The chemical re-design of native protein scaffolds as artificial metalloproteins through the attachment of organometallic moieties offers tantalising opportunities not only in (bio)catalysis but also for the delivery of organometallic-based drugs. In one example, an artificial metallohydrodase featuring two different metal ions, Zn(II) and Hg(II), which are required for catalysis and structural stability, respectively, was shown to catalyse p-nitrophenyl acetate (pNPA) hydrolysis with an efficiency comparable to that of human carbonic anhydrase (CA)-II and much higher than similar synthetic complexes. In another example, a bifunctional artificial streptavidin metalloenzyme, displaying both an engineered carboxylate side-chain and a docked biotinylated rhodium(ni) complex, enabled catalytic asymmetric C-H activation. In addition to catalysis, artificial metalloproteins have also shown promise for the in vivo delivery of organometallic-based drugs. For example, Pt(iv) prodrugs have been designed to specifically bind in a non-covalent manner to human serum albumin (HSA) as a delivery vehicle, enhancing significantly its stability in whole human blood.

Anchoring of a non-native organometallic moiety into a protein template is a challenging task and it is usually achieved using dative, covalent or supramolecular strategies. Among these, covalent modification of cysteine (Cys) residues with complexes bearing reactive electrophilic handles (namely prosthetics) has received considerable attention. Cys bioconjugation with n-haloacetamide functionalised complexes has been used to create, for example, an artificial Diels–Alderase and an artificial metalloenzyme for olefin metathesis. While the covalent conjugation of metal complexes equipped with reactive handles to nucleophilic side chains on proteins has been already demonstrated, the direct metallation of a native or non-native pre-determined site on a protein scaffold by creating a stable side chain–metal bond is less common. In this context, the non-selective metallation of histidine (His), a privileged metallophilic residue, has been exploited in coordination-based protein modification strategies using the nickel(Ni)(II)–His-tag pair as well as in other metallation reactions with cobalt (Co) and ruthenium (Ru) complexes. Unlike these remarkable examples, His-selective metallation of proteins leading to stable, fully functional materials is to the best of our knowledge unprecedented. Alternatively, one could consider employing genetic encoding methods to introduce an unnatural amino acid with a designer side chain for metal binding. This approach has been recently utilized by Roelfes and co-workers to create an artificial metalloprotein for catalytic asymmetric Friedel–Crafts alkylation reactions.

We have previously described the interactions of the Ru carbonyl complex \([	ext{fac-RuCl}[(\kappa^2-\text{H}_2\text{NCH}_2\text{CO}_2)]\text{(CO)}_3]\) (CORM-3; Fig. 1A) with proteins. X-ray crystallography showed the formation of a di-carbonyl Ru protein complex by modification of histidine (His)-15 in hen egg white lysozyme (HEWL) with the fragment cis-[Ru(CO)]\(^{2+}\). This fragment is likely to arise from the hydrolytic decomposition of CORM-3 in aqueous solution through a water-gas shift reaction (Fig. S1, ESI†). In this work, we hypothesised whether the reactivity of the cis-[Ru(CO)]\(^{2+}\) fragment would allow for direct selective His metallation of proteins and thus provide a robust strategy for the construction of synthetic Ru dicarbonyl metalloproteins. We envisioned that the cis-[Ru(CO)]\(^{2+}\) could be generated directly from an aquacarboxyl Ru(n) dication cis-[Ru(CO)]\(^{2+}\) as a fragment (Fig. 1A). The expected chemically defined, artificial Ru dicarbonyl metalloproteins are interesting targets as these have been demonstrated to carry and deliver therapeutic active CO carriers in vivo.
As a protein scaffold we chose the CXC chemokine interleukin-8 (IL-8), which is associated with the promotion of neutrophil chemotaxis and degranulation upon inflammatory stimuli. IL-8 activates multiple intracellular signalling pathways downstream of two G protein-coupled receptors (CXCR1 and CXCR2), and over-expression of IL-8 and/or its receptors has been described in cancer and endothelial cells, infiltrating neutrophils and tumour-associated macrophages, demonstrating the key roles of IL-8 as a regulatory factor within the tumour microenvironment.22 Structurally, IL-8 features two His residues, His18 and His33, that in solution display lower $pK_a$ values (3.7 and 4.9, respectively) than His usually does ($pK_a \sim 6.0$).23–25 The unique reactivity of each of the His residues present in IL-8 makes it an ideal scaffold to achieve His-selective metallation with the cis-$[\text{Ru}(\text{CO})_2]^{2+}$ fragment.

We began by synthesising the aquacarbonyl Ru(n) dication cis-$[\text{Ru}(\text{CO})_2(\text{H}_2\text{O})_8]^{2+}$ complex (Fig. 1). Although our data strongly suggest that the cis-$[\text{Ru}(\text{CO})_2]^{2+}$ fragment is generated in situ as the result of the hydrolytic decomposition of CORM-3, we decided to synthesise the complex aquacarbonyl Ru(n) dication cis-$[\text{Ru}(\text{CO})_2(\text{H}_2\text{O})_8]^{2+}$ (Fig. 1), which would be a more direct source of the fragment cis-$[\text{Ru}(\text{CO})_2]^{2+}$. The synthesis and characterization of the tosylate (OTs) salt of this complex has been described by Merbach and co-workers.26 The reported preparation is, however, lengthy and far from straightforward, so we decided to prepare the cis-$[\text{Ru}(\text{CO})_2]^{2+}$ containing species in aqueous medium, through the reaction of the readily accessible $[\text{Ru}(\text{CO})_2\text{Cl}_2]_2$ and Ag(OTs).27 After removing solid AgCl, the FTIR spectrum (ATR) of the solution showed two CO stretching bands at 2081 and 2009 cm$^{-1}$. The $^{13}$C NMR spectrum of the residue obtained after evaporation displays a single resonance at $\delta$ 192 ppm (Fig. S3, ESI$^\dagger$). Spectroscopic data are in accordance with what was previously published,28 and indicate the presence of a single cis-$[\text{Ru}(\text{CO})_2]^{2+}$ species.

With the aquacarbonyl Ru(n) dication cis-$[\text{Ru}(\text{CO})_2(\text{H}_2\text{O})_8]^{2+}$ in hand we started the metallation reaction by reacting 50 equivalents of this complex with IL-8 for 30 minutes at room temperature in phosphate buffer solution (PBS) pH 7.4 (Fig. 2B). The same reaction conditions were also tested using CORM-3 instead of the Ru aquacarbonyl complex. After purification by size exclusion chromatography to remove excess of reagent, denatured nanoESI MS$^\dagger$ shows the presence of a major species (> 80% conversion), IL-8 in complex with one cis-$[\text{Ru}(\text{CO})_2]^{2+}$ fragment (component B; Fig. 1C). In addition, minor species corresponding to IL-8 only (component A; see also Fig. S4, ESI$^\dagger$ for denatured nanoESI MS of IL-8) and IL-8 in complex with two cis-$[\text{Ru}(\text{CO})_2]^{2+}$ fragments (component C) could also be detected. The use of additional equivalents of both reagents and increasing the temperature and reaction time resulted in additional labelling and/or protein loss. This result indicates potential selective labelling of a single His residue but also shows that, in solution, CORM-3 rapidly decomposes to form a highly reactive cis-$[\text{Ru}(\text{CO})_2]^{2+}$ fragment that is captured by exposed, reactive His residues on proteins.

Next, to determine the labelling site, we subjected the IL-8-Ru$^{\text{II}}$(CO)$_2$ conjugate to proteolysis and analysed the digested fragments by nano LC-MS/MS. 19 unique peptides were identified covering 70% of the sequence. The peptide containing amino acids 27–42 [VIESGPHCANTEIIVK] has a cis-$[\text{Ru}(\text{CO})_2]^{2+}$ modification at His33, which was further corroborated in the MS/MS spectrum (Fig. S5, ESI$^\dagger$). No modification could be found at His18, demonstrating that His33 is the primary site of metallation. Interestingly, this result also shows that the His–Ru bond is stable under processing/analysis conditions similar to other His–Ru complexes.15 The stability of the present site- and residue (His)-selective strategy opens up the development of orthogonal double His modification protocols for the installation of dual labels that will ensure probe homogeneity unlike other nonselective metallation protocols.

Having shown that it is possible to achieve selective metallation of His33 in IL-8, we next studied whether the new artificial chemically defined metalloproteins could spontaneously release one CO ligand from the introduced cis-$[\text{Ru}(\text{CO})_2]^{2+}$ motif. Our group is interested in innovative methods for the in vivo delivery of therapeutic CO6,28 and has recently demonstrated that Ru$^{\text{III}}$(CO)$_2$-albumin complexes are able to carry and release CO in aqueous solution, live cells and mice.21 The viability of HeLa cells in the presence of the artificial IL-8-Ru$^{\text{II}}$(CO)$_2$ metalloprotein (Fig. 2A) was
verified by MTT assay and confocal microscopy. Furthermore, the selective CO fluorescent turn-on probe (COP-1) was used to check for CO release from IL-8-RuII(CO)2 in aqueous buffered (PBS) solution at physiological pH 7.4. The results reflect a robust fluorescence response of COP-1 in the presence of an IL-8-RuII(CO)2 solution that increased in a time-dependent manner while COP-1 alone showed very weak fluorescence (Fig. 2B). Using this assay, we could visualize cytosolic CO release (associated with COP-1 staining), which seems to be intensely associated with the perinuclear region (Fig. 2C), in accordance to what we previously reported. Interpreted as a whole, these data further corroborate the benefits associated with the use of protein scaffolds for carrying and delivering CO.

IL-8 is a chemokine produced and secreted by macrophages and other cell-types including epithelial cells. In addition to its role as a promoter of angiogenesis in humans, IL-8 acts mainly as a chemotactic factor, by inducing migration of neutrophils and other granulocytes towards infection sites. To assess if IL-8 retained its chemotactic activity when conjugated to the cis-[Ru(CO)2]2+ motif, a neutrophil migration assay was performed, using human neutrophils isolated from freshly collected blood. Neutrophil migration induced by the artificial metalloprotein IL-8-RuII(CO)2 was compared against that which
native IL-8 can induce. In the migration assay, IL-8-Ru[1](CO)₂ was able to decrease neutrophil retention in the Transwell membrane at similar levels to those induced by IL-8 alone, i.e. both unmodified and modified IL-8 increase neutrophil migration (Fig. 3). Previous modification of IL-8 by introduction of the natural posttranslational modification citrullination had led to a considerable reduction in chemotactic activity.³⁰ Importantly, our natural posttranslational modification citrullination had led to a (Fig. 3). Previous modification of IL-8 by introduction of the unmodified and modified IL-8 increase neutrophil migration at similar levels to those induced by IL-8 alone, i.e.

**Notes and references**

† General procedure for chemical His metallation of IL-8 with the cis-[Ru(CO)₂]²⁺ fragment: typically, a solution of IL-8 was prepared as 1 mg mL⁻¹ solution in PBS pH 7.4. cis-[Ru(CO)₂(H₂O)₄]²⁺ or CORM-3 (50 equivalents) was added as a solid to the protein solution (1 mL, c = 1.0 mg mL⁻¹) in PBS pH 7.4 in a plastic tube and the mixture was vortexed to homogenize. The reaction was maintained 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 under the conditions described in the ESI.‡


