The Chemistry, Biology and Design of Photochemical CO Releasing Molecules and the Efforts to Detect CO for Biological Applications.

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Abstract:

Several beneficial biological effects of carbon monoxide (CO) have been identified. CO contributes to vascular tone, is anti-inflammatory and antiapoptotic, and protects tissue against hypoxia. In response, a number of novel CO-releasing systems, mostly transition-metal carbonyl compounds, have been reported. Recent research in the area of photochemical CO-releasing molecules (PhotoCORMs) is reviewed. The photophysical and chemical properties of these PhotoCORMs are described, along with studies of biological activity in some cases. Spectral data and quantum yields, when available, are compared. In addition, various methods for the detection and quantification of CO are discussed.

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Key Words: CORMs, PhotoCORMs, carbon monoxide, CO detection

1. Introduction

Carbon monoxide (CO) plays a dual role as friend and foe in human biology. It has long been considered a foe to human health because of its lethal nature, causing death by asphyxiation. Due to its lack of color, taste and odor, it has

been labeled the silent killer. Also contributing to its notoriety is its ubiquity in the environment. Carbon monoxide is produced as a by-product in the combustion of fossil fuels, in metabolism, and in the burning of tobacco [1]. In many regions, carbon monoxide detectors are required in any dwellings that use fuel-burning appliances. The toxicity of CO is attributed in part to its high affinity for hemoglobin, forming carboxyhemoglobin (COHb) and thus reducing the availability of O₂ to tissues. With half of the tetramer's binding sites occupied by CO, hemoglobin can still bind oxygen reasonably well, but the release of oxygen to tissues is markedly reduced. Low levels of CO, with less than 10% of hemoglobin bound by CO, do not present acute toxicity in humans [2, 3]. As these levels increase, people can experience headaches, then dizziness, nausea, and visual disturbances, and finally, at COHb levels of 50% or greater, death [2]. Hemoglobin has a higher affinity for CO than O₂, and to make CO less of a problem nature has created a unique buffering system which allows for reversible binding of CO, as well as a possible buffering system to protect tissue [1]. Formation of COHb, however, cannot alone account for CO's toxicity. Reduction of oxygen carrying capacity by means other than CO inhalation is much less likely to be lethal, indicating that CO has an additional toxic effect, perhaps in its interaction with cytochrome C [4, 5]. Regardless of its exact mode of action, the deleterious health aspects of CO are well known, and it is understandable that CO is primarily regarded as a foe to human wellbeing.

However, CO has more recently drawn attention in a therapeutic role [3], making the issue of friend or foe complicated. This is a familiar story. Nitric oxide (NO) was also once considered only a foe to human health and an environmental pollutant, but it is now well known as an essential physiological small molecule. Along with NO and hydrogen sulfide (H₂S), CO is now recognized as a "gasotransmitter" [6, 7]. All of these small molecules are toxic at high levels but are important at low concentrations as signaling agents. However, relatively little is known about the details of how CO reacts physiologically for beneficial effects. Unlike its cousin NO, CO is fairly stable in air, with an activation energy of 213 kJ/mol for reaction with O₂, and there are no known biologically relevant interactions of oxygen and carbon monoxide [6]. The main biological target for CO is transition metals, due to CO's ability to act as a weak Lewis base and a strong π -acid, and coordination to transition metals greatly alters its reactivity [6].

Carbon monoxide is produced physiologically during heme catabolism by heme oxygenase (HO). During this process heme *b* is converted to biliverdin, CO and Fe²⁺, while two equivalents of NADPH and three equivalents of molecular oxygen are consumed (Scheme 1) [8, 9]. Three types of heme oxygenases have been identified: HO-1, which is inducible and generated in response to stress; HO-2, which is associated with neuronal activity and vascular tone; and HO-3, which does not break down heme or produce CO [1, 10]. The rate and amount of CO production by HO-1 is substantial at about 16 mL/hr in the human body but with cellular concentrations suggested to be at nanomolar levels [1]. The production of CO is associated with a variety of physiological roles, such as anti-apoptotic,

anti-inflammatory, anti-proliferation, and anti-coagulative effects, and therapeutic applications such as reduced organ graft rejection and protection against ischemia reperfusion injury [11-16]. Lack of CO production is associated with several disease states, including sepsis, diabetes, colitis and vascular inflammation [1, 15, 17]. A number of beneficial biological roles of CO, formed by endogenous heme degradation or provided pharmacologically, have been observed *in vivo*. These include anti-inflammatory, vasodilation, and anti-apoptotic effects [18]. In spite of the literature describing the biological importance of CO, the chemical mechanisms by which CO regulates these biological processes are still poorly understood.

Scheme 1. Catabolism of heme by heme oxygenase, producing biliverdin, iron(II) and CO as products [8].

Inspired by the need for greater understanding of CO biochemistry and by the promise of therapeutic applications, development of CO releasing molecules (CORMs) for controlled and directed application of CO is a fertile research area [19-22]. One advantage that CORMs have over gaseous CO as a therapeutic agent is the potential for controlled release of CO to a specific target; current CO therapies include the direct use of CO gas inhalation, an approach that is not specific and could lead to asphyxiation, as is also the case for the other two

“gasotransmitters” [23]. In addition, CORMs have application in elucidating the biological targets for this small molecule signaling agent [24]. Several general approaches to controlled CO release are being investigated. A number of compounds release CO thermally in solution [25-27]. Researchers in Germany have explored enzyme triggered CO release, making use of iron carbonyls, η -coordinated by an enol ligand, that thermally decomposed to release CO [28-31]. The iron compounds were stabilized by transformation of the enols to esters, which could be degraded by esterases, thus generating the unstable enol either *in vitro* or *in vivo*. Another CO delivery method was developed by attaching known CORMs to magnetic iron oxide nanoparticles which, after electromagnetic excitation, released CO [32]. This review will focus on the development of photochemical CORMs (PhotoCORMs) – compounds that release CO upon photochemical excitation – in the context of biological applications, as well as detection and quantification of CO. Other reviews of both CORMs and PhotoCORMs have been published [3, 19, 20, 33-35], and it is our intention to complement those reviews with this contribution.

2. PhotoCORMs

The majority of PhotoCORMs consist of metal carbonyl complexes. A generalized model for photochemical CO release from a metal carbonyl complex and formation of a solvento species is shown in Scheme 2. Such compounds have been studied for many years, but only recently have they been considered for possible therapeutic applications. In order for a PhotoCORM to be useful in a biological context, it must be relatively non-toxic, and it is desirable that its photoproducts are also non-toxic [19]. In addition, these molecules must be stable and soluble under physiological conditions. In addition to biocompatibility, the wavelengths to which they are sensitive are also important. One area of research is to enhance absorption of red light by photoCORMs to facilitate irradiation through the skin and to reduce damage by UV light [19, 36].

Scheme 2. Generalized photo-initiated reaction of a metal carbonyl complex to produce CO in solution.

Ford and coworkers have been studying the release of small molecules such as NO and CO from transition metal centers for many years [19, 37, 38]. Design of a water soluble tungsten carbonyl complex (Figure 1) as a CO donor led to introduction of the term PhotoCORM in 2010 [39], and two years later Ford et al. published a review of the existing PhotoCORMs [19].

Figure 1. Water-soluble tungsten carbonyl PhotoCORM.

About a year later Schatzschneider and coworkers published a second review, focusing mainly on manganese carbonyl complexes [20].

2.1 Manganese based PhotoCORMs

Schatzschneider et al. have synthesized a variety of manganese tricarbonyl compounds containing multi-dentate amine ligands (such as tpm = tris(2-pyrazolyl)methane, Figure 2) and demonstrated that these tripodal compounds were stable in aqueous solution in the dark but released CO when irradiated [20]. The tripodal-Mn compounds were taken up by colon cells without observed toxicity, and $[\text{Mn(CO)}_3\text{tpm}]^+$ showed promise as a water soluble, thermally stable, non-toxic PhotoCORM. This complex decomposed photochemically upon 365 nm irradiation, releasing CO as confirmed spectrophotometrically upon trapping with myoglobin. In addition, photolysis of $[\text{Mn(CO)}_3\text{tpm}]^+$ in cancer cells reduced cell viability. These results inspired efforts to structurally modify the complex with a peptide to induce specific cell targeting or with silica nanoparticles for a possible delivery strategy for solid tumor eradication [40]. The manganese tricarbonyl moiety was also used in the synthesis of the first CO-releasing metallodendrimers [41], which consisted of polypyridyl-containing dendrimers bound to 4 or 8 $\text{Mn(CO)}_3\text{Br}$ units. These were stable in the dark for several hours, but upon 410 nm irradiation released CO. One possible therapeutic advantage of complexes such as these is that dendrimers accumulate in tumor tissue [42]. In addition, ligands based on bis(pyrazolyl)ethylamine (bpea) are being actively explored for easier and more flexible synthetic modifications for bioavailability [43].

To further elucidate the mechanism of photochemical CO release from $[\text{Mn(CO)}_3(\text{tpm})]^+$, ultrafast time-resolved studies using a UV pump/mid-IR probe were carried out in combination with density functional theory (DFT) and time-dependent (TD) DFT calculations [44]. The three highest energy occupied MOs were nearly degenerate and centered on the metal, while the first several LUMOs, accessible through 264 or 347 nm excitation (the pump wavelengths used in the time-resolved studies) are of different natures; some are centered on the tpm ligand and are not expected to give rise to CO photodissociation, while others are delocalized to varying degrees over the CO ligands and are consistent with photodissociation. Spectroscopic studies, performed in D_2O and using 264 or 347 nm excitation along with mid-IR detection, were consistent with formation of a dicarbonyl solvento species. The authors note that although the pump

photons had twice as much energy as was necessary to bring about M-CO bond breakage, there was no evidence for loss of two CO molecules. UV excitation led to ultrafast CO loss followed by either geminate recombination of CO or coordination by solvent (D₂O). Excitation into the higher energy band may have induced states that did not lead to photodissociation. To investigate the possibility of breaking two M-CO bonds within the complex using multiple photons, photolyzed solutions were subjected to re-excitation with a second UV pulse; however, a monocarbonyl species was not observed.

The manganese PhotoCORM platform is also being investigated by Mascharak et al., who are actively working to design ligands that will red-shift the UV-vis spectrum [36]. Beginning with [Mn(CO)₃(tpa)]⁺ (tpa = tris(pyridyl)amine; Figure 2), a combination of rational design methods and computational chemistry indicated that modification of the amine would alter the MLCT band. Substitution of tpa by dpa (N, N'-bis(2-pyridylmethyl)amine) shifted the absorbance from 330 to 350 nm. To build on this proof of concept, ligand conjugation was increased (pqa, pyridylmethyl(2-quinolylmethyl)amine; Figure 2), which red-shifted the MLCT band another 10 nm. It was also suggested that additional ancillary ligands would transfer electron density away from the metal center and could reduce the amount of metal-CO back-bonding, influencing both the rate and quantum yield of CO release. Figure 2 shows several of the ligands incorporated into these manganese complexes, and Table 1 compares the spectral properties, rates for CO release, and quantum yields that have been reported. Notably the replacement of MeCN by Br⁻ resulted in an additional red shift, confirming the influence of a stronger σ donor. Computational studies complimented the experimental results, showing that the extended conjugation of the nitrogen based ligands can lower the energy of the LUMO, while other ancillary ligands like Br⁻ can influence the energy of the occupied molecular orbitals. This synergistic effect can be used to design new ligand systems to tune the photophysics of CO release.

In an investigation of cellular uptake of metal carbonyl complexes, compounds of rhenium and manganese moieties bound to cyanocobalamin (B12) were synthesized [18]. The rhenium complex, B12-ReCORM-2, consisted of a Re(CO)₂(Br)₂(H₂O) moiety attached to cyanocobalamin (B12) through a bridging CN. In B12-MnCORM-1, the Mn unit, consisting of Mn(CO)₃ and a tridentate amine, was appended to B12 through the 5'-OH ribose group. The manganese complex released CO upon irradiation with an LED (emission λ_{max} 470 nm) or a green Ar laser (496.5 nm). To study cellular uptake, 3T3 cells were grown on CaF₂ slides, which were then washed with the B12-ReCORM-2 and the B12-MnCORM-1 and studied with synchrotron FTIR spectromicroscopy. No cellular accumulation of the ReCORM was observed, while cellular uptake of the MnCORM occurred within 1 h. The cytoprotective effect of the MnCORM was investigated using 3T3 fibroblasts; the complex itself showed no toxicity, and its presence, along with irradiation, protected fibroblasts from dying under hypoxic conditions.

Figure 2. Some of the ligands used to make PhotoCORMs discussed in this review. [20, 36]

2.2 Iron based PhotoCORMs

A small number of PhotoCORMs based on iron have been reported, and two of these were discussed in recent reviews [19, 45-47]. Iron-iron hydrogenase model compounds photochemically generate CO when irradiated with 365 nm light [48, 49]. These molecules contain two irons and up to six CO groups that can be liberated photochemically. The details of the photochemistry are currently under investigation. However, this general class of molecules shows promise for photoCORM applications because it has a scaffold that can be synthetically modified with a desired biocompatibility property. One of the simplest iron-iron hydrogenase model compound is μ -pdt-[Fe(CO)₃]₂ (Figure 3), which has an absorbance at 337 nm that is fairly insensitive to solvent. The optical spectral changes that occur during 365 nm irradiation of μ -pdt-[Fe(CO)₃]₂ in acetonitrile are shown in Figure 4. A nearly identical UV-vis spectrum was reported for a water soluble derivative recently synthesized by Fan et al. [48] that contains an open structure, as opposed to the more basket-like apex of μ -pdt-[Fe(CO)₃]₂. For μ -pdt-[Fe(CO)₃]₂, FTIR headspace analysis, the myoglobin assay, and GC-TCD, as well as detection of CO by its coordination to a rhodium binuclear complex (described below) were used to confirm CO release. In addition, the details of the thermal back reaction of the photoproducts with CO have been studied. Solvento species are formed upon irradiation, and these react with CO to regenerate the parent compound [49].

Figure 3. Some of the photoCORMs discussed in this review [36, 48-50].

Fan et al. carried out cell viability studies using $[\mu\text{-(SCH}_2\text{CH}_2\text{COO)-Fe(CO)}_3\text{]}_2$ on human mammary epithelial cells (MCF-10A) to represent normal human cells [48]. Treatment of cells with 1-40 $\mu\text{mol/L}$ of the iron complex over 24 h. did not induce toxicity. Although each of these types of derivatives should be screened for toxicity, the initial results are promising for potential use of iron-iron hydrogenase model compounds as PhotoCORMs. The quantum yield for this compound was estimated to be 0.4 ± 0.1 at 390 nm. It is unclear if all six CO ligands are lost upon photolysis. However, prior work on $\mu\text{-pdt-[Fe(CO)}_3\text{]}_2$ supports sequential loss.

One strategy for increasing water solubility and shifting the absorbance spectrum of the diiron complexes is to chemically modify the bridging thiol. This can be done through two different synthetic routes: first, reaction of triiron dodecacarbonyl directly with a thiol, or second, reduction of $\mu\text{-S}_2\text{-[Fe(CO)}_3\text{]}_2$ followed by reaction with an alkyl halide [51]. These two routes allow for numerous combinations, but thus far we have produced the methyl ester dithiol, propane dithiol, mercaptoethanol and benzene dithiol derivatives. The photochemistry of many of these derivatives has been explored, and the quantum yields were measured for the methyl ester dithiol and the propane dithiol. On average the quantum yields for these types of iron-iron model complexes are 0.15 ± 0.05 at 365 nm and exhibit little solvent dependence. This is the quantum yield for the loss of one CO, and current work is focused on

understanding the complete photochemical reaction and subsequent thermal reactions.

Figure 4. Spectral changes occurring upon 365 nm photolysis of μ -pdt- $[\text{Fe}(\text{CO})_3]_2$ in acetonitrile. Spectra were recorded every 30 seconds in both the optical and the IR (inset). [52]

2.3 Rhenium based PhotoCORMs

Ford and coworkers reported the synthesis, characterization, and photochemical behavior of the water-soluble photoCORM $\text{fac-}[\text{Re}(\text{bpy})(\text{CO})_3(\text{thp})]^+$ (thp = tris-(hydroxymethyl)phosphine) [50]. This luminescent and biocompatible PhotoCORM (Figure 3) was characterized by X-ray diffraction, MS, IR, and UV-vis techniques [50]. Upon 345 nm irradiation in water, this complex lost CO to form an aqua complex, as indicated by a red-shift of the electronic absorption band, shifts of IR bands to lower frequency (as well as a reduction in the number of IR bands), and GC-TCD analysis of the headspace, indicating ~one CO formed for each complex lost. Both the starting complex and the photoproduct were strongly luminescent, with luminescence maxima at 515 nm for $\text{fac-}[\text{Re}(\text{bpy})(\text{CO})_3(\text{thp})]^+$ and 585 nm for the photoproduct. Neither the starting complex nor its photoproduct showed immediate toxicity in cellular studies, and confocal fluorescence microscopy showed that the starting complex was incorporated into the cellular cytosol without penetrating the nuclear envelope. Within the cell, photolysis of $\text{fac-}[\text{Re}(\text{bpy})(\text{CO})_3(\text{thp})]^+$ generated the CO-loss product, as studied with fluorescence microscopy.

2.4 Ruthenium based PhotoCORMs

Ruthenium carbonyl complexes have primarily been investigated as thermal CORMs. A ruthenium-based PhotoCORM was synthesized by Mascharak et al. by modification of $[\text{RuCl}_2(\text{CO})_3]_2$ (known as CORM-2) with ligands designed for the manganese system described above [36]. A potentially tridentate ligand (qmtpm; 2-quinoline-N-(2'-methylthiophenyl)-methylenimine, Figure 2) was used that coordinated to the metal through nitrogen but not sulfur in the manganese derivative. However, in the ruthenium analog, the ligand completely coordinated to the metal, resulting in a complex $[\text{Ru}(\text{qmtpm})(\text{CO})_2\text{Cl}]^+$ having a MLCT band at 405 nm. Synthetically modifying the complex by replacing one CO with PPh_3 resulted in a red shift of the MLCT band to 465 nm. This allowed experimental demonstration of the effects that σ donation and π accepting abilities have on the position of the MLCT band. Additionally, computational studies were conducted to explain the electronic effects on these frontier orbitals. Thermal stability of these compounds was not mentioned, but they were reported to be photochemically active, exhibiting CO release.

Recently Schatzschneider and coworkers reported the synthesis of a series of ruthenium(II) carbonyl polypyridyl complexes [53]. Seven ruthenium(II) complexes were made with bi- or tridentate polypyridyl ligands including a peptide nucleic acid (PNA) coupled to a 2-(pyridine-2-yl)pyrimidine ligand. These complexes had the general formula $\text{RuCl}_2(\text{L})(\text{CO})_2$ or $\text{RuCl}(\text{L})(\text{CO})_2$. The structures of these complexes are shown in Figure 5. All of these ruthenium polypyridyl compounds showed MLCT bands with large extinction coefficients that dominated the optical spectra in the 299-326 nm range, similar to the $[\text{Ru}(\text{bpy})_3]^{2+}$ base compound. Complexes $\text{RuCl}_2(\text{CO})_2$ -**3-7** and $\text{RuCl}(\text{CO})_2$ -**6** were stable in aqueous solution in the dark for at least 24 h. Most of the compounds had observable optical spectral changes upon irradiation at 365 nm, and all of the compounds released CO, as shown using the myoglobin assay. Compounds $\text{RuCl}(\text{CO})_2$ -**6** and $\text{RuCl}_2(\text{CO})_2$ -**7** released CO thermally in the dark over the course of an hour, but with minor decomposition. Complexes $\text{RuCl}_2(\text{CO})_2$ -**1** and $\text{RuCl}_2(\text{CO})_2$ -**3** and $\text{RuCl}_2(\text{CO})_2$ -**7** had small quantum yields for photochemical CO release, on the order of 2×10^{-4} (irradiated at 365 nm) with small variations between compounds. The authors conclude that compounds $\text{RuCl}_2(\text{CO})_2$ -**1**, $\text{RuCl}_2(\text{CO})_2$ -**3** and $\text{RuCl}(\text{CO})_2$ -**6** are promising PhotoCORMs. These molecules allow for a flexible platform for synthetic modifications, are photochemically active, and, in some cases, thermally stable. Quantum yields may be low due to interference of myoglobin in solution during photolysis, which could decrease the fraction of light absorbed by the PhotoCORMs, and additional studies could clarify these numbers.

Figure 5. Ruthenium(II) polypyridyl based PhotoCORMs [53].

Table 1. Some of the quantitative properties of recently reported PhotoCORMS.

PhotoCORM	Quantum Yield (Φ)	Rate of CO release (s^{-1})	λ_{max}	Ref
$[\text{Mn}(\text{CO})_3(\text{tpa})]^+$	NA	NA	330 nm	[54]
$[\text{Mn}(\text{CO})_3(\text{dpa})]^+$	NA	NA	350 nm	[36]
$[\text{Mn}(\text{CO})_3(\text{pqa})]^+$	0.06-0.09 (360 nm)	$8.1\text{-}11.7 \times 10^{-2}$	360 nm	[36]
$\text{fac-}[\text{Re}(\text{bpy})(\text{CO})_3(\text{thp})]^+$	0.21 (365 nm) 0.11 (465 nm)	NA	348 nm	[50]
$\mu\text{-pdt-}[\text{Fe}(\text{CO})_3]_2$	0.15 (365 nm)	NA	327 nm	[55]
6-hydroxy-3-oxo-3H-xanthene-9-carboxylic acid	6.8×10^{-4} (500nm)	NA	488 nm	[56]

2.5 Molybdenum based PhotoCORMs

A series of molybdenum tricarbonyl compounds containing alkynyl ligands was synthesized, and one of these was active as a PhotoCORM [57]. This complex, $(\text{Mo}(\text{C}\equiv\text{CCH}_2\text{O}-\beta\text{-D-fructopyranose})(\eta^5\text{-C}_5\text{H}_5)(\text{CO})_3)$ (Figure 6), was water soluble and released CO very slowly in the dark, as shown by the myoglobin assay. Upon 325 nm irradiation, CO release was markedly accelerated compared to the thermal reaction. Half-lives for photochemical CO release were on the order of hundreds of seconds and varied with concentration; quantum yields were not reported. Based on the amount of Mb-CO formed compared to the amount of molybdenum complex degraded, multiple CO molecules were released.

Figure 6. Water soluble molybdenum PhotoCORM, releases 2.2 CO per molecule [57].

2.6 Organic PhotoCORMs

Recently two organic PhotoCORM systems have been reported, one based on a fluorescein analogue [56] and another based on a cyclic α -diketone [58]. The former compound, 6-hydroxy-3-oxo-3H-xanthene-9-carboxylic acid (Scheme 3), has a λ_{max} at 488 nm at pH 7.4 and is stable in water and in the dark for at least a month. Upon 500 nm irradiation in water at pH 7.4, CO is released with a quantum yield of $(6.8 \pm 3.0) \times 10^{-4}$ as determined by a hemoglobin assay. A higher quantum yield was obtained at lower pH, demonstrating the pH dependence of the photochemistry.

Scheme 3. Photochemical reaction of 6-hydroxy-3-oxo-3H-xanthene-9-carboxylic acid, a fluorescein analogue PhotoCORM [56].

The latter compounds, cyclic diketones (DK), are unstable towards hydrolysis. Encapsulation of DK in Pluronic F127 micelles allowed biological compatibility and functionality [58]. Three derivatives of varying degrees of hydrophobicity were reported (Scheme 4). Irradiation into $n\text{-}\pi^*$ bands using light from 400 – 550 nm resulted in release of two equivalents of CO and formation of an anthracene

derivative (Scheme 4). Since the diketones are reactive with dithionite, a reagent in the conventional myoglobin assay, a two-system myoglobin assay was used to confirm the generation of CO. Photolysis of the DK was done in an airtight syringe, followed by the introduction of a myoglobin solution into the syringe. Using the spectral changes corresponding to formation of MbCO, 84% of CO from DK3 was calculated to bind to myoglobin. This was correlated to a 90% yield of CO release calculated from UV-vis spectral changes corresponding to formation of the anthracene product. Fluorescence of the anthracene-like product allowed for observation of cellular uptake and confirmation of CO release. In addition, cell viability studies did not demonstrate adverse results from either the PhotoCORM or the anthracene derivative.

Scheme 4. Structure of α -diketone analogs and their photochemical reactivity to generate CO [58].

3. Detection of CO

In order to evaluate the effectiveness of CO release from PhotoCORMs and to understand the role of CO in biological systems, the ability to quickly and selectively detect CO at low concentrations is crucial. Several methods have been explored. The most commonly used method is the myoglobin assay, by which free CO is trapped by deoxymyoglobin, resulting in a spectral shift [26]. A rhodium-based system can be used as a colorimetric detector [59-62] and its advantages and disadvantages compared to the traditional myoglobin assay will be discussed. Electrochemical methods have been developed for simultaneous detection of NO and CO in solution, but no commercial electrode is yet available for direct CO detection [63]. Another approach is to analyze headspace above solutions in which CO has been generated. FTIR, GC-TD, and Fe^{II}TPPS flash photolysis have all been employed for headspace analysis [19, 20]. Lastly, fluorescent probes, based on both small molecules and on proteins, are currently being developed for CO detection [64-66]. Specific details and examples of the application of these techniques are described below.

3.1 Myoglobin Assay

The colorimetric myoglobin assay has been used since the development of CORMs [26]. Binding of CO to Mb occurs with a second order rate constant of

0.38 $\mu\text{M}^{-1}\text{s}^{-1}$ and a binding constant of 16.9 μM^{-1} and results in a spectral shift from 560 nm to 540 and 575 nm. The assay is conducted in aqueous solutions at physiological pH, and traditionally spectral changes at 540 nm are monitored. Quantification of CO using this method relies on pure protein, and impurities can cause problems with isosbestic behavior. In addition, there can be competing absorbances from the CORM that is being studied. This is likely to be the case for all PhotoCORMs. Lastly the assay must take place in anaerobic conditions because the iron in the myoglobin is sensitive to oxygen, forming oxygen adducts. It is standard practice to use sodium dithionite to generate the deoxymyoglobin and either introduce the CORM or measure CO *in situ*. However, dithionite is very reactive and causes the release of CO from certain CORMs [67, 68]. Detailed guidelines to address several of the issues concerning the quantification of CO using this technique are published [26].

3.2 Rhodium Assay

A promising alternative for detecting CO in solution is a binuclear rhodium (II) derivative with two ortho-metalated phosphine ligands in a head to tail arrangement, as shown in Scheme 5 [60, 61]. The rhodium scaffold allows synthetic flexibility and could lead to increased water solubility. It is robust in air and has defined spectral changes in both the UV-vis and the IR for monitoring CO attachment. The system is selective toward CO compared to O_2 , carbon dioxide, or N_2 . Some binding of NO has been observed, but only in the presence of high concentrations (1320 ppm). Binding of CO is fully reversible, but the rate of release is slow, over 17 hours in solution, which allows for quantification and detection.

These molecules have been used in biological systems as slow CO donors [62]. They have an absorbance maximum near 560 nm (with ϵ ranging from 157-711 $\text{M}^{-1}\text{cm}^{-1}$), which shifts to 510 nm upon binding of one CO and finally to 390 nm (ϵ ranging from 421-7040 $\text{M}^{-1}\text{cm}^{-1}$) when a second CO binds. The absorbance maxima are slightly sensitive to solvent. Two derivatives of these binuclear rhodium complexes have been tested for toxicity and were not toxic toward RAW 264.7 macrophages [62].

Scheme 5. Reaction of binuclear rhodium(II) complex with CO, where L may be acetate or water. Various phosphines and/or acetate groups can be used to direct solubility [59].

Figure 7. Changes in absorbance of binuclear rhodium complexes in CH_2Cl_2 as CO is added [69].

3.3 Fluorescent Probes

Fluorescent probes have proven useful for the detection of small molecule signaling agents such as NO and H_2S [70, 71], but just recently has this avenue been explored for detection of CO. One approach by Chang et. al. uses a cyclometallated palladium probe and takes advantage of palladium catalyzed carbonylation chemistry [66]. The probe uses a borondipyrromethene difluoride (BODIPY) core, the fluorescence of which is quenched by coordination to palladium. Upon reaction with CO, the carbonylated fluorescent core is released along with palladium(0) (Scheme 6). Because this reaction is unique to CO, this method shows strong selectivity for detection of CO over other biologically important small molecules such as NO. To date only the first generation of this carbon monoxide probe (COP) has been released; it is therefore referred to as COP-1. COP-1 is weakly fluorescent in phosphate buffer at physiological pH, but the fluorescence switches on when reacted with a well-characterized thermal CORM, as indicated by comparison to the fluorescence of the independently synthesized BODIPY core.

COP-1 was also studied *in vivo* and tested for cellular toxicity. Cells treated with both COP-1 and CORM showed increased fluorescence as compared to cells incubated with only COP-1 and no CORM. Cellular toxicity was tested by evaluating the nuclei of cells, which were both intact and viable. In addition, cell proliferation studies were performed with a range of COP-1

concentrations with and without CORMs. Showing no toxicity issues, this is a promising small molecule fluorescent approach for detecting CO.

Scheme 6. Formation of a strongly fluorescent carbonylated product upon reaction of a Pd-BODIPY complex with CO.

Schatzschneider recently utilized COP-1 for the detection of CO from PhotoCORMs based on manganese(I) tricarbonyl [43]. This opportunity allowed for the exploration of COP-1 to detect CO from a PhotoCORM. A non-linear fluorescent response of COP-1 was observed upon reaction with PhotoCORM and 365 nm irradiation. No fluorescence was detected in the non-irradiated control. In addition, the analogous experiment was done in living cells. COP-1 and the PhotoCORM were incubated with cells in the dark. Upon 365 nm irradiation of cell cultures, only modest fluorescence was observed inside the cells compared to the supernatant, suggesting poor cell permeation or a cellular impedance. Regardless, this fluorescent probe shows promise as a CO detector for non-cellular applications, and the next generation may have increased cellular viability.

Another approach used circularly permuted yellow fluorescent protein (cpYFP) as a fluorescent probe tethered to a modified CooA protein [64, 65]. CooA is a homodimer regulatory CO binding protein and was obtained from *Rhodospirillum rubrum*. CO binds to an iron-heme in the regulatory domain and displaces a proline residue, which induces a conformational change that allows the F-helix to bind DNA. He and coworkers used this natural selectivity to create a fusion protein using cpYFP and CooA, which fluoresces when the protein experiences structural modification after binding CO (Scheme 7). The fusion protein was made using PIPE cloning methods and was named COSer for its new use as a CO sensor. The protein was purified using standard methods and was selective for CO over other small biological molecules (NO, H₂S, imidazole, CN⁻, and GSH) *in vitro*. It also showed promise for *in vivo* selective CO determination after HeLa cells were transfected with a COSer expression vector.

When the HeLa cells were treated with a solution equilibrated with CO, fluorescence was weak, but it significantly increased after equilibration with a thermal CORM. This system has potential for real time, cellular CO determination.

Scheme 7. COser fusion protein made by combining CO binding protein, CooA, and a yellow fluorescent protein. When CO binds to the heme, a conformation change occurs and the yellow fluorescent protein is switched on [64].

3.4 Electrodes

Simultaneous detection of CO and NO was reported with a new dual electrode system, consisting of two platinum microelectrodes, one plated with tin, covered with a PTFE gas permeable membrane and working in conjunction with a Ag/AgCl reference/counter electrode [63]. The diameters of the platinum working electrodes were ~250 and 25 μm . Both electrodes were sensitive to both CO and NO; however, one was relatively more sensitive to CO than the other, so that simultaneous measurements of responses for both electrodes, along with calibration curves, allowed for calculation of [CO] and [NO] individually. Response times were relatively quick: 14 - 23 s for NO and 25 - 38 s for CO. Detection limits were ~1 nM for NO and <5 nM for CO, and real-time, direct measurement of CO and NO generated from mouse kidneys was reported.

3.5 FTIR Analysis of Headspace

UV photolysis of $\text{W}(\text{CO})_5(\text{TPPTS})^{3-}$ (TPPTS = tris(sulphonatophenyl)phosphine) resulted in CO loss to form a solvento species, and quantification of CO loss was accomplished using headspace analysis [39]. Solutions of $\text{W}(\text{CO})_5(\text{TPPTS})^{3-}$ were subjected to 308 or 313 nm photolysis in a Schlenk cuvette, and samples of

headspace were withdrawn through the septum using a gastight syringe. Gas samples were then analyzed by FTIR, which was the first attempt to use this method to detect CO from a CORM. After injecting samples into a standard IR gas cell (7.1 cm pathlength), total gas pressure was brought to 1 atm using air because the intensity of the CO band depends strongly on total pressure. The concentration of CO was then determined from the absorbance at 2168.1 cm^{-1} . Analysis by FTIR was complimented by two other methods for headspace analysis: gas chromatography with a thermal conductivity detector (GC-TCD), and flash photolysis of $\text{Fe}^{\text{II}}(\text{TPPS})$, which is described further in Section 3.6. Results for all three techniques indicated a yield of just under 1 CO for each molecule of W complex decomposed by the initial photoreaction, and then between 1 and 2 molecules of CO per molecule of W complex for the subsequent thermal reaction in aerated media.

The use of IR analysis of headspace was further developed in investigations of CORM-2, $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$, which releases CO thermally when dissolved in DMSO, and CORM-S1, $[\text{Fe}(\text{CO})_2(\text{SCH}_2\text{CH}_2\text{NH}_2)_2]$, which releases CO upon irradiation [72]. Modifications of a standard IR gas cell were employed. Solutions of CORM were placed in a cup in the bottom of the cylindrical gas cell, and CO (along with other gases) was detected in the gas phase above. For analysis of the photo-CORM, an in-house constructed cell was used, which allowed for continuous irradiation of the sample while it was in the instrument. Integration of the CO peaks in the gas phase, insensitive to total pressure, was used to quantify the amount of CO produced. Notably, the decomposition of CORM-2 was dependent on the concentration of dithionite, which is significant because dithionite is commonly used to reduce myoglobin. To characterize the metal-containing products, solutions were dried and analyzed using ATR-FTIR.

3.6 $\text{Fe}^{\text{II}}(\text{TPPS})$ Flash Photolysis

The water-soluble porphyrin complex $\text{Fe}^{\text{II}}(\text{TPPS})$ (TPPS = tetrakis(sulphonatophenyl)porphyrinato) reacts with CO to form $\text{Fe}^{\text{II}}(\text{TPPS})(\text{CO})$, and the photochemistry of this system has been well characterized [73]. In the laser flash photolysis of $\text{Fe}^{\text{II}}(\text{TPPS})$ equilibrated with excess CO, k_{obs} for the reaction of the photoproduct with CO is linearly [CO]-dependent. Thus measurement of k_{obs} , in combination with a calibration curve, can be used to calculate CO concentration. To quantify the amount of CO released from irradiated solutions of $\text{W}(\text{CO})_5(\text{TPPTS})^{3-}$, headspace samples were injected into solutions of $\text{Fe}^{\text{II}}(\text{TPPS})$, and measurement of k_{obs} after flash photolysis was used to determine [CO] in the sample [39]. Results from this method were consistent with those obtained using FTIR and GC-TCD.

Summary:

As our understanding of the biochemistry of CO increases, so too does our need for specialized CO detection methods. Chromatographic or spectroscopic analysis of gas samples detects CO directly and quantitatively, but does not give

spatial information about the source of CO. Similarly, electrochemical methods are promising for direct, fast, and sensitive detection of CO, but they may be limited in terms of spatial resolution. Thus for information regarding the location of CO activity, molecular probes, either colorimetric or fluorescent, are crucial. Colorimetric probes present some inherent difficulties in photoCORM systems, as absorbance changes for the probe may be obscured by other species in solution, or the probe may interfere with the absorption of light by the PhotoCORM. In a highly absorbing medium, fluorescence that is activated by reaction with CO is a distinct advantage. Currently developers of molecular probes for CO face some of the same challenges as developers of photoCORMs: solubility, biocompatibility, cellular uptake. Recent developments in this field have been remarkably innovative.

4. Conclusion

It is well accepted that CO is produced endogenously and is correlated with beneficial physiological effects and is therefore considered an important biological small signaling molecule. The exact biological targets that determine these beneficial effects are not known and are currently an active area of research. Unlike NO, CO is relatively inert toward reactions with thiols and oxygen containing species. Therefore it is likely that the biological target is a transition metal complex. One possible target is soluble guanylyl cyclase, but there is only a slight increase in activation compared to NO, and this would not explain all of the observed physiological effects that are correlated with CO [10]. Many biological pathways have been proposed for interaction with CO, and this research is likely in its infancy. In an analogous manner as NO releasing molecules provided insight into both the reactivity of NO and its biological targets, CORMs could have a prominent role in answering many questions regarding the chemical biology of CO.

Metal carbonyl complexes have been known and studied for many years, and it is exciting to see them utilized for potentially therapeutic applications. They are currently being considered as both thermal and photochemical CO releasing molecules, and their proposed application in biological systems implies certain desirable characteristics regarding solubility, toxicity, stability, and light absorption. Thus there exists immense opportunity for chemists to exercise innovation in the design and synthesis of new systems. Computational work in combination with ligand design is being used to tune the redox and photochemical properties of these molecules, while metal-containing moieties are being combined with macromolecules and bio-molecules of well-characterized biological activity. In addition, the challenge of observing and quantifying CO release in solution and in cells has led to innovations in analytical technique. Much progress has been made in the synthesis and study of PhotoCORMs, and it will be interesting to watch the development of this rich, expanding area of research.

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