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FOCUS REVIEW

Capture of carbon monoxide using a heme protein model: from biomimetic chemistry of heme proteins to physiological and therapeutic applications

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Abstract

Hemoglobin (Hb) and myoglobin (Mb) are heme proteins that capture and transport molecular oxygen (O_2) in living organisms. Although mimicking the O_2-binding function of Hb and Mb using synthetic iron-porphyrin has been extensively studied for over 40 years, mimicking the function of Hb/Mb in vivo or 100% aqueous solutions has been minimally studied. Hence, a supramolecular complex termed "hemoCD1" was constructed as the aqueous synthetic Hb/Mb model using a 1:1 inclusion complex of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron(II) (Fe^{II}TPPS) with a per-*O*-methylated β-cyclodextrin dimer with a pyridine linker (Py3CD). Selective carbon monoxide (CO) depletion in vivo is beneficial for studying the physiological roles of endogenous CO as a gaseous signal messenger. HemoCD1 reversibly binds O_2 and CO in aqueous solution at ambient temperature. Hence, hemoCD1 was used as a selective CO scavenger in vivo due to its extremely high binding affinity to CO. In addition, hemoCD1 was utilized to quantify the accumulation of endogenous and exogenous CO in organs/tissues. Finally, hemoCD1 was employed as an antidote for CO poisoning in animals. Thus, recent developments in hemoCD1 have revealed that the aqueous biomimetic heme protein model has significant physiological and therapeutic application potential.

Introduction

Heme (a protoporphyrin IX iron complex or its analog) is ubiquitous in living systems and an essential cofactor of heme proteins (Fig. 1) [1–4]. Heme bound to apoproteins forms highly functional heme proteins, such as myoglobin (Mb) in muscles, hemoglobin (Hb) in red blood cells, and cytochrome *c* oxidase (C*c*O) in mitochondria, which are primarily essential in the O_2-dependent energy conversion process [1, 5–7]. However, heme itself (without a protein) is a nonfunctional and harmful molecule because it tends to self-aggregate in vivo owing to its hydrophobic nature and generates reactive oxygen species (ROS) from oxygen (O_2) through a Fenton-type reaction [8–12]. Thus, a protein matrix provides appropriate environments for heme to exhibit physiologically important functions in living systems.

We constructed artificial biomimetic models of heme proteins using a porphyrin/cyclodextrin dimer supramolecular complex (Fig. 2) [13, 14]. A supramolecular 1:1 inclusion complex, hemoCD1, was constructed using 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron(II) (Fe^{II}TPPS) and a per-*O*-methylated β-cyclodextrin dimer with a pyridine linker (Py3CD). Fe^{II}TPPS and Py3CD were utilized as alternatives for the heme molecule and Hb/Mb apoprotein matrix, respectively, and formed a stable supramolecular complex in water. Analogous to Hb and Mb, the iron(II) center of Fe^{II}TPPS is encapsulated and isolated by Py3CD from the aqueous bulk phase, which is essential for forming a stable O_2 complex in water. Thus, hemoCD1 functions as a synthetic heme protein model in aqueous solution at room temperature [14, 15].

In this focused review, we overview the characteristics of hemoCD1 and its recent development as a unique artificial heme protein model that functions in aqueous biological media. Among the intriguing characteristics of hemoCD1, we focused on highly specific binding to carbon monoxide (CO), which can be utilized for physiological/therapeutic applications in vivo.

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Biomimetic chemistry of myoglobin in water

In 1973, Collman et al. reported the first synthetic Mb model, known as "Picket-fence porphyrin," which reversibly binds O_2 in absolute toluene [16, 17]. This compound has four *o*-pivalamidephenyl groups at the *meso*-positions of porphyrin, preventing the formation of a μ-oxo dimer of iron(III)porphyrin. Following this discovery, many synthetic metalloporphyrins with bulky *meso*-substituents have been reported as synthetic Hb/Mb models [5, 18–25]. However, these synthetic models capture O_2 in anhydrous (absolute) organic solvents, and fewer models function in aqueous media, whereas native Hb/Mb can bind O_2 in water. Furthermore, trace contamination with water must be eliminated from the solution to detect the O_2 complexes of the synthetic porphyrins. Therefore, based on the molecular design of the picket-fence porphyrin, it was considered that preventing μ-oxo dimer formation is the key to constructing synthetic Hb/Mb model complexes in homogenous solutions. Nevertheless, few synthetic models can bind O_2 stably in aqueous solutions [26–29]. Stable and reversible O_2 complex formation in aqueous media has been realized by synthetic porphyrins embedded into hydrophobic lipid membranes, as demonstrated by Tsuchida, Komatsu, and their coworkers [30–32].

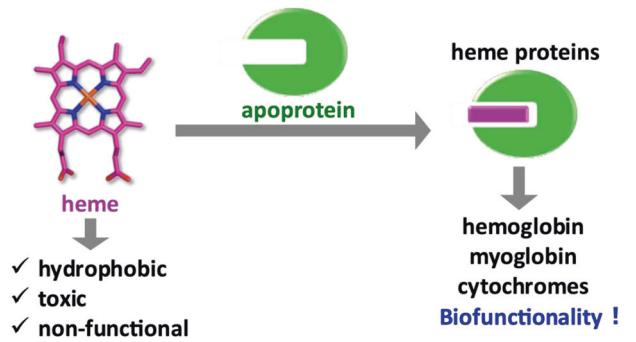

Fig. 1 Heme, a ubiquitous cofactor in living systems, performs its biofunctionality when it is bound with apoproteins

Tetrakis(4-sulfonatophenyl)porphyrin (TPPS) forms a very specific 2:1 inclusion complex with 2,3,6-tri-*O*-methyl-β-cyclodextrins (TMe-β-CD) in aqueous solution (Fig. 3) [33, 34]. The binding constant for the formation of this inclusion complex was estimated to be over $10^{16}\,M^{-2}$ ($=K_1K_2$). Furthermore, Kano et al. reported a significant decrease in the pK_a of protonated TPPS when included by TMe-β-CD ($\Delta pK_a = -5.4$). This decrease in the pK_a indicated that TPPS encapsulation by TMe-β-CD isolated the porphyrin ring to the hydrophobic cavity, thereby preventing protonation of the inner nitrogen of TPPS. Moreover, in 2004, complexation between TMe-β-CD and Fe^{III}TPPS was characterized [35]. Interestingly, anion species such as azide (N_3^-) strongly bind to the iron(III) center of Fe^{III}TPPS when complexed with TMe-β-CD in aqueous solutions. These anions do not coordinate with Fe^{III}TPPS in the absence of TMe-β-CD because of strong hydration in water. The iron (III) center of the Fe^{III}TPPS/TMe-β-CD inclusion complex was placed in the hydrophobic pocket and thus showed analogous anion-binding ability to native ferric Mb (met-Mb) [36]. Therefore, the Fe^{III}TPPS/TMe-β-CD complex can be regarded as a biomimetic met-Mb model complex in water.

Our group was the first to report an aqueous biomimetic Mb model complex hemoCD1 composed of Fe^{II}TPPS and Py3CD (Figs. 2 and 4a) [13]. HemoCD1 reversibly binds O_2 in aqueous solution at room temperature because of the tight encapsulation of Fe^{II}TPPS in the hydrophobic pocket of Py3CD. The O_2 adduct of hemoCD1 (oxy-hemoCD1) formed in aqueous solution readily converted to its deoxy and CO-adduct (CO-hemoCD1) when the atmosphere of the solution was changed (Fig. 4b). The resonance Raman spectrum for oxy-hemoCD1 in water at room temperature showed the Fe^{II}–O_2 bond stretching band at 569 cm^{-1}, which is almost the same as that of the O_2 complex of native

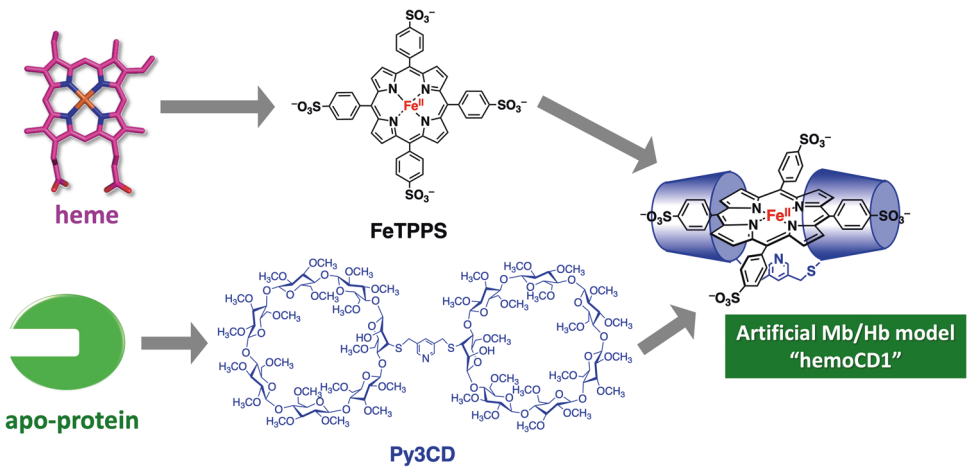

Fig. 2 Construction of the aqueous heme protein model complex "hemoCD1" comprising 5,10,15,20-tetrakis (4-sulfonatophenyl) porphinatoiron(II) (Fe^{II}TPPS) and a per-*O*-methyl-β-cyclodextrin dimer (Py3CD)

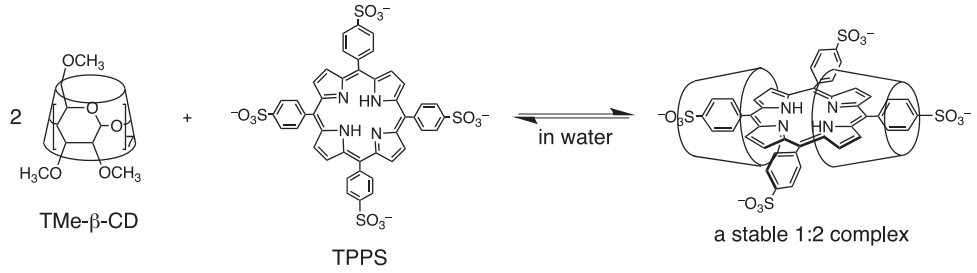

Fig. 3 Highly specific host-guest interaction between 2,3,6-tri-*O*-methyl-β-cyclodextrin (TMe-β-CD) and 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrin (TPPS)

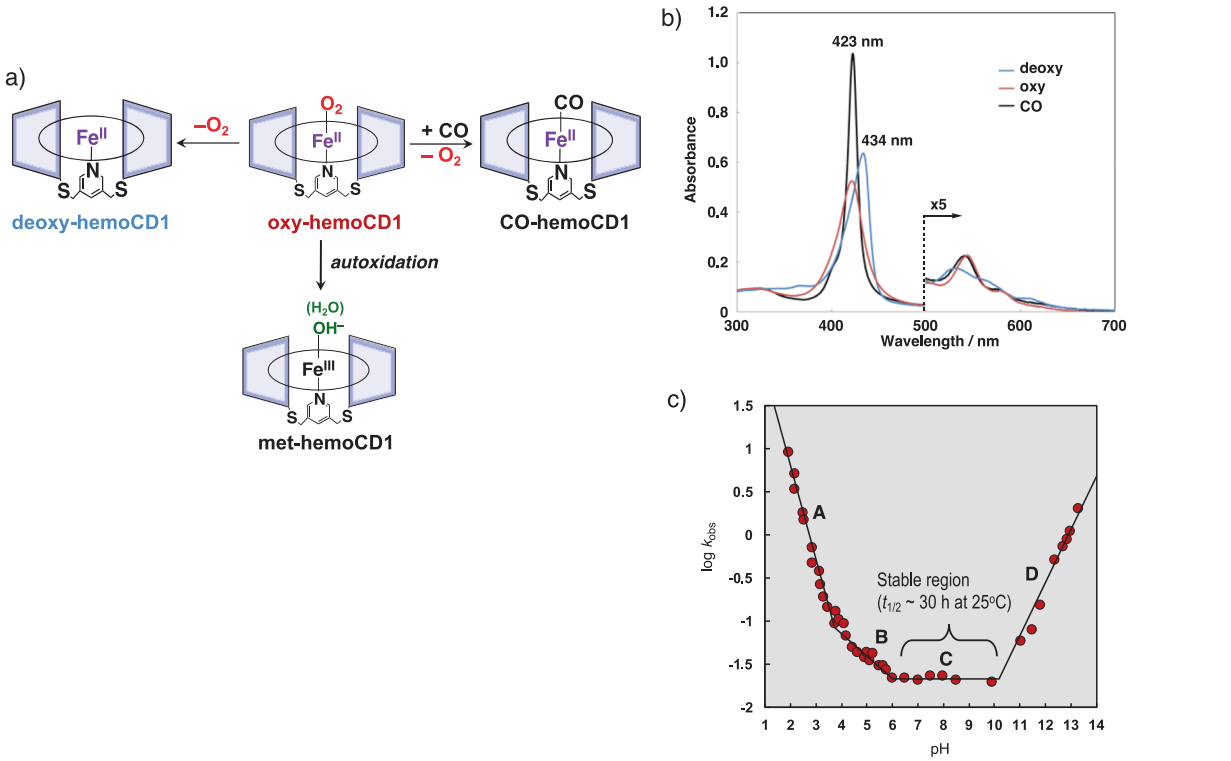

Fig. 4 **a** Molecular oxygen (O_2) and carbon monoxide (CO) binding and autoxidation of hemoCD1. **b** UV-vis spectra of deoxy-, oxy-, and CO-hemoCD1 in phosphate buffer at pH 7 and 25 °C. **c** The pH-rate profile for the autoxidation of oxy-hemoCD1 at 25 °C. Reproduced from ref. [15] with permission. Copyright (2006) American Chemical Society

Mb (horse hart Mb; 571 cm^{-1}) [13, 37], indicating the formation of an O_2-Fe^{II} complex similar to the native heme-O_2 complex in Mb in water. Oxy-hemoCD1 gradually autoxidized to its iron(III) met-form (met-hemoCD1) with a half-life of 30 h in the pH 6–10 region at room temperature (Fig. 4c) [14]. Thus, hemoCD1 can be used as an artificial O_2-carrying molecule at neutral pH in vivo.

Selective removal of CO in vivo

The injection of hemoCD1 into animals was first tested in rats [38]. This experiment was initiated to employ hemoCD1 as an alternative to Hb, which is a synthetic O_2 carrier in blood; however, hemoCD1 acted as a selective CO scavenger in vivo. After intravenous injection of hemoCD1 into the vein of a rat, it was excreted in the urine within an hour. The excreted hemoCD1 contained the CO complex CO-hemoCD1 (Fig. 5a). Because the animals were not exposed to CO, the CO excreted with hemoCD1 must originate from an endogenous biological source. CO is endogenously produced in mammals during heme metabolic reactions catalyzed by heme oxygenase (HO) enzymes [2–4, 9–12]. Because the CO-binding affinity of hemoCD1 is much higher than that of Mb, Hb, and other heme proteins in living systems [15, 39], the injected hemoCD1 quantitatively eliminated endogenous CO from these heme proteins. Thus, the injection of hemoCD1 produced an endogenous CO-depleted state in the animals.

CO is generally known as a toxic gas generated by incomplete combustion during fire accidents. However, CO produced endogenously is an important signaling gas

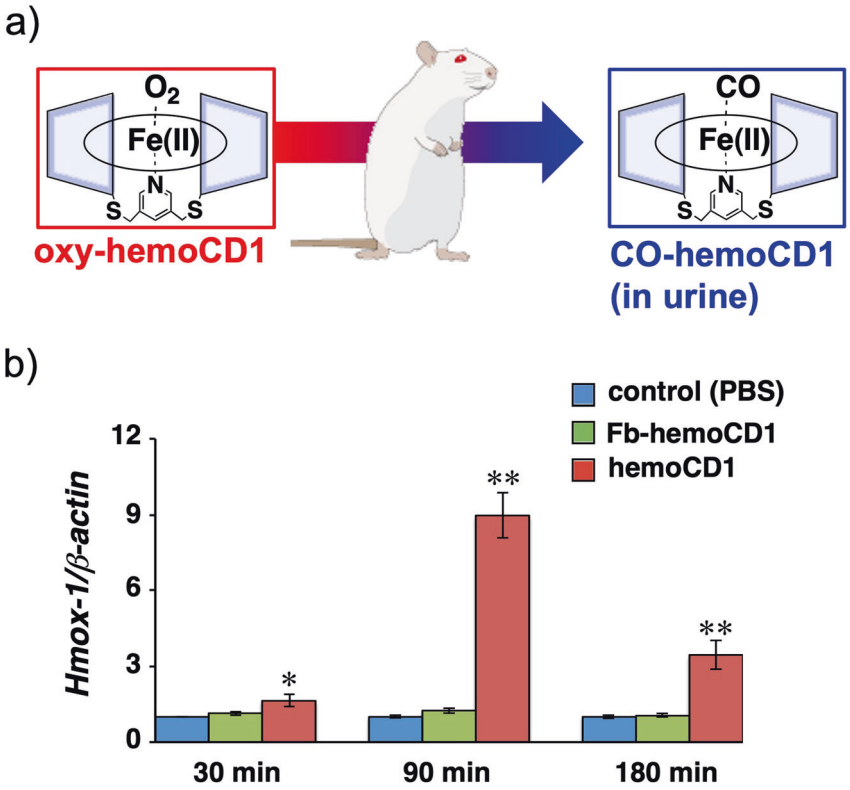

Fig. 5 **a** Selective removal of endogenous carbon monoxide (CO) from the animal body by injecting oxy-hemoCD1. **b** Induction of heme oxygenase-1 mRNA (*Hmox-1*) upon injecting oxy-hemoCD1. Phosphate-buffered saline (PBS) and the iron-free complex (Fb-hemoCD1) were utilized as controls. Reproduced from ref. [49] with permission. Copyright (2016) American Chemical Society

molecule that exerts various physiological functions, such as anti-inflammatory, antiapoptotic, and antiproliferative effects [3, 40–42]. In addition, CO is regarded as a medical gas [43–45]. Recently, CO-releasing molecules (CORMs) have been used as therapeutic agents that can deliver appropriate amounts of CO into the body [45–48]. Moreover, the physiological functions of CO have been extensively studied using CORMs [46–48]. Nevertheless, there is no approach to selectively deplete endogenous CO; therefore, the removal of CO by hemoCD1 can contribute to the study of the biological function of endogenous CO in vivo.

When oxy-hemoCD1 was intraperitoneally injected into mice, a marked reduction in CO-Hb levels in blood from 0.2% to 0.02% was observed [49, 50]. Interestingly, the CO-Hb level quickly recovered to the normal level within 30 min after the excretion of endogenous CO by hemoCD1, indicating that the feedback system responded to maintain CO homeostasis. Consistently, the removal of endogenous CO by oxy-hemoCD1 significantly induced heme oxygenase-1 (HO-1) expression in the murine liver (Fig. 5b). Overexpressed HO-1 can produce additional endogenous CO via heme degradation, compensating for the temporary CO depletion caused by oxy-hemoCD1 injection. This is the first example that reveals feedback regulation for maintaining the homeostasis of endogenous CO in mammals, which was first revealed by a synthetic biomimetic heme protein model compound.

Some genes exhibit rhythmic expression in an approximately 24 h cycle in living systems, known as the "circadian clock." Several transcription factors regulate the circadian clock. Among them, circadian locomotor output cycles kaput (CLOCK) and neuronal PAS domain protein 2 (NPAS2) are the main transcription factors, containing heme proteins that bind CO in its heme cofactor [3, 4, 51–53]. Therefore, endogenous CO has been considered to play a role in circadian clock regulation [53–56]. HemoCD1 was used to remove endogenous CO to show CO involvement in the circadian clock system, and the effect of CO depletion on clock genes was investigated [57]. A temporal decrease in endogenous CO by oxy-hemoCD1 injection in mice showed marked disruption of the main clock genes (*Per1*, *Per2*, *Cry1*, and *Cry2*). The disruption lasted over 19 h and subsequently returned to normal rhythmic expression. This disruption was ascribed to changes in the transcriptional activity of CLOCK and NPAS2. In addition, inflammation caused by a decrease in endogenous CO is also involved in circadian clock disruption. These findings support the hypothesis that endogenous CO participates in circadian clock regulation in the mammalian body.

HemoCD1 was conjugated with octaarginine (R8) as a cell-penetrating peptide to capture CO inside the cells (Fig. 6) [58]. Maleimide-modified hemoCD1 (mal-hemoCD1) was conjugated with Cys-R8 peptide through a 1,4-Michael addition reaction in water. The resulting

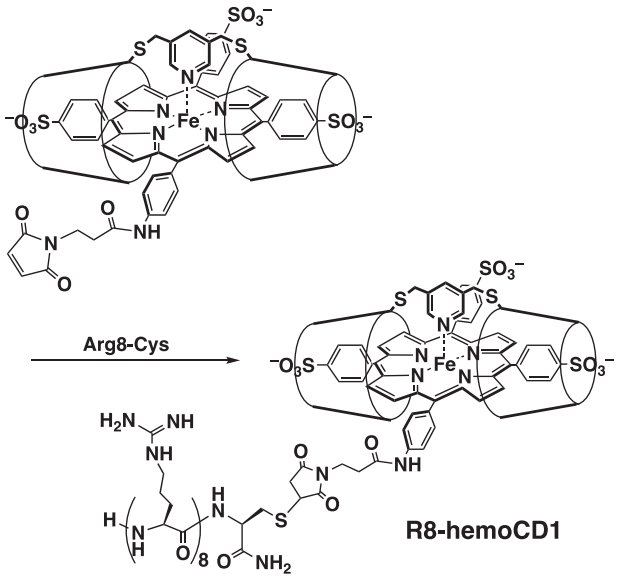

Fig. 6 Synthesis of R8-hemoCD1 as an intracellular selective carbon monoxide (CO) scavenger bearing a cell-penetrating octaarginine (R8) peptide

compound (R8-hemoCD1) was internalized into living cells, resulting in selective depletion of CO in the cells. Furthermore, the removal of endogenous CO in the cells significantly increased intracellular ROS levels, suggesting a relationship between endogenous CO levels and inflammation caused by ROS, although a detailed mechanistic study has not yet been performed.

Selective removal of endogenous CO was achieved in vivo using hemoCD1, contributing to the elucidation of the unknown physiological roles of CO in living systems. This "pseudoknockdown" approach using synthetic compounds is a novel concept researching the roles of biologically related small molecules. Thus, highly specific molecular recognition in vivo by synthetic models will be in high demand to further determine regulatory mechanisms involving small molecules in living systems.

Therapeutic approaches to CO poisoning

It is generally believed that exogenously inhaled CO prevents the O_2 transportation of Hb in red blood cells. However, this finding may be controversial. In a study published in 1975 [59], a CO poisoning animal model using dogs was reported. When dogs were exposed to 13% CO gas for 15 min, they died within an hour. In contrast, they survived when CO-Hb-saturated blood was transfused into healthy recipient dogs. In both cases, the CO-Hb levels reached 60–70%. Moreover, only CO gas exerted significant toxicity, whereas CO-Hb did not show any toxic effect. Therefore, the diffusion of gaseous CO to organs/tissues might be the direct cause of lethal toxicity. Hence, it is essential to determine the distribution of gaseous CO into organs/tissues in the body to understand the true mechanism of CO poisoning.

As mentioned above, hemoCD1 showed the highest CO-binding affinity among the reported CO-binding heme proteins. Therefore, hemoCD1 can remove CO from other heme proteins present in organs/tissues. Moreover, the amount of CO can be quantified using hemoCD1 [39, 58]. The protocol for CO quantification in the tissues using hemoCD1 is shown in Fig. 7. The hemoCD1 solution was mixed with the tissue sample and homogenized by sonication. A clear filtrate was obtained after removing the insoluble materials by centrifugation and filtration. Excess $Na_2S_2O_4$ acted as a reducing agent for hemoCD1 and a precipitant for soluble proteins. Therefore, only the absorbance owing to hemoCD1 was detected, and the amount of endogenous CO was determined simply based on the Lambert–Beer law [39]. The amount of CO in animal tissues was previously determined using gas chromatography (GC) [60, 61]. Comparing the amount of CO determined by GC to that determined by hemoCD1 showed that more CO was detected by hemoCD1, indicating that the assay using hemoCD1 was more sensitive than GC. Thus, the assay using hemoCD1 could become a powerful and convenient tool for sensitive CO quantification in animal tissues and organs, thereby contributing to the elucidation of the CO poisoning mechanism.

CO diffused into the blood and tissues of the rats exposed to CO gas. The amount of CO in tissues before and after CO exposure was quantified by the assay using hemoCD1, revealing that CO contained in the tissues first significantly increased and then reached a plateau. After ventilation by air or O_2, the amount of CO in the tissues decreased; however, a significant amount of CO was detected in the tissues, and it was difficult to remove it by ventilation in the brain tissues alone. Thus, the residual CO in the brain quantified by the assay using hemoCD1 could cause sequelae after CO poisoning.

Finally, we tested hemoCD1 as an antidote for CO poisoning (Fig. 8a) [39]. After exposure to 400 ppm CO gas for 5 min, oxy-hemoCD1 solution (3.5 mM, 1.0 mL) was intravenously injected into the rats during O_2 ventilation. The increased CO-Hb levels (60%) in the rats returned to normal more rapidly in the hemoCD1-injected rats, with excretion of CO in the urine as CO-hemoCD1. Residual CO in the brain tissue was considered the cause of serious sequelae after CO poisoning [62, 63]. After CO exposure, a significant amount of CO accumulated in the brain tissue of the rat and was present even after 30 min of O_2 ventilation (Fig. 8b). In contrast, the amount of residual CO in the brain decreased markedly in hemoCD1-injected rats (Fig. 8b). Thus, this finding suggests that hemoCD1 can be used as an

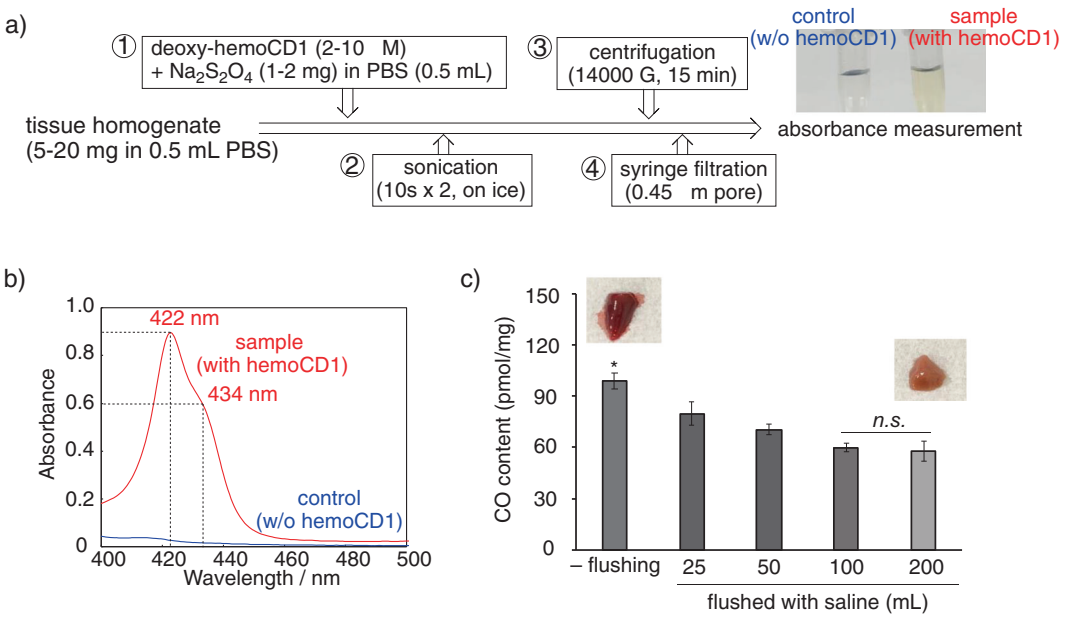

Fig. 7 **a** Experimental procedure describing the various steps in the hemoCD1 assay for measuring carbon monoxide (CO) in tissue samples. **b** Typical spectra for supernatant solutions of the liver sample and control obtained at the end of the hemoCD1 assay. **c** Amounts of CO quantified in liver tissue without (–) or following flushing with 25–200 mL saline. Adapted from ref. [39] with permission

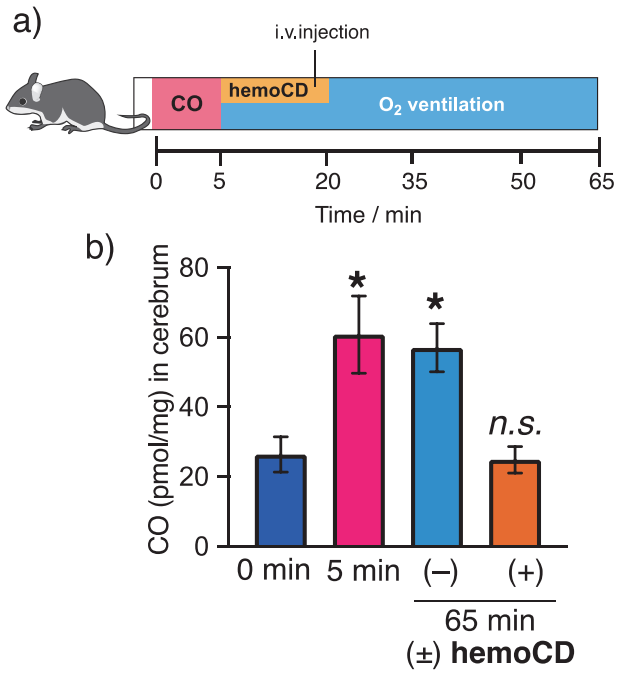

Fig. 8 **a** Experimental procedure for the test of hemoCD1 as an antidote for carbon monoxide (CO, 400 ppm)-exposed animal models. **b** Changes in CO levels detected in the cerebrum samples before (0 min) and after CO exposure without (–) and with hemoCD1 injection (+). Adapted from ref. [39] with permission

effective antidote to reduce CO in blood and brain tissues, although the removal mechanism of CO in brain tissues needs to be further investigated. Nevertheless, the development of a novel antidote to CO poisoning based on hemoCD1 is currently in progress in our laboratory.

Conclusion and outlook

This review focuses on the synthesis and recent development of our biomimetic Hb/Mb model complex hemoCD1. HemoCD1 is continuously synthesized in our laboratory using $Fe^{II/III}$TPPS and Py3CD for several biomedical applications. HemoCD1 is used for the following purposes: (1) selective removal of endogenous CO in vivo to study endogenous CO as a gaseous signal mediator, (2) quantification of endogenous/exogenous CO accumulated in organs/tissues to study the CO biodistribution, and (3) the creation of an injectable antidote for CO poisoning, which frequently occurs in fire accidents.

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Compliance with Ethical Standard

Conflict of interest The authors declare no competing interests.

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