Supporting Information

An artificial CO-releasing metalloprotein built by histidine-selective metallation

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Supporting Figure 1

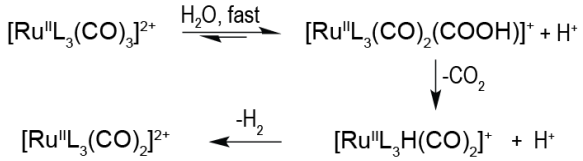


Fig. S1 Proposed water-gas shift reaction for the generation of the $[Ru(CO)_2]^{2+}$ fragment from CORM-3.

1. Synthesis of *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$

$[Ru(CO)_2Cl_2]_n$ was prepared according to the literature.[1] AgOTs and tosylic acid (TsOH) were purchased from Sigma–Aldrich. A suspension of $[Ru(CO)_2Cl_2]_n$ (200 mg; 0.88 mmol) in H_2O (25 mL) acidified with 25 mg of TsOH was treated with dropwise addition of a solution of AgOTs (489 mg; 1.76 mmol) in H_2O (7.5 mL) acidified with 25 mg of TsOH. The mixture was vigorously stirred at 50 ºC for 1 hour. The grey precipitate (AgCl) was filtered off and the solution concentrated to *ca.* 3 mL. The FTIR spectrum (ATR) of this solution presents two vibrations at 2081 and 2009 cm^{-1} in close agreement to literature for *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$.[2] Further concentration and cooling yielded an off-white precipitate, which was separated by filtration and dried in vacuum, to give a waxy, hygroscopic solid that was used without any further purification.

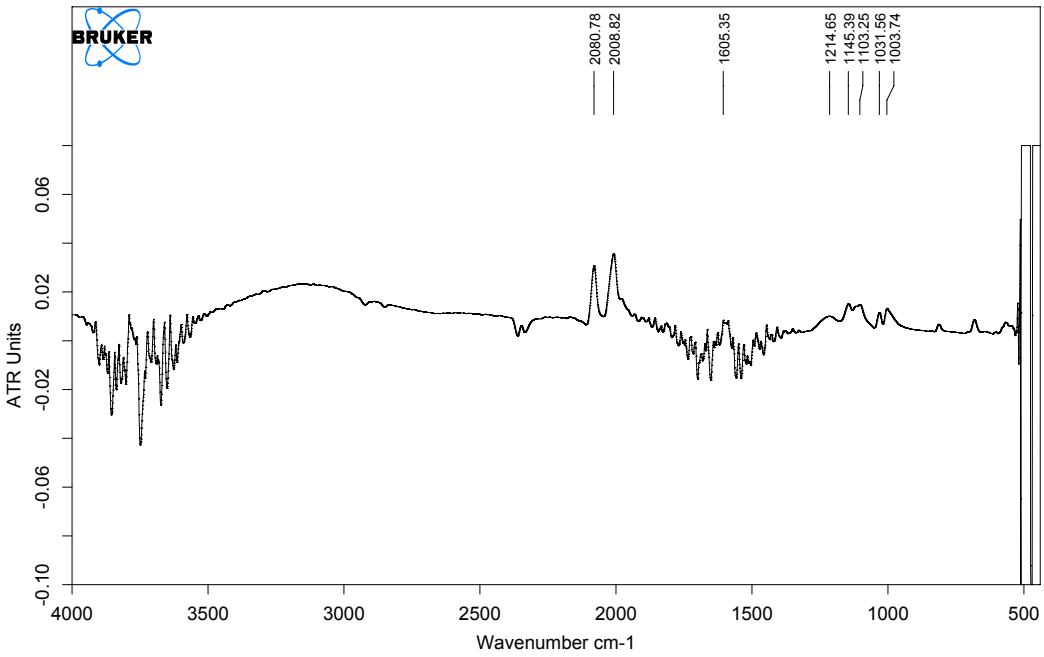


Fig. S2 FTIR (ATR) spectrum of *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$.

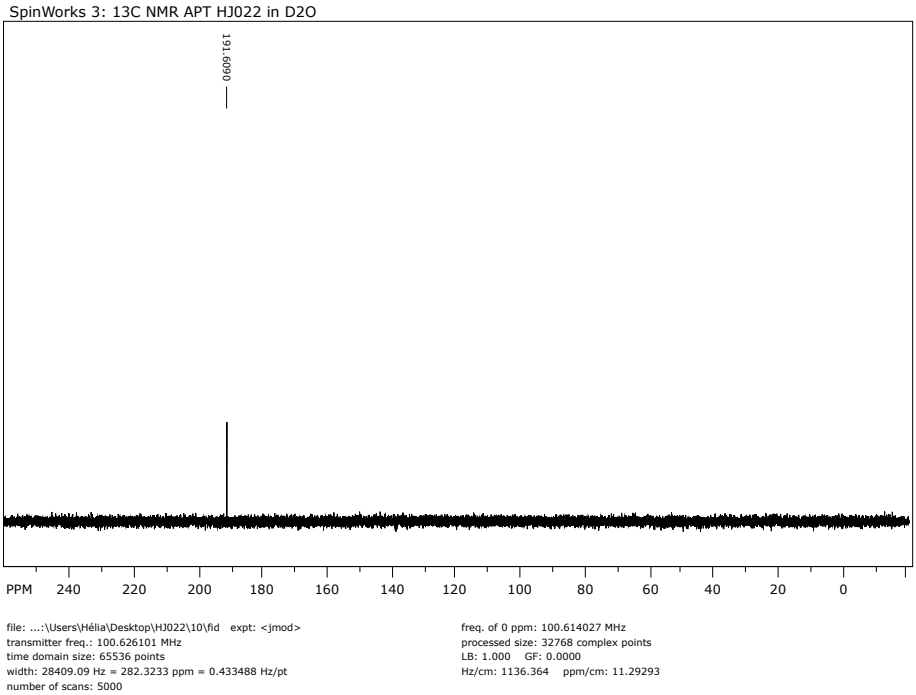


Fig. S3 ^{13}C NMR spectrum (D_2O; δ ppm) of *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$.

2. General procedure for chemical His metallation of proteins using *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$ or CORM-3

Protein used in this study: Interleukin (IL)-8 was purchased from Sigma–Aldrich. Typically, a solution of IL-8 was prepared as 1 mg/mL solution in PBS pH 7.4. CORM-3 (Sigma-Aldrich; 50 equivalents) (Sigma-Aldrich) or *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$ (50 equivalents) was added as a solid to the protein solution (1 mL, c = 1.0 mg/mL) in PBS pH 7.4 in a plastic tube and the mixture vortexed to homogenize. The reaction was left standing for 30 min at room temperature. Purification of the metallated protein was achieved by size exclusion chromatography using a HiTrap desalting column (GE Healthcare) to remove excess reagents. Purified samples were used for mass spectrometry analysis using the conditions described in section 3.

3. Native mass spectrometry

Interleukin (IL)-8 was dissolved in 200 mM ammonium acetate and denatured using ZipTip pipette tip c18 (Millipore). The purified product of the reaction between IL-8 and *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$ or CORM-3 was denatured using ZipTip C18. Mass spectra were acquired on a high-mass Q-TOF-type instrument Xevo G2-S (Waters, Manchester, UK). Mass spectrometry experiments were performed at a capillary voltage of 1990 V, cone voltage of 80 V and source offset voltage of 80 V. Spectra were processed using MassLynx V4.1 (Waters).

Sequence:

~~MTSKLAVALL AAFLISAALC EGAVLPR~~SAK ELRCQCIKTY SKPFHPKFIK 23

ELRVIESGPH CANTEIIVKL SDGRELCLDP KENWVQRVVE KFLKRAENS 72

Calculate average isotopic mass (mature form – highlighted in yellow): 8385.7510 Da

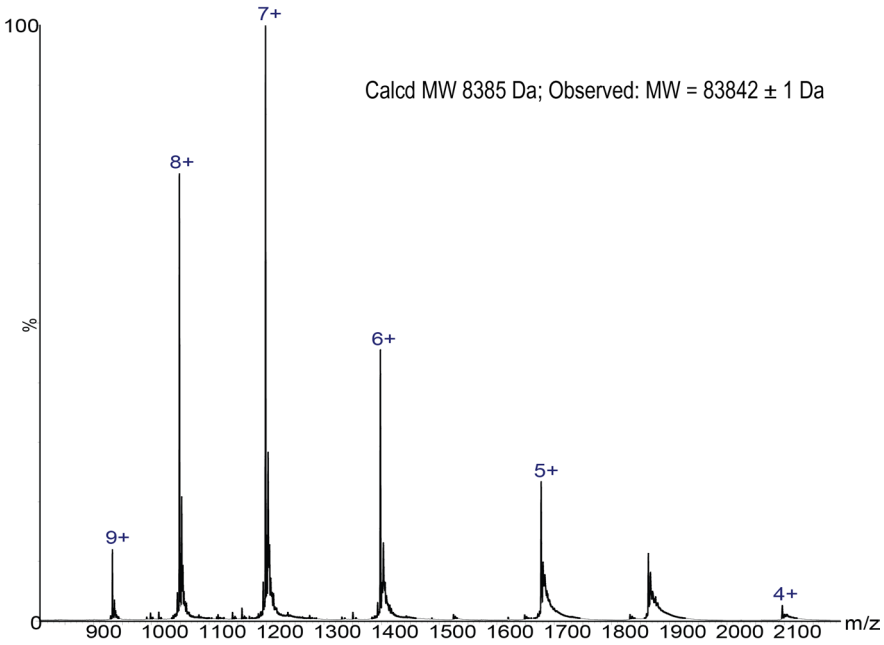


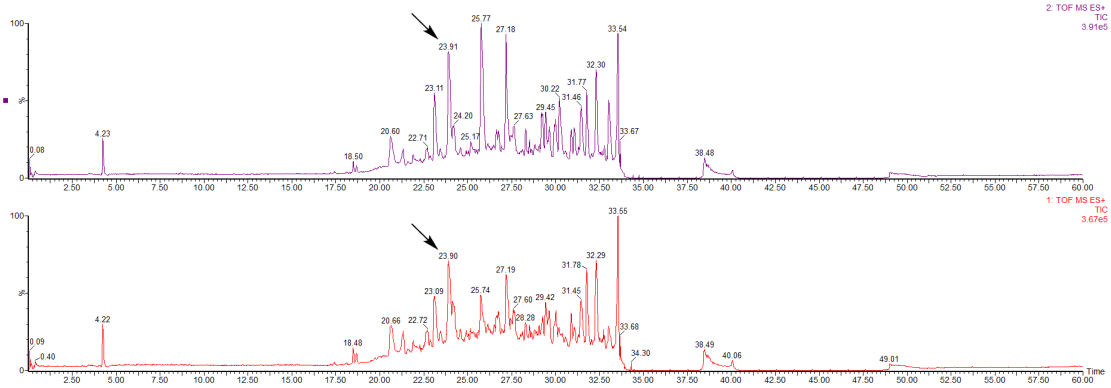
Fig. S4 Denatured nanoESI MS[‡] of Interleukin-8.

[‡] In denatured nanoESI MS the protein is denatured prior to electrospray ionization mass spectrometry experiment. The electrospray ionization (ESI) is a soft ionization technique extensively used for production of gas phase ions of thermally labile large supramolecules.

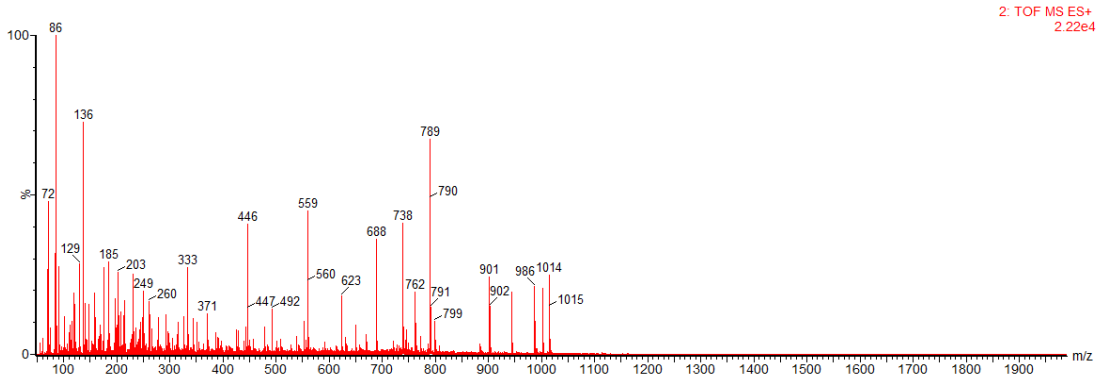
4. Peptide mapping

The purified product obtained from the reaction of IL-8 with *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$ (25 μL in 50 mM ammonium bicarbonate pH 8.0) was digested with 2 μL of a trypsin solution (100 ng/μL, Promega, UK) for a ratio of 1/50 enzyme/protein (*w*/*w*). The resulting digestion mixture was carried out overnight at 37 °C. After extraction, the resulting peptides were analyzed by nano-scale capillary LC–MS/MS. LC–MS/MS data were then analysed using MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

A) MS1 and MS2 chromatograms – the arrow indicates the retention time at which the modified peptide was found



B) MS spectrum of peptide: 27–VIESGPHCANTEIIVK–42 obtained after tryptic digestion of the purified product and containing the modification at histidine 33 (B = $[Ru(CO)_2]^{2+}$). m/z value of the triply charged peptide = 622.93



C) MS/MS spectrum of peptide: 27–VIESGPHCANTEIIVK–42 obtained after tryptic digestion of the purified product and containing the modified histidine 33 (B = $[Ru(CO)_2]^{2+}$). m/z value of the triply charged peptide = 622.93. Score = 1567.

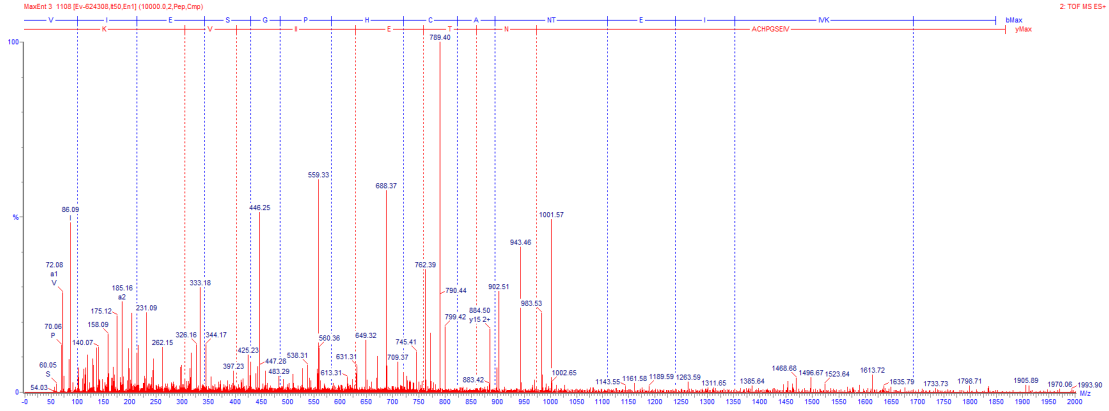


Fig. S5 LC–MS/MS analysis of the peptides resulting from trypsin digestion of the purified product obtained from the reaction of IL-8 with *cis*-$[Ru(CO)_2(H_2O)_4](OTs)_2$. Predicted peptide fragments containing the $[Ru(CO)_2]^{2+}$ fragment modification were determined by MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions. Matched peptides included peptide T7: 27–VIESGPHCANTEIIVK–42; no match was found for a peptide containing a modification at histidine 18.

5. Methods

5.1 Cell culture

HeLa cells (ECACC; passage 10–22) were routinely grown in a humidified incubator at 37 ºC under 5% CO_2 and split twice a week before reaching confluence, using TrypLE Express. HeLa cells were grown on MEM medium supplemented with 10% heat-inactivated FBS, 1% Glutamax, 10 mM HEPES, 10 mM NEAA, 200 units/mL penicillin and 200 μg/mL streptomycin. All reagents were bought from Gibco, Life Technologies (USA), unless otherwise stated.

5.2 COP-1 fluorescence response by confocal microscopy imaging

COP-1 was synthesized according to the literature.[3] HeLa cells were cultured as described above and seeded in Lab-Tek 8 well-chamber slides (Electron Microscopy Sciences, USA) at a concentration of 15 000 cells/300 μL/well, and were maintained at 37 ºC and 5% CO_2, in a humidified incubator throughout the course of the experiment. After 48 hours, medium was removed and the cells were incubated at 37 ºC with Hoechst 33482 (Life Technologies, USA), according to the manufacturer's instructions, for 30 min. After washing twice with DPBS 1x (Gibco, Life Technologies, USA), cells were incubated with complete medium supplemented either with IL-8 or IL-8-$Ru(CO)_2$, for 30 min, after which they were taken for observation under a Zeiss LSM 7 Live confocal microscope. When the sample was ready for observation, 1 μM of COP-1 was added and carefully mixed with the medium. A photo was taken immediately before adding COP-1, so as to determine background noise. Photos were then taken at 5, 15, 30, 45 and 60 min after addition of COP-1. All photos were taken using a 40x oil objective lens and a numerical aperture of 1.3. COP-1 was excited with a 488 nm and a Hoechst with a 33342 with a 405 nm laser, for detection of green (497–558 nm) and blue (420–480 nm) emission spectra, respectively. Transmitted light was also captured to help confirm cell surface-areas, used for data analysis. Analysis was done in ImageJ v.1.46r. The area of each cell was carefully delimited and the mean of green fluorescence intensity for each cell was determined and then normalized for cell-size. Statistical analysis was done using a two-way ANOVA. Data are represented as mean of normalized fluorescence intensity ± SEM.

5.3 Neutrophils migration assay

Blood samples from two voluntary human donors were used for isolation of neutrophils, using Lympholyte-poly cell separation media (Cedarlane Laboratories, USA), according to the manufacturer's instructions. Briefly, 5 mL of fresh blood were carefully pipetted onto 5 mL of the isolation medium and centrifuged for 33 min, at 500 g, at 20 ºC. The monolayer containing the neutrophils was then transferred into a new Falcon tube and an equal volume of 0.45% NaCl solution was added to re-equalize osmolarity. Another two volumes of complete medium were added and the sample was centrifuged again. After two steps of washing with complete medium, cells were re-suspended in 1 mL of complete medium and the concentration of each sample was determined using a Neubauer chamber. Smears with samples before and after isolation were done, to later determine purity of the final suspension. Cells were then diluted to a concentration of 80 000 cells/100 µL. 600 µL of either treated or non-treated medium were added to the bottom of 3 µm-pore 24 well Transwell plates (Corning, USA) and 100 µL of the neutrophils suspension was pipetted into the insert placed on each well. After 3 hours, inserts were removed, cells were fixed with methanol and then stained with a 5% Crystal Violet solution for analysis, using a Leica DM2500 brightfield microscope. Five random fields at 10x and 40x magnification were photographed per membrane and triplicates were performed per condition, per donor. The estimate of the number of cells that were retained in the membrane was assessed by counting the number of events/area of each field and multiplying by the total area of the membrane. Results are shown as percentage of control (complete culture medium). A negative control (complete culture medium without FBS) was included to further confirm validity.

5. References

1. P. A. Anderson, G. B. Deacon, K. H. Haarmann, F. R. Keene, T. J. Meyer, D. A. Reitsma, B. W. Skelton, G. F. Strouse and N. C. Thomas, *Inorg. Chem.*, 1995, **34**, 6145-6157.
2. U. C. Meier, R. Scopelliti, E. Solari and A. E. Merbach, *Inorg. Chem.*, 2000, **39**, 3816-3822.
3. B. W. Michel, A. R. Lippert and C. J. Chang, *J. Am. Chem. Soc.*, 2012, **134**, 15668-15671.