Photoactivatable CO release from engineered protein crystals to modulate NF-κB activation†

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Photoactivatable CO releasing protein crystals were developed by immobilization of Mn carbonyl complexes in polyhedral crystals, which are spontaneously formed in insect cells. The photoactivatable CO release from the engineered protein crystals activates nuclear factor kappa B (NF-κB) upon stimulation by visible light irradiation with suppression of cytotoxicity of the Mn complex.

Carbon monoxide (CO) is one of several gaseous cellular-signaling molecules operating in living cells. CO mediates anti-inflammation and vasoactive response, and has been used to control immune responses during transplantation.1,2 CO signaling involves interactions with other gaseous signaling molecules such as nitric oxide and hydrogen sulfide, which are endogenously produced by the specific enzymes.3,4 Recently, the therapeutic applications of CO have attracted significant attention as a result of rapid development of a number of CO-releasing molecules (CORMs).5 Metal carbonyl complexes such as [Ru(CO)3Cl2]2 (CORM-2) and Ru(CO)3Cl(glycinate) (CORM-3), have been used to deliver CO into living cells.6 Synthetic and natural carrier molecules have been developed with the objective of providing safe and controlled delivery of CORMs into cells, tissues, and animals with effective CO-releasing properties.7–13 Moreover, a wide variety of transition metals and synthetic ligands have been developed for stimulus-induced CO release.14–16 These efforts represent promising strategies for providing controlled CO delivery.

Photoactivation is one of the most favorable external stimuli for CORMs. Other external stimuli include pH change, and enzymatic reactions.14–16 photoactivatable CORMs (photoCORMs) are transition metal carbonyl complexes of Mn, Fe, Ru, Re,18,24 among others. Since Motterlini et al. reported that Mn3(CO)10 (CORM-1) can release CO with triggering by light irradiation,75 design of photoCORMs has been achieved by development of complexes based on organic ligands coordinating to Mn ions.17–19 Recently, Mascharak and co-workers have reported photoCORMs of Mn carbonyl complexes with polypyridine-based ligands to promote rapid CO release in the visible/near infrared region.20 For biotechnological applications of photoCORMs, cytotoxicity arising from free metal ions, organic ligands, or by the reaction of photoCORMs with light irradiation should be considered.27,28 One of the possible solutions is provided by incorporation of photoCORMs into biocompatible scaffolds to prevent release of metal ions or synthetic organic ligands.26 Nanoparticles, polymers, and porous materials have been utilized as molecular matrices for stable accumulation of photoCORMs.8,11,26,29,30 Schatzschneider and co-workers have utilized silica nanoparticles as photoCORM carriers.15 [Mn(CO)5(pqa)]ClO4 incorporated into Al-MCM-41 nanoparticles were found to promote vasorelaxation of rat aorta muscle rings through light-induced CO delivery.26 PhotoCORMs have been non-covalently and covalently embedded into polymers.30 However, their CO releasing properties have slow rates of ca. 20 min. Thus, improvement of carrier matrices for photoCORMs is required for design of photoCORMs with rapid responses to stimuli.

It has been recently reported that proteins and protein assemblies can serve as biocompatible carriers of CORMs.31–37 Composites of protein assemblies with CORMs can stably deliver CO into living cells and effectively release them to activate signal transduction.33–37 Protein crystals are also expected to be appropriate candidates for extracellular matrices (ECMs) for immobilization of CORMs because protein crystals represent precise protein assemblies in the solid state with inner pores that act as “solvent channels.”33,34 In general, when most of the crystals are
stabilized by cross-linking, the crystals are utilized as vessels for preparation of metal nanoparticles or catalytic reactions by immobilization of metal compounds under various reaction conditions. It has been reported that cross-linked hen egg white lysozyme crystals can serve as extracellular matrices for CORM-2. However, inconvenient procedures which include crystallization and cross-linking are required to obtain the materials. To address this issue, we have used protein crystals, which are spontaneously synthesized in insect cells, as matrices for immobilizing CORMs. Polyhedral crystals (PhCs) are directly produced from polyhedrin monomer (PhM) expressed in insect cells after infection by cypovirus. PhCs are highly stable over a wide range of pH, temperature, and in organic solvents, and can be frozen or dried because their original function is to provide protective material to store replicated viruses produced during the viral infection cycle. PhCs have served as extracellular matrices for growth factors and CORMs due to their biocompatibility. In this article, we describe the immobilization of photoCORMs of Mn carbonyl complexes in PhCs, which can be used as ECM without cross-link treatment. To modulate the amounts of CO released from the composite, we increased the number of photoCORMs accumulated in PhCs by using a mutant of PhM with a hexa-histidine tag (His-tag) at its C-terminus (HTPhM). The crystal of HTPhM (HTPhC) containing Mn carbonyl complexes (Mn-HTPhC) has twice the number of Mn carbonyl complexes of WTPhC containing Mn carbonyl complexes (Mn-WTPhC). Mn-HTPhC was found to be capable of acting as an ECM that can release CO gas into living cells with a more rapid response than that of previously reported ECM-containing photoCORMs and activate nuclear factor kappa B (NF-κB) with visible light irradiation (Fig. 1).

HTPhCs were prepared as reported previously using an insect cell expression system of Spodoptera frugiperda 21 (Sf21). To immobilize Mn(CO) moieties into HTPhCs, HTPhCs (7.8 × 10^7 crystals) were soaked in 10 mM HEPES buffer (pH 7.0, 500 μL) containing 0.8% v/v acetonitrile and 1 mM Mn(CO)Br. After gentle stirring for 24 hours at room temperature in the dark, the suspension was washed twice with Milli-Q water.

Fluorescence X-ray analysis of Mn-HTPhC showed that the number of Mn ions per HTPhM was 5.6 ± 1.2. The Mn complexes were fully dispersed in Mn-HTPhC as confirmed by a scanning transmission electron microscopy-energy dispersive X-ray spectrum (STEM-EDX) (Fig. 2a). The IR spectrum of Mn-HTPhC has two bands at 1921 and 2028 cm⁻¹. This set of bands is assigned to the CO stretching vibrations of the θac-Mn(CO)₆(H₂O)₆(His) moiety. Mn-WTPhCs were also prepared under the same conditions. The number of Mn per WTPhM in Mn-WTPhC (2.6 ± 0.3) was found to be almost half the value of that of Mn-HTPhC with homogeneously-dispersed accumulation (Fig. S1, ESIF). The IR spectrum of Mn-WTPhC indicates the presence of Mn carbonyl moieties with a coordination structure similar to that of Mn-HTPhC (Fig. 2b).

The crystal structure of Mn-HTPhC was determined at a resolution of 1.8 Å (ESIF). The structure has no electron density for the extended sequence after Gly245, the carboxy-terminal residue in the wild-type protein. Moreover, an anomalous difference map does not identify densities assignable to Mn atoms in the structure although fluorescence X-ray analysis and STEM-EDX indicate the presence of Mn atoms in the composite. There is no electron density accounting for the presence of Mn in the crystal structure of Mn-WTPhC at a 1.7 Å resolution. These results suggest that the His-tag and Mn moieties can be oriented in several directions at the conjugation sites, as observed previously for Ru complexes conjugated in protein crystals.

The photoactivatable CO-releasing properties of Mn-HTPhC were evaluated using the myoglobin (Mb) assay (Fig. 3 and Fig. S3, ESIF). The data processing steps correct for the uneven absorption of the CORM at the Q-bands in both deoxy-Mb and carbonmonoxy-Mb (MbCO) (Fig. 3a). Mn-HTPhC (4.0 × 10^7 crystals with immobilized Mn ions determined by ICP-MS is 6.6 × 10⁻⁴ μmol) was dispersed in 1 mL of a PBS buffer (pH 7.4) containing deoxy-Mb (6.2 μM) and sodium dithionite (30 mM). The reaction was accompanied by light irradiation at 456 nm using a blue LED light (130 mW, 33.2 mW cm⁻², Fig. S2, ESIF). The total released amount of CO per Mn of Mn-HTPhC with light irradiation was found to be 2.9 ± 0.4. Significantly less CO released from Mn-HTPhC was detected without irradiation (Fig. 3b). The amount of CO released is consistent with the coordination structure.
The half-life ($t_{1/2}$) of bands assignable to CO stretching (Fig. 2b, dotted line). Bioluminescence intensity of HEK293 cells after 12 hours NF-kB activation assay using these composites. The activation of NF-kB by suppressing the cytotoxicity of the Mn(CO)₅Br moieties in the crystals.

In conclusion, light-triggered CO-releasing ECMs were constructed by immobilizing Mn(CO)₅ moieties in PhCs. When a His-tag fragment is fused to the C-terminus of PhM, the resulting composite HTPhC gains the ability to accumulate twice the number of Mn moieties relative to the unmodified WTPhC. Moreover, Mn HTPhC releases a stoichiometric amount of CO molecules per Mn ion. The release effectively contributes to activation of NF-kB by suppressing the cytotoxicity of the precursor complex, Mn(CO)₅Br. These results suggest that photoactivatable CO release from engineered protein crystals will be adaptable to provide information regarding cellular events involving CO gas. Since CO release is expected to be regulated at a specific time point during the course of cellular proliferation, we are investigating the detailed mechanisms of CO biology using these composites.

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