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Supporting Information

Photoactivatable CO Release from Engineered Protein Crystals to Modulate NF-κB Activation

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Experimental Section

Materials: Reagents were purchased from TCI, Wako, Nacalai Tesque, and Sigma–Aldrich and were used without further purification. The expression and purification of sperm whale myoglobin were performed according to reported procedure.[1] Preparation of WTPhC and HTPhC were performed as previously reported. [2]

Immobilization of Mn carbonyls in PhC. HTPhC (7.8×10^7 crystals) were soaked in 10 mM HEPES buffer (pH7.0, 500 μL) containing 1.0 mM $Mn(CO)_5Br$ and 10 vol% acetonitrile (MeCN) at room temperature for 24 h to obtain **Mn•HTPhC**. After washing the crystals at least three times with distilled water, **Mn•HTPhC** were obtained with light yellow crystals. **Mn•WTPhC** were obtained in the same prodecure.

Elemental Analysis. The atomic ratio of Mn and S in **Mn•HTPhC** and **Mn•WTPhC** were determined using a Rigaku EDXL-300 X-ray fluorescence spectrometer.

STEM-EDX measurement. High-resolution images of **Mn•HTPhC** and **Mn•WTPhC** were obtained using a scanning transmission electron microscope (STEM, JEOL JEM-2200FS) equipped with a field-emission gun at 200 kV. After ultra-sonication of a water suspension of **Mn•HTPhC**, a drop containing thin pieces of **Mn•HTPhC** was fixed on an electron microscope microgrid with Cu mesh. The locations of Mn, C, S in **Mn•HTPhC** were determined using a Rigaku ZSX-100S energy dispersive X-ray fluorescence spectrometer.

Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) measurement. Metal concentrations in PhCs were determined using an inductively coupled plasma-Mass Spectrometer (Perkinelmer, ELAN DRC-e). Mn in Multielement Standard Solution W-IV, Wako was used as calibration standard.

Myoglobin (Mb) assay. The assay was performed as previously reported with the slight modification.[3, 4] **Mn•HTPhC** (4.0×10^5 crystals) were dispersed in 1 mL of PBS buffer (pH 7.4) in the presence of Mb (6.2 μM) and sodium dithionite (30 mM) under Ar atmosphere with light irradiation at 456 nm using a blue LED light (130 mW, 33.2 mW/cm^2, RelyOn Ltd., Japan) positioned perpendicular to a cuvette at a distance of 4 cm (Figure S2). The amount of Mn in **Mn•HTPhC** (6.6×10^{-4} μmol) was determined by ICP-MS. The visible spectrum at each time point ware measured using a Shimadzu

UV-2600 UV-vis spectrophotometer. Upon CO releasing, the corresponding visible spectrum showed the formation of the carbonmonoxy-Mb (MbCO) at the peak of 542 nm and 578 nm. Quantification of CO release was calculated from the spectra. The concentration of MbCO was calculated as described previously.[5] The experiments were performed in triplicate and the data represent mean ± SEM. The calculated datasets were fitted to first-order kinetics by using Microsoft Excel and Solver.[5] The same assay was carried out for **Mn•WTPhC** (4.0×10^5 crystals). The amount of Mn of **Mn•WTPhC** was 3.4×10^{-4} μmol. Quantum yields were determined by absorbance of **Mn•HTPhC** in 1.0 cm cuvettes and amount of CO released from each composite.

HEK293/κB-Fluc cell experiment. The assay was performed as previously reported with the slight modification.[2] κB-Fluc reporter-transfected human embryonic kidney 293 (HEK293/κB-Fluc) cells were seeded in a 96-well plate (1.0×10^4 cells/well in 100 μL DMEM medium containing 5% heat-inactivated FBS) and then incubated for 12 h in 5% CO_2 incubator at 37˚C. 10μL of **Mn•HTPhCs** suspension (1.0×10^5 crystals/well) was added to each well (amount of Mn in each well was 1.7×10^{-4} μmol) and then the cells were cultured for 1 h. Light irradiation was carried out for 10 min at 456 nm with a Blue LED desklight (6.62 mW/cm^2, RelyOn Ltd., Japan) positioned perpendicular to the plate at a distance of 10 cm. Then 10 μL of TNF-α solution (120 μg/mL in MilliQ) was added to each well with additional cultivation for 12 h. The protein crystals locate on the cell surface during the incubation in cell culture (Figure S4). The assays for **Mn•WTPhC** (1.0×10^5 crystals/well, amount of Mn in each well was 0.83×10^{-4} μmol), and HTPhC were carried out with the same procedure. For the assay of $Mn(CO)_5Br$, 10 μL of a PBS buffer solution containing $Mn(CO)_5Br$ (15.2 μM) and 1 % MeCN was added to each well (amount of Mn in each well was 1.7×10^{-4} μmol). The components of the luciferase assay kit (Promega, ONE-Glo™ Luciferase Assay System) were added to each well. Photoluminescence spectroscopy was measured with a Promega GloMax® -Multi Detection System. The experiments were carried out in triplicate. The data represent mean ± SEM.

MTT assay. An assessment of cell viability was undertaken using In vitro toxicology assay kit MTT based (Sigma-Aldrich). The amounts of live cells were determined after collecting cells from a 96-well plate. The experiments were carried out in triplicate. The data represent mean ± SEM.

Statistical analysis. Statistical analyses were carried out with a Student's t-test. Values of $P < 0.05$ were considered statistically significant.

X-ray crystal structure analysis

Crystals were mounted on a micromesh support, cryoprotected with 50% ethylene glycol and flash frozen in liquid nitrogen as described before. [6] Data were collected at 100K on the micro crystallography beamline (MX2) at the Australian Synchrotron with a collimated X-ray beam of 10µm x 10µm. Using this setting, the flux is approximately 3.6 x 10^{11} photons s^{-1} at 13 keV. Data were collected at a wavelength of 1.4586Å without attenuation. Diffraction data were processed with HKL2000[7] and statistics are summarized in Table S1.

Table S1.

Data collection	Mn•HTPhC	Mn•WTPhC
Beamline	AS MX2	AS MX2
Wavelength (Å)	1.4586	1.4586
Number of crystals	7	9
Space group	I23	I23
Unit cell parameters	a=b=c=103.2	a=b=c=102.9
(Å)	α=β=γ=90°	α=β=γ=90°
Resolution (Å)	30-1.8 (1.86-1.80)	30-1.7 (1.76-1.70)
Measured reflections	103,442	255,705
Redundancy	6.3 (2.3)	12.8 (3.8)
<I>/<sigmaI>	9.3 (2.1)	14.6 (2.5)
Completeness (%)	96.5 (66.5)	99.0 (90.1)
Rmerge (%)	22.1 (65.2)	21.4 (76.3)
$CC_{1/2}$ (%)	(44.6)	(58.1)

Table S2. PhotoCORM immobilized in materials that works as CO-releasing extracellular scaffold for living cells.

CORM support materials	**CORM**	**$t_{1/2}$ (min.)**	**Target cells**
HPMA based polymer[8]	$Mn(CO)_3$	20	Hct116 cells
non-wovens[9]	$Mn(CO)_3$	5~21	3T3 cells
Al-MCM-41[10]	$Mn(CO)_3$	Not reported	rat aorta muscle rings

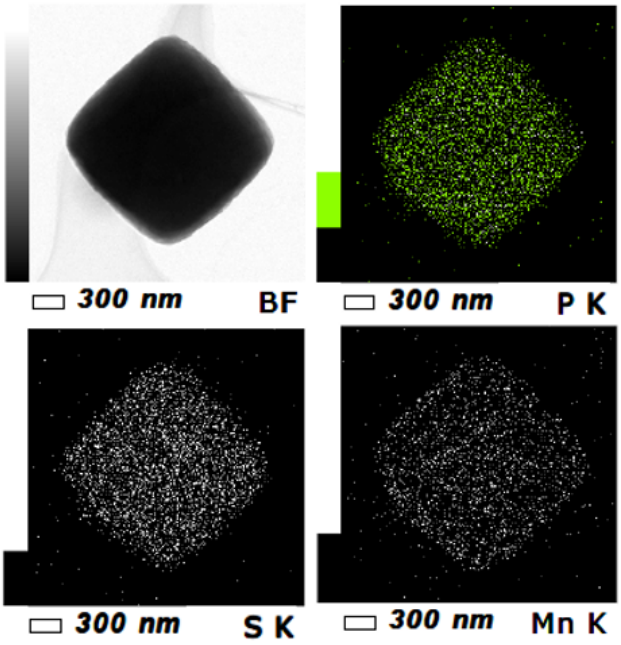

Figure S1. STEM-EDX images of **Mn•WTPhC**: STEM BF image (upper left), elemental mapping images of P-K (upper right), S-K (bottom left) and Mn-K (bottom right).

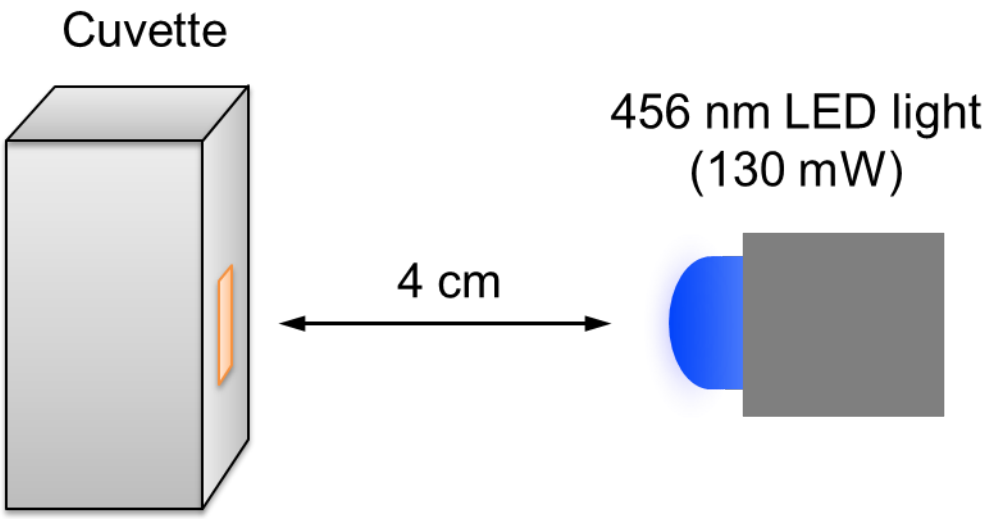

Figure S2. Detection of CO gas using the Mb assay with the light irradiation.

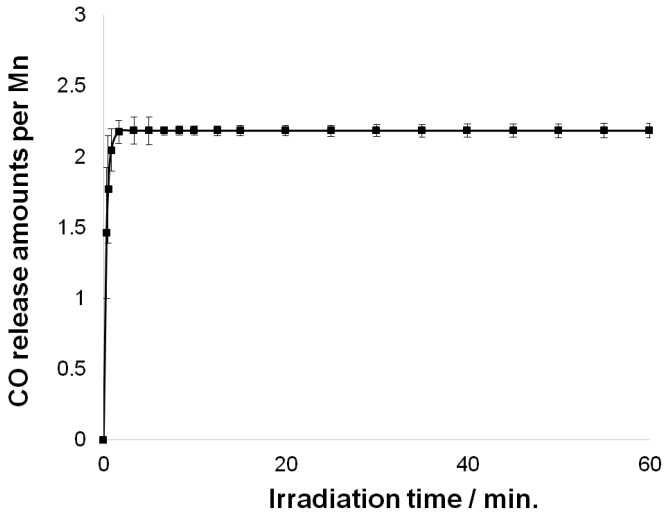

Figure S3. Mb assay of $Mn(CO)_5Br$: Detection of CO release by plotting the concentration of MbCO. The absorption spectral change of MbCO was monitored for $Mn(CO)_5Br$ with light irradiation. All the experiments were performed in 500 μL of 10 mM PBS buffer (pH7.4) containing deoxy-Mb (5.4μM) and $Na_2S_2O_4$ (10 mM). The Mn concentration was 1.0 μM in the PBS solution. The suspended solution was exposed to the LED light at 456 nm. The spectra were corrected at 550 nm to determine the conversions of deoxy-Mb to MbCO by using calculation reported in a previous literature. The experiments were performed three times and the data represent mean ± SEM.

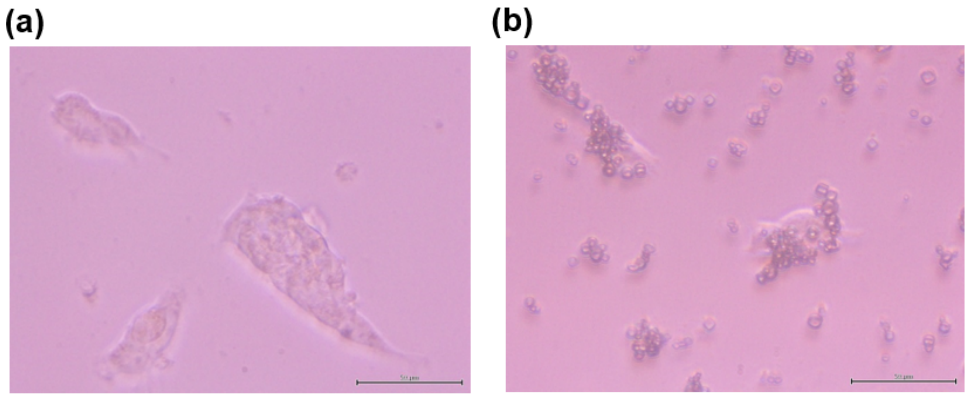

Figure S4. HEK293/κB-Fluc cells were cultured in DMEM medium containing 5% heat-inactivated FBS: (a) without **Mn•HTPhC** and (b) with **Mn•HTPhC** incubated for 12 hrs.

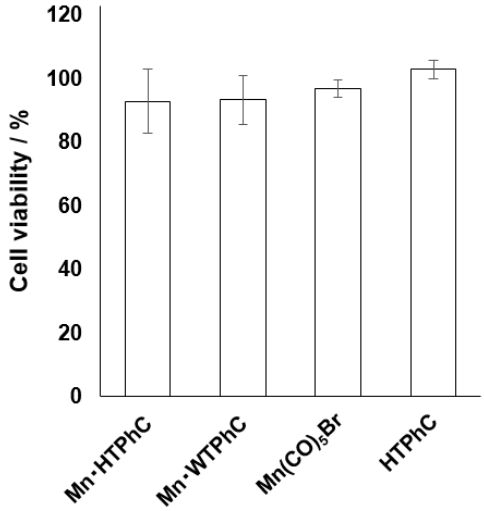

Figure S5. Evaluation of **Mn•HTPhC**, **Mn•WTPhC**, HTPhC, and $Mn(CO)_5Br$ without the light irradiation as a CORM-ECM for cell viability. MTT assay for the evaluation of NF-κB activity of HEK293/κB-Fluc cells in the presence of 10 ng/mL TNF-α after incubation for 12 hours. $*P < 0.05$ vs. PBS buffer. Each experiment was performed in triplicate and the data represent mean ± SEM.

References

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