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Preparation of a Cross-Linked Porous Protein Crystal Containing Ru Carbonyl Complexes as a CO-Releasing Extracellular Scaffold

Hiroyasu Tabe,[†,‡] Kenta Fujita,[§] Satoshi Abe,[§] Masahiko Tsujimoto,[‡] Takahiro Kuchimaru,[§] Shinae Kizaka-Kondoh,[§] Mikio Takano,[‡] Susumu Kitagawa,*[,†,‡] and Takafumi Ueno*[,‡,§]

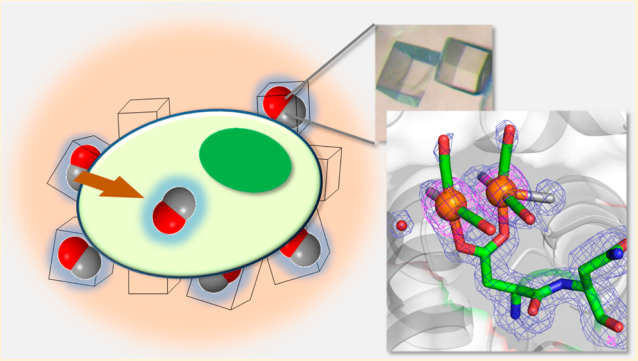
[†]Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

[‡]Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan

[§]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, 4259-B55, Midori-ku, Yokohama 224-8501, Japan

Supporting Information

ABSTRACT: Protein crystals generally are stable solid protein assemblies. Certain protein crystals are suitable for use as nanovessels for immobilizing metal complexes. Here we report the preparation of ruthenium carbonyl-incorporated cross-linked hen egg white lysozyme crystals (**Ru·CL-HEWL**). **Ru·CL-HEWL** retains a Ru carbonyl moiety that can release CO, although a composite of Ru carbonyl-HEWL dissolved in buffer solution (**Ru·HEWL**) does not release CO. We found that treatment of cells with **Ru·CL-HEWL** significantly increased nuclear factor kappa B (NF-κB) activity as a cellular response to CO. These results demonstrate that **Ru·CL-HEWL** has potential for use as an artificial extracellular scaffold suitable for transport and release of a gas molecule.



INTRODUCTION

Protein assemblies are a class of highly organized biomolecules that hold promise for development materials in a number of applications in bionanotechnology.[1−7] In particular, the hierarchical assemblies that are formed from protein monomers as building blocks have recently been designed and developed as supramolecular platforms.[8,9] One of the general methods involves encapsulation of non-natural metal cofactors within the protein scaffolds.[9−14] This method provides the means to construct powerful tools for development of bionanomaterials such as artificial metalloenzymes, drug delivery systems, and imaging reagents.[15−17]

In particular, design of the composites of proteins and organometallic complexes represents one of the most challenging effort in the fields of bioinorganic chemistry and protein engineering.[7,17−20] For example, proteins modified with organoruthenium or organorhodium complexes can catalyze selective reactions of organic molecules.[17,18,20] Recently, metal carbonyls have been conjugated to proteins to improve interactions with proteins and activate small molecules such as H_2.[21] Metal carbonyls also serve as CO-releasing molecules (CORMs). Attempts have been made to deliver CO into living cells using CORMs because CO is a pleiotropic signaling gas molecule with biological functions such as cytoprotection, modulation of inflammation, redox control, and vasoactive response.[22−28] Romão et al. reported excellent progress in evaluating the reactivity between HEWL and ruthenium carbonyls, although the composite was found to be incapable of releasing CO gas.[29,30] CO-releasing biomaterials have been prepared by immobilizing CORMs in inorganic particles, polymer, peptide gels, and metal−organic frameworks (MOFs).[31−35] Several of these examples are utilized as extracellular scaffolds that can deliver CO into living cells to control cellular functions such as apoptosis and alleviation of oxidative stress.[31,33,35]

Protein crystals represent precise protein assemblies in the solid state and are also appropriate candidates for development of robust biomaterials that immobilize CORMs.[11,13,20,36−42] Each protein crystal has inner pores that act as solvent channels.[36] The structures of the solvent channels are retained by cross-linking treatment of protein crystals.[37] Cross-linked protein crystals (CLPCs) have been adapted to serve as reactor vessels for enzymes and as adsorbents of separation columns.[43,44] Postengineering of CLPCs has recently been performed to convert solvent channels into nanovessels for preparation of metal particles or immobilization of organometallic catalysts.[20,41,42] The success of these efforts indicates that protein crystals have further potential to provide new structures and improve the reactivity of CORMs when used as extracellular scaffolds for supporting CO-releasing molecules.

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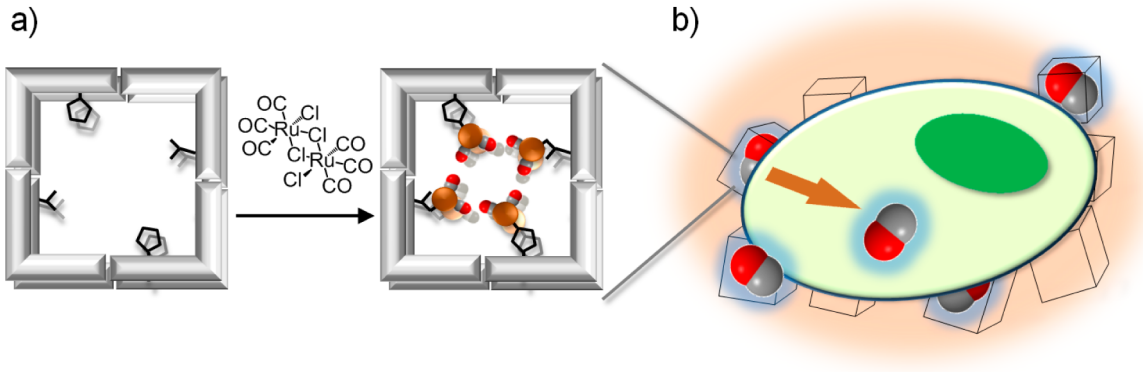
Scheme 1. Schematic Representation of the Preparation of Ru·CL-HEWL (a) and CO Release in the Vicinity of Cells (b)



Herein, we report the storage and release of CO gas by immobilizing ruthenium carbonyls in cross-linked hen egg white lysozyme crystals (CL-HEWLs) because HEWL crystals have the capability to accommodate various metal carbonyls.[29,30,45−48] We found that $[cis\text{-}Ru(CO)_2X_4]^{2-}$ (X = anionic ligands), preserved in the solvent channels of CL-HEWL and releases CO under physiological conditions, Scheme 1. Then the CO molecules can activate nuclear factor kappa B (NF-κB). These results indicate that porous protein crystals have potential for use as extracellular scaffolds for delivery of signaling gases.

EXPERIMENTAL SECTION

Materials. Hen egg white lysozyme (HEWL) was purchased from Sigma-Aldrich. Other reagents were purchased from TCI, Wako, Nacalai Tesque, and Sigma−Aldrich and used without further purification. Expression and purification of sperm whale myoglobin were performed according to the reported procedure.[49]

Preparation of the Composite of CL-HEWL with Ru Carbonyl (Ru·CL-HEWL). HEWL crystals were obtained using a batch crystallization method followed by a cross-linking treatment using 100 mM acetate buffer (pH 7.0, 1.0 mL) containing 1.0% glutaraldehyde and 1.0 M NaCl according to the previously reported procedure.[20] CL-HEWL crystals (150 mg) were soaked in 100 mM acetate buffer (pH 7.0, 1.0 mL) containing 5.0 mM CORM-2 and 1.0 M NaCl at room temperature for 24 h to immobilize the Ru complexes in the CL-HEWL crystals. The composite of CL-HEWL with Ru carbonyl (**Ru·CL-HEWL**) was obtained as dark yellow crystals.

Preparation of Ru·HEWL Dissolved in Buffer Solutions. HEWL solutions (10 mg/mL) prepared in 10 mM HEPES buffer (pH 7.0) were incubated at room temperature for 24 h with CORM-2 (3.5 mM) and DMF (5 vol %). Dialysis of the samples was performed at room temperature with a desalting column with 10 mM HEPES buffer (pH 7.0) to obtain **Ru·HEWL**.

Elemental Analysis. The atomic ratio of Ru and S in **Ru·CL-HEWL** was determined using a Rigaku EDXL-300 X-ray fluorescence spectrometer.

STEM-EDX Measurement. High-resolution images of **Ru·CL-HEWL** were obtained using a scanning transmission electron microscope (STEM, JEOL JEM-2200FS) equipped with a field-emission gun at 200 kV. After ultrasonication of a water suspension of **Ru·CL-HEWL**, a drop containing thin pieces of **Ru·CL-HEWL** was fixed on an electron microscope microgrid with Cu mesh. The locations of Ru, C, O, and S in **Ru·CL-HEWL** were determined using a JEOL JED-2300T energy dispersive X-ray analyzer.

ICP-OES Measurement. Metal concentrations of the buffer solutions were determined using an inductively coupled plasma optical emission spectrometer (Shimadzu ICPE-9000). $Ru(NO_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was used as a calibration standard, and $Y(NO_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was used as an internal standard.

Myoglobin (Mb) Assay. The Mb assay was performed as previously reported with slight modificaton.[23,50] For the CO release assays of **Ru·CL-HEWL**, **Ru·CL-HEWL** (Ru = 0.025 μmol) was dispersed in PBS buffer (pH 7.4) at 25 °C for 4 h in the presence of myoglobin (35 μM) and sodium dithionite (10.0 mM). This solution was quickly transferred to a cuvette at each measurement point, and visible spectra were measured at room temperature using a JASCO FP-8500 diode array spectrophotometer, measuring between 700 and 350 nm at 2 nm steps. Quantification of CO release was calculated from the spectra. The concentration of MbCO was calculated as described previously.[23] Experiments were performed in triplicate, and data represent the mean ± SEM. The calculated data set was fitted to first-order kinetics using Excel and Solver.[50] For control assays, CORM-2 or **Ru·HEWL** (Ru = 0.025 μmol) was dissolved in PBS buffer (pH 7.4) at 25 °C for 1 h in the presence of myoglobin (35.0 μM) and sodium dithionite (10.0 mM).

Crystal Structure Analysis. CL-HEWLs were prepared by the hanging drop vapor diffusion method according to the reported procedure.[51] CL-HEWL was soaked in 100 mM acetate buffer (pH 7.0) containing 5.0 mM CORM-2 and 1.0 M NaCl for 1 day to obtain crystals containing Ru complex (**Ru·CL-HEWL**). For snapshot analysis of CO release, crystals were immersed in PBS buffer (pH 7.4) containing 35.0 μM myoglobin and 10.0 mM $Na_2S_2O_4$ for 2 h. Prior to data collection, crystals were continuously immersed in the precipitant solutions containing 10% (w/w) and 25% glycerol and subsequently frozen in liquid nitrogen. X-ray diffraction data sets for each **Ru·CL-HEWL** sample were collected at 100 K at beamline BL38B1 at SPring-8 using X-ray wavelengths of 1.0 Å. Data were processed with the program *HKL2000*.[52]

Crystal parameters and data collection statistics are summarized in Table S1, Supporting Information. Structures were solved by molecular replacement with *MOLREP*[53] using HEWL structures (PDB ID 193L). Refinement of the protein structure was performed using *REFMAC5*[54] in the *CCP4* suite.[55] Rebuilding was performed using *COOT*[56] based on sigma-weighted $(2F_o - F_c)$ and $(F_o - F_c)$ electron density maps. After rigid-body refinement, coordination structures of Ru binding sites were determined using anomalous Fourier difference maps and geometric parameters. Water molecules were positioned to fit residual $(F_o - F_c)$ density peaks with a lower cutoff of 3σ. Models were subjected to quality analysis during the various refinement stages with omit maps and *PROCHECK*.[57] Refinement statistics are summarized in Table S1, Supporting Information. Atomic coordinates are deposited in the Protein Data Bank under accession numbers 4W94 and 4W96 for **Ru·CL-HEWL** and **Ru·CL-HEWL** after Mb assay, respectively.

HEK293/κB-Fluc Cell Experiment. 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 incubated for 15 h in 5% CO_2 incubator at 37 °C. Then, 15 μL of **Ru·CL-HEWL** suspension

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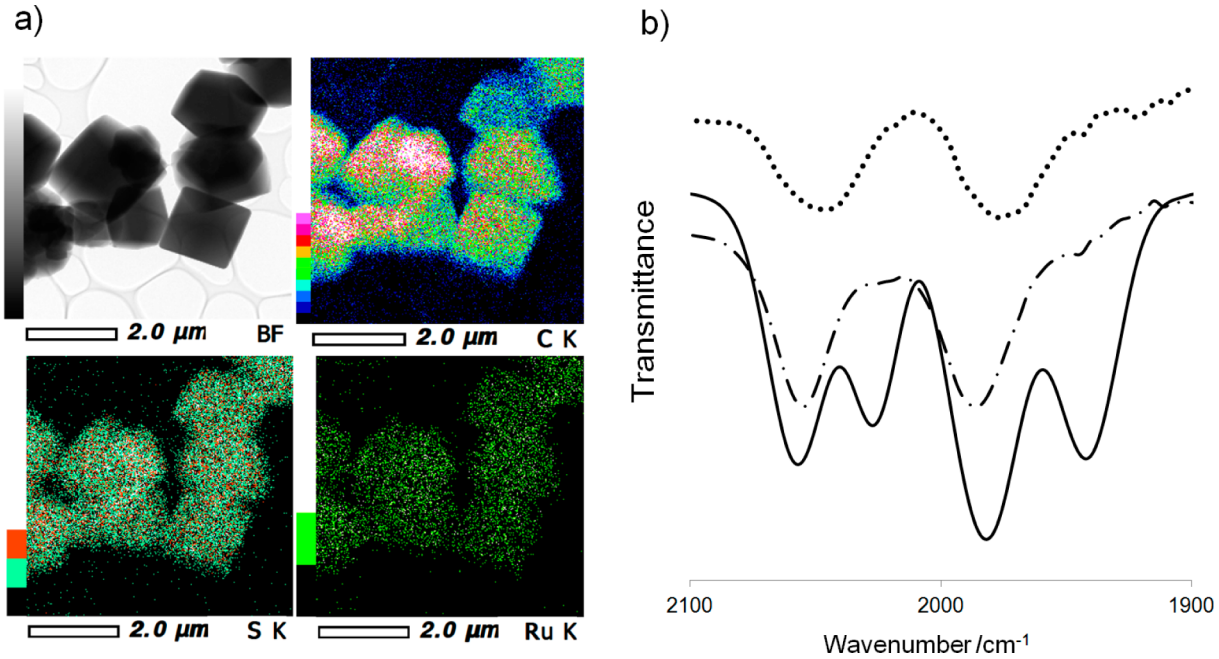

Figure 1. STEM-EDX images of **Ru·CL-HEWL** (a): TEM image (upper left), elemental mapping images of C−K (upper right), S−K (bottom left), and Ru−K (bottom right). Infrared spectra (b): **Ru·CL-HEWL** (KBr method, solid line), **Ru·CL-HEWL** after releasing CO in the Mb assay (KBr method, chained line), and **Ru·HEWL** in 10 mM HEPES buffer (pH 7.0) (ATR method, dotted line).

(2.0×10^5 crystals/well) or 15 μL of a PBS buffer solution containing CORM-2 (40 μM) with 0.04% DMF was added to each well and cells were cultured for 1 h in an incubator under 5% CO_2 at 37 °C. Then, 5.0 μL of 12 μg/mL TNF-α solution was added to each well with additional culture for 23 h. The components of the luciferase assay kit (WAKO PicaGene MelioraStar-LT Luminescence Reagent) were added to each well. Photoluminescence spectroscopy was measured with an ATTO AB-2100 luminometer. An assessment of cell viability was undertaken using a solution of 0.5% trypan blue stain (Nacalai Tesque). The amounts of live cells were determined after collecting cells from a 96-well plate. Experiments were performed in triplicate, and data represent mean ± SEM. Statistical analyses were carried out with a Student's t test. Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Preparation and X-ray Crystal Structure Analysis. Quantitative elemental analysis of **Ru·CL-HEWL** by X-ray fluorescence spectroscopy showed that the number of Ru per HEWL monomer for **Ru·CL-HEWL** is 10.1 ± 0.8. Diffraction data of **Ru·CL-HEWL** indicated that the crystal lattice and solvent channels are maintained after all procedures. A scanning transmission electron microscopy-energy dispersive X-ray spectrum (STEM-EDX) showed that the Ru complexes are well distributed within **Ru·CL-HEWL** (Figure 1a). **Ru·CL-HEWL** was characterized by infrared (IR) spectroscopy (Figure 1b). The IR spectrum of **Ru·CL-HEWL** has four bands at 2055, 2025, 1981 and 1940 cm^{-1} (Figure 1b, solid line). A pair of bands at 2055 and 1981 cm^{-1} is assigned to the CO stretching vibrations of the *cis*-$Ru(CO)_2$(His) moiety in proteins.[29] The other pair at 2025 and 1940 cm^{-1} is proposed to arise from the CO stretching vibrations of negatively charged $[cis\text{-}Ru(CO)_2X_4]^{2-}$ (X = anionic ligands).[58−60] The composite of HEWL and CORM-2 in buffer solution (**Ru·HEWL**) showed two bands at 2047 and 1977 cm^{-1} that are assigned to the *cis*-$Ru(CO)_2$(His) moiety (Figure 1b, dotted line). Difference IR spectra between **Ru·CL-HEWL** and **Ru·HEWL** indicate that $[cis\text{-}Ru(CO)_2X_4]^{2-}$ moieties exist only in **Ru·CL-HEWL**.

The coordination structure of **Ru·CL-HEWL** was determined by X-ray crystal structure analysis at a resolution of 1.55 Å (Figure 2 and Table S1, Supporting Information). The binding positions of the Ru atoms were distinguished by anomalous difference maps (Figure 2c−h). Each of the Ru atoms is bound to a coordinating residue such as His, Asp, and Lys with a bond length of 2.2−2.5 Å. Such bond lengths are the same as typical coordination bond lengths for ruthenium ligated to amino acid residues.[20,61,62] Ru_a is coordinated to His15 as reported for HEWL crystals with other organometallic complexes.[20,29,30,61] Ru_a has an occupancy of 0.7 and adopts an octahedral geometry with Nε of His15, two CO molecules, one water molecule, and two Cl ions to form $[cis\text{-}Ru(CO)_2(H_2O)Cl_2(His15)]$ (Figure 2c). This structure gives rise to IR bands of 2055 and 1981 cm^{-1} according to the literature[29] and is surrounded by Arg14, Asp87, and Thr89. Oδ of Asp87 and Oγ of Thr89 stabilize the coordination structure by participating in hydrogen-bonding networks with $[cis\text{-}Ru(CO)_2(H_2O)Cl_2(His15)]$ as previously reported for $[Ru(benzene)Cl_2(His15)]$ in cross-linked HEWL crystals.[20] An additional nine anomalous densities were identified near the carboxylate groups of Asp18, Asp52, Asp101, Asp119, and Leu129 of the C-terminal and assigned to Ru ions (Figure 2d−h). These structures are expected to have IR bands at 2025 and 1940 cm^{-1} according to the literature.[58−60] The anomalous difference maps of Ru_b and Ru_c imply that Ru_b and Ru_c each form individual mononuclear Ru sites coordinated to each of the Oδ atoms of Asp18 because the occupancy level for each of these Ru ions was found to be 0.5 (Figure 2d). Although anomalous densities of Ru_d−Ru_j were observed as shown in Figure 2e−h, the carbonyl ligands could not be determined at the sites due to the low occupancy of the Ru ions whose value is below 0.5.

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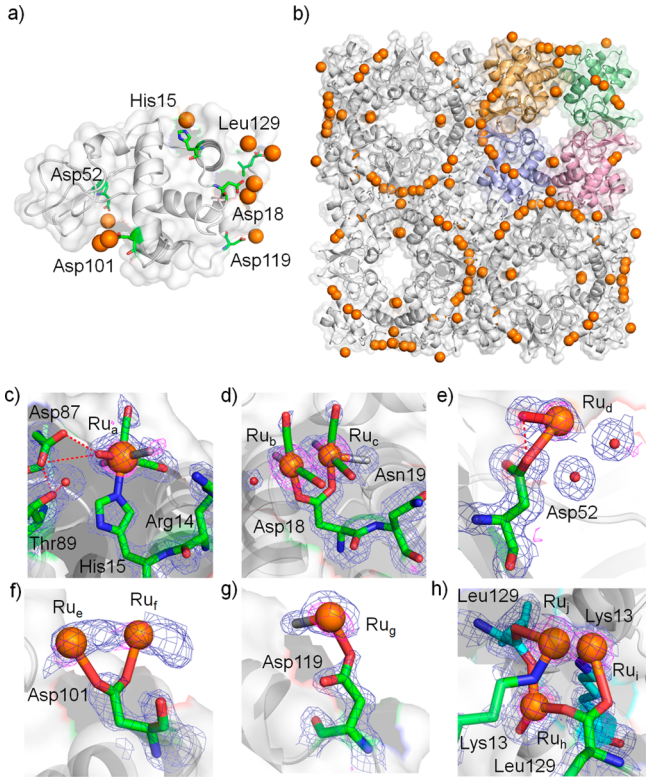

Figure 2. Coordination structures of Ru complexes (Ru_a–Ru_j) in **Ru·CL-HEWL** for a monomer (a) and lattice (b), and magnified structures of coordination sites of His15 (c), Asp18 (d), Asp52 (e), Asp101 (f), Asp119 (g), and Leu129 of the C-terminal (h). Residues of the neighboring HEWL monomer are shown in light blue. Hydrogen bonds are indicated by red dashed lines. These images were produced by Pymol.[63]

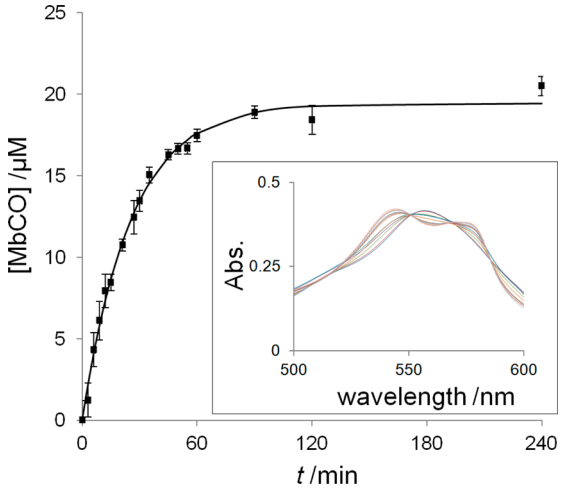

Figure 3. Detection of CO release by monitoring the changes of the Q-band region of the UV–vis spectra of Mb (35 μM) in the presence of **Ru·CL-HEWL** (Ru = 0.025 μmol) and $Na_2S_2O_4$ (10 mM) in 500 μL of PBS buffer at 25 °C (inset), and plot of the formation of MbCO. Conversion of Mb to MbCO was monitored with a spectrophotometer. After calculation of the equivalents of CO/Ru, $t_{1/2}$ was determined by fitting to first-order kinetics. Experiments were performed three times, and the data represent mean ± SEM.

CO Release Assay. To quantify the amount of CO released from **Ru·CL-HEWL** into the aqueous medium, a spectrophotometric assay based on conversion of deoxy-myoglobin (Mb) to carboxy-Mb (MbCO) was conducted.[22] **Ru·CL-HEWL** was suspended in a buffer solution containing Mb and sodium dithionite ($Na_2S_2O_4$). Formation of MbCO was quantified by UV–vis measurements.[64] In the presence of **Ru·CL-HEWL**, the Mb peak at 556 nm decreased while the MbCO peaks at 542 and 578 nm increased with isosbestic points at 550, 571, and 586 nm during the reaction (Figure 3). The released amount of CO of **Ru·CL-HEWL** was found to be 0.38 ± 0.02/Ru. The half-life ($t_{1/2}$) of CO release was found to be 19.4 ± 0.8 min. The $t_{1/2}$ value was about 10-fold greater than the $t_{1/2}$ value of CORM-2 (1.9 ± 1.4 min) estimated under the same conditions. Since the CO-releasing reaction is caused by a ligand exchange reaction of carbonyl ligands with $S_2O_4^{2-}$ or SO_2^- under the conditions of the Mb assay,[64] the solvent channels of CL-HEWL are expected to affect the restricted diffusion of these anions eventually to extend the $t_{1/2}$ value of **Ru·CL-HEWL.**[38,65,66]

The number of Ru ions per HEWL monomer retained in **Ru·CL-HEWL** after the Mb assay is 10.4 ± 1.1. This value is similar to that of **Ru·CL-HEWL** before the assay (10.1 ± 0.8). The IR spectrum of **Ru·CL-HEWL** after the Mb assay showed that the peaks at 2025 and 1940 cm^{-1} disappear and the peaks at 2055 and 1981 cm^{-1} assigned to $[Ru(CO)_2(H_2O)Cl_2(His15)]$ are retained (Figure 1b, chained line). This is supported by X-ray crystal structural analysis of **Ru·CL-HEWL** after the Mb assay. Electron densities of the CO ligands of Ru_a are preserved after the Mb assay, although the electron densities of CO and Cl ligands of Ru_b and Ru_c disappear (Figure S1, Supporting Information). This is expected because $[cis\text{-}Ru(CO)_2(H_2O)Cl_2(His15)]$ is stabilized by steric hindrance and hydrogen-bonding interactions with the surrounding amino acid residues to prevent the access of trigger molecules for the ligand exchange reaction with CO (Figure 2c). For the **Ru·HEWL** dissolved in buffer solutions (control), **Ru·HEWL** was found to be incapable of releasing CO because $[cis\text{-}Ru(CO)_2X_4]^{2-}$ cannot be formed in **Ru·HEWL**. Moreover, the Mb assay could not be confirmed because **Ru·HEWL** precipitates as a dark red aggregate. As reported previously, these results suggest that $[cis\text{-}Ru(CO)_2X_4]^{2-}$ preserved in CL-HEWL is the CO releasing site.[29]

Cellular Assay. To assess the CO-releasing effect of **Ru·CL-HEWL** as an extracellular scaffold, the activity of nuclear factor kappa-B (NF-κB) in living cells was evaluated. Exposure to the exogenous CO gas at a level of 250 ppm is known to activate NF-κB in the presence of tumor necrosis factor-α (TNF-α).[67,68] HEK293/κB-Fluc cells were cultured in the medium containing **Ru·CL-HEWL** (2.0×10^5 crystals, [Ru] = 5.0 μM in the medium) and 1.0 ng/mL of TNF-α (Figure S2, Supporting Information). After incubation for 24 h, the luciferase levels in the cells were analyzed by adding a luminescent substrate.[69] The control assay was carried out using CORM-2 solution, **Ru·HEWL** ([Ru] = 5.0 μM), and CL-HEWL (2.0×10^5 crystals). Figure 4 shows the bioluminescence intensity of the cells with each composite. In the presence of **Ru·CL-HEWL**, a 20% increase of NF-κB activity was observed relative to the assay with PBS buffer only. The results suggest that the activation is caused by (1) effective delivery of CO from **Ru·CL-HEWL** with cell surface adhesion and (2) slower release of CO from the interior space constructed in solvent channels of CL-HEWL as reported previously.[28,70] According to the literature, dozen micromolar levels of CORM induce activation of NF-κB in the presence of LPS, but this effect is accompanied by a decrease in

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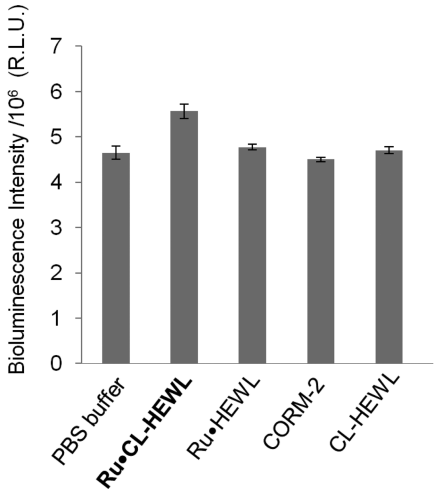

Figure 4. Bioluminescence intensity in the luciferase reporter assay for evaluation of NF-κB activity of HEK293/κB-Fluc cells in the presence of 1.0 ng/mL TNF-α after incubation with PBS buffer (as control), **Ru•CL-HEWL**, **Ru•HEWL**, CORM-2, and CL-HEWL for 24 h. Experiments were performed three times under each set of conditions. Data represent mean ± SEM.

cellular viability due to the cytotoxicity of the high concentration of metal ions.[23] There is no difference of the cell viability for the cultivation with **Ru•CL-HEWL** or other probes as revealed by the trypan blue tests (Figure S3, Supporting Information). It appears that **Ru•CL-HEWL** is a suitable tool for investigating CO release using Ru carbonyl moieties.

CONCLUSIONS

We established a new method for constructing a CO-releasing extracellular scaffold by mixing a member of the CORM-2 class of CO-releasing molecules with CL-HEWL. The CO-releasing reaction was achieved by immobilization of $[cis\text{-}Ru(CO)_2X_4]^{2-}$ moieties in **Ru•CL-HEWL**. We used **Ru•CL-HEWL** as an extracellular scaffold to induce the activation of NF-κB. Our approach represents a new method for constructing extracellular scaffolds using organometallic complexes, and it is expected that the method will provide a useful tool for molecular biology research. We are currently immobilizing various metal complexes in porous protein crystals and evaluating reactions involved in control of living cells.

ASSOCIATED CONTENT

Supporting Information

Additional information on the crystal structure, cellular assay, and crystallography data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*Phone: +81-75-753-9869. Fax: +81-75-753-9820. E-mail: kitagawa@icems.kyoto-u.ac.jp.

*Phone: +81-45-924-5806. Fax: +81-45-924-5806. E-mail: tueno@bio.titech.ac.jp.

Notes

The authors declare no competing financial interest.

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