ChemComm

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Journal:	*ChemComm*
Manuscript ID	CC-COM-09-2021-005542.R3
Article Type:	Communication

COMMUNICATION

A water-soluble iron-porphyrin complex capable of rescuing CO-poisoned red blood cells†

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

We describe herein a small-molecule platform that exhibits key properties needed by an antidote for CO poisoning. The design features an iron-porphyrin complex with bulky substituents above and below the macrocyclic plane to provide a hydrophobic pocket for CO binding and to prevent the formation of inactive oxo-bridged dimers. Peripheral charged groups impart water solubility. We demonstrate that the Fe(II) complex of a porphyrin with 2,6-diphenyl-4-sulfo-phenyl *meso* substituents can bind CO, stoichiometrically sequester CO from carboxyhemoglobin, and rescue CO-poisoned red blood cells.

Carbon monoxide is celebrated as an endogenous gasotransmitter and low doses of CO have demonstrated salutary effects in health conditions ranging from cancer to coronary heart disease.[1] Nevertheless, high levels of CO exposure are harmful and the toxicity of CO is attributed in part to its ability to bind hemoglobin (Hb), forming carboxyhemoglobin (COHb) and inhibiting O_2 transport.[2] Hb binds CO approximately 200-250-fold more strongly than O_2, and the distinctively red-shifted Soret band of COHb is used clinically to assess CO exposure.[3,4]

In the United States alone, over 50,000 emergency department visits each year are attributed to CO exposure.[5] Despite the prevalence of CO poisoning, there is no clinically-approved antidote available. Current best practices involve placing the afflicted individual in fresh air, delivering 100% O_2, or administering superatmospheric levels of O_2 in a hyperbaric chamber.[6] The typical half-life of COHb in the bloodstream is 5.3 h, but hyperbaric O_2 (1.5-3 atm) can decrease this half-life to < 1 h.[7] Unfortunately, these large chambers are generally located in tertiary care centers to which patients must be transported. Moreover, hospitals typically house only a few such chambers, which could be rapidly overwhelmed in the event of a mass exposure.

We appreciate, therefore, that there is a pressing need for a rapid and effective antidote for CO poisoning. An important recent development in this area was the discovery that a mutant form of neuroglobin can function as a CO-poisoning antidote.[8-10] The supramolecular hemoCD complexes formed from iron(II) porphyrins and modified cyclodextrins have also demonstrated a high affinity for CO.[11] The chemistry of these compounds has been exploited to probe CO physiology,[12-15] and could also have utility in binding toxic molecules.[16] Finally, a series of synthetic peptides has been explored for their ability to allosterically enhance the release of CO from COHb.[17] We sought to develop a modular small-molecule platform that exploits the CO-affinity of Fe(II) porphyrin compounds. We have prepared a proof-of-principle compound that forms a strong CO adduct and we demonstrate that this affinity allows it to stoichiometrically remove CO from COHb. Moreover, we demonstrate that this compound can rescue CO-poisoned red blood cells (RBCs) by quantitatively sequestering CO while leaving the cells intact.

Our design is inspired by the seminal bioinorganic studies on picket-fence porphyrins,[18, 19] which bind CO more strongly than O_2.[20] Steric bulk can prevent the formation of deactivated μ-oxo species, but the atropisomeric purity of the original picket-fence porphyrins is unnecessary for CO sequestration. We turned, therefore, to symmetrically substituted bis-pocket porphyrins featuring 2,6-disubstituted *meso*-phenyl groups.[21] We reasoned that the 4-position of the *meso*-phenyl unit could be functionalized with a charged group to impart water solubility (Figure 1A). We emphasize that the large, highly charged complex will have limited-to-no cellular uptake by design; cellular uptake is not required because the complex will not need to interact directly with COHb to function. We stress that the thermodynamic stability of COHb does not preclude kinetic lability, which has been exploited to transfer CO between heme proteins.[8] If a metalloporphyrin complex has a CO affinity

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† Electronic Supplementary Information (ESI) available: Experimental details, spectra, and tables. CCDC 2075089-2075090, 2127883. See DOI: 10.1039/x0xx00000x

sufficiently greater than that of Hb, transfer will proceed (Figure 1B).

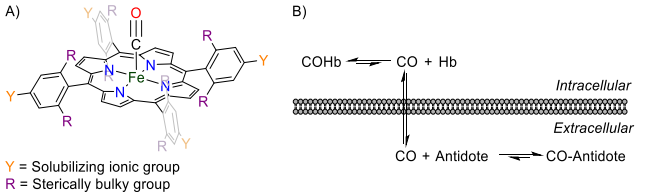
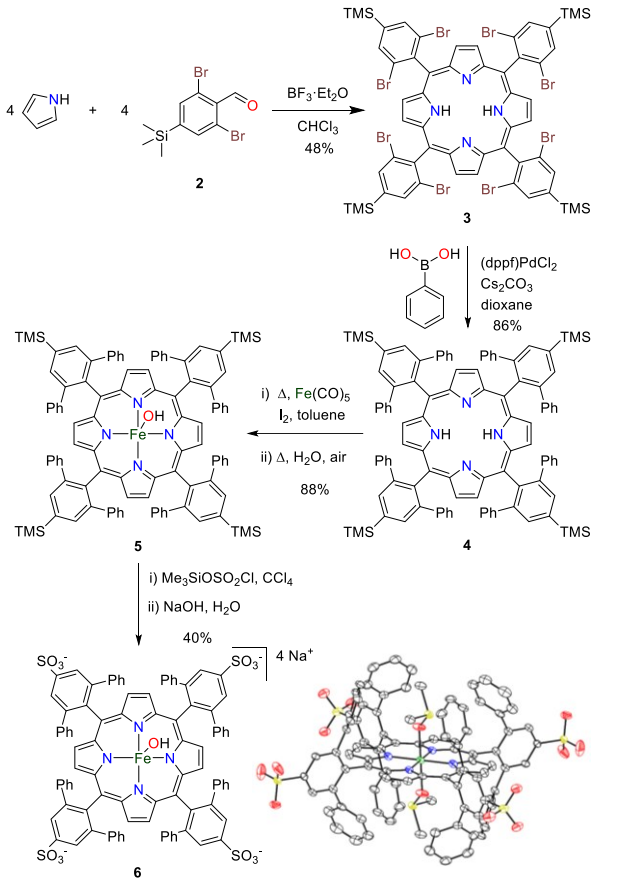


Fig. 1 A) Schematic overview of the proposed small-molecule for CO sequestration. B) Proposed mechanism of action.

To validate our design, we targeted the synthesis of iron-porphyrin complex **6** with 2,6-diphenyl-4-sulfophenyl *meso* substituents (Scheme 1). The *ortho*-phenyl groups will prevent μ-oxo dimer formation and create a hydrophobic CO-binding pocket. The *para*-sulfonate groups will ensure water solubility at physiological pH. The bis-pocket porphyrin with 2,4,6-triphenylphenyl *meso* substituents was previously accessed from pyrrole and 2,4,6-triphenylbenzaldehyde using a standard Adler synthesis, but macrocyclization was inhibited by the extreme steric congestion in the product; the final yield was 1%.[21] We instead targeted 2,6-dibromophenyl-containing *meso* substituents so that steric bulk can be incorporated via Pd-catalyzed cross-coupling reactions *after* macrocyclization. Finally, we avoided the complications associated with harsh electrophilic aromatic sulfonation by incorporating a trimethylsilyl group that can undergo facile late-stage conversion to a sulfonyl chloride, which can then be hydrolyzed to a sulfonate.[22]

(3,5-Dibromophenyl)trimethylsilane (**1**) was prepared from 1,3,5-tribromobenzene via sequential reaction with *n*-BuLi and Me_3SiCl. Conversion to aldehyde **2** was achieved via deprotonation with LDA and carbonylation with DMF. $BF_3{\cdot}OEt_2$-catalyzed condensation of **2** and pyrrole proceeded readily to give brominated porphyrin **3**, which is sparingly soluble in MeCN. Washing the crude product with MeCN until the filtrate is colorless afforded analytically pure material in 48% yield. Eight phenyl rings were installed on the porphyrin via Suzuki-Miyaura coupling in 20:1 1,4-dioxane/water using a three-fold excess of $PhB(OH)_2$ (per Ar–Br bond), Cs_2CO_3 as a base, and 12.5 mol% $(dppf)PdCl_2$ (per Ar–Br bond). Silica gel chromatography afforded bulky porphyrin **4** as a deep purple solid in 86% yield. Single-crystal X-ray diffraction confirms the formation of the desired hydrophobic pocket (Figure S20).

Iron was inserted into **4** by refluxing it with $Fe(CO)_5$ and a catalytic amount of I_2 in toluene under N_2 for 4 h; an additional hour of reflux in open air followed by an alkaline aqueous work-up ensured oxidation to the Fe(III) state. ^{1}H NMR spectroscopy in $CDCl_3$ confirms that product **5** is paramagnetic, with broadened phenyl resonances appearing in the 15-20 ppm range and the β-pyrrole protons characteristically resonating at 82.16 ppm.[23] Crystals of the reaction product grown from MeCN confirmed the proposed connectivity and the presence of an apical hydroxide ligand (Figure S21).



Scheme 1 Synthesis of **6** (details provided in ESI) and molecular structure (50% ellipsoids) of the anion obtained upon slow recrystallization of **6** from $DMSO/CHCl_3$. H atoms, solvent, and counterions omitted for clarity. Color code: Fe green, O red, S yellow, N blue, C grey.

Compound **5** was treated with Me_3SiOSO_2Cl in CCl_4 followed by hydrolysis with 1 M NaOH(aq). The intensely colored porphyrin complex was extracted from the reaction mixture with water and subsequently purified by reverse-phase chromatography. The final tetrasodium salt **6** was isolated as a very dark purple solid in 40% yield. The purity of the product was established using analytical HPLC (Figure S16). Weakly diffracting crystals confirmed the proposed connectivity, including the apical hydroxide ligand (Figure S22). High-quality crystals that were grown from $DMSO/CHCl_3$ permitted atomic-resolution refinement that confirmed the proposed structure, albeit following axial ligand substitution (Scheme 1). In the crystal, the complex sits on an inversion center and, although planarity of the ligand is not crystallographically required, the RMSD of the atoms of the porphyrin core from the plane of best fit is only 0.024 Å.

Compound **6** (λ_{max} = 431 nm in PBS, pH 7.4) can be reduced *in situ* using $Na_2S_2O_4$ to afford an Fe(II) complex (Figure 3). Upon reduction, the Soret band red shifts to 448 nm (Figure 3B), consistent with reduction to an Fe(II) porphyrin.[11] The ^{1}H NMR spectrum of the *in situ* generated reduction product in deuterated PBS (PBS-*d*) exhibits a signal at –3.63 ppm, which we tentatively assign as the β-pyrrole protons, and Evans' method measurements return a μ_{eff} of 3.69 μ_B (Figure S14). These data

are consistent with the formation of a four-coordinate intermediate-spin (S = 1) Fe(II) complex.[24-26] We highlight that an anomalously high μ_{eff}, as compared to the predicted spin-only magnetic moment for an intermediate-spin Fe(II) porphyrin, has also been reported for Fe(TPP) and has been rationalized in terms of spin-orbit coupling.[27]

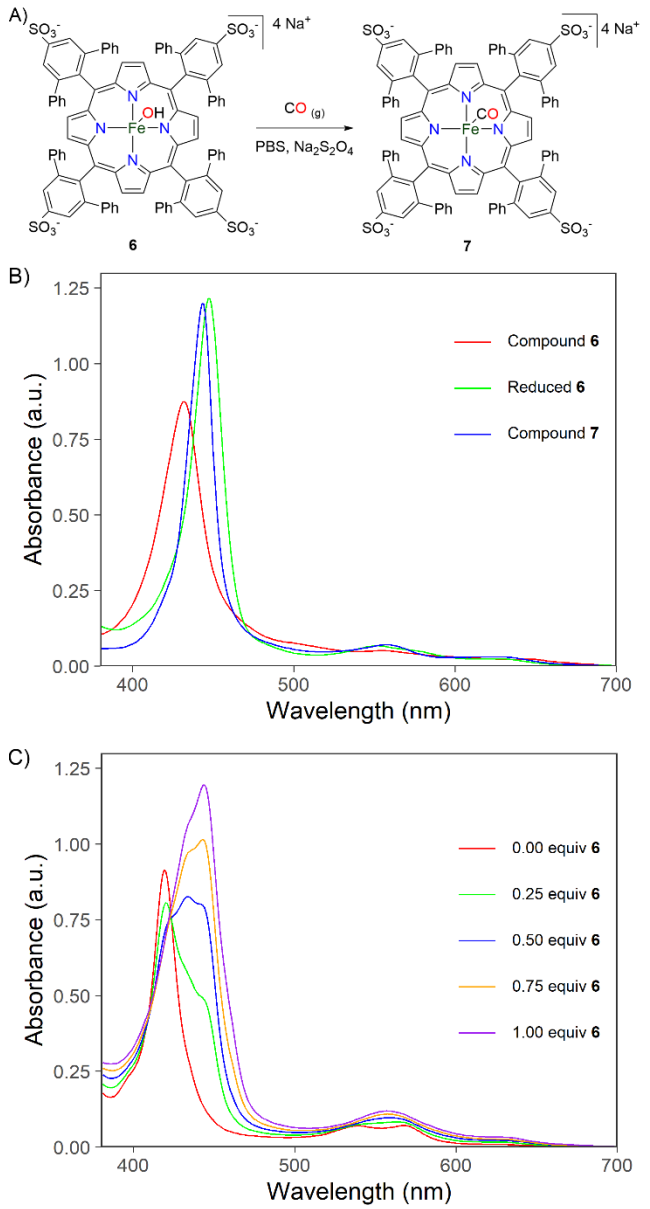


Fig. 3 A) Reaction of **6** with CO under reducing conditions to produce **7**. B) Electronic absorption spectra of 10 μM solutions of **6**, reduced **6**, and **7** in PBS (pH 7.4). For reduced **6** and **7**, the solutions also contain 5.7 mM $Na_2S_2O_4$. C) Titration of bovine COHb (2.5 μM) with **6** in PBS (pH 7.4, 5.7 mM $Na_2S_2O_4$).

Under an inert atmosphere, PBS solutions of this Fe(II) complex containing $Na_2S_2O_4$ are stable for days. When the solutions are opened to air, the complex reverts to **6** ($t_{½}$ ≈ 30 min) following aerial oxidation of the dithionite (Figure S17). Addition of CO to solutions of reduced **6** produces the CO adduct **7** (Figure 3A). Compound **7** is characterized by a Soret band at 444 nm in PBS (Figure 3B); this hypsochromic shift is consistent with formation of an Fe(II)–CO complex.[11] In PBS, solutions of **7** that have been opened to air revert to **6** with $t_{½}$ ≈ 120 min (Figure S18). If the reduction of **6** under a CO atmosphere is performed in PBS-*d*, ^{1}H NMR spectroscopy clearly shows **7** to be diamagnetic and Evans' method measurements return a μ_{eff} of 0 μ_B (Figure S15). These results are consistent with the formation of a low-spin Fe(II) complex, which would be expected upon complexation of CO. Salt metathesis, effected by addition of excess $(Ph_4P)Cl$ to an aqueous solution of **7**, results in rapid precipitation of a red solid. IR spectroscopic analysis of this solid revealed a ν_{CO} of 1970 cm^{-1} (Figure S19). The higher value of ν_{CO} for **7** as compared to COHb (1951 cm^{-1})[28] suggests that the small-molecule porphyrin complex will bind CO more strongly than Hb.[20] This prediction was tested by titrating COHb with **6** in PBS under reducing conditions (5.7 mM $Na_2S_2O_4$). We observed a dose-dependent and stoichiometric transfer of CO to form **7** and deoxyHb with tight isosbestic points, indicating a clean transition from COHb to (deoxyHb + **7**) (Figure 3C). The stoichiometric transfer with each aliquot reveals that the CO binding constant is appreciably greater than that of Hb ($P_{½}^{CO}$ < 0.004 torr).[20] We also titrated a solution of deoxyHb and **6** in 5.7 mM $Na_2S_2O_4$ with CO-saturated water. The first equivalent of CO converts reduced **6** to **7** but leaves deoxyHb unchanged. Addition of a second equivalent of CO results in conversion of deoxyHb to COHb (Figure S23). These experiments confirm that reduced **6** can bind CO preferentially in the presence of Hb and sequester CO from COHb. Although characterization of the kinetics of CO binding will be the topic of future studies, we note that in these experiments, all changes were complete between the time of mixing and spectral acquisition (20-30 s). We also note that titration of a 5.7 mM $Na_2S_2O_4$ solution of COHb with 5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrinatoiron(III), i.e. the derivative of **6** in which the phenyl groups above and below the porphyrin plane are replaced with H atoms, does not result in CO transfer (Figure S24).

Finally, we tested whether reduced **6** could sequester CO from poisoned bovine RBCs after confirming that it does not induce hemolysis (Figure S25). We performed our spectroscopic titrations directly on suspensions of CO-treated RBCs in PBS containing 5.7 mM dithionite. We note that the intensity of the Soret band of COHb (λ_{max} = 420 nm) is decreased from the concentration-predicted absorbance because of an inner filter effect introduced by concentration of the Hb into the RBCs. Accurate concentrations of COHb were obtained by measuring the absorbance of lysed aliquots of the suspension. Titration of the cell suspension with **6**, which is reduced *in situ*, resulted in a dose-dependent decrease in the intensity of the COHb signal, and an increase of the signal for **7** (λ_{max} = 444 nm) (Figure 4). To confirm that CO had indeed been abstracted from Hb, additional CO was bubbled through the suspension of RBCs that had been treated with 1 equiv of **6**; the COHb signal was regenerated. Although a detailed analysis of the kinetics of CO transfer and sequestration will be the subject of future work, we gained preliminary insight by monitoring absorbance at 420 nm of a quiescent suspension of CO-poisoned RBCs in a

buffered dithionite solution following addition of 1 equiv of **6**. The reaction was essentially complete within 3 min (Figure S26).

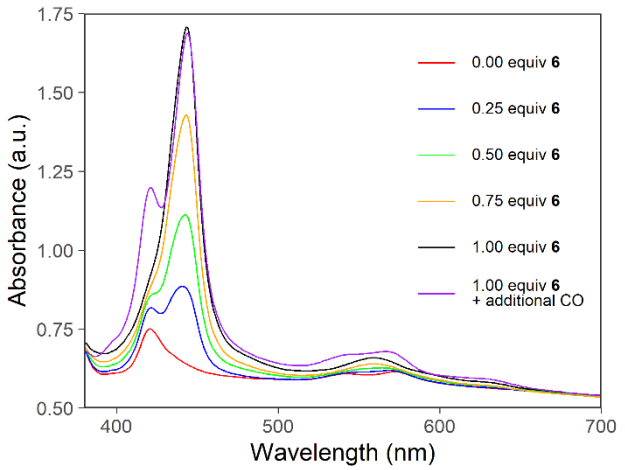


Fig. 4 Titration of a PBS suspension (pH 7.4, 5.7 mM $Na_2S_2O_4$) of CO-treated bovine RBCs with **6** (reduced *in situ*). Final trace obtained after bubbling CO through the suspension treated with 1.00 equiv of **6**.

We note that Fe-porphyrin compounds have been observed to bind 2 equiv of CO,[29] but our titration experiments reveal that CO transfer from COHb to reduced **6** occurs to form **7** with a 1:1 stoichiometry and that no change in the UV-vis spectrum of **7** is observed under 1 atm CO. Moreover, the ν_{CO} of **7** (1970 cm^{-1}) agrees much better with that of FeTPP(CO) (1973 cm^{-1}) than that of $FeTPP(CO)_2$ (2042 cm^{-1}), where TPP = tetraphenylporphyrin.[29] The UV-vis spectrum of **7** also agrees better with FeTPP(CO) than $FeTPP(CO)_2$. These results indicate that **7** is a monocarbonyl complex.

In summary, we have reported the design of a small-molecule platform for CO sequestration that could have applications in the development of a CO-poisoning antidote. A proof-of-principle compound, **6**, has demonstrated the viability of the synthetic strategy. Upon reduction, **6** has an affinity for CO, an ability to remove CO from COHb, and the capacity to sequester CO from poisoned RBCs. This activity is performed without any damage to the cells. The increased affinity of reduced **6** for CO as compared to Hb is expected to arise from a combination of increased electron density at the metal center, increased hydrophobicity of the CO-binding pocket, and decreased steric hinderance to linear binding; ongoing studies with the derivatives that can be readily accessed from **3** will shed light on the relative importance of these factors. We are continuing to investigate derivatives of **6** to uncover compounds with the greater stability, selectivity, and O_2 tolerance needed to function as CO-poisoning antidotes.

We thank the Hellman Foundation for awarding a Hellman Fellowship to T.C.J. and the UCSC Committee on Research for a Special Research Project Grant. X-ray diffraction studies were performed on an instrument purchased with NSF MRI grant #2018501. MALDI-TOF experiments were performed with assistance from Gordon T. Luu and Prof. Laura M. Sanchez using instrumentation supported by UCSC start-up funds to L.M.S.

There are no conflicts to declare.

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