bars represent three independent biological repeats ± SD. Statistical t-tests were used to compare the rate of uptake at each time-point to the initial rate of uptake at 5-minutes (right).*

S3m. *E. coli* EC958 STED imaging

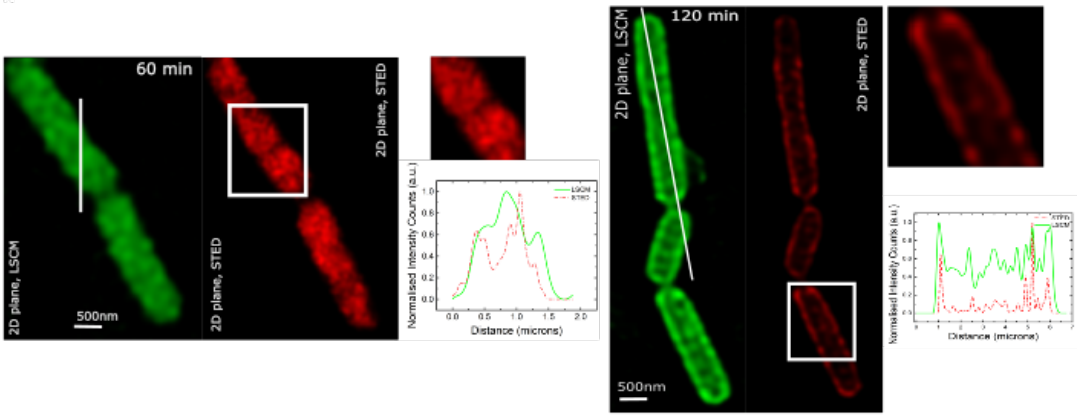


Figure 15. *Localization of* $\mathbf{4}^{2+}$ *in E. coli EC958 cells visualized through laser scanning confocal microscopy (LSCM) and stimulated emission depletion (STED) nanoscopy at 60 and 120 min. LSCM: Cells imaged using the emission of* $\mathbf{4}^{2+}$ *on excitation at 470 nm with a White Light Laser and a 470 nm notch filter. STED: cells imaged with the same excitation and emission settings; STED effect was obtained employing a 775 nm depletion laser, and a 780 nm vortex phase plate. Both deconvoluted diffraction-limited images (d-LSCM) and super-resolution (d-STED) images were processed using commercial Huygens software (SVI). Normalized emission intensity profiles along the solid white lines are given drawn solid green lines represent the d-LSCM; dotted red lines represent d-STED. Conditions: after treatment with 3.2 μM* $\mathbf{4}^{2+}$*, cells were washed with nitric acid before fixing with paraformaldehyde (4 %).*

S3n. *S. aureus* SH1000 STED imaging

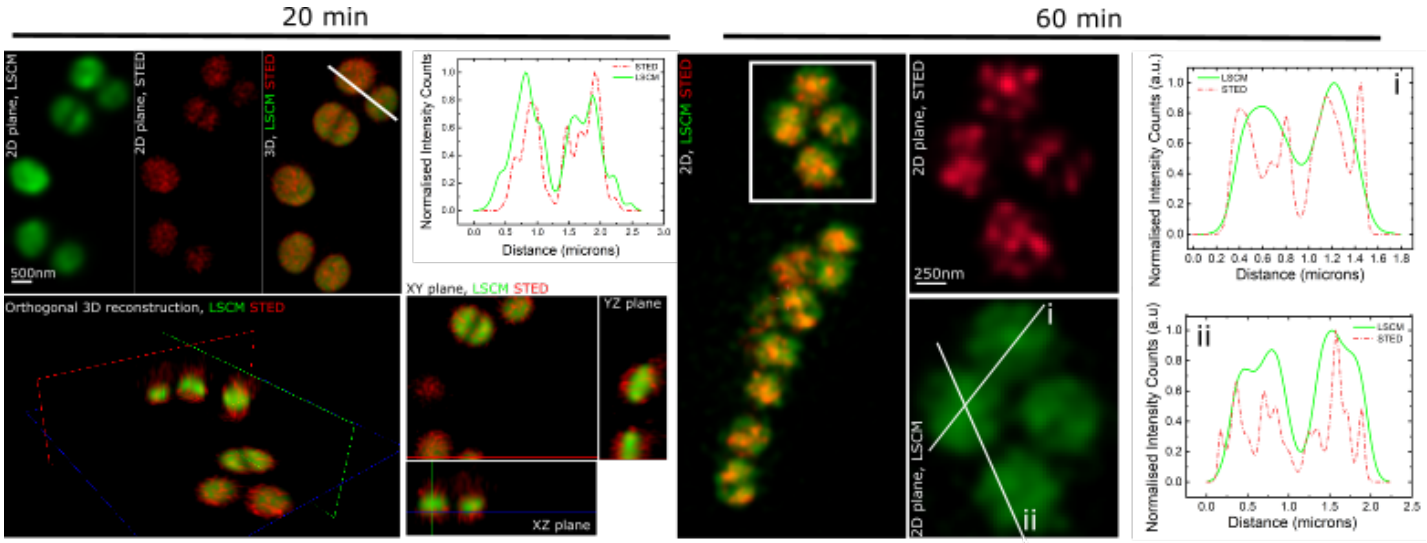


Figure 16. *Representative images showing section planes of full-volume deconvoluted diffraction-limited cells and super-resolution images (d-LCSM and d-3D STED). Normalised emission intensity profile of the solid white lines drawn on top of selected regions of cells are plotted. Solid green lines represent the d-LCSM, dashed red lines represent the d-3D STED. Time-points are shown above the images, as well as the orthogonal representation of every axis (XY, XZ and YZ), where the increased resolution and better localisation of* 4^{2+} *is shown in red. All cells show accumulation at the centre of the cell where DNA is located. Conditions used are identical to those employed in Figure 3 of the main manuscript.*

S4. Instrumentation

S4a. Microscopy

Super resolutions microscopy images were taken on a DeltaVision/GE OMX optical microscope (version 4) for structured illumination (SIM) at the Wolfson Microscope Facility in the University of Sheffield. Images were reconstructed using Delta-Vision OMX softWoRx 6.0 software. All images were analysed using Fiji and Image J software.

S4b. Photochemistry

Absorption spectra at 200-800 nm for the compounds were obtained with a Cary 50 Scan UV-vis-NIR Spectrophotometer, double beam mode (spectral band width = 2 nm), medium scan speed – 600 nm.

Emission spectra and DNA binding studies for the compound were conducted on the Jobin Yvon Hariba Group FluoroMax®-3 Fluorimeter. DNA binding data was fitted using Origin Software.

UV-Vis absorbance spectroscopy for LogP values were conducted via a SpectraMax M2 Microtitre Plater Reader (Molecular Devices UK).

Turbidity measurements were performed on a Jenway Cary 7350 Spectrophotometer.

S4c. Mass Spectroscopy

All Mass Spectroscopy was conducted at the Mass Spectroscopy Facility at the University of Sheffield

Mass spectra and accurate mass for the compounds were conducted using a Krato MS80 mass spectrometer working in positive ion mode.

Uptake experiment data was collected using Spectro CirosCCD (Spectro Analysis) Inductively-Coupled Plasma-Atomic Emission Spectrophotometer.

S4d. NMR Spectroscopy

^{1}H NMR spectra were taken on a Bruker AV400 machine and a Bruker AVIIIHD400 machine, both working in Fourier transform mode. All spectra were analysed using “Mestrenova” software.

S4e. Transmission electron microscopy

Samples were examined on a FEI Tecnai instrument operating at 80 kV equipped with a Gatan 1 K CCD camera. Images were processed and analyzed using FIJI ImageJ software.

REFERENCES

1 K. L. Smitten, H. M. Southam, J. Bernardino de la Serna, M. R. Gill, P. J. Jarman, C. G. W. Smythe, R. K. Poole and J. A. Thomas, *ACS Nano*, 2019, **13**, 5133–5146.

2 D. L. Reger, T. C. Grattan, K. J. Brown, C. A. Little, J. Lamba, A. L. Rheingold and R. D. Sommer, *J. Organomet. Chem.*, 2000, **607**, 120–128..

3 J. Bolger, A. Gourdon, E. Ishow and J.-P. Launay, *Inorg Chem*, 1996, **35**, 2937–2944.

4 B. M. Forde, N. L. Ben Zakour, M. Stanton-Cook, M.-D. Phan, M. Totsika, K. M. Peters, K. G. Chan, M. A. Schembri, M. Upton and S. A. Beatson, *PLoS ONE*, 2014, **9**, e104400–13.

5 I. T. Paulsen, L. Banerjei, G. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty and C. M. Fraser, *Science*, 2003, **299**, 2071–2074.

6 A. Lundin, *Methods. Enzymol.* 2000, **305**, 346-370.

7 C. R. Harding, G. N. Schroeder, J. W. Collins, G. J Frankel, *Vis. Exp*. 2013, **81**, e50964.