

**Studies on Functionalization of
Porous Protein Crystals by
Immobilizing Organometallic Complexes**

Hiroyasu Tabe

2015

Preface

This thesis is based on research in coordination chemistry and protein engineering for developing protein crystals functionalized with organometallic complexes at during April 2009 – March 2015 under the supervision of Prof. Susumu Kitagawa. The research took place at the Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University. The author thanks my colleagues at Institute for Integrated Cell-Material Science (iCeMS), Kyoto University for an inspiring research environment and with the working facilities and the pleasant atmosphere.

The author expresses my deep gratitude to my supervisor Prof. Susumu Kitagawa for guidance, support, and providing the didactical background on which this work is based. The author also thanks my second supervisor, Prof. Takafumi Ueno (Tokyo Institute of Technology) for his instruction, advice and reviewing the manuscripts of this thesis. The author would like to acknowledge the discussion and the suggestion of Prof. Masaaki Ohba (Kyushu Univ.), Dr. Takashi Uemura, Dr. Satoshi Horike, Dr. Shuhei Furukawa, Dr. Ryotaro Matsuda, Dr. Masakazu Higuchi.

The author is extremely grateful to Dr. Satoshi Abe (Tokyo Institute of Technology) and Dr. Tomomi Koshiyama (Kyushu University) for their instruction of experiments and their assistance in the many research concerns.

Warm thanks go to Prof. Hajime Mori (Kyoto Institute of Technology) and Dr. Hiroshi Ijiri (Tokyo Institute of Technology) for assisting works on *in-vivo* protein crystal engineering. The author wishes to express his gratitude to Prof. Shinae Kizaka-Kondo (Tokyo Institute of Technology) and Dr. Takahiro Kuchimaru (Tokyo Institute of Technology) for their collaboration for cellular experiments. The author also expresses his gratitude to Dr. Kunio Hirata (RIKEN), Dr. Nobuhiro Mizuno (JASRI) and Dr. Seiki Baba (JASRI) for their support for the crystallographic data collection at Spring-8.

The thanks are extended to many colleagues at Kitagawa Laboratory and Ueno Laboratory (Tokyo Institute of Technology), with whom the author has had longer or shorter enlightening discussions about the various topics of this work. Among the many, I wish to mention Mr. Kenta Fujita, Mr. Masaki Akai and Mr. Takuya Shimoi. Without

their continued support, the research would not have been possible. The author expresses his extreme gratitude to Dr. Nusratjahan Sanghamitra, Dr. Hiroshi Inaba and Mr. Yu Tokura for daily experiments, discussions and supports. The author would also like to acknowledge the assistance and collaboration by Dr. Yuya Tanaka, Dr. Basudev Maity, Mr. Mitsuhiro Okamoto and Mr. Hashiru Negishi. The author is thankful to Ms. Yoko Takahara, Ms. Shoko Ibuki, Ms. Mieko Yoshida and Ms. Kiyo Yamashita for their expert assistance in the many practical concerns.

The author acknowledges financial support of Research Fellowship of the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

Finally the author wishes to express my gratitude to his parents, Koshi Tabe, Hiroko Tabe and his sister Chiaki Tabe. Most of all, the author thanks his father, Koshi Tabe, who passed away in March 2013 without seeing the completed thesis. The author dedicates this thesis to him.

Hiroyasu Tabe

Department of Synthetic Chemistry and Biological Chemistry
Graduate School of Engineering, Kyoto University
March, 2015

Table of Contents

Preface

General Introduction

1

Chapter 1.

Porous Protein Crystals as Catalytic Vessels for Organometallic Complexes

27

Chapter 2.

Preparation of a Cross-linked Porous Protein Crystal Containing Ru Carbonyl Complexes as a CO-releasing Material

51

Chapter 3.

Design of a CO-releasing Extracellular Scaffold using *in-vivo* Protein Crystals

73

Chapter 4.

Light-triggered CO Releasing from Extracellular Scaffold Constructed by *in-vivo* Protein Crystals

89

Chapter 5.

In-vivo Crystal Engineering for Preparing Porous Protein Frameworks

107

List of Publications

127

General Introduction

Metalloproteins

Metal cofactors are functionalized to promote various enzyme reactions by appropriate immobilization in proteins. Composites of synthetic metal complexes and proteins represent one of the ultimate goals in the fields of bioinorganic chemistry and protein engineering. These composites have been providing unnatural functions to proteins. One of the first examples of artificial metalloproteins was developed by Whitesides *et al.* in 1978.¹ This metalloprotein holds Rh^I complex covalently conjugated to biotin and has a function of asymmetric hydrogenation catalysis when it forms a complex with avidin. This system has been extended by Ward *et al.* for various catalytic reactions with high chemoselectivity (Figure 1).²⁻⁵ Moreover, other design methods of artificial metalloproteins, such as replacement of natural cofactors, and noncovalent modification, have been reported to prepare artificial metalloenzymes (Figure 1).⁶⁻¹³ These examples show the importance of protein scaffolds for controlling the coordination number, geometry and stability of individual metal cofactors for various functions.

Each metalloprotein discussed above possesses a single metal center. Conjugation of proteins containing metal cofactors has been reported by polymerization of proteins. Hayashi *et al.* reported the polymerization of hemeprotein by alternation between natural cofactors and synthetic ones with linker molecules.¹⁴⁻¹⁶ The polymers of artificial metalloproteins are utilized to accumulate and display metal cofactors and achieve functions such as high-efficient cathodic current and an O₂ binding.^{17, 18} It is expected that the construction of protein conjugated system is well-suited to create functional bionanobiomaterials beyond the original function obtained by metalloprotein monomers.¹⁹⁻²¹

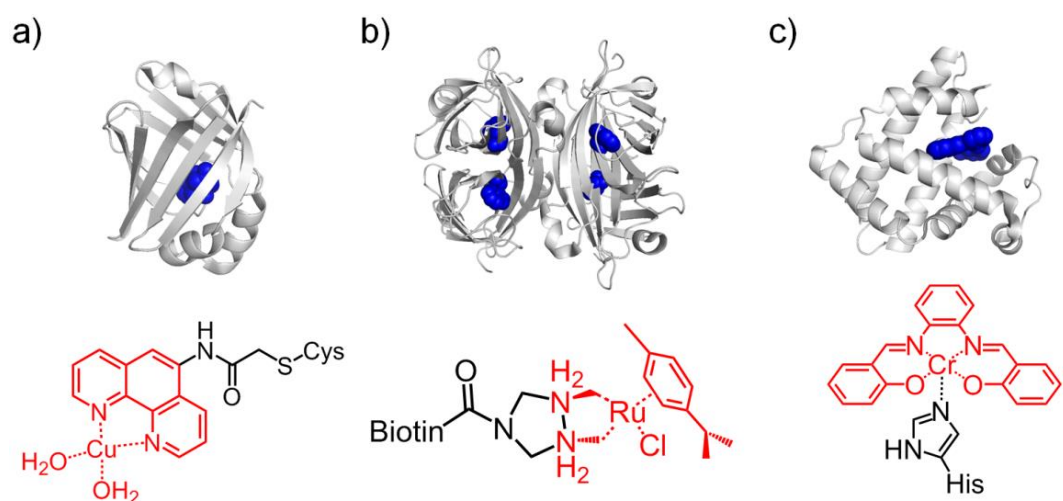


Figure 1. Crystal structure and synthetic metal cofactors of artificial metalloproteins (a) $\text{Cu}^{\text{II}}(\text{phen})\text{-ALBP}$, (b) $\text{Ru}^{\text{II}}(\eta^6\text{-benzene})\text{Cl}(\text{Biotin})\text{-streptavidin}$ and (c) $\text{Cr}^{\text{III}}(\text{salophen})\text{-apo myoglobin}$.

Protein Assemblies

Protein supramolecules have more sophisticated structure compared with simple polymers of proteins.²²⁻³⁸ These compounds are known as protein assemblies and proceed the folding into specific 3D shapes such as oligomer, cages, filaments and layers. Coordination bondings and hydrogen bondings are essential for non-covalent interactions for constructing protein assemblies. Moreover this interaction helps to determine the interactions between proteins and metal cofactors.

Protein assemblies often have metal cofactors with unique coordination structures. One typical example is photosystem II (PSII) that can promote reactions of inactive small molecules. The high-resolution structure of PSII in the 1.9 Å resolution showed the key structure for catalytic functions (Figure 2a).^{39, 40} PSII is composed by 20 kinds of proteins containing metal complexes, organic compounds and water molecules. Several metal cofactors such as Mn_4CaO_5 cluster, chlorophylls, heme and non-heme irons are coordinated to amino acid residues whose location is suit for the multistep catalysis. Mn_4CaO_5 cluster, which is a main moiety for the O_2 production, is distorted compared to the cuban-shaped structure. The distortion is stabilized by the coordination to Glu, Asp, His and Arg residues and coordinated waters in proteins to obtain the reactivity of H_2O splitting and O-O bond formation (Figure 2b). Other metal cofactors are also coordinated to amino acid residues and immobilized in the protein assembly. The organization of metal cofactors is utilized to achieve energy migration and electron transfer to assist O_2 production.

Accumulating large amounts of metal compounds is another typical function of protein assemblies. Ferritin is known to an intracellular protein for metal accumulation and storage.⁴¹ Ferritin is composed by 24 homoprotein subunits and forms a hollow cage structure with an 8 nm inner diameter and a 12 nm outer diameter (Figure 3a). Fe ions can penetrate the 3-fold axis channels located at the intersections of three subunits of ferritin.⁴² Thousands of iron ions can be accommodated by formation of iron oxide inside ferritin (Figure 3b).

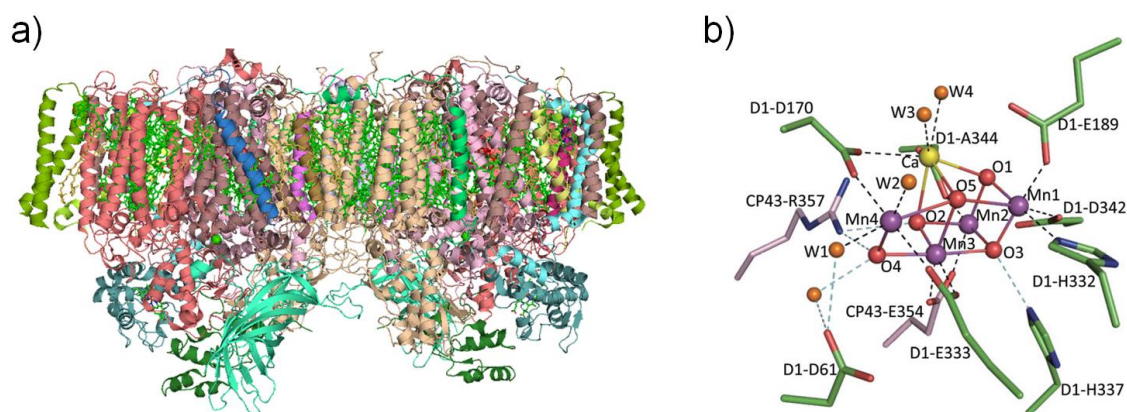


Figure 2. (a) Overall structure of Photosystem II dimer obtained at a resolution of 1.9 Å. Protein subunits are colored individually. (b) structure of the Mn_4CaO_5 cluster in the ligand coordination structure.⁴⁰

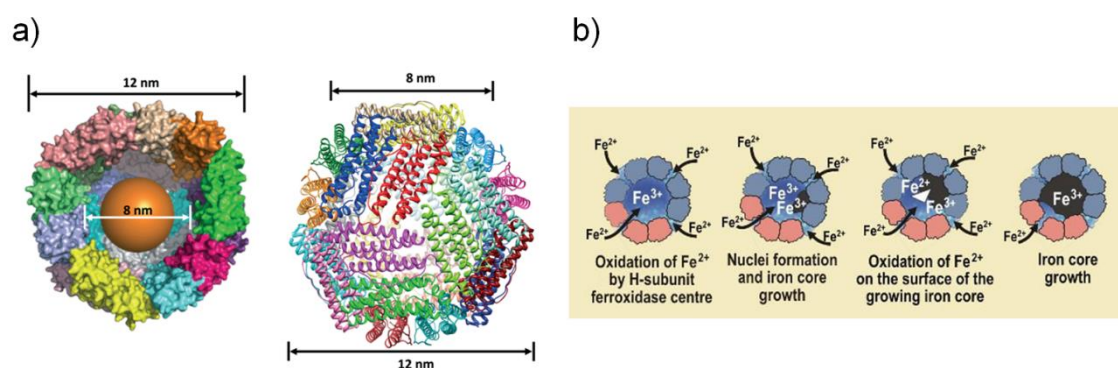


Figure 3. (a) the cross section drawing of the inner cavity (left) and whole structure (right) of 24mer of recombinant L-chain apo-ferritin. (b) Schematic representation of the mechanism of iron accumulation and immobilization.⁴²

Protein Crystals

Protein assemblies have a potential for further engineering toward artificial bionanomaterials using unnatural metal cofactors. However, there are limited numbers of natural protein assemblies for this aim because of their low stability, low yield and difficulty in structure analysis. Protein crystallization is a versatile method to obtain the assembled structure. Proteins can be crystallized when preserved in optimized conditions. A lot of protein crystals have been reported since the first protein crystal of hemoglobin was reported in 1840.⁴³⁻⁴⁵ Research efforts related to crystallization have focused mainly on purification processes, structural analysis and researches in the pharmaceutical and chemical industries.⁴⁶⁻⁴⁸ Single crystals of proteins provide structural information in atomic resolutions using X-ray crystal structure analysis. To date, more than 100,000 structural data are deposited in Protein Data Bank. Protein crystals are constructed in highly ordered 3D structures assembled hierarchically.⁴⁹ Neighboring molecules in protein crystals are tightly bound by non-covalent bonding due to multimetric contacts in lattice structures.⁵⁰ These interactions make protein crystals stable and densely packed (1.3~1.6 g/mL) than other protein assemblies.^{51, 52}

Several protein crystals are found in nature.⁵³ The proinsulin is directed into the regulated secretory pathway and converted to insulin and stored as crystals (Figure 4a).^{54, 55} Six insulin molecules assembled by the histidine chelating to Zn^{II} located in the center of hexamer for the function of the storage and the slow release of insulin monomers. Several kinds of alcohol oxidase form crystals in yeasts. They can catalyze enzymic reactions within the active octamers in the crystal lattice.^{56, 57} Crystalline cell surface layers (S-layer) are found in bacteria and archaea (Figure 4b).^{58, 59} S-layers are composed of a single homogeneous protein or glycoprotein with molecular weights ranging from 40 to 200 kDa. They have several lattice types are found as oblique (p1, p2), square (p4) or hexagonal (p3, P6) subunits that have different type of packing structure of monomers. These structures can be utilized for cell adhesion, surface recognition, cell fission process as well as molecular sieves and molecular and ion traps, because channels are present with diameters in the range of 2–8 nm in most S-layers.

Cypoviruses and baculoviruses particles embedded in polyhedra crystals (PhC) can survive against dehydration, freezing and enzymatic degradation (Figure 4c).^{60, 61} In addition, PhC tolerate chemical treatments that would denature most proteins, including incubation in concentrated urea, acid and detergents. They maintain their molecular order in a crystal structure but are dissolved readily when exposed to pH over 10.5. This

system helps viruses remain infectious for years and release viruses after taken into insects. This method has been expanded to the *in-vivo* crystallization of other recombinant proteins.^{62, 63} There are more examples such as plant seeds and filamentous fungi have protein crystals for the protein storage and damage sealing, respectively.⁶⁴⁻⁶⁶

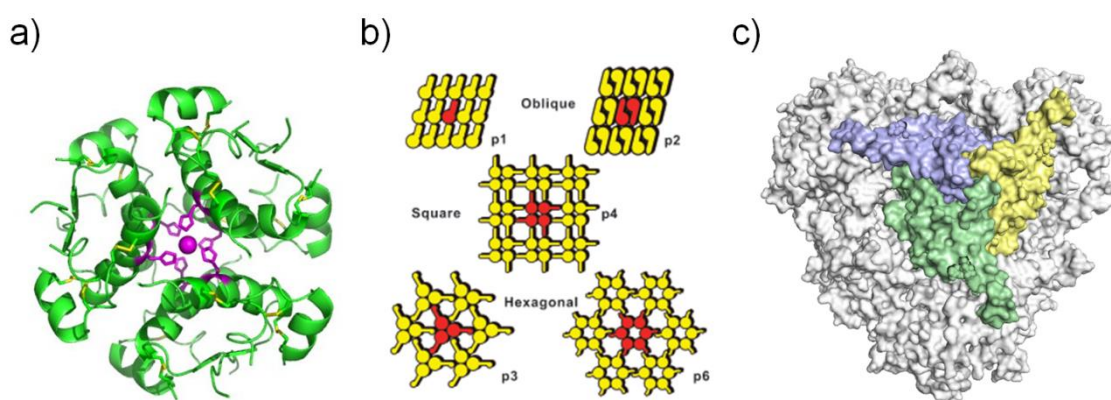


Figure 4. Examples of protein crystals found in nature. (a) insulin crystals formed by the Zn^{II} -His coordination and the trimerization. (b) S-layer in forms of p1, p2, p3, p4 and p6 structures. (c) cypovirus polyhedra with an extraordinary stability by a tightly packed structure.

Metal-Mediated Protein Crystals

Coordination bonds between proteins and metals are convenient for construction and modification of protein crystals. Ferritin forms crystals in the presence of Cd^{II} ion.^{67, 68} His, Glu and Lys residue located at the outer surface of ferritin cage are connected by the Cd^{II} coordination to form 2D and 3D crystals (Figure 5a). This method has been expanded to prepare artificial metal-protein frameworks.²⁹ Schulz et al. reported the crystallization of L-rhamnulose-1-phosphate aldolase (RhuA) and streptavidin depending on the concentration of Ca^{II} ions (Figure 5b).²³ Tezcan *et al.* reported the introduction metal chelating sites to architect protein assemblies like metal-organic frameworks (Figure 5c).^{29, 69-71} They optimized the crystallization procedure to make from 1D to 3D crystals by changing pH or the precursor concentration.⁷⁰ They also immobilized functional groups such as small metalloenzymes into the metal-protein frameworks although catalytic functions have not been reported.⁷² These examples show that it is possible to prepare protein crystals with desired structures by genetic and synthetic methods.

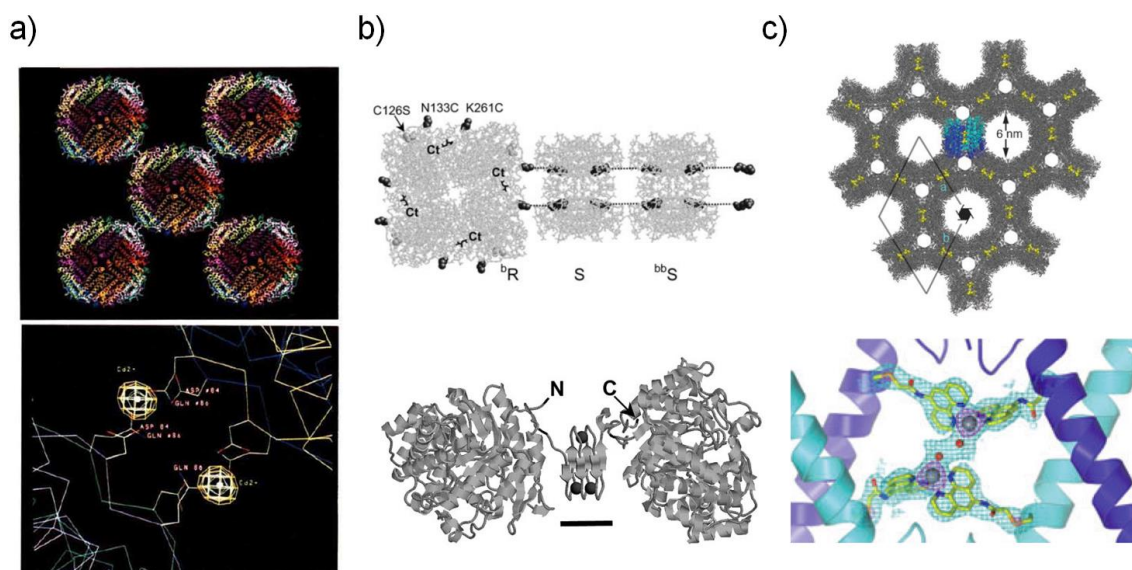


Figure 5. Lattice structure (upper) and local coordination sites (lower) obtained by X-ray crystal structure analysis of a Ferritin- Cd^{II} crystal (a), an L-rhamnulose-1-phosphate aldolase (RhuA)/streptavidin- Ca^{II} crystals (b) and an MBPPhen- Cu^{II} crystals (c).

Protein crystals as Reactive Nanovessels

Since 1990, the application of protein crystals as bionanomaterials is widely reported for functions such as solid enzyme reactors and molecular sieves.⁷³⁻⁸⁵ This is because each protein crystal possesses inner pores called “solvent channels”. The sizes and environment of solvent channels vary according to the lattice of each individual crystal (Figure 6). A given channel occupies 30–65% of the total crystal volume and provides a large surface area (800~2000 m²/g) with a wide range of channel sizes (10~100 Å).⁴⁹ These channels traverse the crystals and facilitate the transport of exogenous molecules in crystals. The amino acid residues are exposed on solvent channels and they can interact with exogenous molecules. The physical properties of exogenous molecules in solvent channels, such as molecular adsorption and diffusion, have been discussed using experimental methods and molecular simulations.⁸⁶⁻⁹²

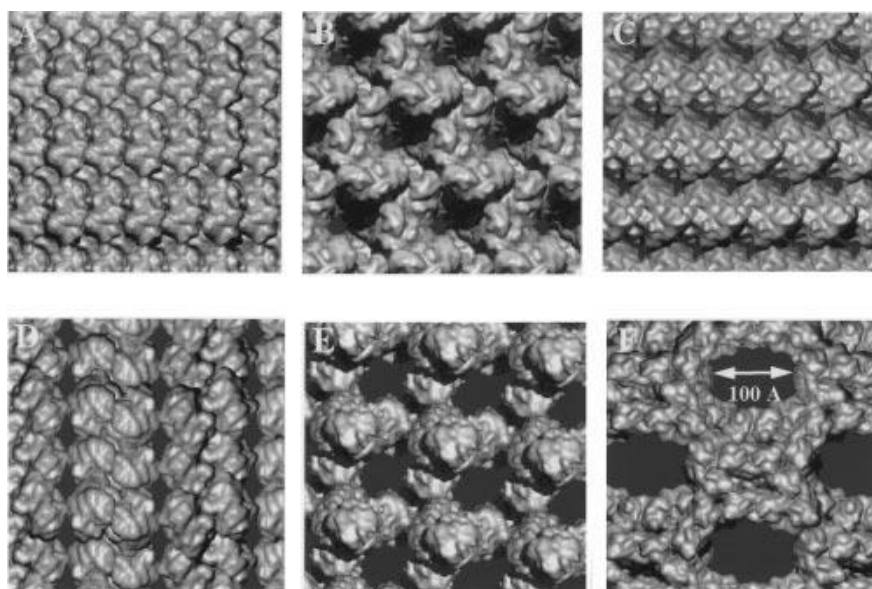


Figure 6. Images of enzyme crystal lattices used as enzyme reactor after cross-linking treatment. (A) superoxide dismutase (PDBID : 1XSO), (B) thermolysin (PDBID : 8TLN), (C) penicillin acylase (PDBID : 1PNL), (D) lipase (PDBID : 1CRL), (E) carboxypeptidase W (PDBID : 1WHS), (F) superoxide dismutase (PDBID : 1SOS). All structures are shown on the same scale.

Cross-linking treatment remarkably stabilizes the crystal lattice against mechanical disruption, elevated temperature and various solvents. Klibanov *et al.* used cross-linked protein crystals for enzyme reactions in organic solvents.⁷³⁻⁷⁶ Navia and Margolin *et al.* expanded this performance to applications of peptide synthesis and molecular separation.⁷⁷⁻⁸⁴ It is reported that the environment of solvent channels highly effect to catalytic functions. For example, when cross-linked γ -chymotrypsin crystals are used as asymmetric synthesis in organic solvents, the solvent dependence can be attributed to changes in the solvation energies of organic substrates in solvent channels for the *pro-R* and *pro-S* (Figure 7).⁷⁵ Lipase crystals have two states of open and close, which are in the different position of a flexible structure that allows substrate to bind to the active site.⁷⁷ The catalytic activity of the open form was almost three times higher than that of the closed form with higher enantioselectivity. Cross-linked protein crystals with cofactors are also applied as enzyme reactor.⁸⁴ This technique provides the information that solvent channels are ideal reaction nanospaces rather than the environment of protein monomers or protein random aggregations. If there is a suitable technique to modify solvent channels, we can design the enzyme reactions more precisely.

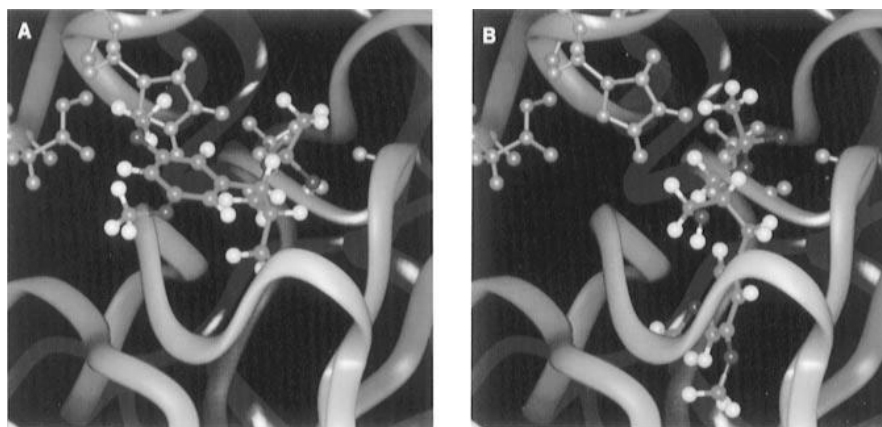


Figure 7. Estimated conformation of 2-(3,5-dimethoxybenzyl)-1,3-propanediol in the *pro-S* (A) and *pro-R* (B) transition states within cross-linked γ -chymotrypsin crystals.⁷⁵

Post-engineering of protein crystals for immobilizing metal complexes

Characterization of metal cofactors in protein crystals has been well reported using X-ray crystal structure analysis.^{9, 93} Observation of intermediates of chemical reaction is available using metalloenzyme crystals.⁹⁴⁻⁹⁶ This system has been expanded to unnatural metal cofactors that proceed a single-turn over reaction by an artificial metal cofactor immobilized in NikA crystals (Figure 8).⁹⁷ Electron transfer reactions in metalloprotein crystals can be triggered by external stimuli or chemical modifications.⁹⁸⁻¹⁰¹

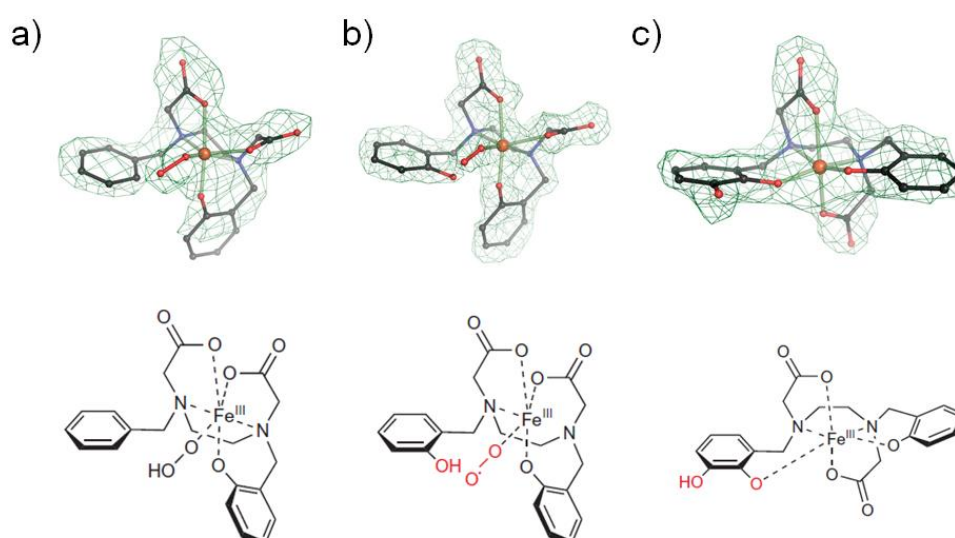


Figure 8. Electron density and structure of non-heme metal complexes under the process of the oxidation of aromatic C-H in NikA crystals. (a) Fe^{II}-O₂ coordination structure, (b) structure of a complex after the monooxidation and (c) structure of a complex after dioxidation.

Solvent channels provide suitable reaction environments for post-engineering of protein crystals.¹⁰² Hen egg white lysozyme (HEWL) crystal is one of the most useful scaffolds for the synthesis of inorganic materials because these crystals are abundantly crystallized and have large solvent channels ranging from 10 to 20 Å.¹⁰³⁻¹¹¹ The first work of preparing metallic nanoparticles was reported by Mann *et al.*¹⁰³ Spontaneous growth of gold nanoparticles within HEWL crystals with disproportionation of Au^I was reported by Lu *et al.*¹⁰⁵ They extended this system to the assess of catalytic activity of gold nanoparticles depending on the particle size.¹⁰⁶ CoPt bimetallic nanoparticles were

prepared by reducing metal ions using NaBH_4 in cross-linked HEWL crystals in tetragonal, orthorhombic and monoclinic forms (Figure 9). The regulation of nanoparticles in each solvent channels was found to have different magnetic coercivity at 5 K.¹⁰⁷

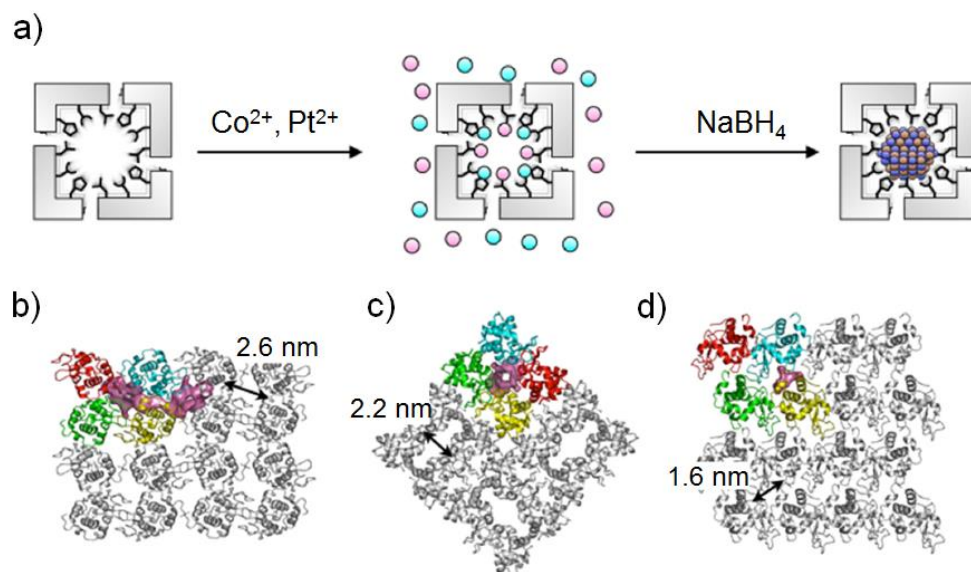


Figure 9. (a) Schematic image describing the preparation of CoPt nanoparticles in a solvent channel of a porous HEWL crystal. Crystal lattice structures and the major solvent channels of (b) O-HEWL, (c) T-HEWL, and (d) M-HEWL obtained from the PDB ID : 1BGI, 193L, and 5LYM, respectively.

The coordination structure of metal ions such as Rh^{III} , Ag^{I} and Ni^{II} in HEWL crystals are observed using X-ray crystal structure analysis.¹¹²⁻¹¹⁴ A large number of metal ions per protein monomers are accumulated in the solvent channels. The snapshot analysis of Rh^{III} coordination in tetragonal HEWL crystals containing different numbers of Rh^{III} provides the first experimental evidence of cooperative behavior between amino acids and hydrogen bonds acting to initiate the coordination of metal ions on the surface of solvent channels in protein crystals (Figure 10).¹¹² Structures of tetragonal HEWL crystals with exogenous metal complexes have been reported as model structures for clarification the interactions between proteins and metallodrugs.¹¹⁵⁻¹²² Mn^{I} , Ru^{II} , and Pt^{II} complexes are immobilized by the coordination to His15, which is exposed on the

largest solvent channels, supported by hydrogen bonding networks with other surrounding amino acid residues and water molecules.

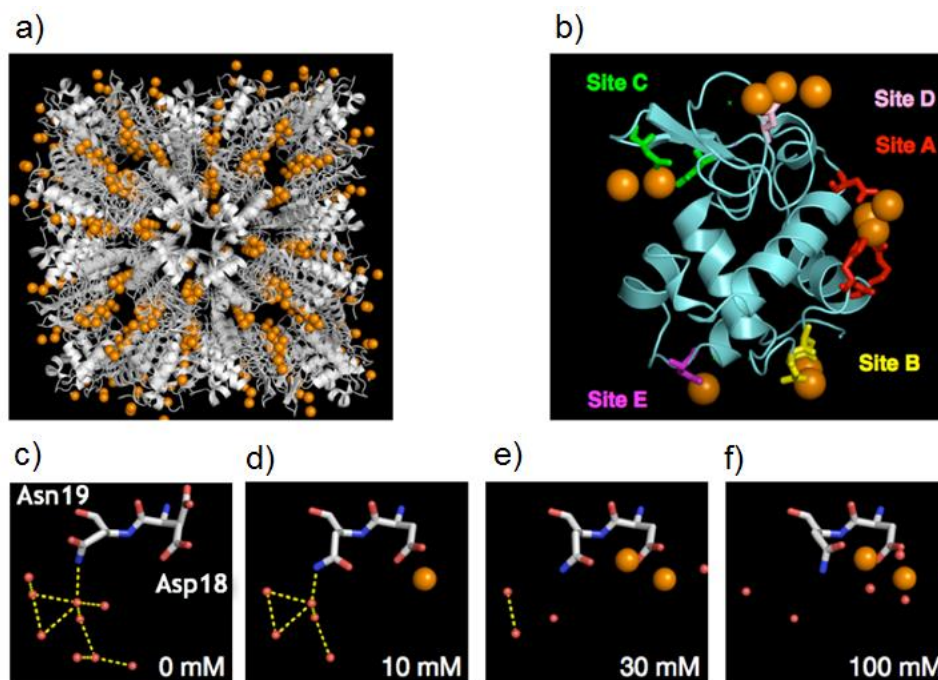


Figure 10. Image of crystal lattice (a) and monomer structures (b) of a porous HEWL crystal after the immobilizing Rh^{III} ions in solvent channels. Snapshot analyses of Rh^{3+} coordinating to Asp18 and Asn19 residues (c-f). Each structure obtained at a different concentration of RhCl_3 in sodium acetate buffer at pH 4.5.

Immobilization of multiple kinds of metal complexes is also reported. The zinc porphyrin-substituted myoglobin crystals containing the oxotetraruthenium clusters (Ru_3O) has been prepared.¹²³ The electron transfer within the solvent channels was observed between the Zn and Ru complexes in the presence of a methyl viologen (MV) (Figure 11). This engineered myoglobin crystal has a charge separated state with a lifetime up to 2,800-fold greater than that of the previously reported system. This is made possible by the accumulation and the site-specific coordination of zinc porphyrin, Ru_3O , and MV in the solvent channel of myoglobin crystals.

Although solvent channels in protein crystals are ideal space for the coordination and the accumulation of metal complexes, there is still few report of the evaluation of chemical reaction of metal complexes.⁴⁸ If we are able to overcome this problem, it is

possible to develop innovative bionanomaterials modified with metal complexes. Recently Ueno *et al.* reported the molecular design of protein crystals using covalent modification to prepare bionanomaterials.^{124, 125} Since this technology can be expanded to various synthetic molecules including metal complexes, it is able to obtain attractive biomaterials with metal complexes, such as extracellular scaffolds for drug delivery systems, metalloenzymes for selective reactions.

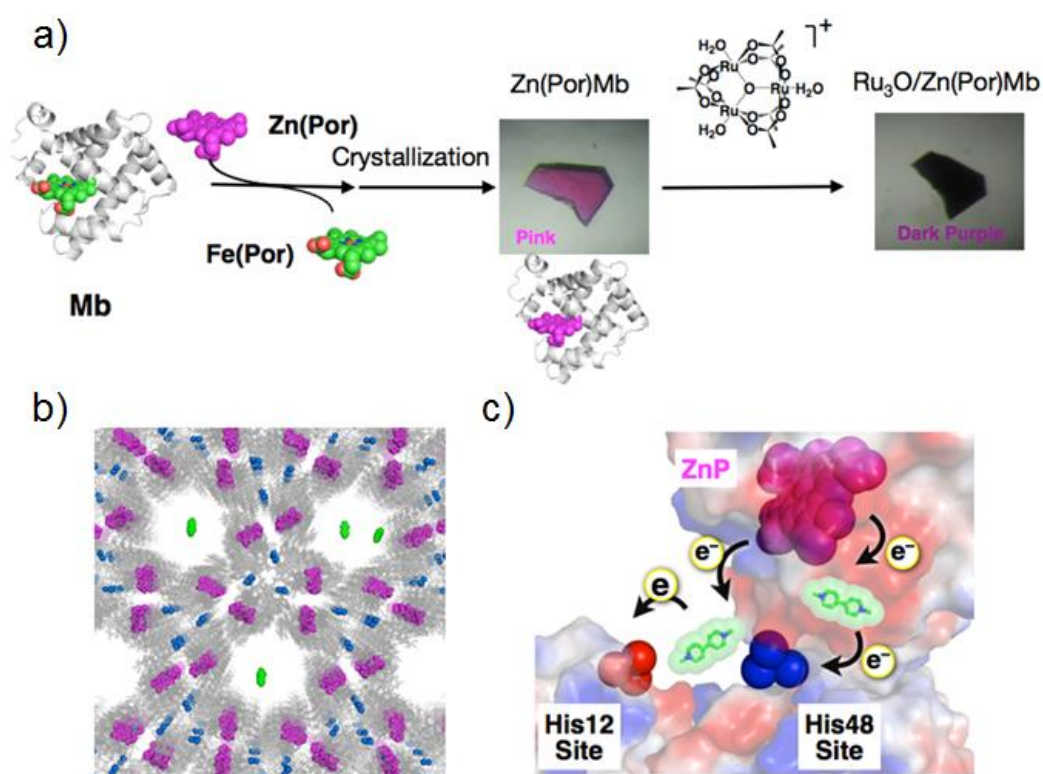


Figure 11. Schematic drawings of (a) Construction of a photoinduced electron transfer system in porous Mb crystals. (b) Lattice structure of myoglobin crystals immobilizing oxotriruthenium cluster (Ru₃O) and Zn porphyrin in myoglobin crystal. (c) Electrostatic potential of the coordination sites of Ru₃O and Zn porphyrin in the crystal. The surface is colored with a spectrum (ranging from red to blue for the negative to positive charge of each residue).

In chapter 1, organoruthenium complexes were immobilized in cross-linked tetragonal HEWL crystals (CL-T-HEWL) and cross-linked orthorhombic HEWL crystals (CL-O-HEWL). X-ray crystal structure analysis showed the different second coordination environment around Ru(benzene)Cl₂-His15 moieties. Both crystals accelerate the hydrogen-transfer hydrogenation (up to 53%) compared with Ru(benzene)Cl₂-HEWL composite dissolved in buffer solution (up to 12%) due to the stabilization of His-Ru coordination supported by hydrogen bonding networks in solvent channels. Ru(benzene)Cl₂ complex immobilized in CL-T-HEWL and CL-O-HEWL showed unique phenomena for enantioselectivity. The phenomena can be discussed by protein-substrate interactions in each solvent channel (Figure 12).

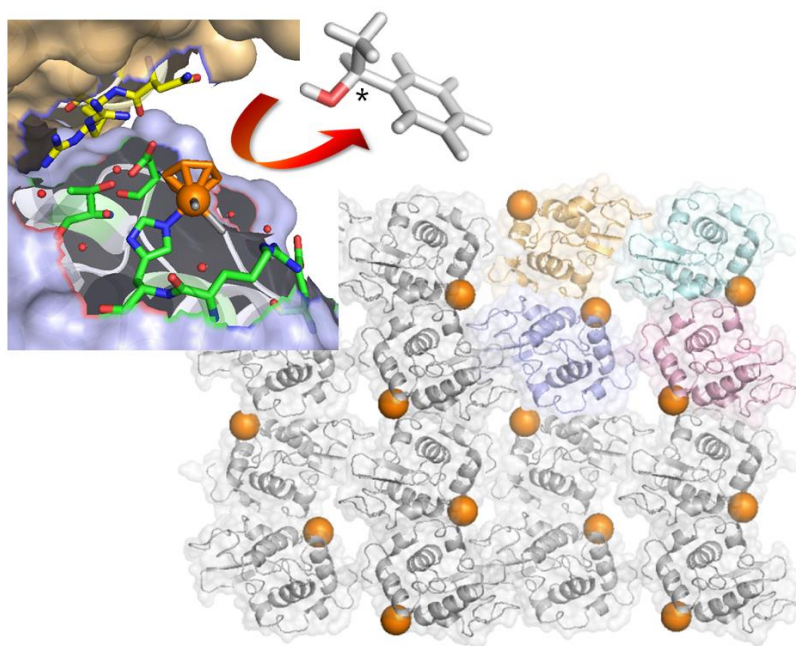


Figure 12. Lattice structure and local coordination structure of cross-linked HEWL crystals containing Ru(benzene)Cl₂ complex (lower right and upper left, respectively) and their catalytic formation of chiral alcohol.

In chapter 2, In chapter 2, the author explores the immobilization of ruthenium carbonyls in CL-HEWL (**Ru•CL-HEWL**). **Ru•CL-HEWL** retains a Ru carbonyl moiety that can release CO gas by ligand liberation although a composite of Ru carbonyl-HEWL dissolved in buffer solution (Ru•HEWL) does not release CO. This result suggested that $[\text{Ru}(\text{CO})_2\text{X}_4]^{2-}$, which is reactive intermediate for CO releasing, is immobilized only within solvent channels of **Ru•CL-HEWL**. **Ru•CL-HEWL** was utilized for an extracellular scaffold to activate the transcriptional factor NF- κ B in living cells. This result represents the utilization of active organometallic intermediates for bioapplication via the immobilization in porous protein crystals (Figure 13).

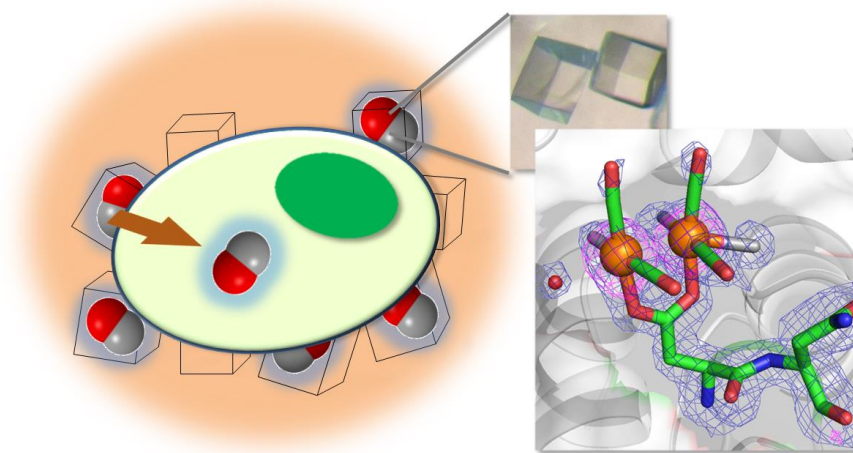


Figure 13. Schematic representation of CO releasing from cross-linked HEWL crystals in the vicinity of cells (left) and the coordination structure of ruthenium carbonyls in cross-linked HEWL crystals (right).

In chapter 3, the author investigated the *iv-vivo* design of protein crystals for promoting the immobilization of organometallic complexes. The molecular design of cypovirus polyhedra crystals (PhC) includes introduction of a hexahistidinetag to the C-terminus to form His-tagged PhC (**HTPhC**). The composite from **HTPhC** and $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ has *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ and $[\text{Ru}(\text{CO})_2\text{X}_3(\text{COO}^-)]^{2-}$ (X =anionic ions). HTPhC provides immobilization of about 2-fold more Ru carbonyls per protein monomer and effectively releases three times more CO for activation of nuclear factor kappa B (NF- κ B) in living cells relative to wild-type PhC with Ru carbonyls (Figure 14).

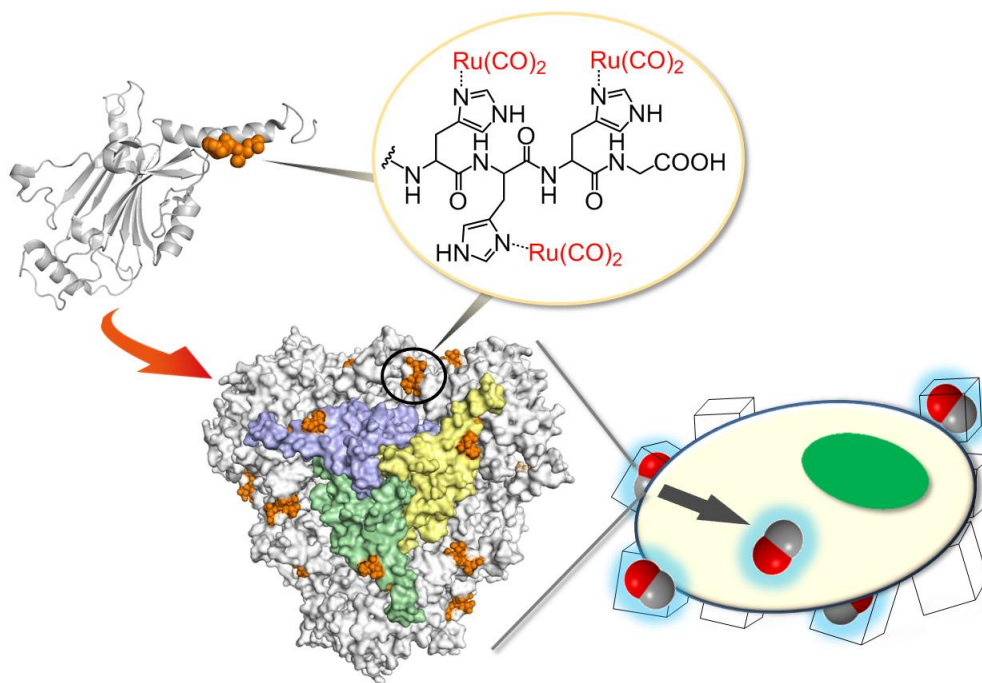


Figure 14. The addition of His-tag into C-terminal of PhM to form **HTPhC** and *cis*- $\text{Ru}(\text{CO})_2$ coordination and CO-releasing into living cells.

The aim of Chapter 4 is to prepare PhC with light-triggered CO releasing ability for quantitative research on bioactivity of CO gas. These crystals are obtained by introducing manganese carbonyl ($\text{Mn}(\text{CO})_5\text{Br}$) into His-tagged PhC (**Mn•HTPhC**). **Mn•HTPhC** possesses *fac*- $\text{Mn}(\text{CO})_3(\text{H}_2\text{O})_2(\text{His})$ moiety that can be a CO releasing site. **Mn•HTPhC** releases three times more CO compared in the presence of light irradiation for activation of nuclear factor kappa B (NF- κ B) in living cells compared to the assay without light irradiation. This strategy offers the possibility of quantitative analysis for bioactivity of CO gas depending on the timing and dosage using this material (Figure 15).

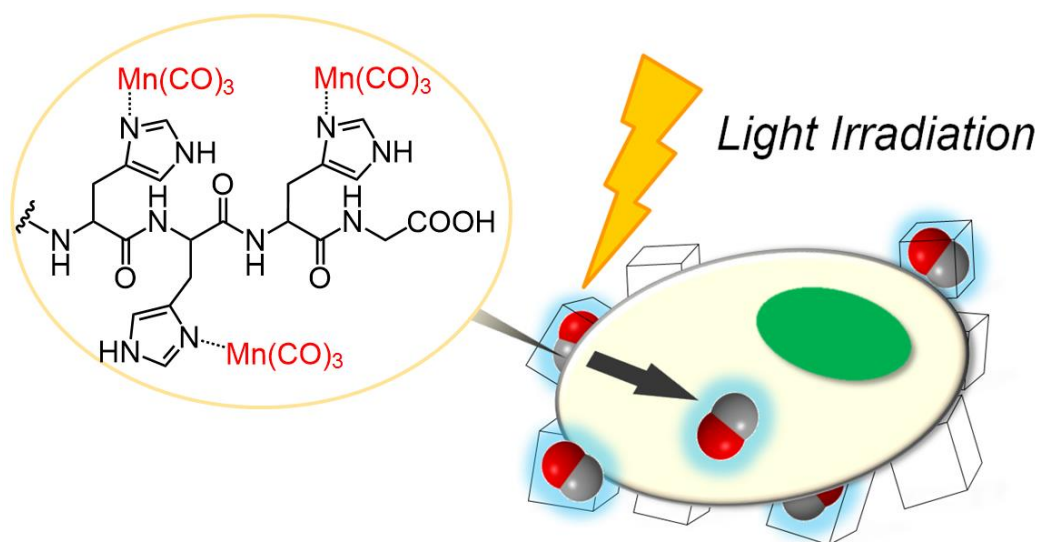


Figure 15. The formation of *fac*- $\text{Mn}(\text{CO})_3(\text{H}_2\text{O})_2(\text{His})$ moiety in **Mn•HTPhC** and light-triggered CO releasing in the vicinity of living cells.

Chapter 5 describes the crystal engineering to reconstruct solvent channels from *in-vivo* protein crystals of cypovirus polyhedra (PhC). Amino acid residues located on the intermolecular contact region are engineered by replacement and deletion of the amino acid residues to enlarge solvent channels with retaining the original crystal lattice structures. The PhM mutants allow the crystalline assemblies to increase solvent contents from 19% to 24%, and to adsorb 16-fold larger amounts of organic fluorescent dyes than wild-type PhC (WTPhC) by increasing the diffusion pathway of the exogenous molecules in solvent channels in the lattice structure due to decreasing non-covalent intra- and inter-molecular interactions. (Figure 16).

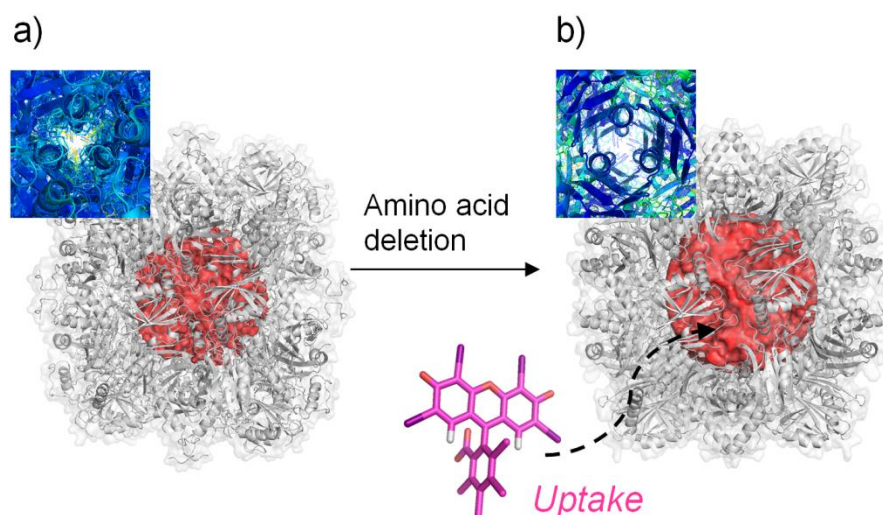


Figure 16. The unit cell structure (lower right) and the structure of 3-fold cavity (upper left) of WTPhC (a) and those of engineered PhC (b) obtained by X-ray crystal structure analysis. The area of solvent channels is shown as red spheres.

Reference

1. M. E. Wilson and G. M. Whitesides, *J. Am. Chem. Soc.*, 1978, **100**, 306-307.
2. C. Letondor, N. Humbert and T. R. Ward, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 4683-4687.
3. C. Letondor, A. Pordea, N. Humbert, A. Ivanova, S. Mazurek, M. Novic and T. R. Ward, *J. Am. Chem. Soc.*, 2006, **128**, 8320-8328.
4. M. Creus, A. Pordea, T. Rossel, A. Sardo, C. Letondor, A. Ivanova, I. Letrong, R. E. Stenkamp and T. R. Ward, *Angew. Chem. Int. Ed.*, 2008, **47**, 1400-1404.
5. T. R. Ward, *Acc. Chem. Res.*, 2011, **44**, 47-57.
6. R. R. Davies and M. D. Distefano, *J. Am. Chem. Soc.*, 1997, **119**, 11643-11652.
7. T. Hayashi and Y. Hisaeda, *Acc. Chem. Res.*, 2002, **35**, 35-43.
8. Y. Lu, N. Yeung, N. Sieracki and N. M. Marshall, *Nature*, 2009, **460**, 855-862.
9. T. Ueno, S. Abe, N. Yokoi and Y. Watanabe, *Coord. Chem. Rev.*, 2007, **251**, 2717-2731.
10. H. J. Yang, T. Matsui, S. Ozaki, S. Kato, T. Ueno, G. N. Phillips, Jr., S. Fukuzumi and Y. Watanabe, *Biochemistry*, 2003, **42**, 10174-10181.
11. T. Ueno, T. Koshiyama, S. Abe, N. Yokoi, M. Ohashi, H. Nakajima and Y. Watanabe, *J. Organomet. Chem.*, 2007, **692**, 142-147.
12. S. Abe, K. Hirata, T. Ueno, K. Morino, N. Shimizu, M. Yamamoto, M. Takata, E. Yashima and Y. Watanabe, *J. Am. Chem. Soc.*, 2009, **131**, 6958-6960.
13. J. Bos, F. Fusetti, A. J. Driessen and G. Roelfes, *Angew. Chem. Int. Ed.*, 2012, **51**, 7472-7475.
14. K. Oohora, S. Burazerovic, A. Onoda, Y. M. Wilson, T. R. Ward and T. Hayashi, *Angew. Chem. Int. Ed.*, 2012, **51**, 3818-3821.
15. H. Kitagishi, K. Oohora, H. Yamaguchi, H. Sato, T. Matsuo, A. Harada and T. Hayashi, *J. Am. Chem. Soc.*, 2007, **129**, 10326-+.
16. H. Kitagishi, Y. Kakikura, H. Yamaguchi, K. Oohora, A. Harada and T. Hayashi, *Angew. Chem. Int. Ed.*, 2009, **48**, 1271-1274.
17. K. Oohora and T. Hayashi, *Curr. Opin. Chem. Biol.*, 2014, **19**, 154-161.
18. K. Oohora, A. Onoda and T. Hayashi, *Chem. Commun.*, 2012, **48**, 11714-11726.
19. S. Hirota, Y. Hattori, S. Nagao, M. Taketa, H. Komori, H. Kamikubo, Z. H. Wang, I. Takahashi, S. Negi, Y. Sugiura, M. Kataoka and Y. Higuchi, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 12854-12859.

20. A. Onoda, Y. Kakikura, T. Uematsu, S. Kuwabata and T. Hayashi, *Angew. Chem. Int. Ed.*, 2012, **51**, 2628-2631.
21. K. Oohora, A. Onoda, H. Kitagishi, H. Yamaguchi, A. Harada and T. Hayashi, *Chem. Sci.*, 2011, **2**, 1033-1038.
22. N. Dotan, D. Arad, F. Frolow and A. Freeman, *Angew. Chem. Int. Ed.*, 1999, **38**, 2363-2366.
23. P. Ringler and G. E. Schulz, *Science*, 2003, **302**, 106-109.
24. M. Uchida, M. T. Klem, M. Allen, P. Suci, M. Flenniken, E. Gillitzer, Z. Varpness, L. O. Liepold, M. Young and T. Douglas, *Adv. Mater.*, 2007, **19**, 1025-1042.
25. E. H. C. Bromley, K. Channon, E. Moutevelis and D. N. Woolfson, *ACS Chem. Biol.*, 2008, **3**, 38-50.
26. I. C. Reinhout, J. J. L. M. Cornelissen and R. J. M. Nolte, *Acc. Chem. Res.*, 2009, **42**, 681-692.
27. P. A. Suci, S. Kang, M. Young and T. Douglas, *J. Am. Chem. Soc.*, 2009, **131**, 9164-9165.
28. M. A. Kostiainen, O. Kasyutich, J. J. L. M. Cornelissen and R. J. M. Nolte, *Nat. Chem.*, 2010, **2**, 394-399.
29. E. N. Salgado, R. J. Radford and F. A. Tezcan, *Acc. Chem. Res.*, 2010, **43**, 661-672.
30. T. O. Yeates, C. S. Crowley and S. Tanaka, *Annu. Rev. Biophys.*, 2010, **39**, 185-205.
31. M. Fukuto, Q. L. Nguyen, O. Vasilyev, N. Mank, C. L. Washington-Hughes, I. Kuzmenko, A. Checco, Y. M. Mao, Q. Wang and L. Yang, *Soft Matter*, 2013, **9**, 9633-9642.
32. J. Mikkila, H. Rosilo, S. Nummelin, J. Seitsonen, J. Ruokolainen and M. A. Kostiainen, *ACS Macro Lett.*, 2013, **2**, 720-724.
33. N. J. M. Sanghamitra and T. Ueno, *Chem. Commun.*, 2013, **49**, 4114-4126.
34. G. Bellapadrona and M. Elbaum, *Angew. Chem. Int. Ed.*, 2014, **53**, 1534-1537.
35. V. Liljestrom, J. Mikkila and M. A. Kostiainen, *Nat. Commun.*, 2014, **5**, 4445.
36. S. K. Saka, A. Honigmann, C. Eggeling, S. W. Hell, T. Lang and S. O. Rizzoli, *Nat. Commun.*, 2014, **5**, 4509.
37. F. Sakai, G. Yang, M. S. Weiss, Y. J. Liu, G. S. Chen and M. Jiang, *Nat. Commun.*, 2014, **5**, 4634.

38. E. M. Quistgaard, *Chem. Commun.*, 2014, **50**, 14995-14997.
39. N. Kamiya and J. R. Shen, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 98-103.
40. Y. Umena, K. Kawakami, J. R. Shen and N. Kamiya, *Nature*, 2011, **473**, 55-65.
41. X. F. Liu and E. C. Theil, *Acc. Chem. Res.*, 2005, **38**, 167-175.
42. R. K. Watt, O. D. Petrucci and T. Smith, *Catal Sci Technol*, 2013, **3**, 3103-3110.
43. J. C. Kendrew, G. Bodo, H. M. Dintzis, R. G. Parrish, H. Wyckoff and D. C. Phillips, *Nature*, 1958, **181**, 662-666.
44. H. Muirhead and M. F. Perutz, *Nature*, 1963, **199**, 633-638.
45. C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma, *Nature*, 1965, **206**, 757-761.
46. A. L. Margolin and M. A. Navia, *Angew. Chem. Int. Ed.*, 2001, **40**, 2204-2222.
47. J. J. Roy and T. E. Abraham, *Chem. Rev.*, 2004, **104**, 3705-3721.
48. T. Ueno, *Chem. Eur. J.*, 2013, **19**, 9096-9102.
49. B. W. Matthews, *J. Mol. Biol.*, 1968, **33**, 491-497.
50. J. Janin and F. Rodier, *Proteins*, 1995, **23**, 580-587.
51. K. M. Andersson and S. Hovmoller, *Acta Crystallogr. D*, 2000, **56**, 789-790.
52. H. Fischer, I. Polikarpov and A. F. Craievich, *Protein Sci.*, 2004, **13**, 2825-2828.
53. J. P. K. Doye and W. C. K. Poon, *Curr. Opin. Colloid Interdace Sci.*, 2006, **11**, 40-46.
54. G. Dodson and D. Steiner, *Curr. Opin. Struct. Biol.*, 1998, **8**, 189-194.
55. M. L. Brader, M. Sukumar, A. H. Pekar, D. S. McClellan, R. E. Chance, D. B. Flora, A. L. Cox, L. Irwin and S. R. Myers, *Nat. Biotechnol.*, 2002, **20**, 800-804.
56. I. J. Vanderklei, G. J. Sulter, W. Harder and M. Veenhuis, *Yeast*, 1991, **7**, 15-24.
57. J. Vonck and E. F. J. Vanbruggen, *J. Bacteriol.*, 1992, **174**, 5391-5399.
58. U. B. Sleytr and M. Sara, *Trends Biotechnol.*, 1997, **15**, 20-26.
59. U. B. Sleytr, C. Huber, N. Ilk, D. Pum, B. Schuster and E. M. Egelseer, *FEMS Microbiol. Lett.*, 2007, **267**, 131-144.
60. F. Coulibaly, E. Chiu, K. Ikeda, S. Gutmann, P. W. Haebel, C. Schulze-Bries, H. Mori and P. Metcalf, *Nature*, 2007, **446**, 97-101.
61. F. Coulibaly, E. Chiu, S. Gutmann, C. Rajendran, P. W. Haebel, K. Ikeda, H. Mori, V. K. Ward, C. Schulze-Bries and P. Metcalf, *Proc. Natl. Acad. Sci. USA*, 2009, **106**, 22205-22210.
62. R. Koopmann, K. Cupelli, L. Redecke, K. Nass, D. P. DePonte, T. A. White, F. Stellato, D. Rehders, M. N. Liang, J. Andreasson, A. Aquila, S. Bajt, M.

- Barthelmess, A. Barty, M. J. Bogan, C. Bostedt, S. Boutet, J. D. Bozek, C. Caleman, N. Coppola, J. Davidsson, R. B. Doak, T. Ekeberg, S. W. Epp, B. Erk, H. Fleckenstein, L. Foucar, H. Graafsma, L. Gumprecht, J. Hajdu, C. Y. Hampton, A. Hartmann, R. Hartmann, G. Hauser, H. Hirsemann, P. Holl, M. S. Hunter, S. Kassemeyer, R. A. Kirian, L. Lomb, F. R. N. C. Maia, N. Kimmel, A. V. Martin, M. Messerschmidt, C. Reich, D. Rolles, B. Rudek, A. Rudenko, I. Schlichting, J. Schulz, M. M. Seibert, R. L. Shoeman, R. G. Sierra, H. Soltau, S. Stern, L. Struder, N. Timneanu, J. Ullrich, X. Y. Wang, G. Weidenspointner, U. Weierstall, G. J. Williams, C. B. Wunderer, P. Fromme, J. C. H. Spence, T. Stehle, H. N. Chapman, C. Betzel and M. Duszynko, *Nat. Methods*, 2012, **9**, 259-U254.
63. L. Redecke, K. Nass, D. P. DePonte, T. A. White, D. Rehders, A. Barty, F. Stellato, M. N. Liang, T. R. M. Barends, S. Boutet, G. J. Williams, M. Messerschmidt, M. M. Seibert, A. Aquila, D. Arnlund, S. Bajt, T. Barth, M. J. Bogan, C. Caleman, T. C. Chao, R. B. Doak, H. Fleckenstein, M. Frank, R. Fromme, L. Galli, I. Grotjohann, M. S. Hunter, L. C. Johansson, S. Kassemeyer, G. Katona, R. A. Kirian, R. Koopmann, C. Kupitz, L. Lomb, A. V. Martin, S. Mogk, R. Neutze, R. L. Shoeman, J. Steinbrener, N. Timneanu, D. J. Wang, U. Weierstall, N. A. Zatsepin, J. C. H. Spence, P. Fromme, I. Schlichting, M. Duszynko, C. Betzel and H. N. Chapman, *Science*, 2013, **339**, 227-230.
 64. G. Jedd and N. H. Chua, *Nat. Cell. Biol.*, 2000, **2**, 226-231.
 65. K. Muntz, *Plant Mol. Biol.*, 1998, **38**, 77-99.
 66. E. Stoger, M. Parker, P. Christou and R. Casey, *Plant Physiol.*, 2001, **125**, 1732-1742.
 67. D. M. Lawson, P. J. Artymiuk, S. J. Yewdall, J. M. A. Smith, J. C. Livingstone, A. Treffry, A. Luzzago, S. Levi, P. Arosio, G. Cesareni, C. D. Thomas, W. V. Shaw and P. M. Harrison, *Nature*, 1991, **349**, 541-544.
 68. H. Yoshimura, T. Scheybani, W. Baumeister and K. Nagayama, *Langmuir*, 1994, **10**, 3290-3295.
 69. R. J. Radford, M. Lawrenz, P. C. Nguyen, J. A. McCammon and F. A. Tezcan, *Chem. Commun.*, 2011, **47**, 313-315.
 70. J. D. Brodin, X. I. Ambroggio, C. Y. Tang, K. N. Parent, T. S. Baker and F. A. Tezcan, *Nat. Chem.*, 2012, **4**, 375-382.

71. J. D. Brodin, J. R. Carr, P. A. Sontz and F. A. Tezcan, *Proc. Natl. Acad. Sci. USA*, 2014, **111**, 2897-2902.
72. T. W. Ni and F. A. Tezcan, *Angew. Chem. Int. Ed.*, 2010, **49**, 7014-7018.
73. N. L. Stclair and M. A. Navia, *J. Am. Chem. Soc.*, 1992, **114**, 7314-7316.
74. M. A. Navia, N. L. Stclair and J. P. Griffith, *Stud. Org. Chem.*, 1993, **47**, 63-73.
75. T. Ke, C. R. Wescott and A. M. Klibanov, *J. Am. Chem. Soc.*, 1996, **118**, 3366-3374.
76. K. Xu and A. M. Klibanov, *J. Am. Chem. Soc.*, 1996, **118**, 9815-9819.
77. J. J. Lalonde, C. Govardhan, N. Khalaf, A. G. Martinez, K. Visuri and A. L. Margolin, *J. Am. Chem. Soc.*, 1995, **117**, 6845-6852.
78. R. A. Persichetti, N. L. Stclair, J. P. Griffith, M. A. Navia and A. L. Margolin, *J. Am. Chem. Soc.*, 1995, **117**, 2732-2737.
79. N. Khalaf, C. P. Govardhan, J. L. Lalonde, R. A. Persichetti, Y. F. Wang and A. L. Margolin, *J. Am. Chem. Soc.*, 1996, **118**, 5494-5495.
80. T. Zelinski and H. Waldmann, *Angew. Chem. Int. Ed.*, 1997, **36**, 722-724.
81. L. Z. Vilenchik, J. P. Griffith, N. St Clair, M. A. Navia and A. L. Margolin, *J. Am. Chem. Soc.*, 1998, **120**, 4290-4294.
82. E. Winfree, F. R. Liu, L. A. Wenzler and N. C. Seeman, *Nature*, 1998, **394**, 539-544.
83. D. Haring and P. Schreier, *Curr. Opin. Chem. Biol.*, 1999, **3**, 35-38.
84. N. St Clair, Y. F. Wang and A. L. Margolin, *Angew. Chem. Int. Ed.*, 2000, **39**, 388-391.
85. J. J. Roy, T. E. Abraham, K. S. Abhijith, P. V. S. Kumar and M. S. Thakur, *Biosens. Bioelectron.*, 2005, **21**, 206-211.
86. A. Cvetkovic, C. Picoreanu, A. J. J. Straathof, R. Krishna and L. A. M. van der Wielen, *J. Phys. Chem. B*, 2005, **109**, 10561-10566.
87. A. Cvetkovic, C. Picoreanu, A. J. J. Straathof, R. Krishna and L. A. M. van der Wielent, *J. Am. Chem. Soc.*, 2005, **127**, 875-879.
88. A. Cvetkovic, A. J. J. Straathof, R. Krishna and L. A. M. van der Wielen, *Langmuir*, 2005, **21**, 1475-1480.
89. Z. Q. Hu and J. W. Jiang, *Biophys. J.*, 2008, **95**, 4148-4156.
90. Z. Q. Hu and J. W. Jiang, *Langmuir*, 2008, **24**, 4215-4223.
91. Z. Hu and J. Jiang, *J. Phys. Chem. B*, 2009, **113**, 15851-15857.

92. J. J. Shou, F. Wang, G. A. Zeng and Y. H. Zhang, *J. Phys. Chem. B*, 2011, **115**, 3708-3712.
93. T. Ueno and Y. Watanabe, *Coordination Chemistry in Protein Cages: Principles, Design, and Applications*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2013.
94. I. Schlichting, J. Berendzen, K. Chu, A. M. Stock, S. A. Maves, D. E. Benson, B. M. Sweet, D. Ringe, G. A. Petsko and S. G. Sligar, *Science*, 2000, **287**, 1615-1622.
95. A. Karlsson, J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund and S. Ramaswamy, *Science*, 2003, **299**, 1039-1042.
96. E. G. Kovaleva and J. D. Lipscomb, *Science*, 2007, **316**, 453-457.
97. C. Cavazza, C. Bochot, P. Rousselot-Pailley, P. Carpentier, M. V. Cherrier, L. Martin, C. Marchi-Delapierre, J. C. Fontecilla-Camps and S. Menage, *Nat. Chem.*, 2010, **2**, 1069-1076.
98. B. R. Crane, A. J. Di Bilio, J. R. Winkler and H. B. Gray, *J. Am. Chem. Soc.*, 2001, **123**, 11623-11631.
99. F. A. Tezcan, B. R. Crane, J. R. Winkler and H. B. Gray, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 5002-5006.
100. S. A. Kang, P. J. Marjawaara and B. R. Crane, *J. Am. Chem. Soc.*, 2004, **126**, 10836-10837.
101. S. A. Kang and B. R. Crane, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 15465-15470.
102. J. C. Falkner, M. E. Turner, J. K. Bosworth, T. J. Trentler, J. E. Johnson, T. W. Lin and V. L. Colvin, *J. Am. Chem. Soc.*, 2005, **127**, 5274-5275.
103. M. Guli, E. M. Lambert, M. Li and S. Mann, *Angew. Chem. Int. Ed.*, 2010, **49**, 520-523.
104. Y. Takeda, T. Kondow and F. Mafune, *Chem. Phys. Lett.*, 2011, **504**, 175-179.
105. H. Wei, Z. D. Wang, J. Zhang, S. House, Y. G. Gao, L. M. Yang, H. Robinson, L. H. Tan, H. Xing, C. J. Hou, I. M. Robertson, J. M. Zuo and Y. Lu, *Nat. Nanotechnol.*, 2011, **6**, 92-96.
106. H. Wei and Y. Lu, *Chem. Asian J.*, 2012, **7**, 680-683.
107. S. Abe, M. Tsujimoto, K. Yoneda, M. Ohba, T. Hikage, M. Takano, S. Kitagawa and T. Ueno, *Small*, 2012, **8**, 1314-1319.

108. M. Liang, L. B. Wang, R. X. Su, W. Qi, M. F. Wang, Y. J. Yu and Z. M. He, *Catal. Sci. Technol.*, 2013, **3**, 1910-1914.
109. O. L. Muskens, M. W. England, L. Danos, M. Li and S. Mann, *Adv. Funct. Mater.*, 2013, **23**, 281-290.
110. H. Wei, S. House, J. J. X. Wu, J. Zhang, Z. D. Wang, Y. He, E. J. Gao, Y. G. Gao, H. Robinson, W. Li, J. M. Zuo, I. M. Robertson and Y. Lu, *Nano Res.*, 2013, **6**, 627-634.
111. Y. Ding, L. Shi and H. Wei, *J. Mater. Chem. B*, 2014, **2**, 8268-8291.
112. T. Ueno, S. Abe, T. Koshiyama, T. Ohki, T. Hikage and Y. Watanabe, *Chem. Eur. J.*, 2010, **16**, 2730-2740.
113. M. J. Panzner, S. M. Bilinovich, W. J. Youngs and T. C. Leeper, *Chem. Commun.*, 2011, **47**, 12479-12481.
114. S. J. Li, *Biopolymers*, 2006, **81**, 74-80.
115. I. W. McNae, K. Fishburne, A. Habtemariam, T. M. Hunter, M. Melchart, F. Wang, M. D. Walkinshaw and P. J. Sadler, *Chem. Commun.*, 2004, 1786-1787.
116. A. Casini, G. Mastrobuoni, C. Temperini, C. Gabbiani, S. Francese, G. Moneti, C. T. Supuran, A. Scozzafava and L. Messori, *Chem. Commun.*, 2007, 156-158.
117. M. Razavet, V. Artero, C. Cavazza, Y. Oudart, C. Lebrun, J. C. Fontecilla-Camps and M. Fontecave, *Chem. Commun.*, 2007, 2805-2807.
118. S. L. Binkley, T. C. Leeper, R. S. Rowlett, R. S. Herrick and C. J. Ziegler, *Metallomics*, 2011, **3**, 909-916.
119. T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romao and M. J. Romao, *J. Am. Chem. Soc.*, 2011, **133**, 1192-1195.
120. G. Santoro, O. Blacque and F. Zobi, *Metallomics*, 2012, **4**, 253-259.
121. M. F. A. Santos, J. D. Seixas, A. C. Coelho, A. Mukhopadhyay, P. M. Reis, M. J. Romao, C. C. Romao and T. Santos-Silva, *J. Inorg. Biochem.*, 2012, **117**, 285-291.
122. F. Zobi and B. Spingler, *Inorg. Chem.*, 2012, **51**, 1210-1212.
123. T. Koshiyama, M. Shirai, T. Hikage, H. Tabe, K. Tanaka, S. Kitagawa and T. Ueno, *Angew. Chem. Int. Ed.*, 2011, **50**, 4849-4852.
124. T. Koshiyama, N. Kawaba, T. Hikage, M. Shirai, Y. Miura, C. Y. Huang, K. Tanaka, Y. Watanabe and T. Ueno, *Bioconjugate Chem.*, 2010, **21**, 264-269.

125. S. Abe, Y. Tokura, R. Pal, N. Komura, Imamura A., K. Matsumoto, H. Ijiri, N. J. M. Sanghamitra, H. Tabe, H. Ando, M. Kiso, H. Mori, S. Kitagawa and T. Ueno, *Chem. Lett.*, 2015, **44**, 29-31.

Chapter 1

Porous Protein Crystals as Catalytic Vessels for Organometallic Complexes

Abstract

A solid artificial metalloenzyme based on HEWL crystals has been designed. Nanovessels constructed by two HEWL crystal forms of tetragonal and orthorhombic systems provide different catalytic environments. The crystals accelerate the catalytic reaction and different enantiomers are obtained based on the crystal structure of each form, which is revealed by X-ray crystallography.

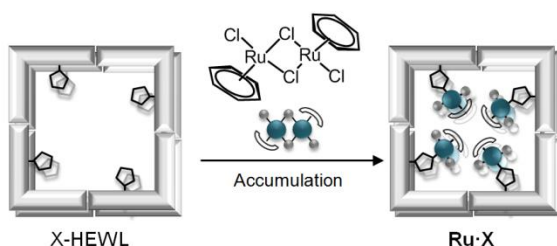
Introduction

The design of artificial metalloenzymes has been the focus of much attention in fundamental research and development of applications in biotechnology.^{1,2} Conjugation of proteins to metal compounds is one of the most convenient methods used in the expansion of function of known proteins.³⁻¹⁵ Since Whitesides et al. reported that a Rh^I-biotin/avidin complex can catalyze asymmetric hydrogenation reactions,³ several artificial metalloenzymes have been developed with non-covalent or covalent conjugation between proteins and synthetic metal cofactors.⁴⁻⁸ Recent progress in structural biology and computational approaches has enabled artificial metalloenzymes to be designed more precisely.⁹⁻¹⁵ These reconstruction methods have been expanded to protein assemblies^{11, 12, 16}. Thus, it is expected that protein crystals, which are protein assemblies in solid state, can also accommodate synthetic organometallics and serve as catalysts because they can provide robust and unique nano environment for catalytic reactions. This is because typical metalloproteins tend to be unstable and can only be used under limited conditions without recyclability.

Protein crystals represent precise protein assemblies in the solid state and are thus appropriate candidates to develop robust catalytic nanovessels.¹⁷⁻³⁵ Each protein crystal is a stable protein assembly with inner pores called solvent channels. The sizes of the solvent channels vary according to the lattice of each individual crystal. Given channels occupy 30% to 65% of the total crystal volume, and provide a great surface area (800 ~ 2000 m²/g) with a wide range of pore sizes (10 ~ 100 Å).^{18, 19} The physical properties of the solvent channels provide unique chemical environments as revealed by theoretical methods.^{20, 21} As they are stable and can be easily prepared, the channels of crystallized natural enzymes have been used as enzyme reactors and separation columns.^{19, 22, 23}

Post-engineering of porous protein crystals has also been carried out to convert solvent channels to catalytic nanovessels for inorganic compounds or metal complexes using the functional groups of amino acid side chains, which are exposed with regularity on the interior surfaces of the channels.²⁷⁻³³ Metal ions, which were accumulated within the solvent channels, were converted to inorganic materials in protein crystals. A deposition of catalytic materials in protein crystals is reported by Lu and his co-workers.^{28, 29} Gold nanoparticles synthesized in the lysozyme crystals by disproportionation of accumulated Au^I can catalyze the reduction of *p*-nitrophenol with NaBH₄. With respect to reactivity of metal complex•protein crystal composites, hydroxylation and electron transfer with

a)



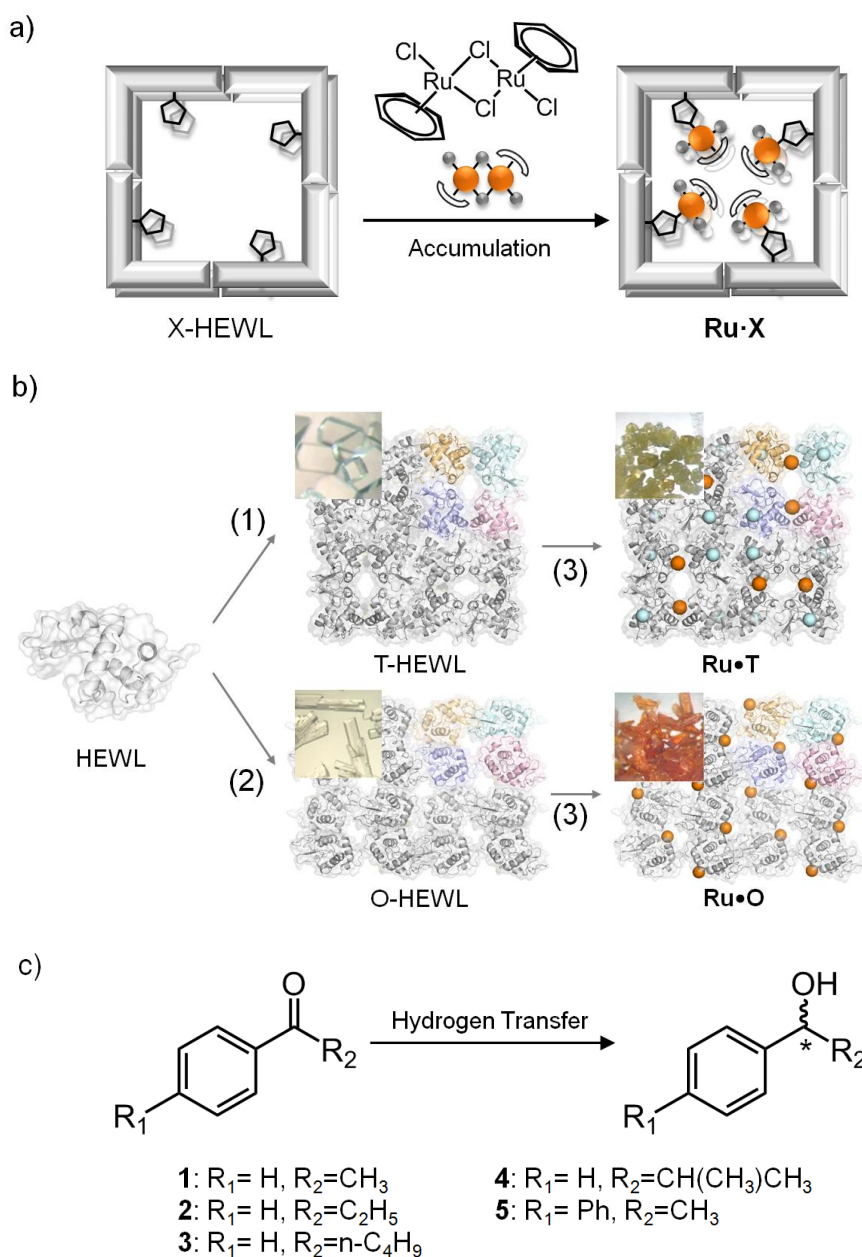
single turnover have also been achieved by metal complexes immobilized in solvent channels of NikA and myoglobin crystals, respectively.^{32, 36} Although Sadler

and co-workers immobilized metal complexes in protein crystals, the catalytic reactions have not been reported.³⁷

Here the author reports on catalytic reactions promoted by organometallic complexes immobilized in porous protein crystals.¹⁹ Cross-linked hen egg white lysozyme crystals (CL-HEWLs) have been engineered to immobilize organoruthenium complexes so that the crystal composites can catalyze enantioselective reduction of acetophenone and its derivatives (Scheme 1a). The following results were obtained:

- (1) Stable coordination of $\text{Ru}(\text{benzene})\text{Cl}_2$ complex to His15 in CL-HEWL crystals provides higher catalytic activity than a $\text{Ru}(\text{benzene})\text{Cl}_2$ -HEWL composite dissolved in a buffer solution.
- (2) A different configuration of enantiomers was obtained as a result of the structure of active sites by altering the lattice structure of HEWL crystal systems to tetragonal and orthorhombic forms (Scheme 1b).

These results demonstrate that porous protein crystals have the potential to serve as suitable catalytic vessels immobilizing organometallic complexes.



1. (a) Schematic representation of the preparation of Ru(benzene)Cl₂•His coordination in a solvent channel of a porous HEWL crystal. (b) Preparation of HEWL crystals in (1) tetragonal form (T-HEWL, PDB code 193L)³⁸, (2) orthorhombic form (O-HEWL, PDB code 1BGI)³⁹, and (3) cross-linking treatment followed by accumulation of [Ru(benzene)Cl₂]₂. (PDB ID : 3W6A and 4J7V, respectively) (c) The reaction scheme of the hydrogen-transfer reduction of acetophenone derivatives.

Results and Discussion

Preparation and X-ray crystal structure analysis.

Crystals of HEWL, which can be routinely and abundantly crystallized to obtain yields of several hundred milligrams, are exploited as templates in construction of catalytic nanovessels. The crystals have the capacity to immobilize organometallic complexes by coordination bonds between His or Asp residues on the surfaces of the solvent channels.^{11, 12, 16} Tetragonal HEWL crystals (T-HEWL) were obtained in batch crystallization method.³⁸ The crystals were soaked into 100 mM acetate solution containing 2.5% glutaraldehyde at room temperature for 48 hours to stabilize crystals by cross-linking (CL-T-HEWLs). CL-T-HEWLs (150 mg) were soaked in 1 mL of 100 mM acetate buffer (pH 7.0) containing 5 mM [Ru(benzene)Cl₂]₂ at room temperature for 24 hours to immobilize Ru complexes in the CL-T-HEWL crystals. Then Ru(benzene)Cl₂•CL-T-HEWL (**Ru•T**) crystals were obtained as dark yellow crystals. Quantitative elemental analysis of **Ru•T** by inductively coupled plasma-optical emission spectrometry (ICP-OES) showed that the number of Ru per HEWL monomer of **Ru•T** was 0.73. The diffraction data of **Ru•T** indicated that the crystal lattice and the solvent channels were maintained after all procedures (Figure 1). Ru(benzene)Cl₂•CL-O-HEWL (**Ru•O**) was obtained as orange crystals by the same procedures.³⁹ The ratio of Ru HEWL monomer, determined by ICP-OES, was 0.60 for **Ru•O**. The X-ray diffraction patterns of the **Ru•O** showed that the original lattice structures were retained. The color change of each crystal was caused by cross-linking treatment before accumulation of Ru(benzene) complexes (Figure 2). It is expected that the color of CL-X-HEWLs is influenced by the number and the location of C=N bonds formed by the coupling of lysine/glutaraldehyde in each crystal lattice.⁴⁰

The coordination structures of the Ru complexes in the solvent channels were determined by X-ray crystal structure analysis at resolutions of 1.77 Å and 1.54 Å for **Ru•T** (PDB ID : 3W6A) and **Ru•O** (PDB ID : 4J7V), respectively (Figure 1 and Table 1). The binding positions of the Ru atoms were distinguished by anomalous difference maps. **Ru•T** has two Ru binding sites, designated Ru_{aT}, and Ru_{bT}, per HEWL monomer with occupancies of 0.6 and 0.3, respectively (Figure 3a). The Ru complexes are located only at these sites found by the crystal structure analysis because the sum of the occupancies corresponds well to the quantitative analysis of the Ru/HEWL ratio estimated by ICP-OES. The Ru (benzene)Cl₂•His15_{T1} moiety is located within the

largest of the solvent channels of the crystal (Figure 3b). The electron density of Ru_{aT1} was observed around His15 with an electron density expected to be a benzene ligand, and two chloride ions (Figure 3c). The observed bond length of Ru_{aT1}-NεHis15_{T1} is 2.5 Å which is within the typical range of Ru-N bonds.^{37, 41} The angle of the benzene plane-Ru_{aT1}-NεHis15_{T1} is 127°. The angle appears to be restricted by participation of the Cl_{aT1} atom in a hydrogen bonding network with a water molecule (Wat_{1T}) and carboxylate of Asp7 (OAsp7_{T2}) of the neighboring HEWL monomer (Figure 3c). Ru(benzene)Cl₂•His15 moiety is stabilized by participating in hydrogen-bonding networks arranged in the solvent channels of **Ru•T** as reported for a Ru₃O cluster in the hexagonal channel of myoglobin crystals (Figure 3c).³² These coordination parameters are essentially identical to those of Ru(*p*-cymene) fixed in a tetragonal-HEWL crystal as reported previously. The Ru_{bT} atom, which is fixed within a narrow channel (Figure 3b), is bound to OδAsp101_{T1} at a distance of 2.2 Å, and does not have non-covalent interactions with neighboring residues. This implies that the lower occupancy of Ru_{bT} relative to Ru_{aT} is due to Ru-OδAsp101 having less stable formation of coordination bondings. Lys13 forms an intermolecular cross-linked bond with Lys13 of the neighboring HEWL through glutaraldehyde conjugation as reported previously⁴⁰. However, the cross-linking is expected not to influence the coordination structure of the Ru center bound to His15 because the side chain of Lys13 is 11 Å away from His15.

In **Ru•O**, electron density of Ru_O was found only in the vicinity of His15_O with ideal occupancy (0.7) (Figure 3d). The Ru(benzene)Cl₂•His15_{O1} moiety is located within the largest solvent channel of **Ru•O** (Figure 3e). The Ru atom is bound to NεHis15_{O1} at a distance of 2.2 Å and accompanied with a benzene group and two chloride atoms (Figure 3f). The angle of the benzene plane-Ru-NεHis15_{O1} (113°) is restrained by intramolecular hydrogen bonding between Cl_{O1} and Arg14_{O1} (Figure 3f).

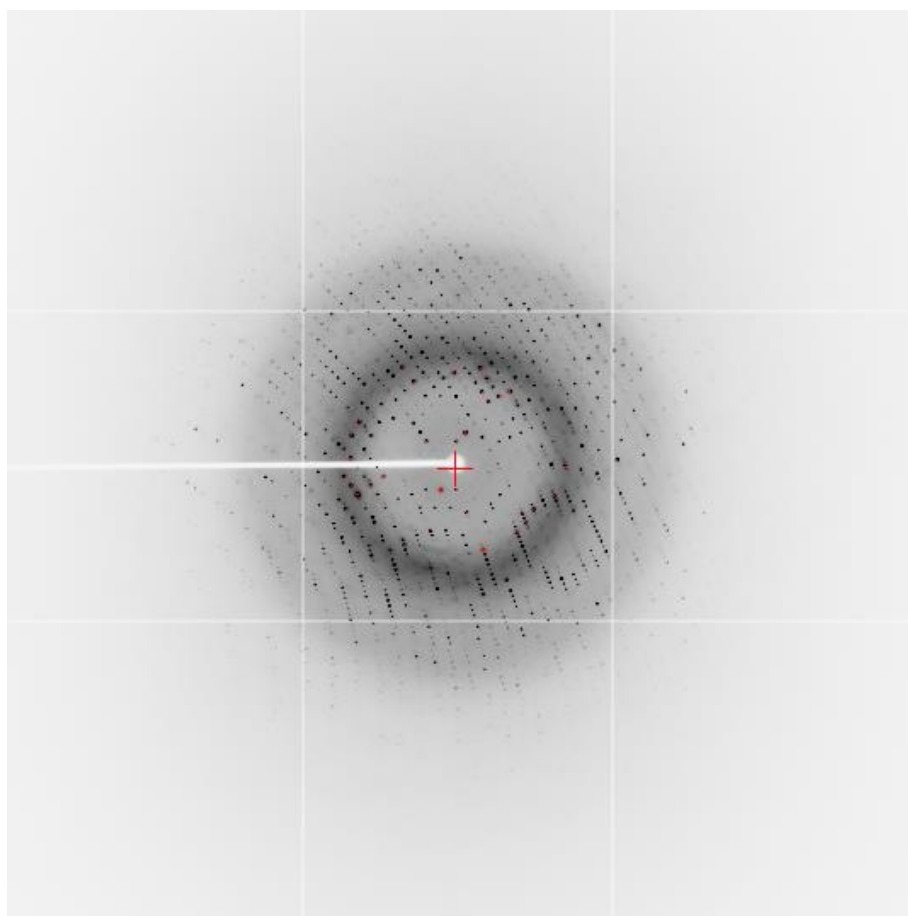


Figure 1. X-ray diffractograms of **Ru•T**.

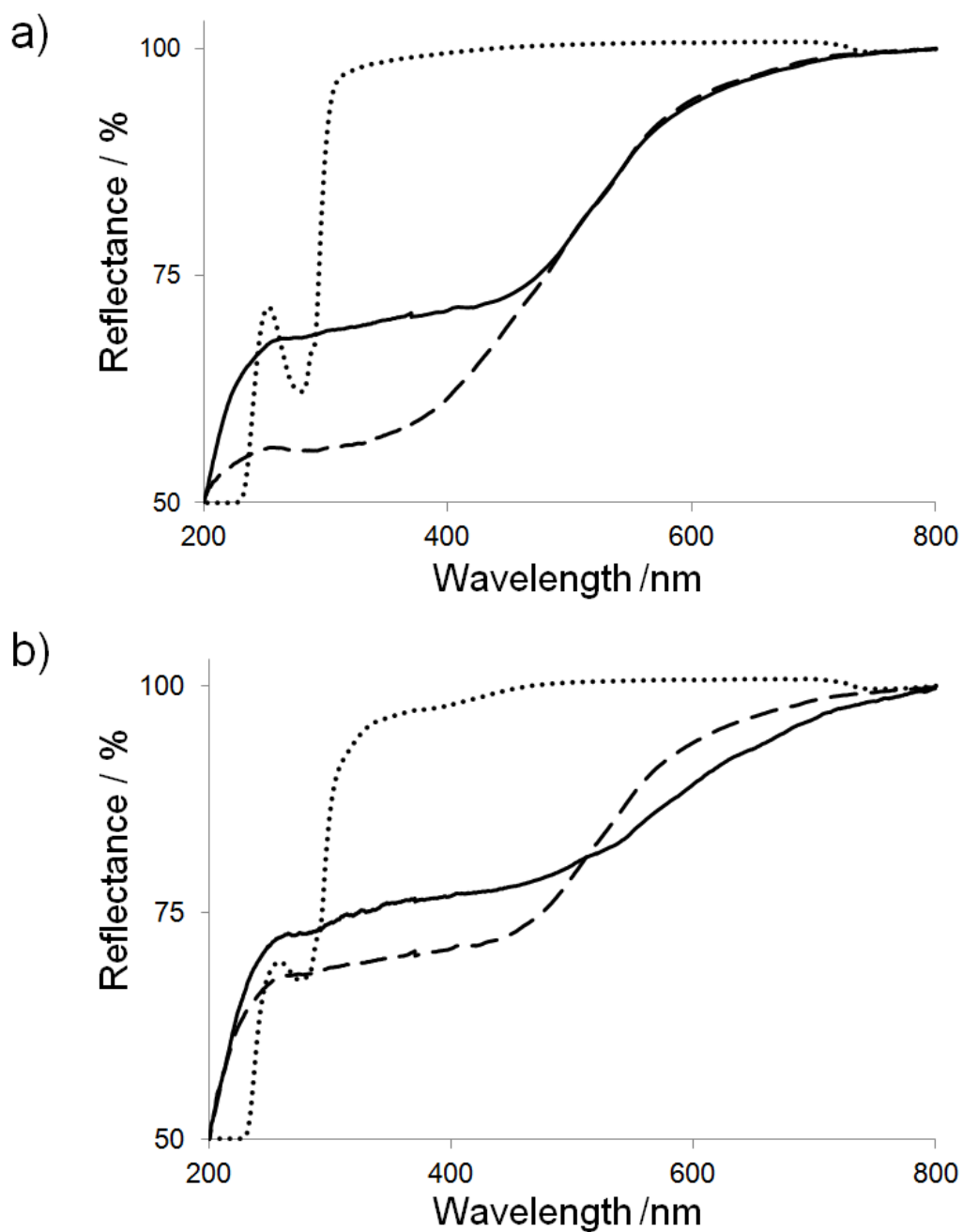


Figure 2. The UV/Vis spectra of composites. (a) CL-T-HEWL (straight line), CL-O-HEWL (dashed line) and HEWL monomer in buffer solution (dotted line). (b) **Ru•T** (straight line), **Ru•O** (dashed line) and HEWL monomer in buffer solution (dotted line).

Table 1. Crystallographic data and refinement statistics of **Ru·T** and **Ru·O**.

PDB ID	Ru·T 3W6A	Ru·O 4J7V
Data Collection Statistics		
X-ray wavelength (Å)	1.0000	1.0000
Space group	P4 ₃ 2 ₁ 2	P2 ₁ 2 ₁ 2 ₁
Crystal cell, (Å)		
a	79.046	30.194
b	79.046	55.572
c	36.358	72.418
α , β , γ	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution Range	30.00-1.77 (1.82-1.77)	40.00-1.54 (1.58-1.54)
Completeness (%)	97.06 (99.34)	97.63 (98.03)
R _{merge} (%)	4.7 (30)	5.2 (73)
I/ σ	58.8 (76.9)	58.8 (66.7)
Refinement statistics		
Resolution range (Å)	27.95-1.77	25.94-1.54
Reflection used	10839	17315
R-factor (%)	20.2	19.5
R _{free} (%)	27.2	23.5
Rms diviation from ideality		
Bonds (Å)	0.025	0.031
Angle (°)	2.338	3.497
Ramachandan plot (%)		
Most favored	99.2	98.4
allowed	0.8	1.6

Values in parentheses are for the highest resolution shell.

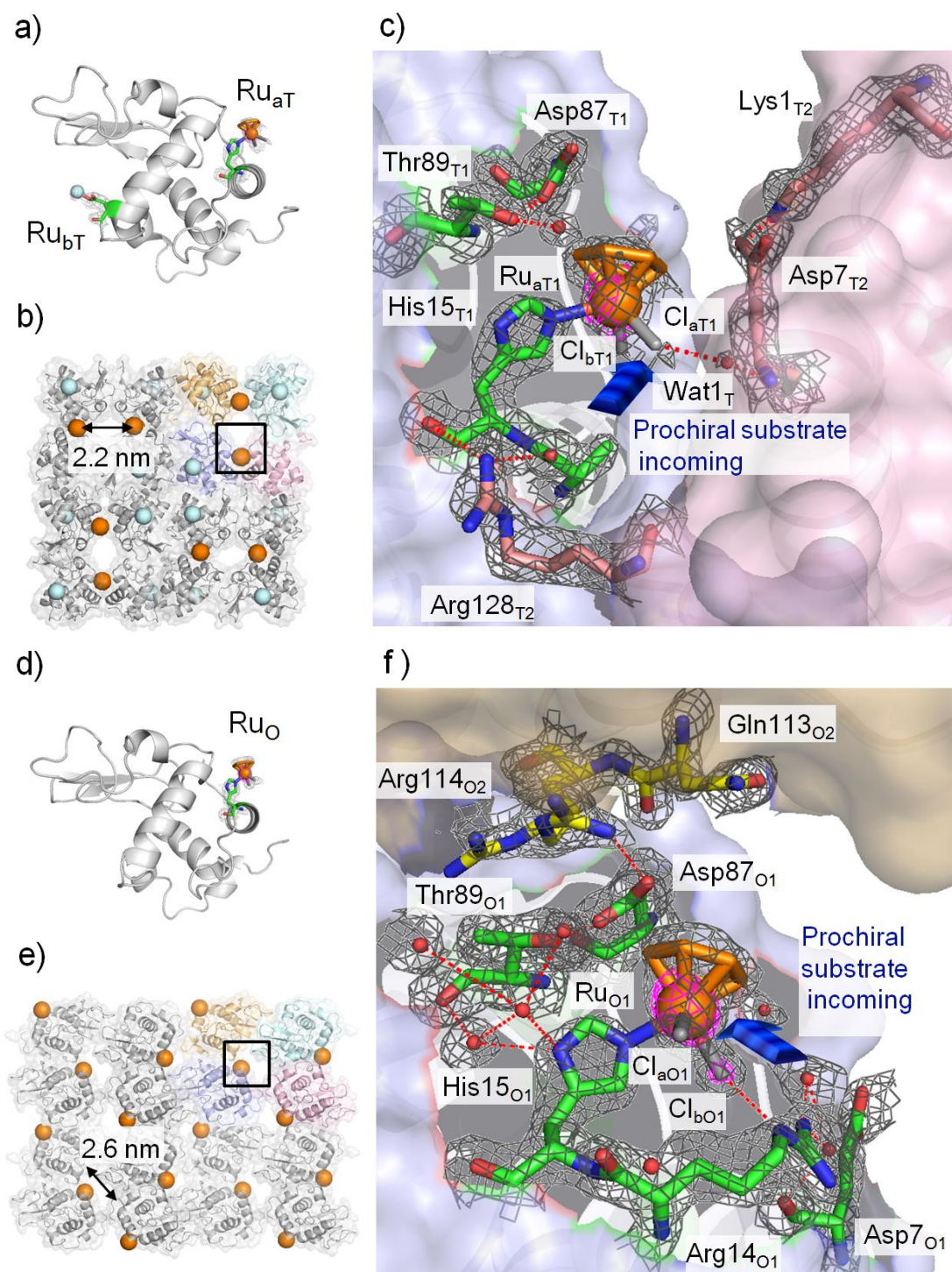


Figure 3. Coordination structures of $\text{Ru}\bullet\text{X}$ for a monomer (a for $\text{Ru}\bullet\text{T}$, and d for $\text{Ru}\bullet\text{O}$), a lattice (b for $\text{Ru}\bullet\text{T}$, and e for $\text{Ru}\bullet\text{O}$), and active site structures with hydrogen-bonding networks (c for $\text{Ru}\bullet\text{T}$, and f for $\text{Ru}\bullet\text{O}$). The coordination structures were magnified from the black square in the lattice structures. HEWL

monomers which are coordinated to Ru(benzene)Cl₂ are assigned as T1 and O1, respectively. The residues in T1 and O1 are green. The next HEWL monomers are assigned as T2 (pink) and O2 (yellow), respectively. Anomalous difference Fourier maps at 3.0 σ indicate the positions of individual Ru atoms colored with magenta. The selected 2 |Fo|-|Fc| electron-density maps at 1.0 σ are colored with gray. Crystallographic images were produced by Pymol.⁴²

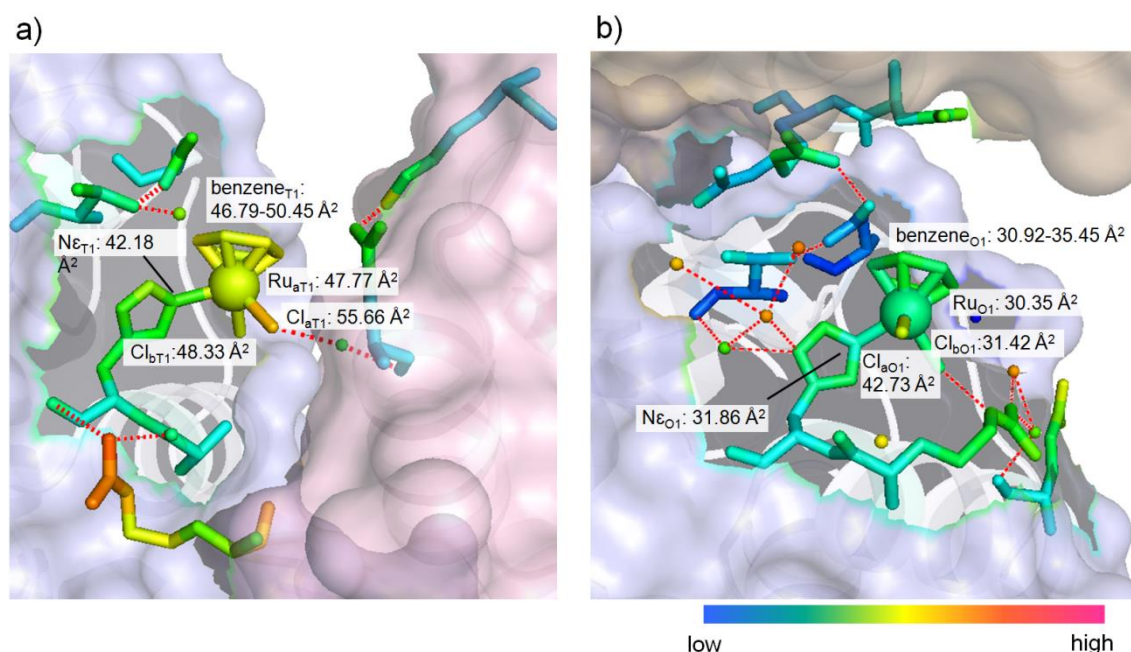


Figure 4. *B*-factor of the coordination sites of (a) **Ru•T** and (b) **Ru•O**.

Catalytic reactions.

The reduction reactions by hydrogen transfer of acetophenone and its derivatives were examined at 35 °C in the presence of **Ru•T** and **Ru•O** (Scheme 1c). After the reaction, the conversion and enantiomeric excess (*ee*) were determined by HPLC equipped with a Daicel Chiralcel OD-H column (Figure 5). No residual compounds were remained in **Ru•X** after catalytic reactions as confirmed by ¹H NMR. All of the composites catalyze the hydrogen transfer reactions listed in Table 2. **Ru•T** exhibits the highest activity for propiophenone (**2**) with a conversion of 53% (entry 4). With respect to the *ee* value of the catalytic reactions, the reaction of isobutyrophenone (**4**) by **Ru•O** has the highest *ee* value; 36 % *ee* *S* (entry 11). The *ee* values for all of the other products obtained from **Ru•O** are greater than those of the products obtained from **Ru•T**. When control experiments were performed with Ru(benzene)Cl₂-HEWL composite dissolved in a buffer solution (Ru•(HEWL)_{sol}), the solution shows 4-8-fold lower conversions than those of **Ru•T**. (Table 2, entry 3, 6, 9, 12, and 15). In the absence of HEWL, or the presence of only L-histidine, the Ru complex shows no catalytic activity of the hydrogen transfer reaction. These results suggest that the coordination of His to the Ru complex is stabilized within the solvent channels of HEWL crystals as shown in Figure 3.

Next, recyclable performance of **Ru•O** was evaluated with the reaction of entry 11, which showed the highest *ee* value (Table 3). The second–fifth runs maintained *ca.* 78 – 57 % conversions of the first reaction. The tenth runs showed 70 % of conversion, 71 % enantioselectivity, respectively, from the first reaction. The quantitative analysis of the Ru/HEWL ratio after the tenth run (0.49) was only 72 % of **Ru•O** before the 1st run (0.60). It suggests that the decreases were mainly caused by partial disruption of the coordination structure of Ru(benzene) and leaching of Ru complexes during the recycle procedure.

Table 2. Catalytic reduction of acetophenone derivatives with Ru-HEWL compounds.^a

Entry	Substrate	Catalyst	Conversion [%]	ee [%]
1	1	Ru•T	48	4 (R)
2	1	Ru•O	44	7 (R)
3	1	Ru•(HEWL) _{sol} ^b	8	14 (S)
4	2	Ru•T	53	8 (R)
5	2	Ru•O	44	31 (R)
6	2	Ru•(HEWL) _{sol} ^b	8	7 (S)
7	3	Ru•T	33	18 (R)
8	3	Ru•O	21	20 (R)
9	3	Ru•(HEWL) _{sol} ^b	4	3 (S)
10	4	Ru•T	38	23 (R)
11	4	Ru•O	46	36 (S)
12	4	Ru•(HEWL) _{sol} ^b	7	24 (R)
13	5	Ru•T	46	8 (S)
14	5	Ru•O	41	29 (R)
15	5	Ru•(HEWL) _{sol} ^b	12	12 (R)

a. Hydrogen transfer reactions were carried out in borate buffer (0.3 M, pH 7.5) at 35 °C for 24 hr in the presence of a catalyst ([Ru] = 1.3 mM), substrate (11.3 mM), and sodium formate (3.3 M).

b. Hydrogenation reactions were carried out in borate buffer (0.3 M, pH 7.5) at 35 °C for 24 hr in the presence of a [Ru(benzene)Cl₂]₂ (1.3 mM), substrate (11.3 mM), HEWL (2.0mg/mL), and sodium formate (3.3 M).

Table 3. Catalytic reduction of substrate **4** with **Ru•O**.^a

Run	Conversion [%]	<i>ee</i> [%]
1	46	36 (S)
2	36	28 (S)
3	31	27 (S)
4	31	23 (S)
5	26	23 (S)
6	25	18 (S)
7	22	15 (S)
8	22	12 (S)
9	20	10 (S)
10	14	11 (S)

a. Hydrogen transfer reactions were carried out in borate buffer (0.3M pH 7.5) at 35 °C for 24 hrs in the presence of substrate **4** (11.3 mM) and sodium formate (3.3M) and **Ru•O** which were filtered off from the previous run.

To the best of our knowledge, this is the first example of a recyclable artificial metalloenzyme. Mechanism of selective catalytic reactions.

As previously reported, the mononuclear half-sandwich Ru(η^6 -arene)-N coordination is the key structure for the catalytic reaction.^{43, 44} Ru(benzene)Cl₂•His15 is stabilized in **Ru•T** and **Ru•O** by hydrogen bonding networks while Ru(benzene)Cl₂•His15 in Ru•(HEWL)_{sol} is expected to have random coordination structure. This is because Ru complexes prefer to coordinate to phenylalanine and tyrosine residues to form sandwich complexes in solution.³⁷ Ru•(HEWL)_{sol} has lower activity than **Ru•T** and **Ru•O** because CL-HEWLs have few aromatic residues exposed on the surfaces of the large solvent channels because those residues are mostly involved in hydrophobic protein-protein interactions to maintain the crystal lattice.³⁷ [Ru(benzene)Cl₂]₂ dissolved in a buffer solution in the absence of HEWL also lacks Ru(benzene)Cl₂•His15 structure. Thus, **Ru•T** and **Ru•O** have higher reactivity relative to Ru•(HEWL)_{sol}.

ee value obtained by **Ru•T** and **Ru•O** is usually higher than that of **Ru•(HEWL)_{sol}** (Table 2). This result can also be interpreted by the stabilization of coordination bondings in the restricted space in solvent channels of **Ru•T** and **Ru•O**. In the case of previously reported artificial transfer hydrogenases with a Ru(η^6 -arene) moiety, when the first coordination sphere around the Ru atom is an achiral ligand, enantioselectivity is determined by the second coordination environment in proteins.¹⁴
¹⁵ The chiral environment of the second sphere in solution is affected only by the intramolecular interactions with neighboring residues. The reaction products obtained with **Ru•T** include the *R*-isomer, which is opposite from the product obtained with **Ru•(HEWL)_{sol}**, except for **5** (entry 13), because the second coordination environment in the crystal is influenced by the adjacent HEWL monomer along the solvent channel. The direction of substrate access toward the active site of **Ru•T** is restricted because Ru(benzene)Cl₂•His15 of **Ru•T** is located at the boundary with the adjacent HEWL monomer and the Cl_{aT1} atom participates in a hydrogen bonding network with OAsp7_{T2} through a water molecule (Figure 3c and Figure 6a). The absolute *ee* values of the products are lower than those produced with **Ru•O**. This appears to be due to the high flexibility of Arg14_{T1} which is indicated by the high *B*-factor value of the side chain (Figure 4a).

In the solvent channel of **Ru•O**, substrates **4** and **5** produce *S*-, and *R*-isomers, respectively, which are the opposite of those produced by **Ru•T**. The direction of substrate access is expected to be different from that of **Ru•T** because the adjacent HEWL monomer extends over the benzene ligand of the Ru binding site and Cl_{bO1} retains an intramolecular hydrogen bond with Arg14_{O1} (Figure 3f and Figure 6b). The *B*-factor of the first Ru coordination sphere is lower than that of **Ru•T** because of the influence of the consistent interaction (Figure 4b). The less flexible environment around Ru(benzene)Cl₂ in the solvent channel of **Ru•O** gives higher *ee* values relative to that of **Ru•T**.

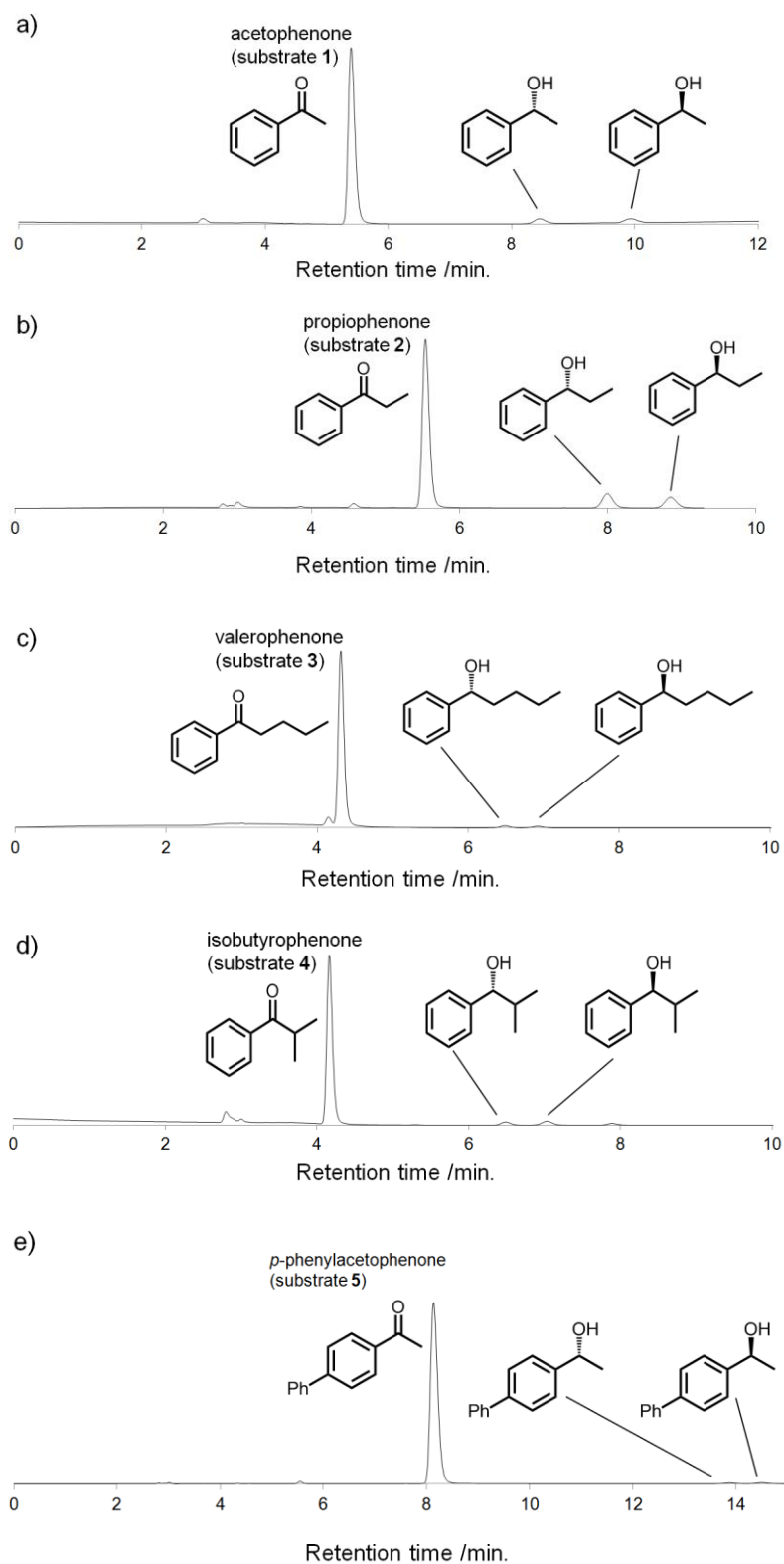
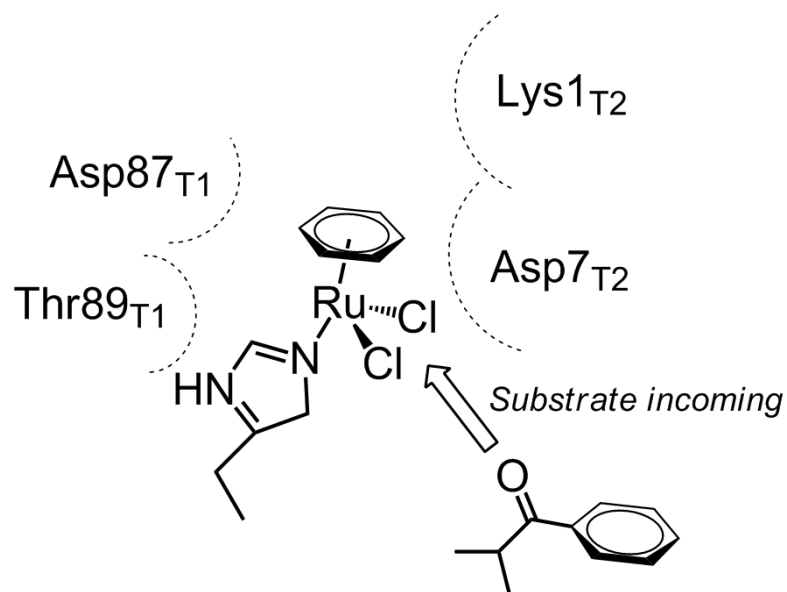


Figure 5. Chiral phase HPLC trace of acetophenone (a), propiophenone (b), valerophenone (c), isobutyrophenone (d) and *p*-phenylacetophenone conversion with **Ru•T** showing the signals of (*R*)- and (*S*)-products, respectively.

a)



b)

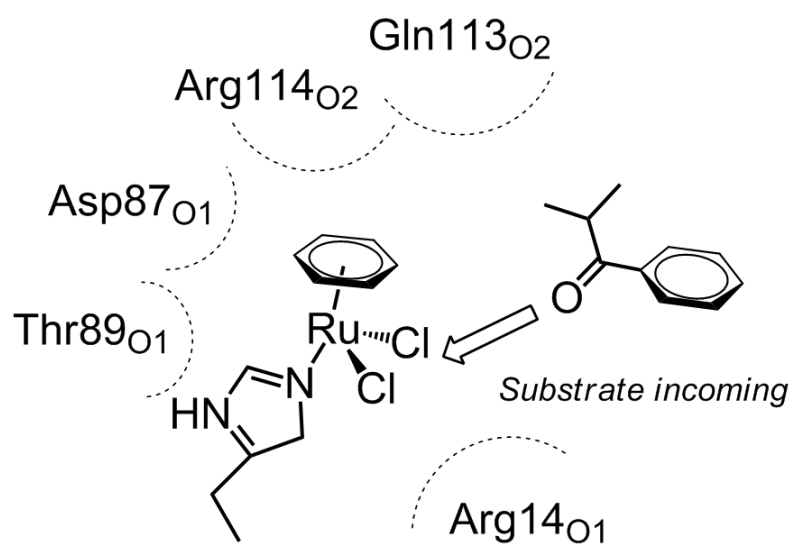


Figure 6. Proposed direction of substrate access in **Ru•T** (a) and **Ru•O** (b).

Conclusions

The author has successfully established a method for the construction of biohybrid catalysts by immobilizing organometallic complexes in solvent channels of cross-linked porous HEWL crystals. The improvement of catalytic activity is attained by stabilization of Ru(benzene)Cl₂ coordination in the solvent channels of CL-HEWLs by hydrogen bonding networks across the neighboring HEWL monomers. The changes of the *ee* values and configurations can be explained by protein-substrate interactions in solvent channels as revealed by X-ray crystal structure analysis. This method represents a new approach for immobilization and activation of organometallic complexes using the environment of solvent channels in porous protein crystals.

solid materials

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 were used without further purification.

Crystal Structure Analysis. CL-X-HEWLs were prepared by the hanging drop vapor diffusion method according to the reported procedure.^{39, 45} CL-T-HEWL and CL-O-HEWL were soaked in a 0.1 M acetate buffer (pH 6.1) containing 5.0 mM [Ru(benzene)Cl₂]₂ and 1.0 M NaCl for 24 hrs to obtain the crystals containing Ru complex. Prior to the data collection, crystals were continuously immersed in a precipitant solution containing 10 % (w/w) and 25 % glycerol and subsequently frozen in liquid nitrogen. X-ray diffraction data of each **Ru•X** were collected at 100 K at beamline BL38B1 at SPring-8 using X-ray wavelengths of 1.0 Å. The data were processed with the program *HKL2000*.⁴⁶

The crystal parameters and the data collection statistic are summarized in Table 1. The structures were solved by molecular replacement with *MOLREP*⁴⁷ using HEWL structures of tetragonal and orthorhombic forms (PDB ID: 193L and 1BGI, respectively). Refinement of the protein structure was performed using *REFMAC5*⁴⁸ in the *CCP4* suite⁴⁹. Rebuilding was performed using *COOT*⁵⁰ based on sigma weighted ($2F_o - F_c$) and ($F_o - F_c$) electron density maps. After rigid-body refinement, the

coordination structures of Ru binding sites were picked up according to anomalous Fourier difference maps and geometric parameters. Water molecules were positioned to fit residual ($F_o - F_c$) density peaks with a lower cut-off of 3σ . The models were subjected to quality analysis during the various refinement stages with omit maps and *PROCHECK*.⁵¹ The refinement statistics are summarized in Table 1. Atomic coordinates are deposited in the Protein Data Bank under accession numbers 3W6A and 4J7V for **Ru•T** and **Ru•O**, respectively.

ICP-OES measurement. **Ru•X** (5.0 mg) were ashed in 2 mL concentrated HNO_3 . The precipitate was dissolved in 0.1 M HNO_3 . Metal concentrations of the solution were determined by using an inductively coupled plasma optical emission spectrometer (Shimadzu ICPE-9000). $\text{Ru}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was used as calibration standard and $\text{Y}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was applied for an internal standard.

Hydrogen transfer reactions by Ru•X. Hydrogen transfer reactions were carried out in borate buffer (0.3 M, pH 7.5) at 35 °C for 24 hrs in the presence of a catalyst ($[\text{Ru}] = 1.3 \text{ mM}$), substrate (11.3 mM), and sodium formate (3.3 M). After the reaction, the reaction mixture was filtrated to remove crystals and the substrates and products remaining were extracted with hexane three times. There were no residual compounds in **Ru•X** after this treatment by ^1H NMR. The solutions were analysed with HPLC equipped with a Daicel Chiralcel OD-H column installed on a HITACHI LC-9000 system (1.0 mL/min, hexane: *i*PrOH = 95:5, column temperature 30°C) (Figure 5).

Recycling performance of Ru•O : Hydrogen transfer reactions were carried out in borate buffer (0.3 M, pH 7.5) at 35 °C for 24 hr in the presence of substrate **4** (11.3 mM), and sodium formate (3.3 M) and **Ru•O** which were filtered off from the 1st run. After the reaction, the reaction mixture was filtrated to remove crystals for the next run.

Typical Procedure for the synthesis of the HPLC standards. NaBH_4 (0.71 g, 19 mmol) dissolved in ethanol (20 mL) was stirred at 10°C for 10 min. A solution of acetophenone (6.0g, 50mmol) in ethanol (10mL) was added dropwise and was refluxed for 20min. The mixture is placed in ice water bath and 5% HCl solution (20mL) is added dropwise to the mixture. After being stirred for 10min at 0 °C, a saturated NaCl solution (30mL) was added and the product was extracted with ethyl acetate (20 mL $\times$ 3). The extract is washed with Na_2CO_3 solution (30mL) and with NaCl solution (30mL)

and dried over with Na_2SO_4 . Concentration of the filtrate with a rotary evaporator affords 1-phenylethanol (3.66 g, 30.1 mmol). The product is analyzed by ^1H NMR (CDCl_3 , 500 MHz) and HPLC. The peak assignment was carried out with commercial (*R*)-1-phenylethanol.

References

1. H.-B. Kraatz and N. Metzler-Nolte, *Concepts and Models in Bioinorganic Chemistry*, WILEY-VCH, Weinheim, 2006.
2. K. D. Karlin, *Progress in Inorganic Chemistry*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2012.
3. M. E. Wilson and G. M. Whitesides, *J. Am. Chem. Soc.*, 1978, **100**, 306-307.
4. R. R. Davies and M. D. Distefano, *J. Am. Chem. Soc.*, 1997, **119**, 11643-11652.
5. T. Hayashi and Y. Hisaeda, *Acc. Chem. Res.*, 2002, **35**, 35-43.
6. T. R. Ward, *Acc. Chem. Res.*, 2011, **44**, 47-57.
7. Y. Lu, N. Yeung, N. Sieracki and N. M. Marshall, *Nature*, 2009, **460**, 855-862.
8. T. Ueno, S. Abe, N. Yokoi and Y. Watanabe, *Coord. Chem. Rev.*, 2007, **251**, 2717-2731.
9. H. J. Yang, T. Matsui, S. Ozaki, S. Kato, T. Ueno, G. N. Phillips, Jr., S. Fukuzumi and Y. Watanabe, *Biochemistry*, 2003, **42**, 10174-10181.
10. T. Ueno, T. Koshiyama, S. Abe, N. Yokoi, M. Ohashi, H. Nakajima and Y. Watanabe, *J. Organomet. Chem.*, 2007, **692**, 142-147.
11. S. Abe, K. Hirata, T. Ueno, K. Morino, N. Shimizu, M. Yamamoto, M. Takata, E. Yashima and Y. Watanabe, *J. Am. Chem. Soc.*, 2009, **131**, 6958-6960.
12. J. Bos, F. Fusetti, A. J. Driessen and G. Roelfes, *Angew. Chem. Int. Ed.*, 2012, **51**, 7472-7475.
13. M. Creus, A. Pordea, T. Rossel, A. Sardo, C. Letondor, A. Ivanova, I. Letrong, R. E. Stenkamp and T. R. Ward, *Angew. Chem. Int. Ed.*, 2008, **47**, 1400-1404.
14. C. Letondor, N. Humbert and T. R. Ward, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 4683-4687.
15. C. Letondor, A. Pordea, N. Humbert, A. Ivanova, S. Mazurek, M. Novic and T. R. Ward, *J. Am. Chem. Soc.*, 2006, **128**, 8320-8328.
16. M. L. Zastrow, A. F. A. Peacock, J. A. Stuckey and V. L. Pecoraro, *Nat. Chem.*, 2012, **4**, 118-123.
17. T. Ueno, *Chem. Eur. J.*, 2013, **19**, 9096-9102.
18. B. W. Matthews, *J. Mol. Biol.*, 1968, **33**, 491-497.
19. A. L. Margolin and M. A. Navia, *Angew. Chem. Int. Ed.*, 2001, **40**, 2204-2222.
20. A. Cvetkovic, C. Picoreanu, A. J. Straathof, R. Krishna and L. A. van der Wielen, *J. Phys. Chem. B*, 2005, **109**, 10561-10566.

21. Z. Hu and J. Jiang, *J. Phys. Chem. B*, 2009, **113**, 15851-15857.
22. R. A. Persichetti, N. L. Stclair, J. P. Griffith, M. A. Navia and A. L. Margolin, *J. Am. Chem. Soc.*, 1995, **117**, 2732-2737.
23. L. Z. Vilenchik, J. P. Griffith, N. St Clair, M. A. Navia and A. L. Margolin, *J. Am. Chem. Soc.*, 1998, **120**, 4290-4294.
24. R. J. Radford, M. Lawrenz, P. C. Nguyen, J. A. McCammon and F. A. Tezcan, *Chem. Commun.*, 2011, **47**, 313-315.
25. J. D. Brodin, X. I. Ambroggio, C. Tang, K. N. Parent, T. S. Baker and F. A. Tezcan, *Nat. Chem.*, 2012, **4**, 375-382.
26. T. Koshiyama, N. Kawaba, T. Hikage, M. Shirai, Y. Miura, C. Y. Huang, K. Tanaka, Y. Watanabe and T. Ueno, *Bioconjugate Chem.*, 2010, **21**, 264-269.
27. M. Guli, E. M. Lambert, M. Li and S. Mann, *Angew. Chem. Int. Ed.*, 2010, **49**, 520-523.
28. H. Wei, Z. Wang, J. Zhang, S. House, Y. G. Gao, L. Yang, H. Robinson, L. H. Tan, H. Xing, C. Hou, I. M. Robertson, J. M. Zuo and Y. Lu, *Nat. Nanotechnol.*, 2011, **6**, 93-97.
29. H. Wei and Y. Lu, *Chem. Asian. J.*, 2012, **7**, 680-683.
30. M. W. England, E. M. Lambert, M. Li, L. Turyanska, A. J. Patil and S. Mann, *Nanoscale*, 2012, **4**, 6710-6713.
31. O. L. Muskens, M. W. England, L. Danos, M. Li and S. Mann, *Adv. Funct. Mater.*, 2013, **23**, 281-290.
32. T. Koshiyama, M. Shirai, T. Hikage, H. Tabe, K. Tanaka, S. Kitagawa and T. Ueno, *Angew. Chem. Int. Ed.*, 2011, **50**, 4849-4852.
33. S. Abe, M. Tsujimoto, K. Yoneda, M. Ohba, T. Hikage, M. Takano, S. Kitagawa and T. Ueno, *Small*, 2012, **8**, 1314-1319.
34. E. N. Salgado, R. J. Radford and F. A. Tezcan, *Acc. Chem. Res.*, 2010, **43**, 661-672.
35. T. Ueno, S. Abe, T. Koshiyama, T. Ohki, T. Hikage and Y. Watanabe, *Chem. Eur. J.*, 2010, **16**, 2730-2740.
36. C. Cavazza, C. Bochet, P. Rousselot-Pailley, P. Carpentier, M. V. Cherrier, L. Martin, C. Marchi-Delapierre, J. C. Fontecilla-Camps and S. Menage, *Nat. Chem.*, 2010, **2**, 1069-1076.
37. I. W. McNae, K. Fishburne, A. Habtemariam, T. M. Hunter, M. Melchart, F. Wang, M. D. Walkinshaw and P. J. Sadler, *Chem. Commun.*, 2004, 1786-1787.

38. J. C. Falkner, M. J. Al-sSomali, J. A. Jamison, S. L. Zhang, S. L. Simpson, M. K. Calabretta, W. Radding, G. N. Phillips and V. L. Colvin, *Chem. Mater.*, 2005, **17**, 2679-2686.
39. H. Oki, Y. Matsuura, H. Komatsu and A. A. Chernov, *Acta Crystallogr. D*, 1999, **55**, 114-121.
40. Y. Wine, N. Cohen-Hadar, A. Freeman and F. Frolow, *Biotechnol. Bioeng.*, 2007, **98**, 711-718.
41. T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. Bernardes, C. C. Romao and M. J. Romao, *J. Am. Chem. Soc.*, 2011, **133**, 1192-1195.
42. <http://www.pymol.org/>.
43. J. S. M. Samec, J. E. Backvall, P. G. Andersson and P. Brandt, *Chem. Soc. Rev.*, 2006, **35**, 237-248.
44. R. Noyori, M. Yamakawa and S. Hashiguchi, *J. Org. Chem.*, 2001, **66**, 7931-7944.
45. M. C. Vaney, S. Maignan, M. RiesKautt and A. Ducruix, *Acta Crystallogr. D*, 1996, **52**, 505-517.
46. Z. Otwinowski and W. Minor, *Method Enzymol.*, 1997, **276**, 307-326.
47. A. Vagin and A. Teplyakov, *J. Appl. Crystallogr.*, 1997, **30**, 1022-1025.
48. A. A. Vagin, R. A. Steiner, A. A. Lebedev, L. Potterton, S. McNicholas, F. Long and G. N. Murshudov, *Acta Crystallogr. D*, 2004, **60**, 2184-2195.
49. E. Potterton, P. Briggs, M. Turkenburg and E. Dodson, *Acta Crystallogr. D*, 2003, **59**, 1131-1137.
50. P. Emsley and K. Cowtan, *Acta Crystallogr. D*, 2004, **60**, 2126-2132.
51. R. A. Laskowski, M. W. Macarthur, D. S. Moss and J. M. Thornton, *J. Appl. Crystallogr.*, 1993, **26**, 283-291.

Chapter 2

Preparation of a Cross-linked Porous Protein Crystal containing Ru carbonyl complexes as a CO-releasing Extracellular Scaffold

Abstract

In the previous chapter, the author investigated the immobilization of reactive organoruthenium complexes in the solvent channels of cross-linked porous HEWL crystals (CL-HEWL). Herein, ruthenium carbonyls are another candidate for an immobilization in CL-HEWL (**Ru•CL-HEWL**). **Ru•CL-HEWL** retains a Ru carbonyl moiety that can release CO gas by the ligand liberation although a composite of Ru carbonyl-HEWL dissolved in buffer solution (Ru•HEWL) does not release CO. The culture of cells with **Ru•CL-HEWL** significantly increased nuclear factor kappa B (NF-κB) activity as a cellular response to CO gas. These results demonstrate that **Ru•CL-HEWL** has potential for use as an artificial extracellular scaffold suitable for transport and release of a signaling small molecule using organometallic complexes.

Introduction

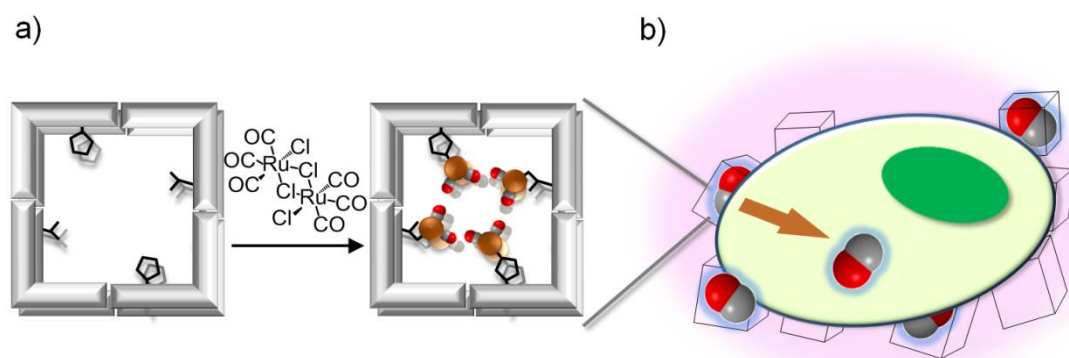
Protein assemblies are a class of highly organized composites that hold promise for development materials in a number of applications in biotechnology.¹⁻⁶ One of the general methods is the encapsulation of non-natural metal cofactors within the scaffolds of protein assemblies.⁷⁻¹² This method provides the means to construct bionanomaterials composed by artificial metalloproteins *i.e.* artificial metalloenzymes, drug delivery systems and imaging reagents.¹³⁻¹⁵ In particular, the design of composites of proteins and organometallic complexes represents one of the most challenging efforts because of the high reactivity of organometallic complexes.¹⁵⁻¹⁸ For example, proteins modified with organoruthenium or organorhodium complexes can catalyze selective reactions for organic synthesis.

One candidate for functional organometallic complexes is metal carbonyls. Iron carbonyls have been conjugated to proteins.¹⁹ They are active for evolution of small molecules as H₂. Metal carbonyls also serve as carbon monoxide (CO)-releasing molecules (CORMs). Delivery of CO into living cells using CORMs is useful because CO gas is a pleiotropic signaling gas molecule such as cytoprotection, modulation of inflammation, redox control and vasoactive response.²⁰⁻²⁶ Although bionanomaterials with CO-releasing ability have been prepared by immobilizing CORMs in inorganic particles, polymer, peptide gels and metal-organic frameworks (MOFs), their structure are not well clarified and their cellular application has been limited.²⁷⁻³¹ As yet, several of these materials are utilized as extracellular scaffolds that can deliver CO into living cells to control cellular functions such as apoptosis and alleviation of oxidative stress.^{27, 29, 31}

Protein crystals represent precise protein assemblies in solid state and are also appropriate candidates for development of robust bionanomaterials that immobilize CORMs.^{8, 11, 32-38} Each protein crystal has inner pores that act as solvent channels. Metal complexes are immobilized in solvent channels by the coordination bonding between amino acid residues.³² The structures of the solvent channels are usually retained by cross-linking treatment of protein crystals.³³ Cross-linked protein crystals (CLPCs) have been adapted to serve as nanovessels for enzyme reactions and molecular separations.^{39, 40} Post-engineering of CLPCs has recently been performed to deposit functional metal compounds in solvent channels.^{37, 38} The success of these efforts indicates that protein

crystals have further potential to provide new structures of CORMs and improve the reactivity of CO-releasing.

Herein, the immobilization of CORMs and release of CO gas using cross-linked HEWL crystals (CL-HEWLs) are demonstrated. Porous HEWL crystals have the capability to accommodate various metal carbonyls.⁴¹⁻⁴⁶ Romão *et al.* reported composites from HEWL and Ru carbonyls although the composite was found to be incapable of releasing CO gas.^{43, 45} The immobilization of Ru carbonyls in porous CL-HEWLs induced the formation of $[cis-Ru(CO)_2X_4]^{2-}$ (X = anionic ligands) to preserve CO gas in solvent channels and to release CO gas under physiological conditions. The CO molecules can activate nuclear factor kappa B (NF- κ B). These results indicate that porous protein crystals have potential for use as extracellular scaffolds for the stable immobilization of bioactive organometallic compounds.



Scheme 1. Schematic representation of the preparation of **Ru•CL-HEWL** (a) and CO release in the vicinity of cells (b).

Results and discussions

Preparation and X-ray Crystal Structure Analysis.

HEWL crystals were obtained using a batch crystallization method followed by a cross-linking treatment as reported in the previous chapter. CL-HEWL were soaked in acetate buffer solution containing 5.0 mM $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$, known as CORM-2, to immobilize the Ru carbonyls in CL-HEWLs (**Ru•CL-HEWL**). The ratio of Ru to the HEWL monomer, as determined by X-ray fluorescence spectroscopy, was $10.1 \pm 0.8:1$ for **Ru•CL-HEWL**. A scanning transmission electron microscopy-energy dispersive X-ray spectrum (STEM-EDX) showed that Ru complexes are fully distributed within **Ru•CL-HEWL** (Figure 1a).

Ru•CL-HEWL was characterized by infrared (IR) spectroscopy (Figure 1b, solid line). The IR spectrum of **Ru•CL-HEWL** has four bands at 2,055, 2,025, 1,981 and $1,940\text{ cm}^{-1}$. A pair of bands at 2,055 and $1,981\text{ cm}^{-1}$ is assigned to the CO stretching vibrations of the *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ moiety in proteins.⁴³ The other pair at 2,025 and $1,940\text{ cm}^{-1}$ is expected to arise from the CO stretching vibrations of $[\text{cis-Ru}(\text{CO})_2\text{X}_4]^{2-}$ (X = anionic ligands).⁴⁷⁻⁴⁹ The composite of HEWL and $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ in buffer solution (**Ru•HEWL**) showed a pair of bands at 2,047 and $1,977\text{ cm}^{-1}$ that are assigned to *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ moiety (Figure 1b, dotted line). The difference IR spectra between **Ru•CL-HEWL** and **Ru•HEWL** indicates that $[\text{cis-Ru}(\text{CO})_2\text{X}_4]^{2-}$ moieties can exist only in **Ru•CL-HEWL**.

Ru carbonyls in **Ru•CL-HEWL** was characterized by X-ray crystal structure analysis at a resolution of $1.55\text{ \AA}$ (Figure 2 and Table 1). The binding positions of the Ru atoms were distinguished by anomalous difference maps (Figure 2a-2b). Each Ru atom is bound to a coordinating residue such as His, Asp and Lys with a bond length of 2.2–2.5 $\text{\AA}$ (Figure 2c-2h). All bond lengths are reasonable compared with the reported bond lengths between Ru and residues.^{50, 51} Ru_a is coordinated to His15 as reported for the other HEWL-organometallic complex composites.^{43, 45, 50} Ru_a has an occupancy of 0.7 and adopts an octahedral structure with N ϵ of His15, two CO molecules in the *cis*-position, one water molecule, and two Cl ions to form the octahedral structure, $[\text{cis-Ru}(\text{CO})_2(\text{H}_2\text{O})\text{Cl}_2(\text{His15})]$ (Figure 2c). This structure gives rise to IR bands of 2,055 and $1,981\text{ cm}^{-1}$ according to the literature⁴³. This moiety is surrounded by O δ of Asp87 and O γ of Thr89 and participating in hydrogen bonding networks. An additional nine anomalous densities assigned to Ru ions were identified near the carboxylate of

Asp18, Asp52, Asp101, Asp119, and the C-terminal (Leu129) and (Figure 2d-2h). These moieties are expected to have IR bands at 2,025 and 1,940 cm^{-1} according to the literature.⁴⁷⁻⁴⁹ The anomalous difference maps of Ru_b and Ru_c imply that each forms individual mono-nuclear Ru carbonyl moiety coordinated to each O δ atom of Asp18 because the occupancy level for each of these Ru ions was estimated to be 0.5 (Figure 2d). Although anomalous difference maps were observed in Ru_d - Ru_j (Figure 2e-2h), carbonyl ligands could not be observed around these Ru ions because of low occupancy of Ru_d - Ru_j whose value is below 0.5.

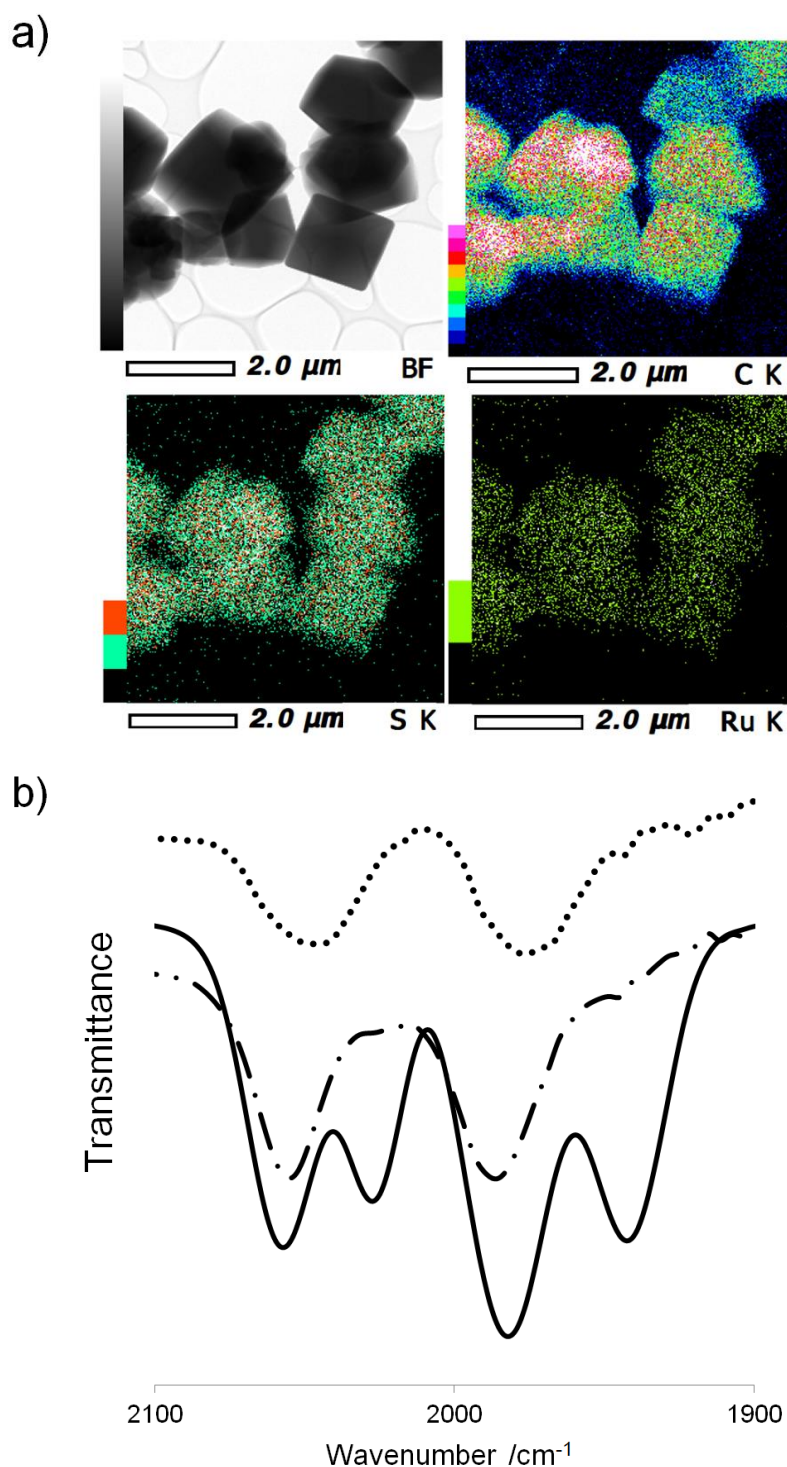


Figure 1. (a) STEM-EDX images of **Ru•CL-HEWL**: STEM Bright-field image (upper left), elemental mapping images of C-K (upper right), S-K (bottom left) and Ru-K (bottom right). (b) Infrared spectra: **Ru•CL-HEWL** (KBr method, solid line), **Ru•CL-HEWL** after releasing CO in the Mb assay (KBr method,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

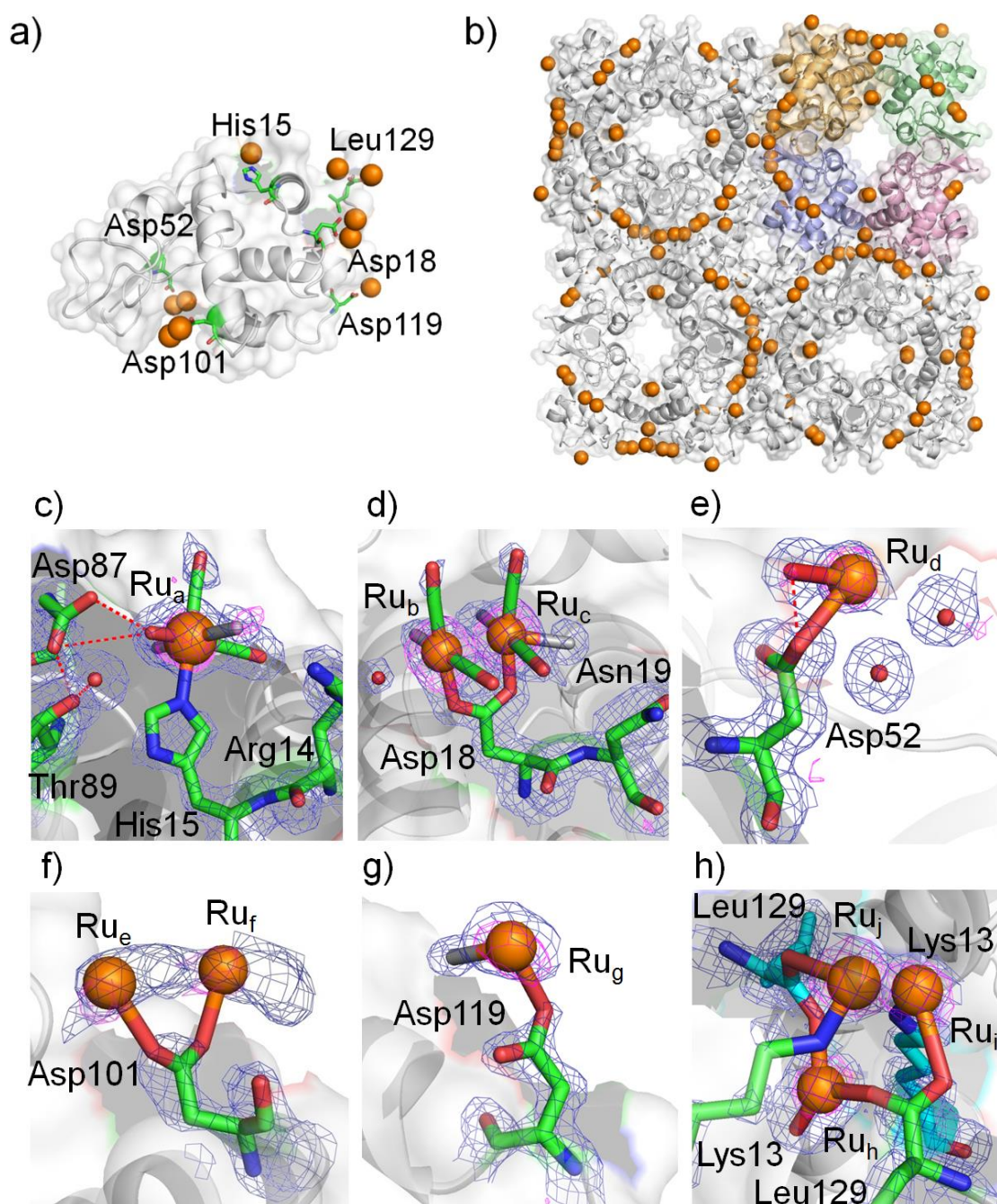


Figure 2. Coordination structures of Ru complexes (Ru_a-Ru_j) in **Ru•CL-HEWL** for a monomer (a), lattice (b) and the magnified structures of coordination sites of His15 (c), Asp18 (d), Asp52 (e), Asp101 (f), Asp119 (g) and C-terminal (Leu129) (h). The residues of the neighboring HEWL monomer are shown in light blue. Hydrogen bonds are indicated by red dashed lines. These images were produced by Pymol.⁵²

Table 1. Crystallographic data and refinement statistics of **Ru•CL-HEWL** and that of after the Mb assay.

	Ru•CL-HEWL	Ru•CL-HEWL after the Mb assay
PDB ID	4W94	4W96
Data Collection Statistics		
X-ray wavelength (Å)	1.0000	1.0000
Space group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2
Crystal cell, (Å)		
a	79.034	79.011
b	79.034	79.011
c	36.920	36.994
α , β , γ	90.00	90.00
Resolution Range	35.00 - 1.55 (1.59 - 1.55)	35.00-1.50 (1.54- 1.50)
Completeness (%)	99.06 (99.76)	93.50 (99.14)
Refinement statistics		
Resolution range (Å)	30.80-1.55	30.84-1.50
Reflection used	16476	17128
R-factor (%)	18.24	19.45
R _{free} (%)	21.43	22.66
Rms diviation from ideality		
Bonds (Å)	0.025	0.025
Angle (°)	2.218	2.154
Ramachandan plot (%)		
Most favored	98.4	97.6
allowed	1.6	2.4

Values in parentheses are for the highest resolution shell.

CO release assay.

The CO-releasing functions of **Ru•CL-HEWL** into the aqueous medium was evaluated by a spectrophotometric assay based on conversion of deoxy-myoglobin (Mb) to carboxy-Mb (MbCO).²⁰ **Ru•CL-HEWL** was suspended in a buffer solution containing Mb and sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). Ru carbonyls are expected to liberate CO gas by ligand exchange reactions with nucleophiles or anions such as $\text{S}_2\text{O}_4^{2-}$ and then coordinate with them.⁵³ The conversion from Mb to MbCO was quantified by UV/Vis measurements. In the presence of **Ru•CL-HEWL**, the MbCO peaks at 542 nm and 578 nm increased during the reaction while the Mb peak at 556 nm decreased with isosbestic points at 550, 571, and 586 nm (Figure 3a). The amount of CO released from **Ru•CL-HEWL** was $0.38 \pm 0.02/\text{Ru}$. The half-life ($t_{1/2}$) of the CO releasing reaction 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 $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (1.9 ± 1.4 min) estimated using the same assay. The solvent channels in **Ru•CL-HEWL** are expected to affect the restricted diffusion of these anions eventually to extend the $t_{1/2}$ value of **Ru•CL-HEWL**.^{34, 54, 55}

The ratio of Ru to the HEWL monomer in **Ru•CL-HEWL** after the Mb assay was $10.4 \pm 1.1:1$. This ratio is similar to that of **Ru•CL-HEWL** before the assay ($10.1 \pm 0.8:1$). The IR spectrum of **Ru•CL-HEWL** after the Mb assay showed that the bands at $2,025$ and $1,940\text{ cm}^{-1}$ disappear while the bands at $2,055$ and $1,981\text{ cm}^{-1}$ assigned to $[\text{cis-Ru}(\text{CO})_2(\text{H}_2\text{O})\text{Cl}_2(\text{His15})]$ are retained (Figure 1b, chained line). Thus, $[\text{cis-Ru}(\text{CO})_2\text{X}_4]^{2-}$ is the CO-releasing site in **Ru•CL-HEWL**. This is supported by the 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 4). This is expected that $[\text{cis-Ru}(\text{CO})_2(\text{H}_2\text{O})\text{Cl}_2(\text{His15})]$ is stabilized by steric hindrance of surrounding amino acid residues. This effect prevents the access of trigger molecules (Figure 2c). **Ru•HEWL** dissolved in buffer solutions was found to be incapable of releasing CO because $[\text{cis-Ru}(\text{CO})_2\text{X}_4]^{2-}$ cannot be formed in **Ru•HEWL**.⁴³ Moreover, the Mb assay could not be confirmed because **Ru•HEWL** precipitates as a dark red aggregate.

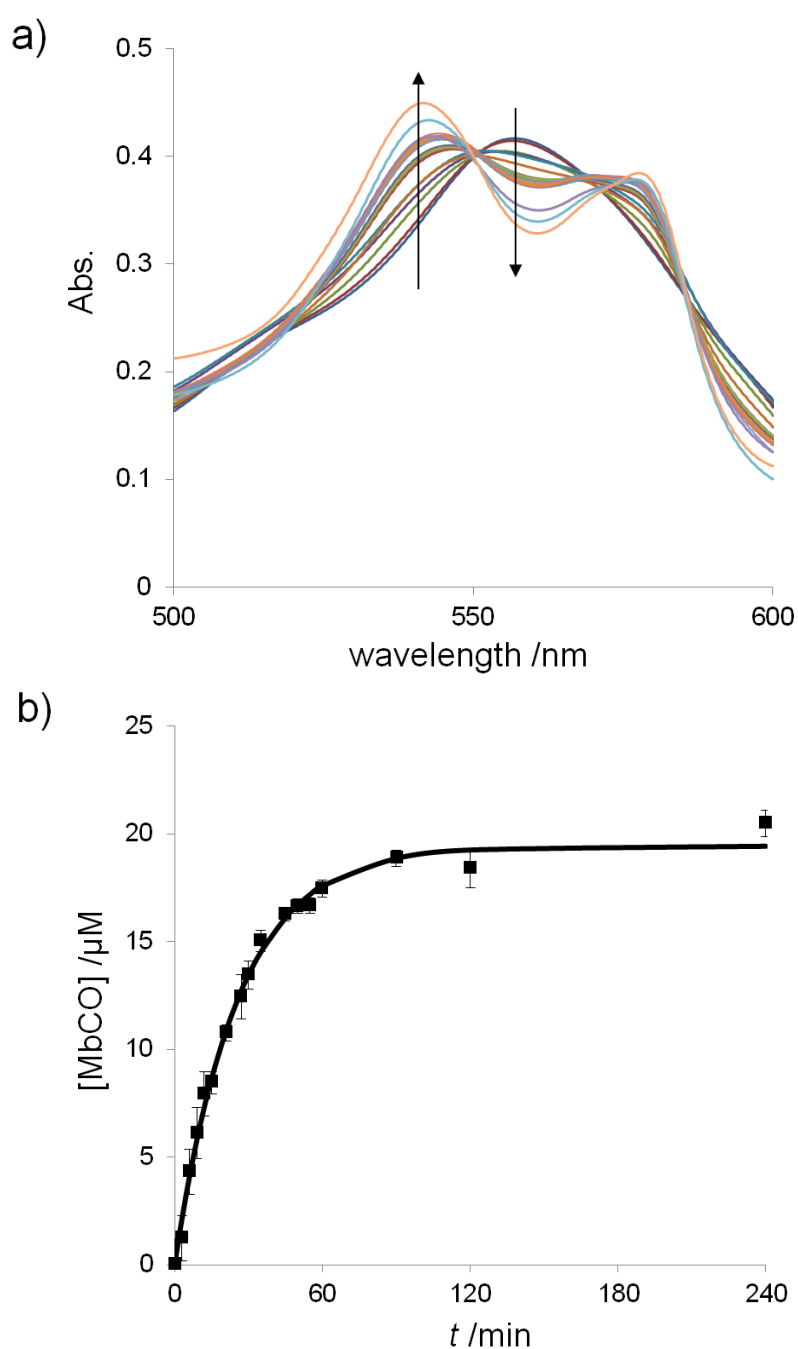


Figure 3. (a) Detection of CO release by monitoring the changes of the Q-band region of the UV/Vis spectra of Mb (35 μM) in the presence of **Ru•CL-HEWL** (Ru = 0.025 μmol) and Na₂S₂O₄ (10 mM) in 500 μL PBS buffer at 25°C. The arrows indicate the change in absorption intensity at 542 nm during the course of the assay ($t = 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 90, 120,$ and 240 minute). (b) The plot of the formation of MbCO. The conversion of Mb to MbCO was monitored with a spectrophotometer. After the calculation of the equivalents

of CO/Ru, $t_{1/2}$ was determined by fitting to first-order kinetics. The experiments were performed three times and the data represent mean $\pm$ SEM.

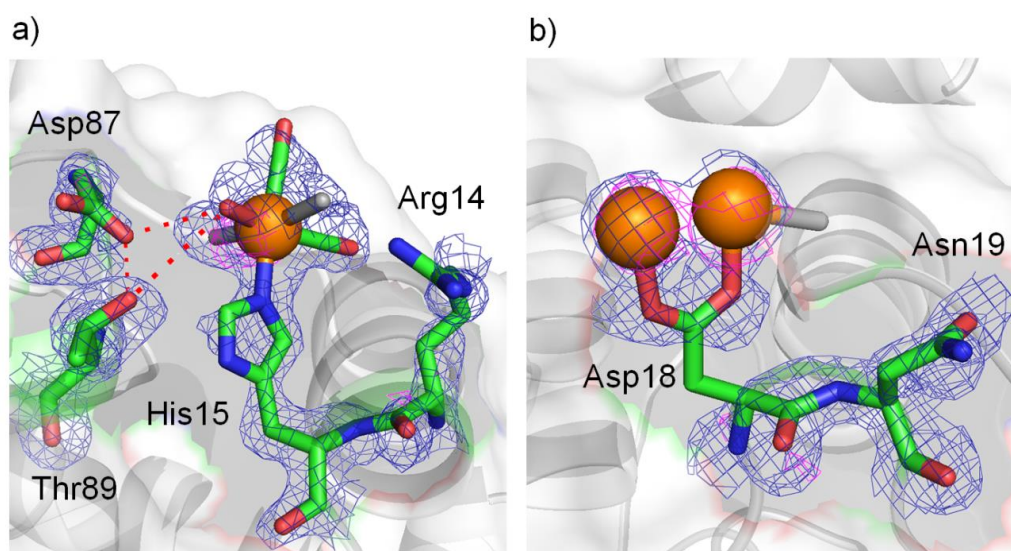


Figure 4. The coordination structures of Ru carbonyls at His15 (a) and Asp18 (b) in **Ru•CL-HEWL** after the Mb assay. Hydrogen bonds are represented with indicated by red dashed lines. These images were produced by Pymol.⁵²

Cellular assay.

To assess the function of CO-releasing from **Ru•CL-HEWL** as an extracellular scaffold, the activity of nuclear factor kappa-B (NF- κ B) in living cells was evaluated. Exposure to the exogenous CO gas is known to activate NF- κ B in the presence of tumor necrosis factor- α (TNF- α).^{56, 57} HEK293/ κ B-Fluc cells were cultured with **Ru•CL-HEWL** (2.0×10^5 crystals, the total amount of Ru is 0.50×10^{-3} μ mol) and 1.0 ng/mL of TNF- α (Figure S2). After incubation for 24 hours, the luciferase level in the cells were quantified by adding a luminescent substrate.⁵⁸ The control assay was carried out using [Ru(CO)₃Cl₂]₂ solution (2.5 μ M, the total amount of Ru is 0.50×10^{-3} μ mol), Ru•HEWL (the total amount of Ru is 0.50×10^{-3} μ mol) and CL-HEWL (2.0×10^5 crystals). Figure 5a shows the bioluminescence intensity of the cell lysate. In the presence of **Ru•CL-HEWL**, a 20% increase of NF- κ B activity was observed relative to the control (assay). 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 solvent channels of **Ru•CL-HEWL** as reported previously.^{26, 59} In the previous report using CORMs, dozen μ M levels of CORMs induce activation of NF- κ B in the presence of LPS, but this effect is accompanied by the cytotoxicity of high concentration of metal ions that decreases cellular viability.²¹ For assays with **Ru•CL-HEWL** or other probes discussed above, there is no difference of the cell viability for the cultivation as revealed by the trypan blue tests because **Ru•CL-HEWL** releases no metal ions (Figure 6). It appears that **Ru•CL-HEWL** is a suitable tool for CO-releasing bionanomaterials that can investigate biofunctions of CO gas.

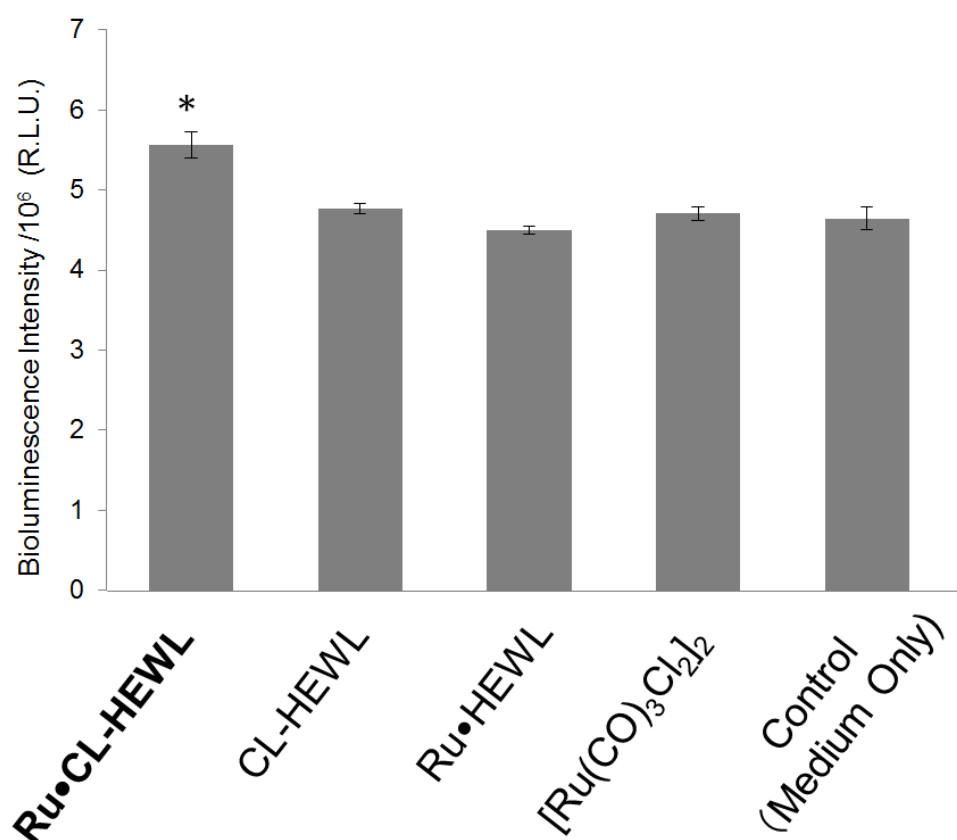


Figure 5. Bioluminescence intensity in the luciferase reporter assay for the evaluation of NF- κ B activity of HEK293/ κ B-Fluc cells in the presence of 1.0 ng/mL TNF- α after incubation with **Ru•CL-HEWL**, CL-HEWL, Ru•HEWL, [Ru(CO)₃Cl₂]₂ and medium only (as control) for 24 hours. Each sample was incubated with HEK293/ κ B-Fluc cells under 5% CO₂ at 37 °C. * P < 0.05 vs. control. The experiments were performed three times under each set of conditions. The data represent mean $\pm$ SEM.

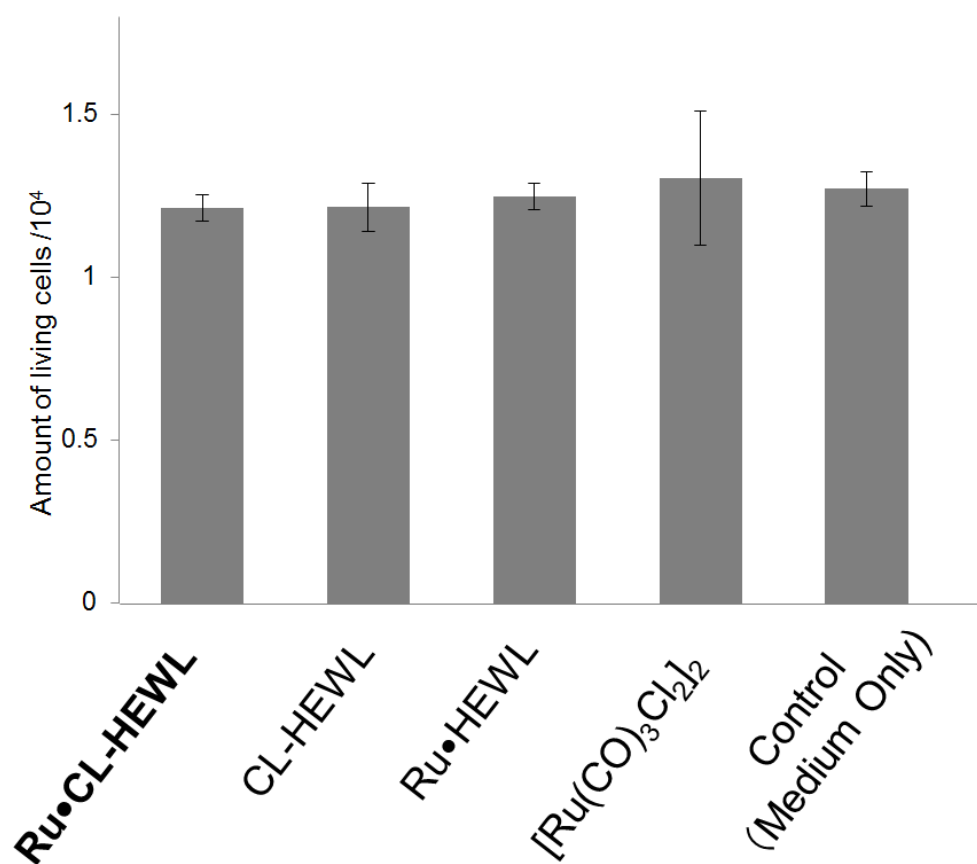


Figure 6. Effects on cell viability of each sample. The viability was evaluated by cell populations after incubation for 24 hours. * $P < 0.05$ vs. control. Each experiment was performed three times in triplicate and the data represent mean $\pm$ SEM.

Conclusions

In summary, a CO-releasing extracellular scaffold was successfully constructed by immobilizing Ru carbonyls in cross-linked porous HEWL crystals. The CO-releasing reaction was achieved by immobilization of $[cis-Ru(CO)_2X_4]^{2-}$ moieties preserved only within **Ru•CL-HEWL**. We used **Ru•CL-HEWL** as a CO-releasing extracellular scaffold to induce the activation of NF- κ B. This approach represents a method for obtaining a bioactive organometallic complex using a specific environment in porous protein crystals. Since organometallic complexes are used in biofunctions such as gas release, and in applications such as development of metallodrugs and bioimaging, it is expected that this method will provide a useful tool for molecular biology research using organometallic complexes.

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 were used without further purification. The expression and purification of sperm whale myoglobin were performed according to reported procedure.⁶⁰

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. CL-HEWL crystals (150 mg) were soaked in 100 mM acetate buffer (pH 7.0, 1.0 mL) containing 5.0 mM $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ and 1.0 M NaCl at room temperature for 24 hours 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 hours with $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (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** were 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 ultra-sonication 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, S in **Ru•CL-HEWL** were determined using a Rigaku ZSX-100S energy dispersive X-ray fluorescence spectrometer.

ICP-OES measurement. Metal concentrations of the buffer solutions were determined using an inductively coupled plasma optical emission spectrometer (Shimadzu ICPE-9000). $\text{Ru}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was used as calibration standard and $\text{Y}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was applied for an internal standard.

Crystal Structure Analysis. T-HEWLs were prepared by the hanging drop vapor diffusion method according to the reported procedure.⁶² T-HEWL was soaked in 100 mM acetate buffer (pH 7.0) containing 5.0 mM $[\text{Ru}(\text{benzene})\text{Cl}_2]_2$ and 1.0 M NaCl for 1 day to obtain the crystals containing Ru complex (**Ru•CL-HEWL**). For the snapshot analysis of CO release, crystals were immersed in PBS buffer (pH 7.4) containing 35.0 μM myoglobin and 10.0 mM $\text{Na}_2\text{S}_2\text{O}_4$ for 2 hours. 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 datasets 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. The data were processed with the program *HKL2000*.⁶³ The crystal parameters and the data collection statistics are summarized in Table S1. The structures were solved by molecular replacement with *MOLREP*⁶⁴ using HEWL structures (PDB ID: 193L). Refinement of the protein structure was performed using *REFMAC5*⁶⁵ in the *CCP4* suite.⁶⁶ Rebuilding was performed using *COOT*⁶⁷ based on sigma-weighted ($2F_o - F_c$) and ($F_o - F_c$) electron density maps. After rigid-body refinement, the 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 cut-off of 3σ . The models were subjected to quality analysis during the various refinement stages with omit maps and *PROCHECK*.⁶⁸ The refinement statistics are summarized in Table S1. Atomic coordinates are deposited in the Protein Data Bank under accession numbers 4W94 and 4W96 for **Ru•CL-HEWL**, **Ru•CL-HEWL** after the Mb assay, respectively.

Myoglobin (Mb) assay. The Mb assay was performed as previously reported with slight modification.^{21, 61} 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 hours 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 nm 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.²¹ The experiments were performed in triplicate and the data represent mean $\pm$ SEM. The calculated dataset was fitted to first-order kinetics by using Excel and Solver.⁶¹ For the control assays, $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ or Ru•HEWL (Ru = 0.025 μmol) were dissolved in PBS buffer (pH 7.4) at 25 °C for 1 hour in the presence of myoglobin (35.0 μM), and sodium dithionite (10.0 mM).

HEK293/ κ B-Fluc cell experiment. κ B-Fluc reporter-transfected human embryonic kidney 293 (HEK293/ κ B-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 hours in 5% CO_2 incubator at 37°C. Then, 15 μL of **Ru•CL-HEWL** suspension (2.0×10^5 crystals/well, the total amount of Ru is 0.50×10^{-3} μmol) or 15 μL of a PBS buffer solution containing Ru•HEWL, $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (the total amount of Ru is 0.50×10^{-3} μmol μM) was added to each well and the cells were cultured for 1 hour in an incubator under 5% CO_2 at 37°C. Then, 5.0 μL of 12 $\mu\text{g/mL}$ TNF- α solution was added to each well with additional culture for 23 hours. 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. The experiments were performed in triplicate and the data represent mean $\pm$ SEM. Statistical analyses were carried out with a Student's t-test. Values of $P < 0.05$ were considered statistically significant.

References

1. C. M. Niemeyer and C. A. Mirkin, *Nanobiotechnology: Concepts, Applications and Perspectives*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2005.
2. D. M. Vriezema, M. C. Aragoes, J. A. A. W. Elemans, J. J. L. M. Cornelissen, A. E. Rowan and R. J. M. Nolte, *Chem. Rev.*, 2005, **105**, 1445-1489.
3. M. Uchida, M. T. Klem, M. Allen, P. Suci, M. Flenniken, E. Gillitzer, Z. Varpness, L. O. Liepold, M. Young and T. Douglas, *Adv. Mater.*, 2007, **19**, 1025-1042.
4. S. Mann, *Nat. Mater.*, 2009, **8**, 781-792.
5. K. Oohora, S. Burazerovic, A. Onoda, Y. M. Wilson, T. R. Ward and T. Hayashi, *Angew. Chem. Int. Ed.*, 2012, **51**, 3818-3821.
6. R. Tang, C. S. Kim, D. J. Solfiell, S. Rana, R. Mout, E. M. Velazquez-Delgado, A. Chompoosor, Y. Jeong, B. Yan, Z. J. Zhu, C. Kim, J. A. Hardy and V. M. Rotello, *ACS Nano*, 2013, **7**, 6667-6673.
7. M. B. Dickerson, K. H. Sandhage and R. R. Naik, *Chem. Rev.*, 2008, **108**, 4935-4978.
8. M. Guli, E. M. Lambert, M. Li and S. Mann, *Angew. Chem. Int. Ed.*, 2010, **49**, 520-523.
9. E. N. Salgado, R. J. Radford and F. A. Tezcan, *Acc. Chem. Res.*, 2010, **43**, 661-672.
10. N. J. M. Sanghamitra and T. Ueno, *Chem. Commun.*, 2013, **49**, 4114-4126.
11. T. Ueno, *Chem. Eur. J.*, 2013, **19**, 9096-9102.
12. H. Wei, Z. Wang, J. Zhang, S. House, Y. G. Gao, L. Yang, H. Robinson, L. H. Tan, H. Xing, C. Hou, I. M. Robertson, J. M. Zuo and Y. Lu, *Nat. Nanotechnol.*, 2011, **6**, 93-97.
13. T. Ueno, S. Abe, N. Yokoi and Y. Watanabe, *Coord. Chem. Rev.*, 2007, **251**, 2717-2731.
14. T. Ueno, H. Tabe and Y. Tanaka, *Chem. Asian J.*, 2013, **8**, 1646-1660.
15. T. R. Ward, *Acc. Chem. Res.*, 2011, **44**, 47-57.
16. M. E. Wilson and G. M. Whitesides, *J. Am. Chem. Soc.*, 1978, **100**, 306-307.
17. T. Matsuo, C. Imai, T. Yoshida, T. Saito, T. Hayashi and S. Hirota, *Chem. Commun.*, 2012, **48**, 1662-1664.

18. T. Ueno and Y. Watanabe, *Coordination Chemistry in Protein Cages: Principles, Design, and Applications*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2013.
19. Y. Sano, A. Onoda and T. Hayashi, *Chem. Commun.*, 2011, **47**, 8229-8231.
20. R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann and C. J. Green, *Circ. Res.*, 2002, **90**, E17-E24.
21. U. Hasegawa, A. J. van der Vlies, E. Simeoni, C. Wandrey and J. A. Hubbell, *J. Am. Chem. Soc.*, 2010, **132**, 18273-18280.
22. R. Motterlini and L. E. Otterbein, *Nat. Rev. Drug Discov.*, 2010, **9**, 728-724.
23. B. E. Mann, *Organometallics*, 2012, **31**, 5728-5735.
24. C. C. Romao, W. A. Blattler, J. D. Seixas and G. J. L. Bernardes, *Chem. Soc. Rev.*, 2012, **41**, 3571-3583.
25. S. H. Heinemann, T. Hoshi, M. Westerhausen and A. Schiller, *Chem. Commun.*, 2014, **50**, 3644-3660.
26. K. Fujita, T. Tanaka, T. Sho, S. Ozeki, S. Abe, T. Hikage, T. Kuchimaru, S. Kizaka-Kondoh and T. Ueno, *J. Am. Chem. Soc.*, 2014, **136**, 16902-16908.
27. N. E. Bruckmann, M. Wahl, G. J. Reiss, M. Kohns, W. Watjen and P. C. Kunz, *Eur. J. Inorg. Chem.*, 2011, 4571-4577.
28. G. Dordelmann, H. Pfeiffer, A. Birkner and U. Schatzschneider, *Inorg. Chem.*, 2011, **50**, 4362-4367.
29. J. B. Matson, M. J. Webber, V. K. Tamboli, B. Weber and S. I. Stupp, *Soft Matter*, 2012, **8**, 6689-6692.
30. M. Ma, H. Noei, B. Mienert, J. Niesel, E. Bill, M. Muhler, R. A. Fischer, Y. Wang, S. U. and N. Metzler-Nolte, *Chem. Eur. J.*, 2013, **19**, 6785-6790.
31. M. A. Gonzales, H. Han, A. Moyes, A. Radinos, A. Hobbs, N. Coombs, S. R. J. Oliver and P. K. Mascharak, *J. Mater. Chem. B*, 2014, 2107-2113.
32. B. W. Matthews, *J. Mol. Biol.*, 1968, **33**, 491-497.
33. A. L. Margolin and M. A. Navia, *Angew. Chem. Int. Ed.*, 2001, **40**, 2204-2222.
34. Z. Hu and J. Jiang, *J. Phys. Chem. B*, 2009, **113**, 15851-15857.
35. T. Koshiyama, N. Kawaba, T. Hikage, M. Shirai, Y. Miura, C. Y. Huang, K. Tanaka, Y. Watanabe and T. Ueno, *Bioconjugate Chem*, 2010, **21**, 264-269.
36. H. Wei, Z. D. Wang, J. Zhang, S. House, Y. G. Gao, L. M. Yang, H. Robinson, L. H. Tan, H. Xing, C. J. Hou, I. M. Robertson, J. M. Zuo and Y. Lu, *Nat. Nanotechnol.*, 2011, **6**, 92-96.

37. S. Abe, M. Tsujimoto, K. Yoneda, M. Ohba, T. Hikage, M. Takano, S. Kitagawa and T. Ueno, *Small*, 2012, **8**, 1314-1319.
38. H. Wei and Y. Lu, *Chem.Asian J.*, 2012, **7**, 680-683.
39. R. A. Persichetti, N. L. Stclair, J. P. Griffith, M. A. Navia and A. L. Margolin, *J. Am. Chem. Soc.*, 1995, **117**, 2732-2737.
40. L. Z. Vilenchik, J. P. Griffith, N. St Clair, M. A. Navia and A. L. Margolin, *J. Am. Chem. Soc.*, 1998, **120**, 4290-4294.
41. M. Razavet, V. Artero, C. Cavazza, Y. Oudart, C. Lebrun, J. C. Fontecilla-Camps and M. Fontecave, *Chem. Commun.*, 2007, 2805-2807.
42. D. Valensin, P. Anzini, E. Gaggelli, N. Gaggelli, G. Tamasi, R. Cini, C. Gabbiani, E. Michelucci, L. Messori, H. Kozlowski and G. Valensin, *Inorg. Chem.*, 2010, **49**, 4720-4722.
43. T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romao and M. J. Romao, *J. Am. Chem. Soc.*, 2011, **133**, 1192-1195.
44. R. S. Herrick, C. J. Ziegler, J. Lopez, N. V. Barone, A. Gambella and C. Masi, *J. Organomet. Chem.*, 2012, **700**, 160-165.
45. M. F. A. Santos, J. D. Seixas, A. C. Coelho, A. Mukhopadhyay, P. M. Reis, M. J. Romao, C. C. Romao and T. Santos-Silva, *J. Inorg. Biochem.*, 2012, **117**, 285-291.
46. F. Zobi and B. Spingler, *Inorg. Chem.*, 2012, **51**, 1210-1212.
47. R. Colton and R. H. Farthing, *Aust. J. Chem.*, 1967, **20**, 1283-1286.
48. R. Colton and R. H. Farthing, *Aust. J. Chem.*, 1969, **22**, 2011-2012.
49. R. Colton and R. H. Farthing, *Aust. J. Chem.*, 1971, **24**, 903-909.
50. I. W. McNae, K. Fishburne, A. Habtemariam, T. M. Hunter, M. Melchart, F. Wang, M. D. Walkinshaw and P. J. Sadler, *Chem. Commun.*, 2004, 1786-1787.
51. T. Koshiyama, M. Shirai, T. Hikage, H. Tabe, K. Tanaka, S. Kitagawa and T. Ueno, *Angew. Chem. Int. Ed.*, 2011, **50**, 4849-4852.
52. <http://www.pymol.org/>.
53. S. McLean, B. E. Mann and R. K. Poole, *Anal. Biochem.*, 2012, **427**, 36-40.
54. A. Cvetkovic, C. Picioreanu, A. J. Straathof, R. Krishna and L. A. van der Wielen, *J. Phys. Chem. B*, 2005, **109**, 10561-10566.
55. A. Cvetkovic, C. Picioreanu, A. J. J. Straathof, R. Krishna and L. A. M. van der Wielent, *J. Am. Chem. Soc.*, 2005, **127**, 875-879.

56. H. S. Kim, P. A. Loughran, P. K. Kim, T. R. Billiar and B. S. Zuckerbraun, *Biochem. Biophys. Res. Commun.*, 2006, **344**, 1172-1178.
57. H. S. Kim, P. A. Loughran, J. Rao, T. R. Billiar and B. S. Zuckerbraun, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2008, **295**, G146-G152.
58. A. R. Brasier, J. E. Tate and J. F. Habener, *Biotechniques*, 1989, **7**, 1116-1122.
59. S. Chlopicki, M. Lomnicka, A. Fedorowicz, E. Grochal, K. Kramkowski, A. Mogielnicki, W. Buczko and R. Motterlini, *N.-S. Arch. Pharmacol.*, 2012, **385**, 641-650.
60. B. A. Springer and S. G. Sligar, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 8961-8965.
61. L. Hewison, S. H. Crook, B. E. Mann, A. J. H. M. Meijer, H. Adams, P. Sawle and R. A. Motterlini, *Organometallics*, 2012, **31**, 5823-5834.
62. M. C. Vaney, S. Maignan, M. RiesKautt and A. Ducruix, *Acta Crystallogr. D*, 1996, **52**, 505-517.
63. Z. Otwinowski and W. Minor, *Method Enzymol.*, 1997, **276**, 307-326.
64. A. Vagin and A. Teplyakov, *J. Appl. Crystallogr.*, 1997, **30**, 1022-1025.
65. A. A. Vagin, R. A. Steiner, A. A. Lebedev, L. Potterton, S. McNicholas, F. Long and G. N. Murshudov, *Acta Crystallogr. D*, 2004, **60**, 2184-2195.
66. E. Potterton, P. Briggs, M. Turkenburg and E. Dodson, *Acta Crystallogr. D*, 2003, **59**, 1131-1137.
67. P. Emsley and K. Cowtan, *Acta Crystallogr. D*, 2004, **60**, 2126-2132.
68. R. A. Laskowski, M. W. Macarthur, D. S. Moss and J. M. Thornton, *J. Appl. Crystallogr.*, 1993, **26**, 283-291.

Chapter 3

Design of a CO-releasing Extracellular Scaffold using *in-vivo* Protein Crystals

Abstract

Crystalline protein assemblies of cypovirus polyhedra were engineered to develop carbon monoxide (CO) releasing extracellular scaffolds by immobilizing ruthenium carbonyls. The molecular design of PhC includes introduction of a hexahistidine moiety (His-tag) to the C-terminus and provides immobilization of about 2-fold more Ru carbonyls per protein monomer compared to wild-type PhC. His-tagged PhC with Ru carbonyls effectively releases three times more CO for activation of nuclear factor kappa B (NF- κ B) in living cells relative to wild-type PhC.

Introduction

Significant efforts are underway to design hybrid materials of proteins and metal complexes to obtain systems for drug delivery or artificial metalloenzymes in the fields of bioinorganic chemistry and protein engineering.¹⁻³ Protein assemblies are a member of highly organized biomolecules which have potential for use as bionanomaterials.⁴⁻⁶ Among them are protein crystals that are hierarchical protein assemblies in solid state.⁷ Protein crystals are an appropriate candidate for bionanomaterials which can immobilize metal ions, particles and complexes.⁸⁻¹³

The author discussed function of metal carbonyls for biological application as carbon monoxide (CO) releasing molecules (CORMs).¹⁴⁻²⁰ The bioactivity of CO gas is evident because CO gas exerts multiple effects in living cells, such as anti-inflammatory, anti-apoptotic and anti-proliferative activities. Thus, CO gas protects cells against hypoxia or injury. Although recently many CORMs are reported as metal carbonyls, there are only a few examples of CO-releasing bionanomaterials.²¹⁻²⁵ HEWL crystals serve as a carrier of metal carbonyls because they have porous structure and immobilize organometallic complexes inside the solvent channels.²⁶⁻²⁹ This suggests the potential of protein crystals for CO-releasing bionanomaterials by immobilizing CORMs. Moreover, fine tuning may be possible by making mutations to immobilize a large amount of CORMs.

Polyhedra crystals (PhC), which are produced in insect cells infected by cypovirus, are candidates to develop a bionanomaterial based on protein crystals.³⁰⁻³² PhCs have very high stability against acid, detergents and various solvents without a cross-linking treatment. PhC can be converted to extracellular scaffold that can deliver proteins into living cells. This system is constructed by immobilizing exogenous proteins genetically fused with an H1 α -helix tag via co-expression in insect cells.^{33, 34} This suggests that PhC can be designed as an extracellular scaffold immobilizing and releasing other synthetic molecules using a suitable tag.

In this chapter the author demonstrates the design of a PhC mutant for immobilization of Ru carbonyls for control of CO releasing reactions (Figure 1a). PhC with a hexahistidine tag (His-tag) at the C-terminus (HTPhC), which is expected to be a metal immobilization site, was prepared in insect cells (Figure 1b). HTPhC with Ru carbonyls (**Ru•HTPhC**) immobilizes 2-fold more Ru carbonyls than polyhedra without His-tag (wild-type PhC, WTPHC). **Ru•HTPhC** was used as an extracellular scaffold for

delivery of CO gas into living cells to achieve the 6-fold higher activity of nuclear factor kappa B (NF- κ B) compared with **Ru•WTPhC**. These results indicate that PhC has potential for use in development of extracellular scaffolds for storage and release of biomolecules at biologically significant levels by immobilizing organometallic compounds.

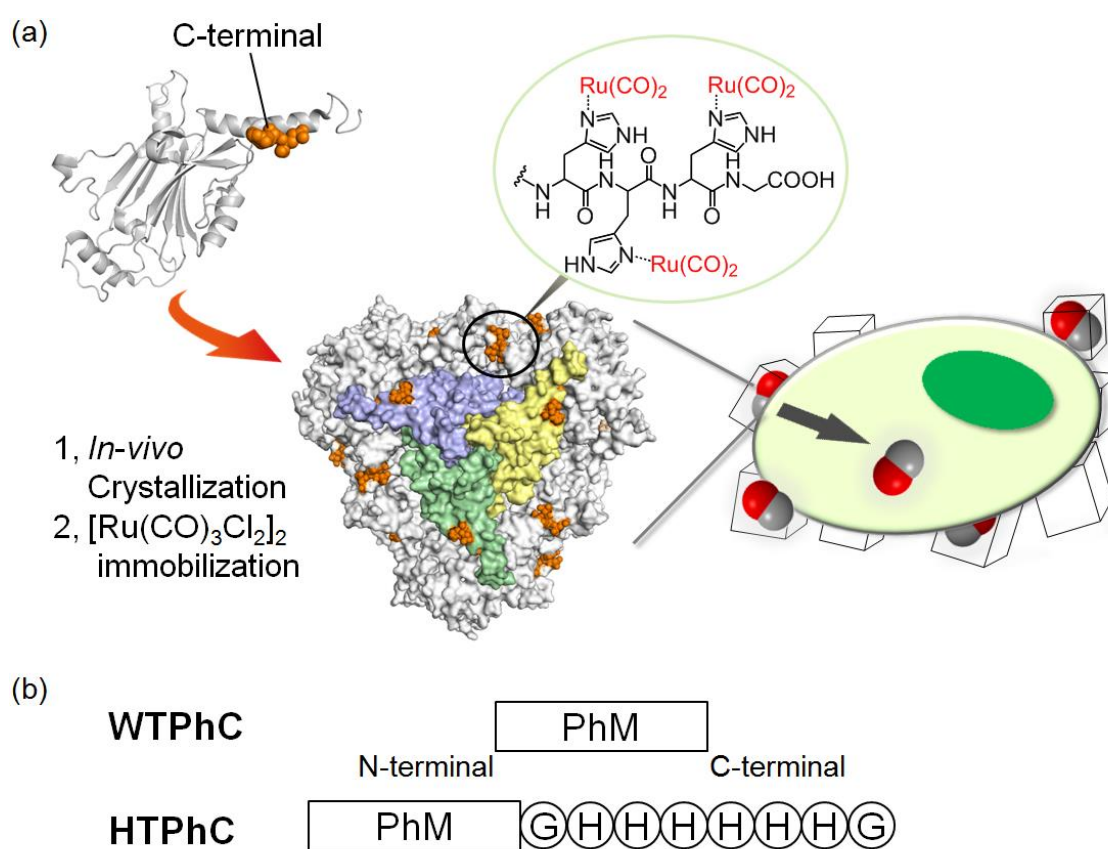


Figure 1. An addition of His-tag into C-terminal of PhM to form HTPhC and immobilization of ruthenium carbonyls (a) and the amino acid sequence of HTPhC (b).

Results and discussions

Preparation and characterization.

Hexahistidine tag (His-tag) was fused to a polyhedrin monomer (PhM) at the C-terminus of wild-type polyhedrin monomer that has nine original histidines. The fusion PhM was crystallized in insect cells and obtained as distorted crystals (HTPhC). The MALDI-TOF mass spectrum of the crystals showed a mass increment of 913 Da, which is assigned to His-tag moiety (calculated 937 Da), from wild-type PhC (WTPHC) (Figure 2). $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (CORM-2), known as a CO-releasing molecule, was immobilized in HTPhC by soaking in a solution of $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ to obtain **Ru•HTPhC**. The ratio of Ru to a PhM, as determined by X-ray fluorescence spectroscopy, was $12.3 \pm 1.6:1$ for **Ru•HTPhC**. Scanning transmission electron microscopy-energy dispersive X-ray spectrum (STEM-EDX) showed that the Ru atoms are fully dispersed in **Ru•HTPhC** (Figure 3a). The IR spectrum of **Ru•HTPhC** has five bands at 2,053, 2,026, 1,995, 1,979 and 1,945 cm^{-1} (Figure 4, solid line). Pairs of bands at 2,053 and 1,995 or 1,979 cm^{-1} are assigned to the CO stretching vibrations of a *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ moiety immobilized in proteins.²⁸ The split of the band into 1,995 cm^{-1} and 1,979 cm^{-1} indicates a loss of symmetry upon coordination to histidine residues at the different positions in PhM.²⁷ The other pair of bands at 2,026 and 1,945 cm^{-1} is expected to arise from the negatively charged $[\text{cis}-(\text{CO})_2\text{X}_4]^{2-}$ (X = anionic ligands) that are expected to coordinate to a carboxylate group of Asp or Glu in PhC.^{35, 36} **Ru•WTPHC** was prepared by the same procedure (Figure 3b, Figure 4 dashed line). The ratio of Ru per PhM was $6.6 \pm 1.8:1$ for **Ru•WTPHC**, which is almost half value of the ratio determined for **Ru•HTPhC**. For **Ru•HTPhC**, the relative peak intensity of *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ is larger than that of $[\text{cis}-(\text{CO})_2\text{X}_4]^{2-}$. This ratio opposite to that of **Ru•WTPHC** suggests that *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ are retained in **Ru•HTPhC** effectively at the His-tag fragment.

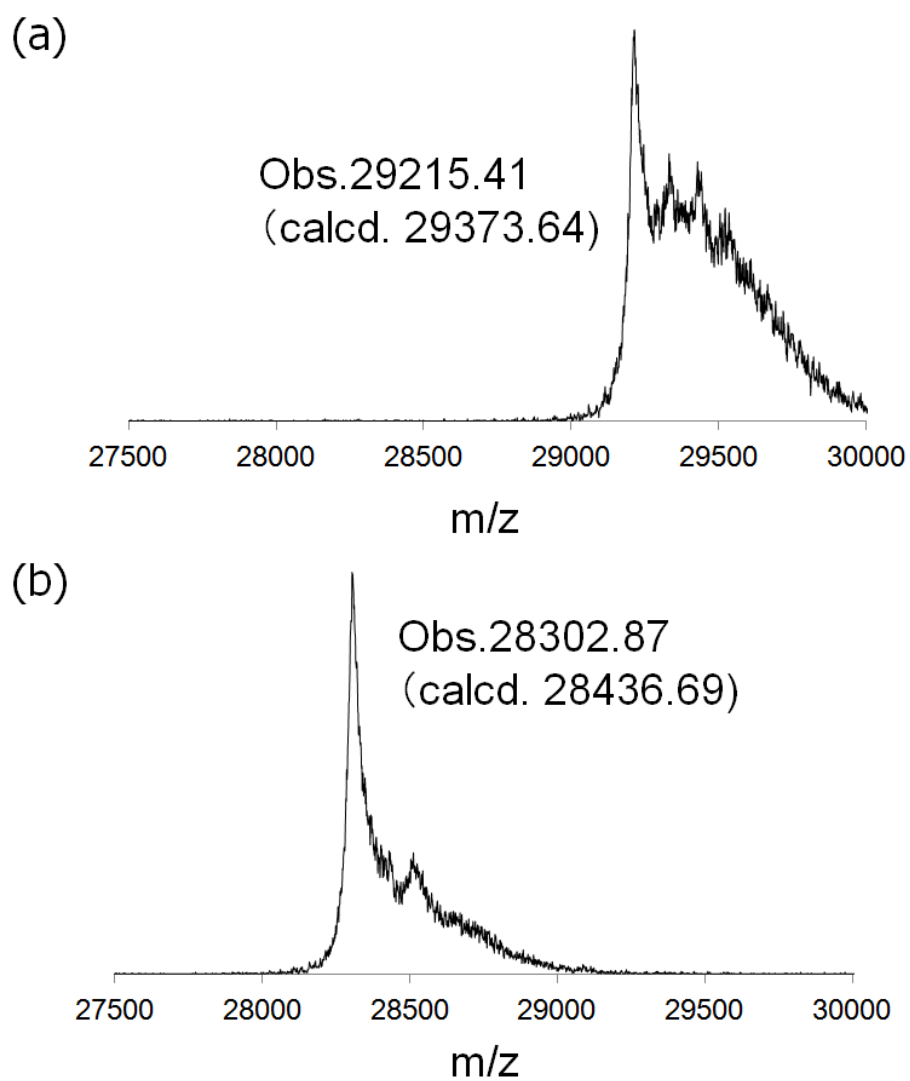


Figure 2. Characterization of HTPhC. MALDI-TOF mass spectra of HTPhC (a), and WTPhC (b).

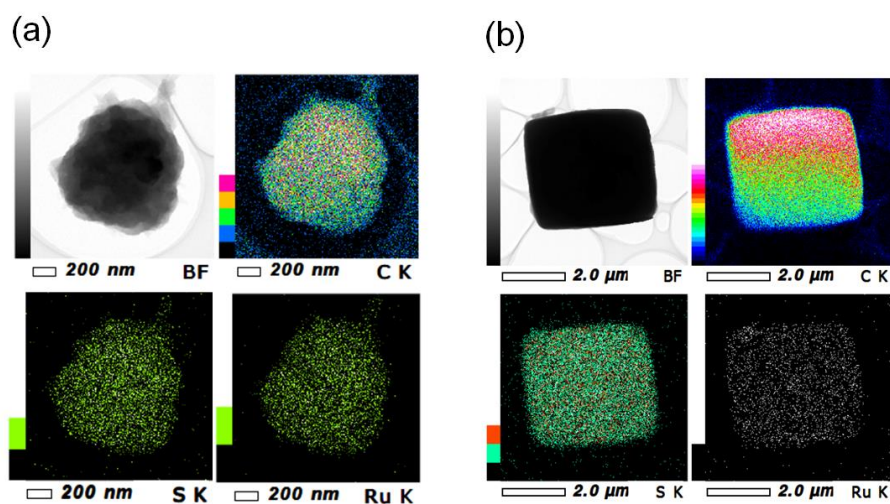


Figure 3. (a) STEM-EDX images of **Ru-HTPhC**. STEM Bright-field (BF) image (upper left), elemental mapping images of C-K (upper right), S-K (bottom left) and Ru-K (bottom right). (b) STEM-EDX images of **Ru-WTPPhC**. STEM BF image (upper left), elemental mapping images of C-K (upper right), S-K (bottom left) and Ru-K (bottom right).

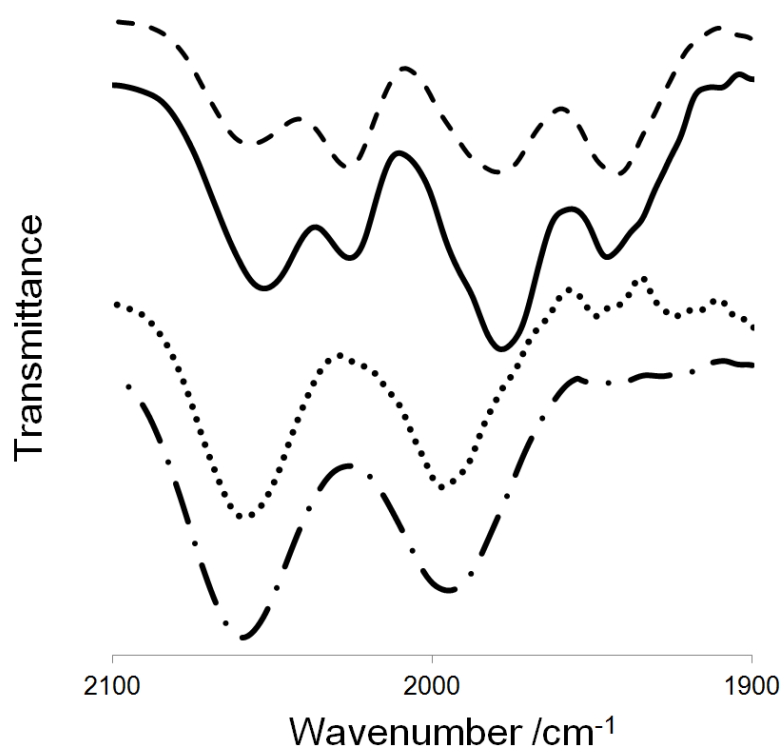


Figure 4. Infrared spectra of **Ru-HTPhC** (solid line), **Ru-WTPPhC** (dashed line), **Ru-HTPhC** after CO releasing in the Mb assay (chained line) and **Ru-WTPPhC** after CO releasing in the Mb assay (dotted line).

CO release assay.

The myoglobin (Mb) assay was carried out to investigate the CO-releasing functions of **Ru•HTPhC** and **Ru•WTPPhC** (Figure 5).^{14, 16} Ru carbonyls are known to release CO gas by ligand exchange reactions with nucleophiles or anions such as $\text{S}_2\text{O}_4^{2-}$ and then the nucleophiles coordinate with Ru centers.^{37, 38} The released amount of CO per PhM of **Ru•HTPhC** was found to be 6.2 ± 0.9 (Figure 5, solid line). The half-life ($t_{1/2}$) of the CO release was found to be 27.7 ± 1.6 min. The $t_{1/2}$ value is about 4-fold greater than that of $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (6.9 ± 2.8 min) obtained under the same assay conditions (Figure 5, dotted line). The amount of CO released per PhM of **Ru•WTPPhC** is about 1/3 of that of **Ru•HTPhC** (2.2 ± 0.1) while the $t_{1/2}$ is similar (26.8 ± 1.2 min) (Figure 5, dashed line). The number of Ru ions per PhM retained in **Ru•HTPhC** after the Mb assay is 11.7 ± 2.0 . This value is similar to the corresponding value determined before the assay (12.3 ± 1.6). The IR spectra of the composites after the Mb assay has peaks at $2,060$ and $1,995 \text{ cm}^{-1}$ (**Ru•HTPhC**, Figure 4, chained line), $2,059$ and $1,996 \text{ cm}^{-1}$ (**Ru•WTPPhC**, Figure 4, dotted line), respectively. These peaks are assigned to portions of the *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ moieties.²⁸ In both cases, four peaks assigned to $[\text{cis}-(\text{CO})_2\text{X}_4]^{2-}$ ($2,026$ and $1,945 \text{ cm}^{-1}$) and a portion of *cis*- $\text{Ru}(\text{CO})_2(\text{His})$ moiety ($2,053$ and $1,979 \text{ cm}^{-1}$) were disappeared. Thus, these coordination sites were used to release CO gas.

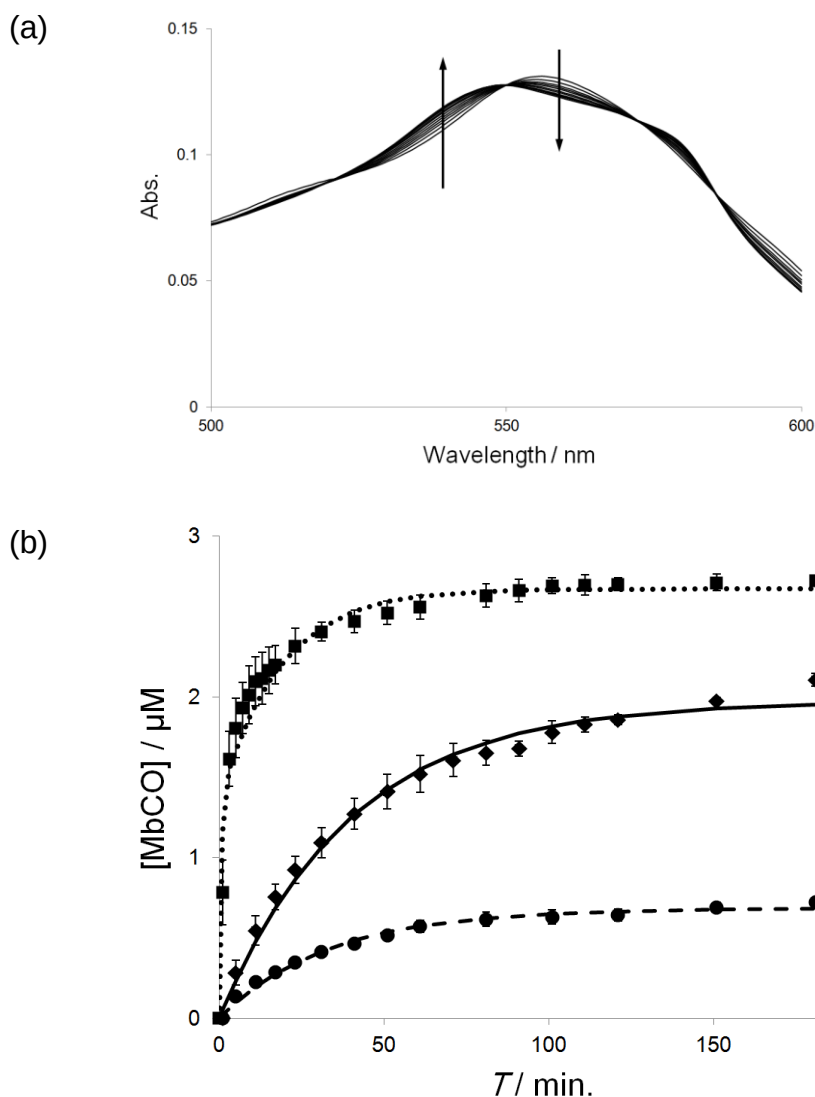


Figure 5. (a) The concentration of MbCO vs. time. UV/Vis spectra of Mb (8.0 μM) in 500 μL PBS buffer containing $\text{Na}_2\text{S}_2\text{O}_4$ (10 mM) was monitored at room temperature in the presence of **Ru•HTPhC** (solid line, 1.0×10^6 crystals, the Ru content determined by ICP-OES is 2.0×10^{-3} μmol), **Ru•WTPPhC** (dashed line, 1.0×10^6 crystals, the Ru content is 1.0×10^{-3} μmol) or $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (the Ru content is 2.0×10^{-3} μmol , dotted line), respectively. The experiments were performed three times and the data represent mean $\pm$ SEM. (b) Detection of CO release by monitoring the changes of the Q-band region of the UV/Vis spectra of Mb (8.0 μM) in the presence of **Ru•HTPhC** (2.0×10^5 crystals, the Ru content is

2.0×10^{-3} μmol) and $\text{Na}_2\text{S}_2\text{O}_4$ (10 mM) in 500 μL PBS buffer at 25°C . The arrows indicate the change in absorption intensity at 542 nm during the course of the assay ($t = 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160$ and 180 minutes).

Cellular assay.

Ru•HTPhC and **Ru•WTPPhC** were utilized as extracellular scaffolds to observe the activity of nuclear factor kappa-B (NF- κ B) in living cells. CO gas at a concentration of about 250 ppm is known to activate NF- κ B in the presence of tumor necrosis factor- α (TNF- α).^{39, 40} The activity of NF- κ B was evaluated using κ B-Fluc-transfected HEK293 (HEK293/ κ B-Fluc) cells cultured with **Ru•HTPhC** or **Ru•WTPPhC** and TNF- α .⁴¹ The assay was also carried out using HTPhC, WTPPhC or $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ solution. The activity of NF- κ B was estimated by the bioluminescence intensity of cells after a 16 hours incubation period (Figure 6). In the presence of **Ru•HTPhC**, a 24% increase of NF- κ B activity was observed compared with the assay with HTPhC. **Ru•WTPPhC** activated NF- κ B about 4% under the same conditions compared with that of WTPPhC. This suggests that three times more CO gas from **Ru•HTPhC** is available for effective activation of NF- κ B, as observed by the intracellular release of CO using ferritin mutants with various amounts of Ru carbonyls.²⁰ $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ showed less NF- κ B activation not only than **Ru•WTPPhC** but also than WTPPhC. The effect of WTPPhC will be clarified with further investigations. The cell viability was also evaluated with trypan blue and indicated that all composites are not cytotoxic (Figure 7).

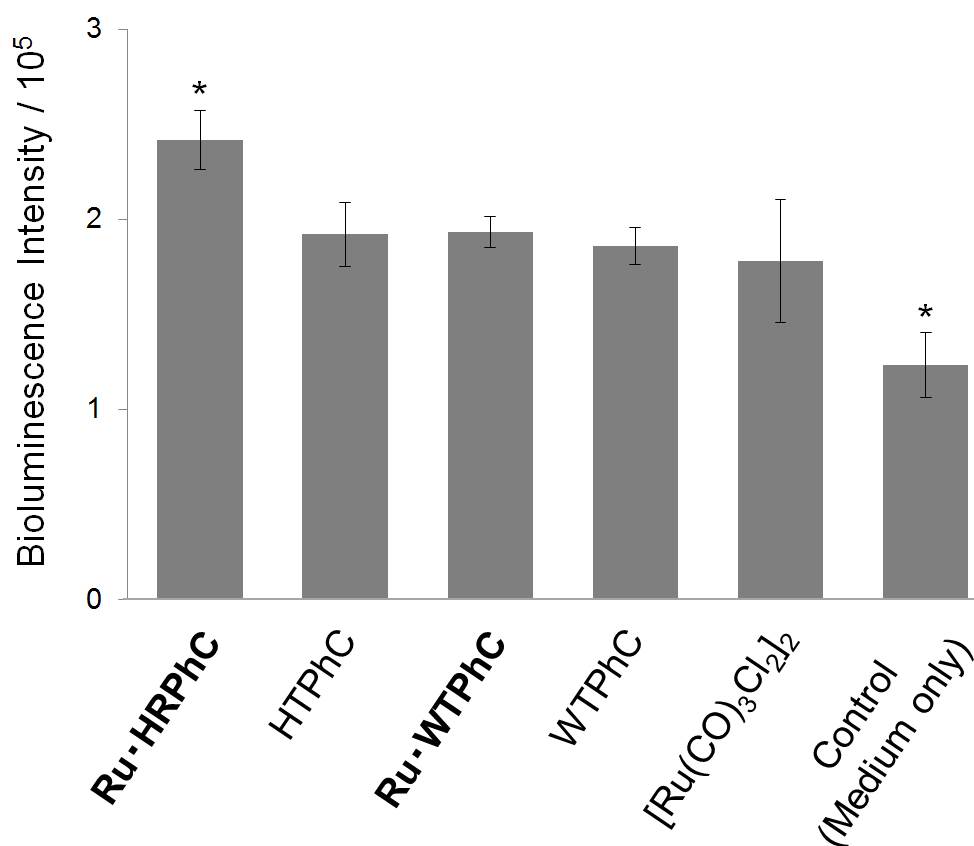


Figure 6. Bioluminescence intensity of the luciferase reporter assay. To evaluate NF- κ B activity, HEK293/ κ B-Fluc cells were incubated in the presence of 1.0 ng/mL TNF- α after incubation for 16 hours with **Ru•HTPhC** (2.0×10^5 crystals/well, the Ru content was 4.0×10^{-4} μ mol), HTPhC (2.0×10^5 crystals/well), **Ru•WTPPhC** (2.0×10^5 crystals/well, the amount of Ru ion is 2.0×10^{-4} μ mol), WTPPhC (2.0×10^5 crystals/well) and $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (the amount of Ru ion was 4.0×10^{-4} μ mol) compared with control (medium only). * $P < 0.05$ vs. control. The experiments were performed three times under each set of conditions. The data represent mean $\pm$ SEM.

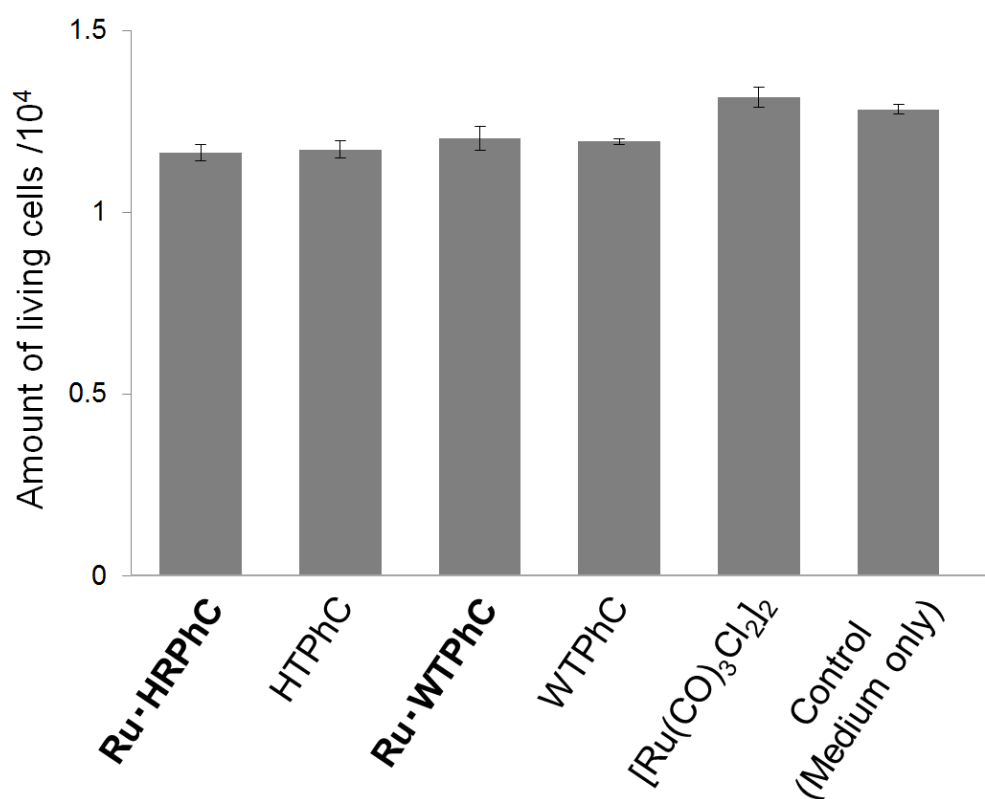


Figure 7. Effects on cell viability of each sample. Each sample was incubated with HEK293/κB-Fluc cells under 5% CO₂ at 37°C. The viability was evaluated by cell populations. Each experiment was performed in triplicate and the data represent mean ± SEM.

Conclusions

A method for constructing a CO-releasing extracellular scaffold by immobilizing Ru carbonyls in PhC was established. With the genetically-added His-tag, twice the amount of Ru carbonyl was immobilized in **Ru•HTPhC** compare to **Ru•WTPhC**. We used **Ru•HTPhC** as a CO-releasing extracellular scaffold to induce the activation of NF- κ B and achieved six-fold more effective activation compared to **Ru•WTPhC**. Recent research indicates the intracellular CO release from ferritin containing CORMs. Comparison of exogenous and endogenous CO release by extracellular scaffolds and the direct delivery system, respectively, provides a greater understanding of CO functions as a signal molecule. Based on our approach, the addition of functional groups to *in-vivo* protein crystals can be used to construct extracellular scaffolds containing foreign molecules. The immobilization of functional molecules in PhC is useful with the aim of controlling biological systems.

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 was performed as previously reported.⁴²

Preparation of WTPhC. The *Spodoptera frugiperda* PLB-Sf21-AE (Sf21) cell was maintained in tissue culture flasks in Grace's medium (Gibco-BRL) at 27°C with 10% fetal bovine serum (MP Biomedicals, Inc.), 2.6 mg/mL tryptose broth, 100 U/mL penicillin, and 100 µg/mL streptomycin.³⁰ Primers for site-directed mutagenesis were obtained from Gene Design Inc. PCR was performed using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The LR reaction was performed using the Gateway® LR Clonase® Enzyme mix (Life Technologies).

Preparation of HTPhC. The mutant plasmid DNAs of HTPhM were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).³³ To produce transfer vectors encoding HTPhC, the open reading frame cloned between attL1 and attL2 sites in the entry vectors was transferred to destination vectors (pDEST) via LR Clonase™ reactions. The resulting transfer vectors were cotransfected into Sf21 cells with linearized Baculogold Baculovirus DNA (BD Pharmingen), and replication-competent baculovirus was rescued by recombination between the transfer vector and the linearized viral DNA and ultimately harvested from the supernatant. Expression and purification of HTPhC were performed by the same procedures with WTPHC.

Immobilization of Ru carbonyls in PhC. HTPhC (7.8×10^7 crystals) were soaked in 10 mM HEPES buffer (pH 7.0, 500 µL) containing 5.0 mM $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (CORM-2) and 10 vol% dimethylformamide (DMF) at room temperature for 24 h to obtain **Ru•HTPhC**. After washing the crystals at least three times with distilled water, **Ru•HTPhC** was obtained as light yellow crystals. **Ru•WTPhC** was obtained according to the same procedure.

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

STEM-EDX measurement. High-resolution images of **Ru•HTPhC** and **Ru•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 **Ru•HTPhC**, a drop containing thin pieces of **Ru•HTPhC** was fixed on an electron microscope microgrid with Cu mesh. The locations of Ru, C, S in **Ru•HTPhC** were determined using a Rigaku ZSX-100S energy dispersive X-ray fluorescence spectrometer.

ICP-OES measurement. Metal concentrations of the buffer solutions were determined using an inductively coupled plasma optical emission spectrometer (Shimadzu ICPE-9000). $\text{Ru}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was used as calibration standard and $\text{Y}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was used as the internal standard.

Myoglobin (Mb) assay. The Mb assay was performed as previously reported with a slight modification.^{14, 16} For the CO release assays of **Ru•HTPhC**, **Ru•HTPhC** (1.0×10^6 crystals, the immobilized Ru ion determined by ICP-OES is 2.0×10^{-3} μmol) was dispersed in PBS buffer (pH 7.4) at 25 °C for 3 h in the presence of myoglobin (8.0 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 nm and 400 nm at 2 nm steps. Upon release of CO, the corresponding UV/Vis spectra showed the formation of the MbCO adduct with peaks at 542 nm and 578 nm. Quantification of CO release was calculated from the spectra. The concentration of MbCO was calculated as described previously. The experiments were performed in triplicate and the data represent mean $\pm$ SEM. The calculated dataset was fitted to first-order kinetics using Microsoft Excel and Solver.⁴³ The same assay was carried out using **Ru•WTPHC** (1.0×10^6 crystals, the immobilized Ru ion determined by ICP-OES is 1.0×10^{-3} μmol) and $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$; the concentration of Ru ion was 2.0×10^{-3} μmol).

HEK293/ κ B-Fluc cell experiment. κ B-Fluc reporter-transfected human embryonic kidney 293 (HEK293/ κ B-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 22 h under 5% CO_2 in an incubator at 37°C. 15.0 μL of **Ru•HTPhC** suspension was added

to each well and the cells were cultured for 1 h (with 2.0×10^5 crystals/well, the amount of Ru ion is 4.0×10^{-4} μmol). Then 5.0 μL of 24 $\mu\text{g/mL}$ TNF- α solution was added to each well to a final concentration of 1.0 ng/mL with additional culture for 15 h. The assay for HTPhC, **Ru•WTPhC** or WTPhC (with 2.0×10^5 crystals/well each) was carried out using the same procedure. An assay for $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (CORM-2), 15 μL of a PBS buffer solution containing $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (13.3 μM) with 0.04 % DMF was added to each well. The components of the luciferase assay kit (Promega ONE-GRO) were added to each well. Photoluminescence spectra were measured with a Promega GLOMAX MULTI+DETECTION SYSTEM. An assessment of cell viability was undertaken using a solution of 0.5% trypan blue stain (Nacalai Tesque). The quantity of live cells was determined after collection of cells from a 96-well plate. The experiments were performed in triplicate and the data represent mean $\pm$ SEM. Statistical analyses were carried out with a Student's t-test. Values of $P < 0.05$ were considered statistically significant.

References

1. M. B. Dickerson, K. H. Sandhage and R. R. Naik, *Chem. Rev.*, 2008, **108**, 4935-4978.
2. T. R. Ward, *Acc. Chem. Res.*, 2011, **44**, 47-57.
3. N. J. M. Sanghamitra and T. Ueno, *Chem. Commun.*, 2013, **49**, 4114-4126.
4. C. M. Niemeyer and C. A. Mirkin, *Nanobiotechnology: Concepts, Applications and Perspectives*, Wiley-VCH, Weinheim, 2005.
5. M. Uchida, M. T. Klem, M. Allen, P. Suci, M. Flenniken, E. Gillitzer, Z. Varpness, L. O. Liepold, M. Young and T. Douglas, *Adv. Mater.*, 2007, **19**, 1025-1042.
6. T. Ueno, H. Tabe and Y. Tanaka, *Chem. Asian J.*, 2013, **8**, 1646-1660.
7. T. Ueno, *Chem. Eur. J.*, 2013, **19**, 9096-9102.
8. M. Guli, E. M. Lambert, M. Li and S. Mann, *Angew. Chem. Int. Ed.*, 2010, **49**, 520-523.
9. H. Wei, Z. D. Wang, J. Zhang, S. House, Y. G. Gao, L. M. Yang, H. Robinson, L. H. Tan, H. Xing, C. J. Hou, I. M. Robertson, J. M. Zuo and Y. Lu, *Nat. Nanotechnol.*, 2011, **6**, 92-96.
10. T. Ueno, S. Abe, T. Koshiyama, T. Ohki, T. Hikage and Y. Watanabe, *Chem. Eur. J.*, 2010, **16**, 2730-2740.
11. T. Koshiyama, M. Shirai, T. Hikage, H. Tabe, K. Tanaka, S. Kitagawa and T. Ueno, *Angew. Chem. Int. Ed.*, 2011, **50**, 4849-4852.
12. S. Abe, M. Tsujimoto, K. Yoneda, M. Ohba, T. Hikage, M. Takano, S. Kitagawa and T. Ueno, *Small*, 2012, **8**, 1314-1319.
13. H. Tabe, S. Abe, T. Hikage, S. Kitagawa and T. Ueno, *Chem. Asian J.*, 2014, **9**, 1373-1378.
14. R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann and C. J. Green, *Circ. Res.*, 2002, **90**, E17-E24.
15. R. Motterlini and L. E. Otterbein, *Nat. Rev. Drug Discov.*, 2010, **9**, 728-U724.
16. U. Hasegawa, A. J. van der Vlies, E. Simeoni, C. Wandrey and J. A. Hubbell, *J. Am. Chem. Soc.*, 2010, **132**, 18273-18280.
17. B. E. Mann, *Organometallics*, 2012, **31**, 5728-5735.
18. C. C. Romao, W. A. Blattler, J. D. Seixas and G. J. L. Bernardes, *Chem. Soc. Rev.*, 2012, **41**, 3571-3583.

19. S. H. Heinemann, T. Hoshi, M. Westerhausen and A. Schiller, *Chem. Commun.*, 2014, **50**, 3644-3660.
20. K. Fujita, T. Tanaka, T. Sho, S. Ozeki, S. Abe, T. Hikage, T. Kuchimaru, S. Kizaka-Kondoh and T. Ueno, *J. Am. Chem. Soc.*, 2014, **136**, 16902–16908.
21. N. E. Bruckmann, M. Wahl, G. J. Reiss, M. Kohns, W. Watjen and P. C. Kunz, *Eur. J. Inorg. Chem.*, 2011, 4571-4577.
22. G. Dordelmann, H. Pfeiffer, A. Birkner and U. Schatzschneider, *Inorg. Chem.*, 2011, **50**, 4362-4367.
23. J. B. Matson, M. J. Webber, V. K. Tamboli, B. Weber and S. I. Stupp, *Soft Matter*, 2012, **8**, 6689-6692.
24. M. Ma, H. Noei, B. Mienert, J. Niesel, E. Bill, M. Muhler, R. A. Fischer, Y. Wang, S. U. and N. Metzler-Nolte, *Chem. Eur. J.*, 2013, **19**, 6785-6790.
25. M. A. Gonzales, H. Han, A. Moyes, A. Radinos, A. Hobbs, N. Coombs, S. R. J. Oliver and P. K. Mascharak, *J. Mater. Chem. B*, 2014, 2107-2113.
26. I. W. McNae, K. Fishburne, A. Habtemariam, T. M. Hunter, M. Melchart, F. Wang, M. D. Walkinshaw and P. J. Sadler, *Chem. Commun.*, 2004, 1786-1787.
27. M. Razavet, V. Artero, C. Cavazza, Y. Oudart, C. Lebrun, J. C. Fontecilla-Camps and M. Fontecave, *Chem. Commun.*, 2007, 2805-2807.
28. T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romao and M. J. Romao, *J. Am. Chem. Soc.*, 2011, **133**, 1192-1195.
29. M. F. A. Santos, J. D. Seixas, A. C. Coelho, A. Mukhopadhyay, P. M. Reis, M. J. Romao, C. C. Romao and T. Santos-Silva, *J. Inorg. Biochem.*, 2012, **117**, 285-291.
30. F. Coulibaly, E. Chiu, K. Ikeda, S. Gutmann, P. W. Haebel, C. Schulze-Bries, H. Mori and P. Metcalf, *Nature*, 2007, **446**, 97-101.
31. F. Coulibaly, E. Chiu, S. Gutmann, C. Rajendran, P. W. Haebel, K. Ikeda, H. Mori, V. K. Ward, C. Schulze-Bries and P. Metcalf, *Proc. Natl. Acad. Sci. USA*, 2009, **106**, 22205-22210.
32. L. Redecke, K. Nass, D. P. DePonte, T. A. White, D. Rehders, A. Barty, F. Stellato, M. N. Liang, T. R. M. Barends, S. Boutet, G. J. Williams, M. Messerschmidt, M. M. Seibert, A. Aquila, D. Arnlund, S. Bajt, T. Barth, M. J. Bogan, C. Caleman, T. C. Chao, R. B. Doak, H. Fleckenstein, M. Frank, R. Fromme, L. Galli, I. Grotjohann, M. S. Hunter, L. C. Johansson, S. Kassemeyer, G. Katona, R. A. Kirian, R. Koopmann, C. Kupitz, L. Lomb, A. V. Martin, S.

- Mogk, R. Neutze, R. L. Shoeman, J. Steinbrener, N. Timneanu, D. J. Wang, U. Weierstall, N. A. Zatsepin, J. C. H. Spence, P. Fromme, I. Schlichting, M. Duszhenko, C. Betzel and H. N. Chapman, *Science*, 2013, **339**, 227-230.
33. H. Ijiri, F. Coulibaly, G. Nishimura, D. Nakai, E. Chiu, C. Takenaka, K. Ikeda, H. Nakazawa, N. Hamada, E. Kotani, P. Metcalf, S. Kawamata and H. Mori, *Biomaterials*, 2009, **30**, 4297-4308.
 34. N. Nishishita, H. Ijiri, C. Takenaka, K. Kobayashi, K. Goto, E. Kotani, T. Itoh, H. Mori and S. Kawamata, *Biomaterials*, 2011, **32**, 3555-3563.
 35. B. F. G. Johnson, R. D. Johnston and J. Lewis, *J. Chem. Soc. A*, 1969, 792-&.
 36. R. Colton and R. H. Farthing, *Aust. J. Chem.*, 1971, **24**, 903-909.
 37. S. McLean, B. E. Mann and R. K. Poole, *Anal. Biochem.*, 2012, **427**, 36-40.
 38. S. Garcia-Gallego and G. J. L. Bernardes, *Angew. Chem. Int. Ed.*, 2014, **53**, 9712-9721.
 39. H. S. Kim, P. A. Loughran, P. K. Kim, T. R. Billiar and B. S. Zuckerbraun, *Biochem. Biophys. Res. Commun.*, 2006, **344**, 1172-1178.
 40. H. S. Kim, P. A. Loughran, J. Rao, T. R. Billiar and B. S. Zuckerbraun, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2008, **295**, G146-G152.
 41. A. R. Brasier, J. E. Tate and J. F. Habener, *Biotechniques*, 1989, **7**, 1116-1122.
 42. B. A. Springer and S. G. Sligar, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 8961-8965.
 43. L. Hewison, S. H. Crook, B. E. Mann, A. J. H. M. Meijer, H. Adams, P. Sawle and R. A. Motterlini, *Organometallics*, 2012, **31**, 5823-5834.

Chapter 4

Light-triggered CO releasing from an extracellular scaffold constructed by *in-vivo* protein crystals

Abstract

Carbon monoxide (CO)-releasing extracellular scaffolds have been successfully established by the immobilization of Ru carbonyls in the solvent channels of protein crystals although they spontaneously release CO and the rate cannot be altered. To overcome these limitations, molecular design toward a light-triggered CO releasing system is an interesting alternative. In this chapter, cypovirus polyhedra crystals (PhC) were engineered to CO releasing extracellular scaffolds by immobilizing manganese (Mn) carbonyls. *Fac*-Mn(CO)₃(H₂O)₂(His) moiety immobilized in PhC have a function of light-triggered CO releasing. The light irradiation contributes to release three-fold larger amount of CO per protein monomer to effectively activate nuclear factor kappa B (NF-κB) in living cells compared with a Mn carbonyls-incorporated PhC without light irradiation.

Introduction

As shown in the previous chapters, recently carbon monoxide (CO) has been well-established as a small signal molecule biological effector in living systems.^{1, 2} Metal carbonyl complexes, known as CO releasing molecules (CORMs) are promising compounds to achieve safe and controlled delivery of CO for therapeutic applications.³⁻⁶ CO releasing materials have achieved significant attention in the last several years for the purpose of extracellular scaffolds.⁷⁻¹¹ It is reported that the amount and rate of CO critically effect for controlling cellular functions. In the previous chapter, the author discussed the introduction of a hexahistidine tag to the C-terminus of cypovirus polyhedra crystals (PhC) to obtain HTPhC and the immobilization of about two-fold larger number of Ru carbonyls per protein monomer than that of wild type PhC (WTPhC). This composite effectively released three times larger number of CO than the composite of WTPhC to induce the activation of NF- κ B in living cells. This method represents that the control of CO amount delivered into living cells is essential for cellular functions. However, the CO release from Ru carbonyls is triggered by ligand exchange reactions with nucleophiles in aqueous medium.¹² Thus, CO release is spontaneously initiated and cannot be interrupted although immobilization of CORMs in protein crystals makes them stable. It shows that CO releasing from protein crystals with a specific timing and amount is still difficult. To overcome these limitations, molecular design for a light-induced CO releasing system is an interesting alternative.¹³⁻¹⁷ This method would provide more information of cellular events depending on signal molecules rather than a spontaneous CO releasing, *i.e.* a CO response at a specific time point in the cellular proliferation.

Manganese carbonyl complexes are known to release CO upon irradiation with a UV or visible light.¹³⁻¹⁷ Light-triggered CORMs, such as $\text{Mn}(\text{CO})_3(2,2'\text{-bipyridine})$ and $\text{Mn}(\text{CO})_3(\text{trispyrazolylmethane})$ are made from $\text{Mn}(\text{CO})_5\text{Br}$ and organic ligands.^{16, 17} These Mn complexes are stable in the dark and in the absence of nucleophiles that triggers ligand exchange reactions. Cavazza *et al.* reported a progress in immobilizing manganese tricarbonyls ($\text{Mn}(\text{CO})_3$) in HEWL crystals and analyzing their crystals structure although this compound have no CO-releasing function.¹⁸ This conjugation technique would provide an insight into a preparation of a light-triggered CO releasing bionanomaterial based on protein crystals. In this chapter, Mn carbonyls were immobilized in Polyhedra crystals (PhC), which are produced in insect cells infected by

cyopovirus. The immobilization of Mn carbonyls in PhC was supported by the extremely high stability of PhC against the immobilization reaction and the application as extracellular scaffolds.¹⁹⁻²¹ PhC with hexahistidine tag (His-tag) at the C-terminal of polyhedrin monomer (HTPhC), which has a high activity for immobilizing Ru carbonyls, were also used in this chapter. HTPhC involving Mn carbonyls (**Mn•HTPhC**) immobilizes larger number of Mn carbonyls compared with WTPHC involving Mn carbonyls (**Mn•WTPHC**). **Mn•HTPhC** is capable of an extracellular scaffold that can release CO gas into living cells to activate nuclear factor kappa B (NF-κB) with the light irradiation. These results indicate that researches on the cellular response to CO gas stress can be observed using a light-triggered CO releasing system at the various amount and rate of CO releasing profiles.

Results and Discussion

Preparation and Characterization.

PhC with a hexahistidine (His-tag) moiety (HTPhC) was obtained from Sf21 cells as shown in the previous chapter. $\text{Mn}(\text{CO})_5\text{Br}$ was immobilized in HTPhC by soaking HTPhC into buffer solution containing 1.0 mM $\text{Mn}(\text{CO})_5\text{Br}$ and 5.0 vol% acetonitrile (**Mn•HTPhC**). Quantitative elemental analysis of **Mn•HTPhC** by X-ray fluorescence showed that the number of Mn per PhM is 5.6 ± 1.2 . The Mn complexes are fully dispersed in **Mn•HTPhC** as revealed by a scanning transmission electron microscopy-energy dispersive X-ray spectrum (STEM-EDX). The IR spectrum of **Mn•HTPhC** has three bands at 1,913, 1,927 and 2,029 cm^{-1} . They are assigned to the CO stretching vibrations of *fac*- $\text{Mn}(\text{CO})_3(\text{H}_2\text{O})_2(\text{His})$ moiety in protein crystals.²² The split of bands (1,913 cm^{-1} and 1,927 cm^{-1}) indicates the loss of symmetry upon coordination to proteins.²² The composite were also prepared using WTPHC by the same procedure (**Mn•WTPHC**). The number of Mn per PhM in **Mn•WTPHC** (2.6 ± 0.3) was almost 1/2 of **Mn•HTPhC**. The IR spectrum and peak values were almost similar to that of **Mn•HTPhC**.

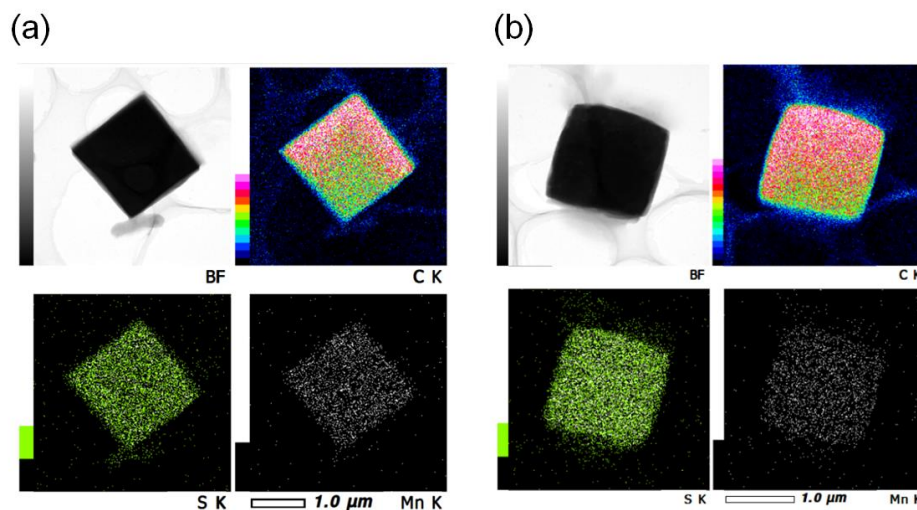


Figure 1. STEM-EDX images of **Mn•HTPhC** (a): STEM Bright-field (BF) image (upper left), elemental mapping images of C-K (upper right), S-K (bottom left) and Mn-K (bottom right). STEM-EDX images of **Mn•WTPHC** (b): STEM BF image (upper left), elemental mapping images of C-K (upper right), S-K (bottom left) and Mn-K (bottom right).

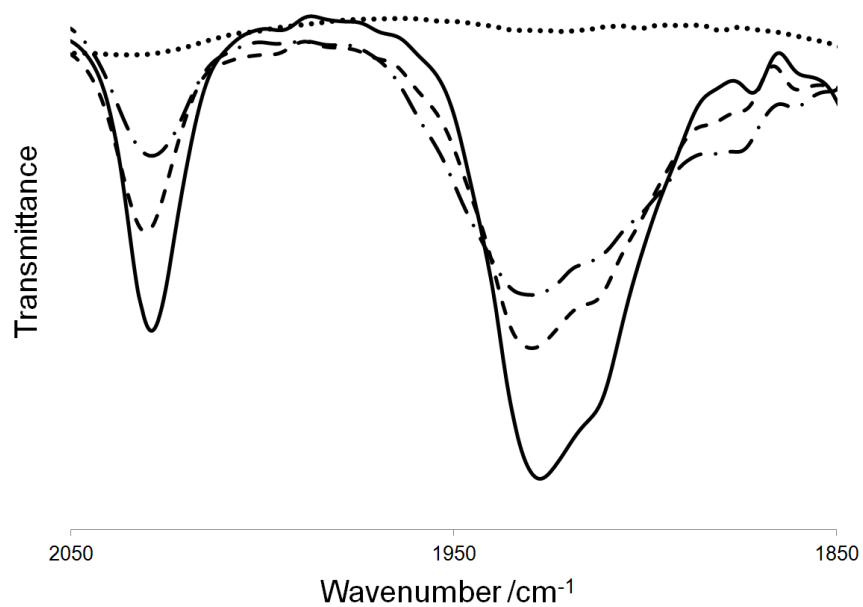


Figure 2. Infrared spectra: **Mn•HTPhC** (solid line), **Mn•WTPPhC** (dashed line), **Mn•HTPhC** after releasing CO in the Mb assay with a light irradiation (chained line) and without a light irradiation (dotted line).

CO release assay.

The CO-releasing property of **Mn•HTPhC** was evaluated using the myoglobin (Mb) assay.^{1, 3} **Mn•HTPhC** (3.0×10^6 crystals, the immobilized Mn ion determined by ICP-OES is 0.60×10^{-3} μmol) was dispersed in a PBS buffer containing myoglobin (5.4 μM) and sodium dithionite (10.0 mM). The reaction was accompanied with light irradiation at 465 nm for 1 hour using a blue LED light (15 W, RelyOn Ltd., Japan) positioned perpendicular to a glass vial at a distance of 5 cm (Figure 3). This solution was quickly transferred to a quartz cell at each measurement point and visible spectra were measured at room temperature (Figure 4, solid line). The released amount of CO per polyhedrin monomer (PhM) of **Mn•HTPhC** was 3.3 ± 0.4 with light irradiation. This value is about 2.8 times higher than that with **Mn•HTPhC** without light irradiation (1.2 ± 0.3) (Figure 4, dotted line). The half-life ($t_{1/2}$) of the CO release from **Mn•HTPhC** was found to be 19.3 ± 2.0 min and 28.4 ± 1.3 min (with and without the light irradiation, respectively). These results suggest that more CO molecules can be rapidly liberated by the light irradiation. In both case the $t_{1/2}$ value is much longer than that of $\text{Mn}(\text{CO})_5\text{Br}$ (less than 1 min) obtained under the same assay conditions (Figure 4, chained line). The released amount of CO per PhM of **Mn•WTPPhC** with light irradiation (1.8 ± 0.6) was almost a half of that of **Mn•HTPhC**. The $t_{1/2}$ (6.5 ± 1.3 min) is shorter than that of **Mn•HTPhC** (Figure 4, dashed line). The number of Mn ions per PhM retained in **Mn•HTPhC** after the Mb assay was 5.8 ± 1.0 , whose value is similar to the corresponding value before the assay (5.6 ± 1.2). The IR spectrum of **Mn•HTPhC** after the Mb assay with light irradiation have the no bands of CO stretching (Figure 2, dotted line). In case of **Mn•HTPhC** after the Mb assay without light irradiation, the bands at 1,911, 1,928 and 2,029 cm^{-1} (Figure 2, chained line), which are assigned to a part of *fac*- $\text{Mn}(\text{CO})_3(\text{H}_2\text{O})_2(\text{His})$ moieties.²²

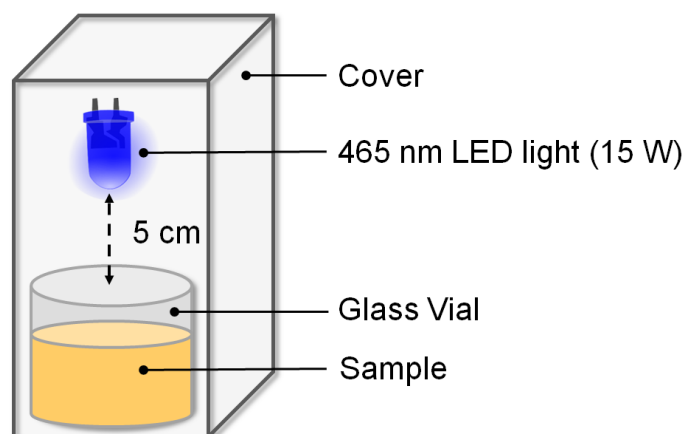


Figure 3. Detection of CO gas using the Mb assay with the photo-irradiation.

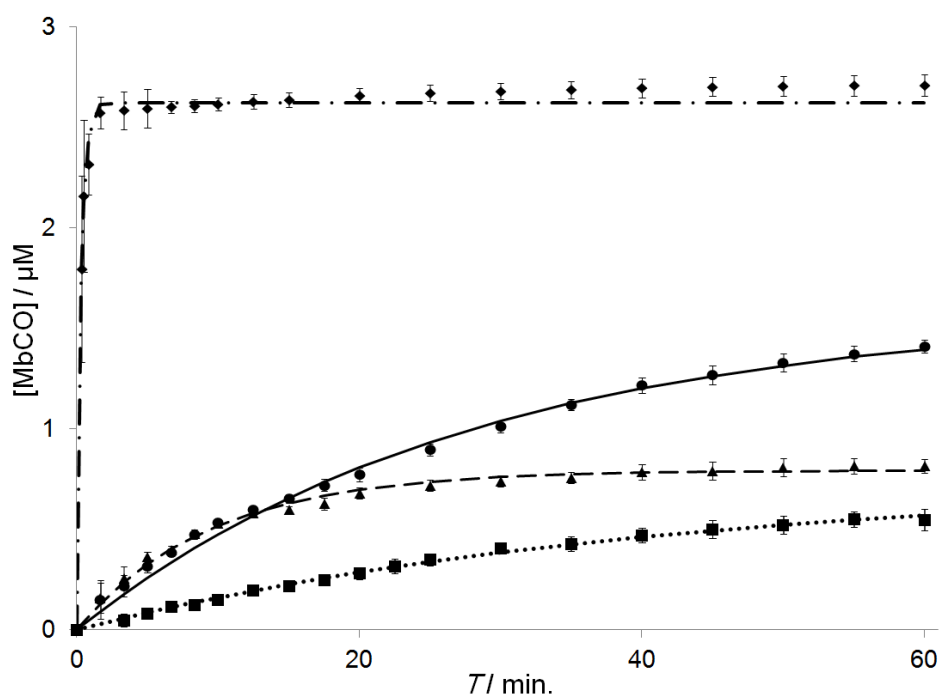


Figure 4. Detection of CO release by plotting the concentration of MbCO. UV/Vis spectra of Mb (5.4 μM) in 500 μL PBS buffer containing $\text{Na}_2\text{S}_2\text{O}_4$ (10 mM) was monitored at room temperature for **Mn•HTPhC** (solid line, 3.0×10^6 crystals, the amount of Mn determined by ICP-OES is 0.60×10^{-3} μmol) with and without light irradiation (solid line and dotted line, respectively). **Mn•WTPPhC** with light irradiation (dashed line, 3.0×10^6 crystals, the amount of Mn determined by ICP-OES is 0.21×10^{-3} μmol), and $\text{Mn}(\text{CO})_5\text{Br}$ with light irradiation (chained line, the amount of Mn is 0.60×10^{-3} μmol). The experiments were performed three times and the data represent mean $\pm$ SEM.

Cellular Assay.

Mn•HTPhC was applied as an extracellular scaffold to evaluate the activity of nuclear factor kappa-B (NF- κ B) by CO gas stress.^{23, 24} It is well known that the amount and the rate of CO induction into living cells are essential for the activation of NF- κ B.²⁵ Thus, the activity of NF- κ B is expected to be controlled by **Mn•HTPhC** in the presence or the absence of light irradiation.²⁶ The activity of κ B-Fluc transfected HEK293 (HEK293/ κ B-Fluc) cultured with **Mn•HTPhC** and TNF- α is assessed by the bioluminescence intensity of HEK293 cells after 16 hours incubation (Figure 5). The assay was also carried out using HTPPhC and Mn(CO)₅Br. In the presence of **Mn•HTPhC**, a 17% increase of NF- κ B activity was observed compared to the assay with HTPPhC by light irradiation (Figure 5a). **Mn•HTPhC** activated NF- κ B 9% under the same condition without light irradiation (Figure 5b). This effect is observed by the difference in the amount of CO gas released from **Mn•HTPhC** in these assays.²⁷ There is no activation effect to NF- κ B in the presence of HTPPhC and Mn(CO)₅Br (Figure 5a and 5b). Mn(CO)₅Br is expected to release CO before the cellular uptake because Mn(CO)₅Br have a fast CO-releasing function ($t_{1/2} < 1$ min). The cell viability test with the trypan blue showed no cytotoxicity of **Mn•HTPhC** as well as other composites (Figure 6).

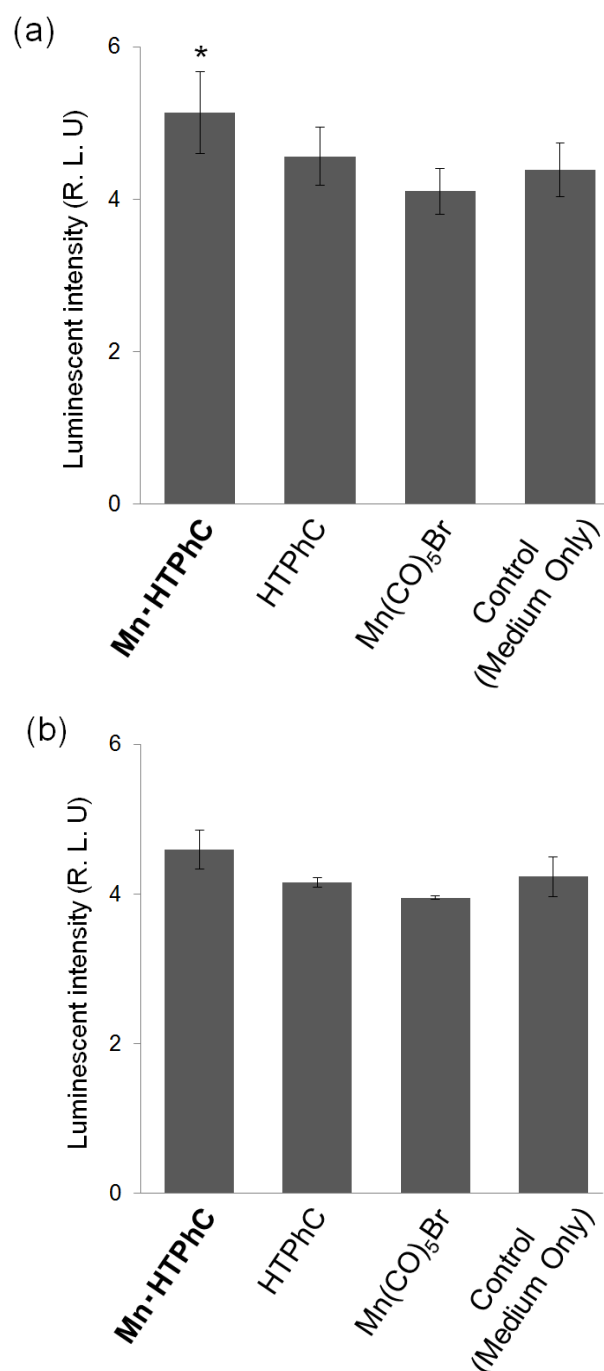


Figure 5. Luminescent intensity in the luciferase reporter assay for the evaluation of NF-κB activity of HEK293/κB-Fluc cells in the presence of 1.0 ng/mL TNF-α after incubation for 16 hours with **Mn•HTPhC**, HTPhC, and Mn(CO)₅Br and PBS buffer (control) with the light irradiation (a) and without the light irradiation (b). **P* < 0.05 vs. control. The experiments were performed three times under each set of conditions. The data represent mean ± SEM.

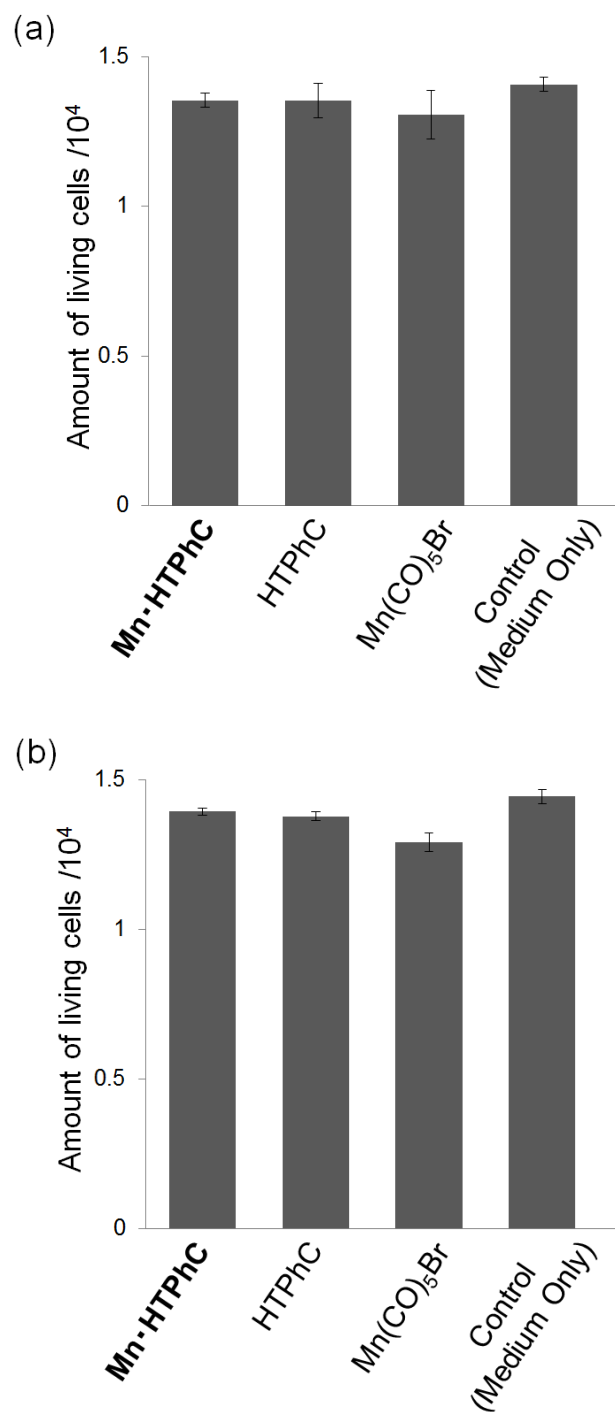


Figure 6. Effects on cell viability of each sample. Each sample was incubated with HEK293/kB-Fluc cells under 5% CO₂ at 37°C. The viability was evaluated by cell populations after incubation for total 16 hours. Each experiment was performed in triplicate and the data represent mean $\pm$ SEM.

Conclusion

To summarize, a light-triggered CO releasing extracellular scaffold by immobilizing $\text{Mn}(\text{CO})_5\text{Br}$ in PhC were successfully developed. About two times larger amount of CO gas was released from **Mn•HTPhC** with a light irradiation with a three-time extension of half-life time of CO releasing. We used **Mn•HTPhC** as a CO-releasing extracellular scaffolds to induce the activation of NF- κ B. The opposite tendency of activation/inactivation of NF- κ B was observed that would be depended on the rate and amount of CO releasing. This result would suggest that the quantitative analysis of cellular response vs biomolecules can be carried out using extracellular scaffolds constructed by PhC because previous research indicated that PhC is also useful for controlled protein releasing.^{28, 29} Co-encapsulation of various biomolecules from gases to proteins and releasing them at the same time would provide the information of their relationship for cellular functions.³⁰⁻³²

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.³³

Preparation of WTPhC. The *Spodoptera frugiperda* PLB-Sf21-AE (Sf21) cell was maintained in tissue culture flasks in Grace’s medium (Gibco-BRL) at 27°C with 10 % fetal bovine serum (MP Biomedicals. Inc.), 2.6 mg/mL tryptose broth, 100 U/mL penicillin, and 100 µg/mL streptomycin.¹⁹ Primers for site-directed mutation were ordered to Gene Design Inc. PCR was done by using QuickChange® Site-Directed Mutagenesis Kit (Stratagene). LR reaction was done by Gateway® LR Clonase® Enzyme mix (Life Technologies).

Preparation of HTPhC. The mutant plasmid DNAs of His-tagged polyhedron monomer was prepared using a QuikChange Site-Directed Mutagenesis Kit (Stratagene).²⁸ To produce transfer vectors encoding HTPhC, the open reading frame cloned between attL1 and attL2 sites in the entry vectors was transferred to destination vectors (pDEST) via LR Clonase™ reactions. The resulting transfer vectors were cotransfected into Sf21 cells with linearized Baculogold Baculovirus DNA (BD Pharmingen), and replication-competent baculovirus was rescued by recombination between transfer vector and the linearized viral DNA and ultimately harvested from the supernatant. Expression and purification of HTPhC were performed by same procedures with WTPHC.

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 $\text{Mn}(\text{CO})_5\text{Br}$ and 10 vol% acetonitrile (MeCN) at room temperature for 24 hours 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 procedure.

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.

ICP-OES measurement. Metal concentrations of the buffer solutions were determined using an inductively coupled plasma optical emission spectrometer (Shimadzu ICPE-9000). MnCl_2 in 1.0 M HCl_2 (1.03 g/L) was used as calibration standard and $\text{Y}(\text{NO}_3)_3$ in 0.1 M HNO_3 (1.03 g/L) was applied for an internal standard.

Myoglobin (Mb) assay. The Mb assay was performed as previously reported with the slight modification.^{1, 3} **Mn•HTPhC** (3.0×10^6 crystals, the immobilized Mn ion determined by ICP-OES is 0.60×10^{-3} μmol) was dispersed in PBS buffer (pH 7.4) at 25 °C for 1 hour in the presence of myoglobin (5.4 μM) and sodium dithionite (10.0 mM) under Ar atmosphere with light irradiation at 465 nm using a blue LED light (15 W, RelyOn Ltd., Japan) positioned perpendicular to the cuvette at a distance of 5 cm (Figure 3). This solution was quickly transferred to a quartz cell at each measurement point and visible spectra were measured at room temperature (Figure 4, straight line). This solution was quickly transferred to a quartz cell at each measurement point and visible spectra were measured at room temperature using a JASCO FP-8500 diode array spectrophotometer, measuring between 700 nm and 400 nm at 2 nm steps. Upon CO releasing, the corresponding UV/Vis spectra showed the evolution of the MbCO adduct 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.³⁴ The experiments were performed in triplicate and the data represent mean $\pm$ SEM. The calculated dataset was fitted to first-order kinetics by using Microsoft Excel and Solver.³⁴ The same assay was carried out using **Mn•WTPHC** (3.0×10^6 crystals, the immobilized Mn ion determined by ICP-OES is 0.21×10^{-3} μmol) and $\text{Mn}(\text{CO})_5\text{Br}$ (Mn ion is 0.60×10^{-3} μmol).

HEK293/ κ B-Fluc cell experiment. κ B-Fluc reporter-transfected human embryonic kidney 293 (HEK293/ κ B-Fluc) cells were seeded in a 96-well plate (1.0×10^4 cells/well in 100 μ L DMEM medium containing 5% heat-inactivated FBS) and incubated for 22 hours in 5% CO₂ incubator at 37°C. 15.0 μ L of **Mn•HTPhC** suspension (1.0×10^5 crystals/well, the amount of Mn ion is 2.0×10^{-5} μ mol) was added to each well and the cells were cultured for 1 h. Light irradiation was carried out for 30 min at 465 nm with a Blue LED desklight (15 W, RelyOn Ltd., Japan) positioned perpendicular to the plate at a distance of 10 cm for 1 hour. Then 5.0 μ L of 24 μ g/mL TNF- α solution was added to each well (final concentration: 1.0 ng/mL) with additional culture for 15 hours. The assay for HTPhC was carried out in the same procedure. An assay for Mn(CO)₅Br, 15 μ L of a PBS buffer solution containing Mn(CO)₅Br was added to each well. 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. The experiments were performed in triplicate and the data represent mean $\pm$ SEM. Statistical analyses were carried out with a Student's t-test. Values of $P < 0.05$ were considered statistically significant.

References

1. R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann and C. J. Green, *Circ. Res.*, 2002, **90**, E17-E24.
2. R. Motterlini and L. E. Otterbein, *Nat Rev Drug Discov*, 2010, **9**, 728-U724.
3. U. Hasegawa, A. J. van der Vlies, E. Simeoni, C. Wandrey and J. A. Hubbell, *J. Am. Chem. Soc.*, 2010, **132**, 18273-18280.
4. B. E. Mann, *Organometallics*, 2012, **31**, 5728-5735.
5. C. C. Romao, W. A. Blattler, J. D. Seixas and G. J. L. Bernardes, *Chem. Soc. Rev.*, 2012, **41**, 3571-3583.
6. S. H. Heinemann, T. Hoshi, M. Westerhausen and A. Schiller, *Chem. Commun.*, 2014, **50**, 3644-3660.
7. N. E. Bruckmann, M. Wahl, G. J. Reiss, M. Kohns, W. Watjen and P. C. Kunz, *Eur. J. Inorg. Chem.*, 2011, 4571-4577.
8. G. Dordelmann, H. Pfeiffer, A. Birkner and U. Schatzschneider, *Inorg. Chem.*, 2011, **50**, 4362-4367.
9. J. B. Matson, M. J. Webber, V. K. Tamboli, B. Weber and S. I. Stupp, *Soft Matter*, 2012, **8**, 6689-6692.
10. M. Ma, H. Noei, B. Mienert, J. Niesel, E. Bill, M. Muhler, R. A. Fischer, Y. Wang, S. U. and N. Metzler-Nolte, *Chem. Eur. J.*, 2013, **19**, 6785-6790.
11. M. A. Gonzales, H. Han, A. Moyes, A. Radinos, A. Hobbs, N. Coombs, S. R. J. Oliver and P. K. Mascharak, *J. Mater. Chem. B*, 2014, 2107-2113.
12. S. McLean, B. E. Mann and R. K. Poole, *Anal. Biochem.*, 2012, **427**, 36-40.
13. K. Splith, I. Neundorff, W. N. Hu, H. W. P. N'Dongo, V. Vasylyeva, K. Merz and U. Schatzschneider, *Dalton Trans.*, 2010, **39**, 2536-2545.
14. U. Schatzschneider, *Inorg. Chim. Acta.*, 2011, **374**, 19-23.
15. F. Mohr, J. Niesel, U. Schatzschneider and C. W. Lehmann, *Z. Anorg. Allg. Chem.*, 2012, **638**, 543-546.
16. R. D. Rimmer, A. E. Pierri and P. C. Ford, *Coord. Chem. Rev.*, 2012, **256**, 1509-1519.
17. J. S. Ward, J. M. Lynam, J. W. B. Moir, D. E. Sanin, A. P. Mountford and I. J. S. Fairlamb, *Dalton Trans.*, 2012, **41**, 10514-10517.
18. M. Razavet, V. Artero, C. Cavazza, Y. Oudart, C. Lebrun, J. C. Fontecilla-Camps and M. Fontecave, *Chem. Commun.*, 2007, 2805-2807.

19. F. Coulibaly, E. Chiu, K. Ikeda, S. Gutmann, P. W. Haebel, C. Schulze-Briese, H. Mori and P. Metcalf, *Nature*, 2007, **446**, 97-101.
20. F. Coulibaly, E. Chiu, S. Gutmann, C. Rajendran, P. W. Haebel, K. Ikeda, H. Mori, V. K. Ward, C. Schulze-Briese and P. Metcalf, *Proc. Natl. Acad. Sci. USA*, 2009, **106**, 22205-22210.
21. L. Redecke, K. Nass, D. P. DePonte, T. A. White, D. Rehders, A. Barty, F. Stellato, M. N. Liang, T. R. M. Barends, S. Boutet, G. J. Williams, M. Messerschmidt, M. M. Seibert, A. Aquila, D. Arnlund, S. Bajt, T. Barth, M. J. Bogan, C. Caleman, T. C. Chao, R. B. Doak, H. Fleckenstein, M. Frank, R. Fromme, L. Galli, I. Grotjohann, M. S. Hunter, L. C. Johansson, S. Kassemeyer, G. Katona, R. A. Kirian, R. Koopmann, C. Kupitz, L. Lomb, A. V. Martin, S. Mogk, R. Neutze, R. L. Shoeman, J. Steinbrener, N. Timneanu, D. J. Wang, U. Weierstall, N. A. Zatsepin, J. C. H. Spence, P. Fromme, I. Schlichting, M. Duszhenko, C. Betzel and H. N. Chapman, *Science*, 2013, **339**, 227-230.
22. T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romao and M. J. Romao, *J. Am. Chem. Soc.*, 2011, **133**, 1192-1195.
23. H. S. Kim, P. A. Loughran, P. K. Kim, T. R. Billiar and B. S. Zuckerbraun, *Biochem. Biophys. Res. Commun.*, 2006, **344**, 1172-1178.
24. H. S. Kim, P. A. Loughran, J. Rao, T. R. Billiar and B. S. Zuckerbraun, *Am. J. Physiol. Gastrointestinal. Liver Physiol.*, 2008, **295**, G146-G152.
25. S. Chlopicki, M. Lomnicka, A. Fedorowicz, E. Grochal, K. Kramkowski, A. Mogielnicki, W. Buczek and R. Motterlini, *N.-S. Arch. Pharmacol.*, 2012, **385**, 641-650.
26. A. R. Brasier, J. E. Tate and J. F. Habener, *Biotechniques*, 1989, **7**, 1116-1122.
27. K. Fujita, T. Tanaka, T. Sho, S. Ozeki, S. Abe, T. Hikage, T. Kuchimaru, S. Kizaka-Kondoh and T. Ueno, *J. Am. Chem. Soc.*, 2014, **136**, 16902-16908.
28. H. Ijiri, F. Coulibaly, G. Nishimura, D. Nakai, E. Chiu, C. Takenaka, K. Ikeda, H. Nakazawa, N. Hamada, E. Kotani, P. Metcalf, S. Kawamata and H. Mori, *Biomaterials*, 2009, **30**, 4297-4308.
29. N. Nishishita, H. Ijiri, C. Takenaka, K. Kobayashi, K. Goto, E. Kotani, T. Itoh, H. Mori and S. Kawamata, *Biomaterials*, 2011, **32**, 3555-3563.
30. S. Brouard, L. E. Otterbein, J. Anrather, E. Tobiasch, F. H. Bach, A. M. K. Choi and M. P. Soares, *J. Exp. Med.*, 2000, **192**, 1015-1025.

31. L. E. Otterbein, F. H. Bach, J. Alam, M. Soares, H. T. Lu, M. Wysk, R. J. Davis, R. A. Flavell and A. M. K. Choi, *Nat. Med.*, 2000, **6**, 422-428.
32. D. Morse, S. E. Pischke, Z. H. Zhou, R. J. Davis, R. A. Flavell, T. Loop, S. L. Otterbein, L. E. Otterbein and A. M. K. Choi, *J. Biol. Chem.*, 2003, **278**, 36993-36998.
33. B. A. Springer and S. G. Sligar, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 8961-8965.
34. L. Hewison, S. H. Crook, B. E. Mann, A. J. H. M. Meijer, H. Adams, P. Sawle and R. A. Motterlini, *Organometallics*, 2012, **31**, 5823-5834.

Chapter 5

In-vivo Crystal Engineering for Reconstruction of Porous Protein Frameworks

Abstract

Porous protein crystals have been utilized as templates in the development of solid protein materials. The properties and reactivity of exogenous molecules are affected by the environments of solvent channels in porous protein crystals. Chemical characters of inorganic nanoparticles and organometallic complexes can be controlled when they are immobilized in cross-linked HEWL crystals in several crystal systems such as tetragonal and orthorhombic as shown in chapter 1. However, it is difficult to design porous environments without using different crystal systems. Here, the author describes a reconstitute of solvent channels in in-vivo protein crystals of cypovirus polyhedra (PhC). Amino acid residues located on the intermolecular contact region are engineered by replacement and deletion of the amino acid residues to enlarge solvent channels with retaining the original crystal lattice structures. The PhM mutants allow the crystalline assemblies to increase solvent contents from 19% to 24%, and to adsorb 16-fold larger amounts of organic fluorescent dyes than wild-type PhC (WTPhC) by increasing the diffusion pathway of the exogenous molecules in solvent channels in the lattice structure due to decreasing non-covalent intra- and inter-molecular interactions.

Introduction

Protein assemblies with highly ordered nano structures have attracted a great deal of interest for molecular templates in materials science and bionanotechnology.¹⁻⁴ Nano-sized chemical environments of protein assemblies such as cages, tubes and two-dimensional arrays can be modified with functional compounds for immobilization of metal complexes and organic molecules with catalytic, magnetic and optical properties. Recently, many researchers reported rational design of the interfaces between protein monomers in efforts to construct specific unique protein assemblies including large cages, two-dimensional layers and three-dimensional crystals.⁵⁻⁹

Protein crystals can be regarded as solid protein materials because they present highly ordered 3D arrangements of protein molecules with inner pores called solvent channels.^{10, 11} The porous environments are obtained by the lattice structures of protein subunits. Solvent channels are utilized as nanovessels to immobilize functional molecules by metal coordination and chemical conjugation for applications in preparation of inorganic materials, heterogeneous catalysis and drug delivery systems.¹²⁻¹⁵ Porous protein crystals can form various polymorphic structures by changing crystallization conditions, indicating that we can modulate the chemical environments of solvent channels using several crystal systems. For examples, the diffusion and adsorption of organic molecules, water, and ions in different crystal systems of hen egg white lysozyme (HEWL), tetragonal, orthorhombic, and monoclinic crystals, were experimentally and theoretically investigated.¹⁶⁻¹⁸ These observations suggest that solvent channels of protein crystal systems can affect the diffusion and adsorption of the small guest molecules due to different pore size, hydrophobicity and electrostatics. Magnetic properties of CoPt nanoparticles synthesized in the solvent channels of protein crystals were also controlled by using different crystal systems of HEWL, indicating that the properties of inorganic nanoparticles can be controlled by chemical environments of solvent channels with depending on crystal systems.¹⁵ However, it is difficult to reconstitute porous environments without using different crystal systems.

Recently, crystallization of proteins in living cell has been investigated in the fields of structural biology and nanotechnology.¹⁹⁻²¹ Cypovirus (cytoplasmic polyhedrosis virus, CPV) produces polyhedrin monomer (PhM) in the infected cell.^{22, 23} The crystalline assemblies of PhM known as polyhedra are produced in the replication

cycle to embed the viruses in the crystals. Polyhedra crystal (PhC) is extremely stable against concentrated urea, acid and detergents because PhM is densely interacted with neighboring molecules. The condense packing of PhM provides low solvent contents (19%) and no continuous porous structures.²² PhC can encapsulate exogenous proteins by co-crystallization in insect cells.²³

Here, the author demonstrates design of solvent channels that allow adsorption of organic molecules by replaced and deletion of amino acid residues located on the intermolecular contact region with retaining crystal lattice structures. The genetically engineered PhM allows the crystalline assemblies to increase solvent contents from 19 % to 24 %. These engineered PhC adsorb maximally 16-fold larger amounts of organic fluorescent dyes than wild-type PhC (WTPhC) using the diffusion pathway of the exogenous molecules in solvent channels due to decreasing non-covalent intra- and inter-molecular interactions. It is possible to construct porous architectures by replacement and deletion of amino acid residues located on the contact positions of building blocks.

Results and discussion

Preparation and X-ray crystal structure analysis.

The crystal structure of WTPhC shows that PhMs form PhTs to retain highly organized crystalline structures (Figure 1a, 1b).²² The dense packing of PhTs provides narrow solvent channels. If the contact between PhTs can be reduced with retaining the original crystal system of PhC, it is expected that porous structures are extended, and small organic compounds can be adsorbed within the PhC. The amino acid residues of Val191-His195 located at the contact interface between PhTs were interacted with neighboring PhT by hydrogen bonding networks (Figure 1d). To decrease the interactions at the interfaces between PhTs in the crystals, three mutants, Δ 1-PhC, Δ 2-PhC and Δ 3-PhC, in which amino acid residues in L4 loop located on the interface of PhTs were replaced or deleted, were prepared. Δ 1-PhC was prepared with deletion of Ala194 from the original PhM and replacement of Ser193 to Gly. Δ 2-PhC and Δ 3-PhC were prepared with deletion of Ser193-Ala194, and Gly192- Ala194 from the original PhM, respectively. All three mutants were successfully crystallized in insect Sf21 cells as well as WTPhC. The MALDI-TOF mass spectrum of the crystals showed mass decrement, which are assigned to deletion or replacement of amino acid residues (Figure 2).

The crystal structures of Δ 1-PhC, Δ 2-PhC and Δ 3-PhC were refined with the resolution of 1.93, 2.15, and 2.18 Å (Figure 3, table 1 and table 2). These structures have the same space group with WTPhC (*I*23), indicating that crystal lattice structures were retained after deletion of amino acid residues at intermolecular contact regions of PhTs. The Δ 1-PhC structure is identical to that of WTPhC with small root-mean-square deviations (rmsd) of 1.76 Å even after deletion of Ala194 in L4 loop. Matthew's coefficient and the solvent content of Δ 1-PhC is 1.52 Å³/Da and 19%, respectively, which are identical to those of WTPhC (1.52 Å³/Da and 19%).^{22, 24} These results suggest that deletion of Ala194 is not affected to retaining the original lattice structure, although several hydrogen bonding networks are broken compared to those of WTPhC (Figure 4a and 4b). The crystal structure of Δ 2-PhC shows that the structures of N-terminal (ACE1-Asn9), L2 and H3 (Leu68-Asn103), L3 (Asn128-Asp134), and L4 (Ser187-Tyr189) and hydrogen bonding networks could not be determined due to disordered density (Figure 3c and 4c). Matthew's coefficient and solvent content of Δ 2-PhC are 1.62 Å³/Da and 24%, respectively. As for Δ 3-PhC, we could not

determine the structure of N-terminal (ACE1-Ser8), L2 and H3 (Leu68-Asn103), L3 (Ala129-Asp134), and L4 (Pro186-Asn196) because the corresponding electron densities were disordered (Figure 3d and 4d). The crystal data of $\Delta 3$ -PhC exhibits the Matthew's coefficient ($1.60 \text{ \AA}^3/\text{Da}$) and solvent content (23%) are almost the same as those of $\Delta 2$ -PhC. These results indicate that the solvent contents of PhC are increased by the deletion of two or three residues of the L4 loop. In addition, the amino acid residues of N-terminal, L2, H3, L3, and L4 are expected to exhibit high flexibility in $\Delta 2$ -PhC and $\Delta 3$ -PhC. In fact, the observable amino acid residues in $\Delta 2$ -PhC and $\Delta 3$ -PhC show also high flexibility as high *B*-factors are observed in the crystals (Figure 3).

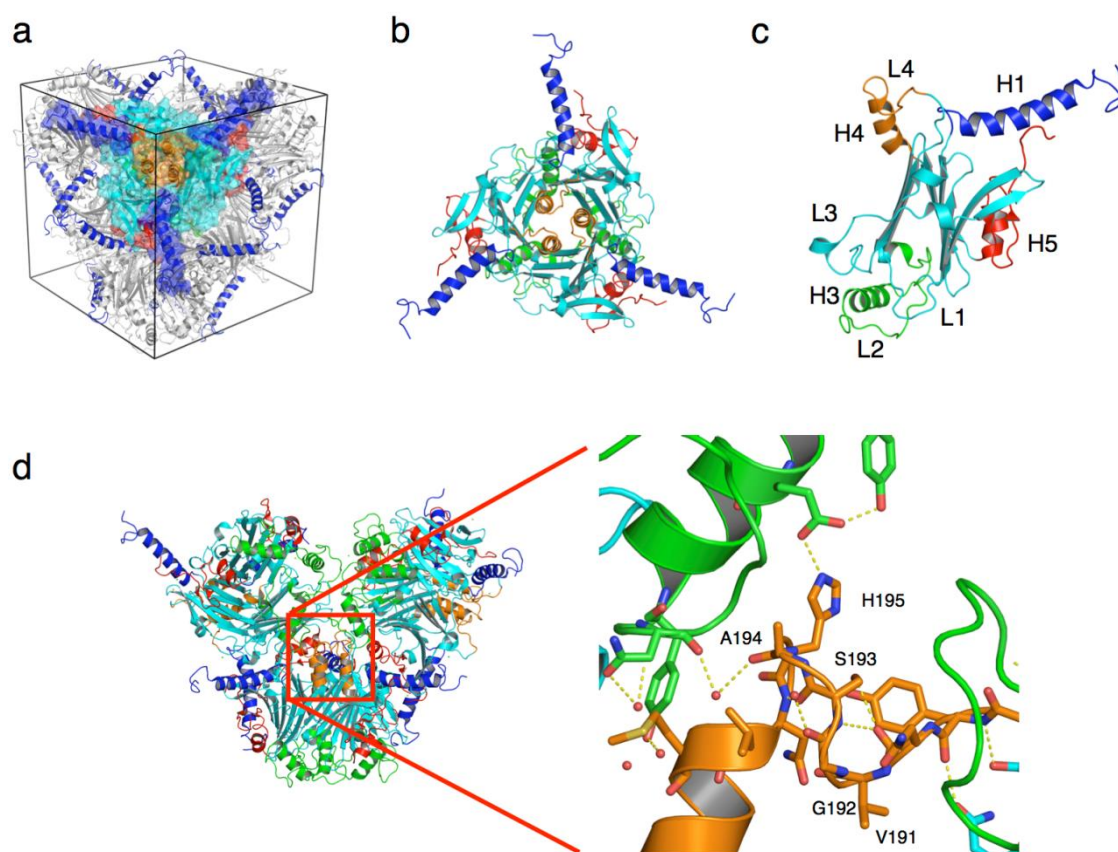


Figure 1. Crystal structure of WTPhC. Molecular assembling of PhT in a unit cell of WTPhC taken from PDB ID: 2OH6.²² One PhT in the unit cell is shown as a sphere (a). Crystal structure of PhT (b) and PhM (c). The intramolecular interactions in L4 loop and intermolecular interactions between PhMs (d).

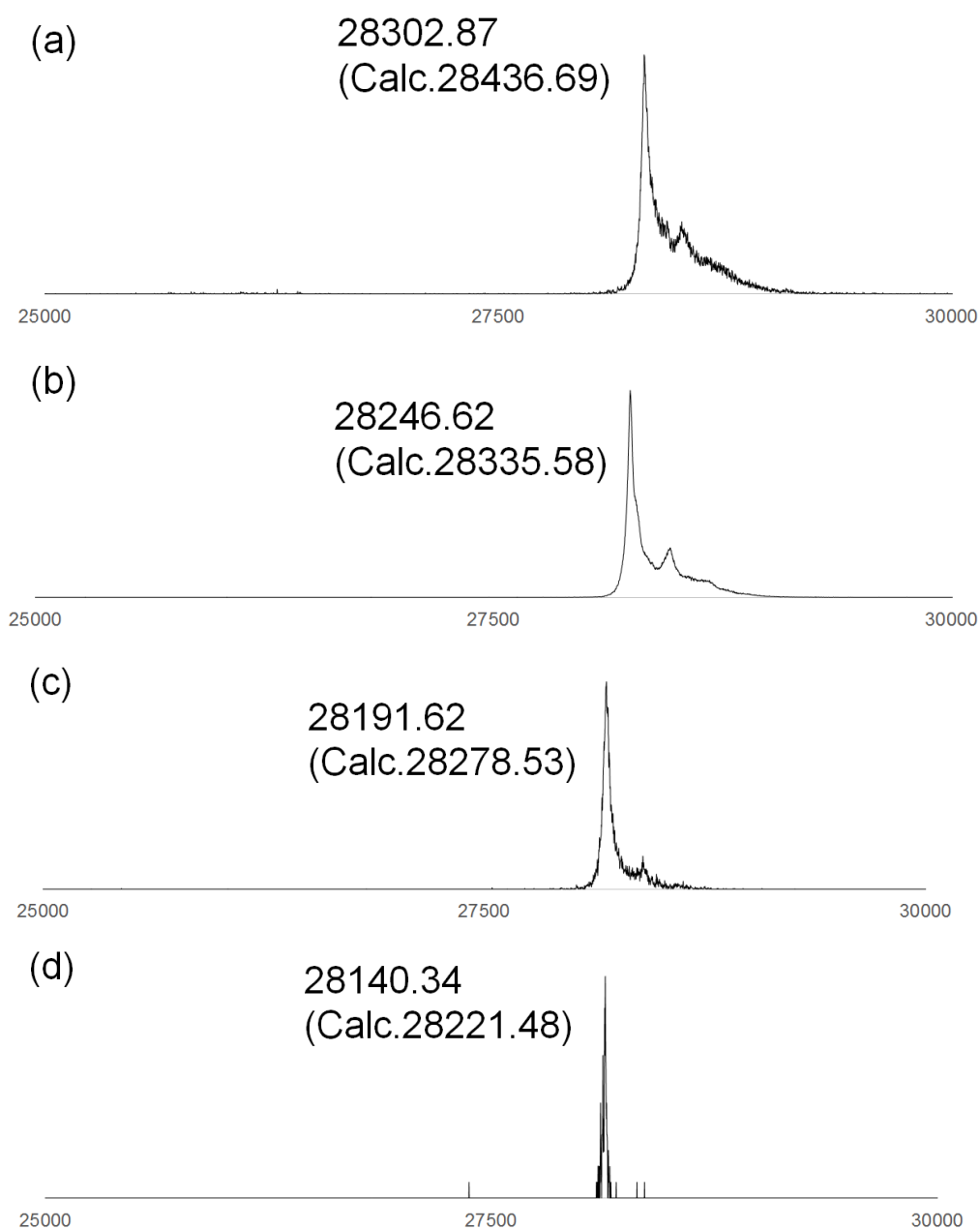


Figure 2. Characterization of HTPhC. MALDI-TOF mass spectra of WTPhC (a), $\Delta 1$ -PhC (b), $\Delta 2$ -PhC (b) and $\Delta 3$ -PhC (c).

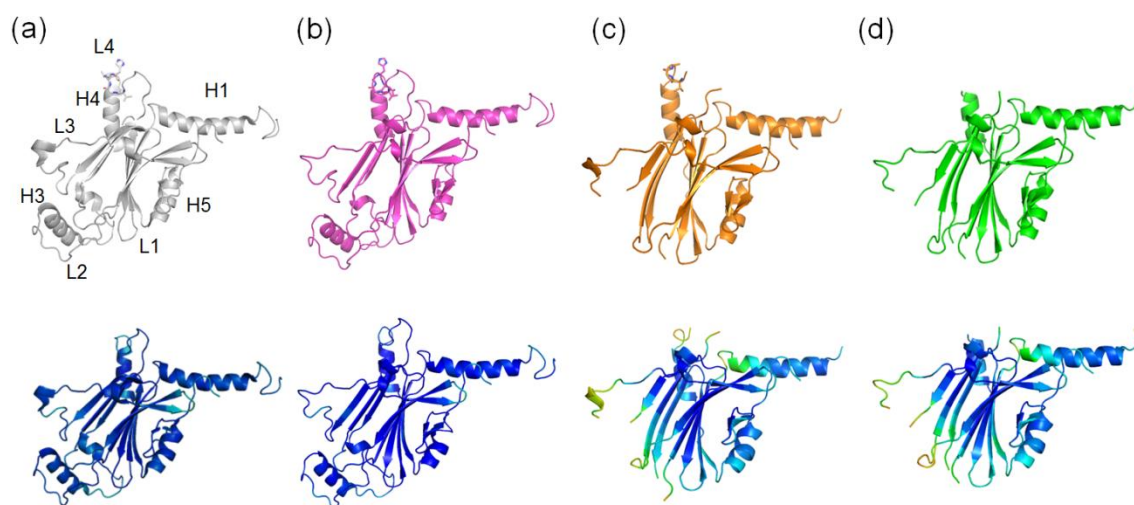


Figure 3. Crystal structures (top) and *B*-factor (bottom) of WTPhC (a), $\Delta 1$ -PhC (b), $\Delta 2$ -PhC (c) and $\Delta 3$ -PhC (d). The bottom figures are represented by a color spectrum extending from red to blue for large to small *B*-factor values of each atom.

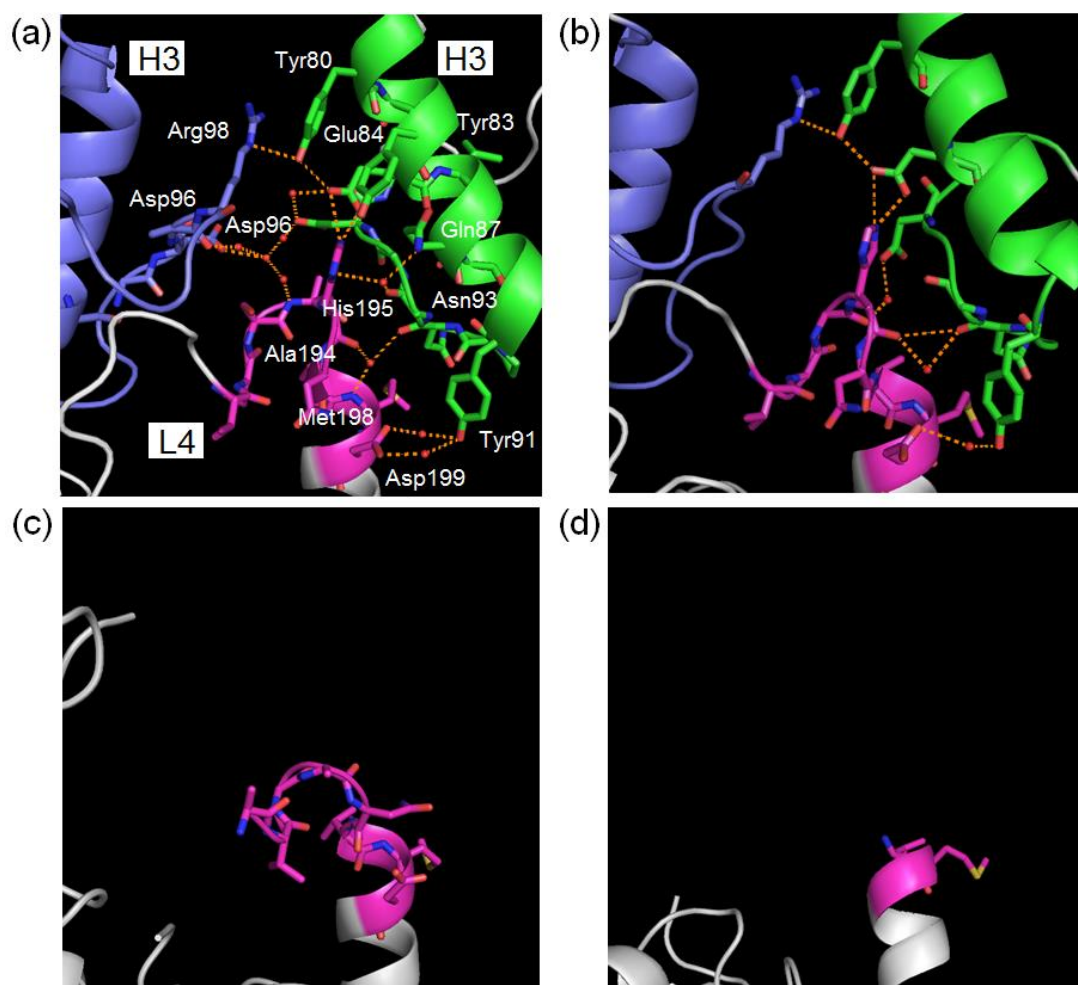


Figure 4. The intramolecular interactions of L4 loop and intermolecular interactions between PhMs of WTPhC (a), $\Delta 1$ -PhC (b), $\Delta 2$ -PhC (b) and $\Delta 3$ -PhC (c). H3 helices in neighboring PhMs are shown in green and blue. Hydrogen bondings are shown in yellow dotted lines.

Table 1. Crystallographic data

Data collection	$\Delta 1$ -PhC	$\Delta 2$ -PhC	$\Delta 3$ -PhC
Space group	<i>I</i> 32	<i>I</i> 32	<i>I</i> 32
Crystal cell (Å)			
$a = b = c$	102.997	105.024	104.421
Resolution range (Å)	32.0-1.93 (1.96-1.93)	40.0-2.15 (2.19-2.15)	30.0-2.18 (2.22-2.18)
Observations	65374	33072	48801
Unique reflections	24568	17978	19075
Completeness (%)	92.5 (96.4)	88.5 (89.1)	99.1 (97.9)
I/σ	6.2 (2.1)	9.2 (2.1)	8.8 (2.1)
R_{merge}	16.3 (44.0)	12.0 (44.9)	13.2 (49.8)
Redundancy	2.8 (2.7)	2.2 (2.2)	2.6 (2.6)

Values in parentheses are for the highest-resolution shell.

Table 2. Refinement statistics.

	$\Delta 1$ -PhC	$\Delta 2$ -PhC	$\Delta 3$ -PhC
Resolution range (Å)	27.53 – 1.93	37.13 – 2.15	27.9 – 2.18
Reflection used	12732	10019	9583
R -factor (%)	14.8	18.2	19.2
Free R -factor (%)	20.0	25.0	28.1
R.m.s. deviations			
Bond length (Å)	0.0199	0.0170	0.0166
Angle (°)	2.0006	1.8725	1.8753
Ramachandran plot			
most favored	96.3	96.7	97.8
allowed	3.7	3.3	2.2

Adsorption of organic compounds.

To assess adsorption of exogenous organic compounds within mutant PhC, PhCs (1×10^6 crystals) were soaked in a 100 μ M HEPES buffer (pH 7.0) containing fluorescent dyes (10 μ M) for 1day (Figure 5a). Eosin Y, rose bengal, rhodamine B, and rhodamine 6G were used as fluorescent dyes. The numbers of adsorbed fluorescent dyes within the crystals were determined based on the fluorescence intensities of the supernatant solution after reaction of PhCs with fluorescent dyes for 1day (Figure 5b). The analyses show that the adsorbed amounts of Eosin Y per PhMs of $\Delta 1$ -PhC, $\Delta 2$ -PhC and $\Delta 3$ -PhC are 1.3-, 12.5-, 13.1-fold larger amounts than that of WTPhC, respectively. In the case of rose bengal, the number of dyes per PhMs of $\Delta 1$ -PhC, $\Delta 2$ -PhC and $\Delta 3$ -PhC are 10.2-, 15.2-, and 16.5-fold larger amounts than WTPhC, respectively, which is same tendency as Eosin Y. The amounts of adsorbed rhodamine B and 6G within the crystals exhibit no large difference among the all mutants of PhCs. These results indicate that $\Delta 2$ -PhC and $\Delta 3$ -PhC can adsorb larger amounts of anionic fluorescent dyes in the crystals than WTPhC, although cationic fluorescent dyes were not adsorbed within the crystals.

Overall, Porous structures of PhC can adsorb anionic fluorescent dyes by deletion of two or three amino acid residues of contact interfaces of PhTs. This is to be expected because PhTs possess positive environment inside of trimer structure, where Lys53, Lys58, Lys73 and Arg209 are located (Figure 6). The contact interfaces of PhTs in $\Delta 2$ -PhC and $\Delta 3$ -PhC exhibit larger flexibility than WTPhC and $\Delta 1$ -PhC because the structures of the amino acid residues of contact regions could not be determined due to disordered electron density. Therefore, these positive amino acid residues in $\Delta 2$ -PhC and $\Delta 3$ -PhC are exposed on the solvent channels, and are expected to interact with anionic dyes. On the other hand, cationic fluorescent dyes were not adsorbed due to both the size-effect and the electrostatic effect. Thus, it is expected that deletion of amino acid residues at contact interfaces affect not only extension of porous structures, but also adsorption of exogenous organic molecules. Further investigation is ongoing to determine diffusion of the organic molecules in the crystals.

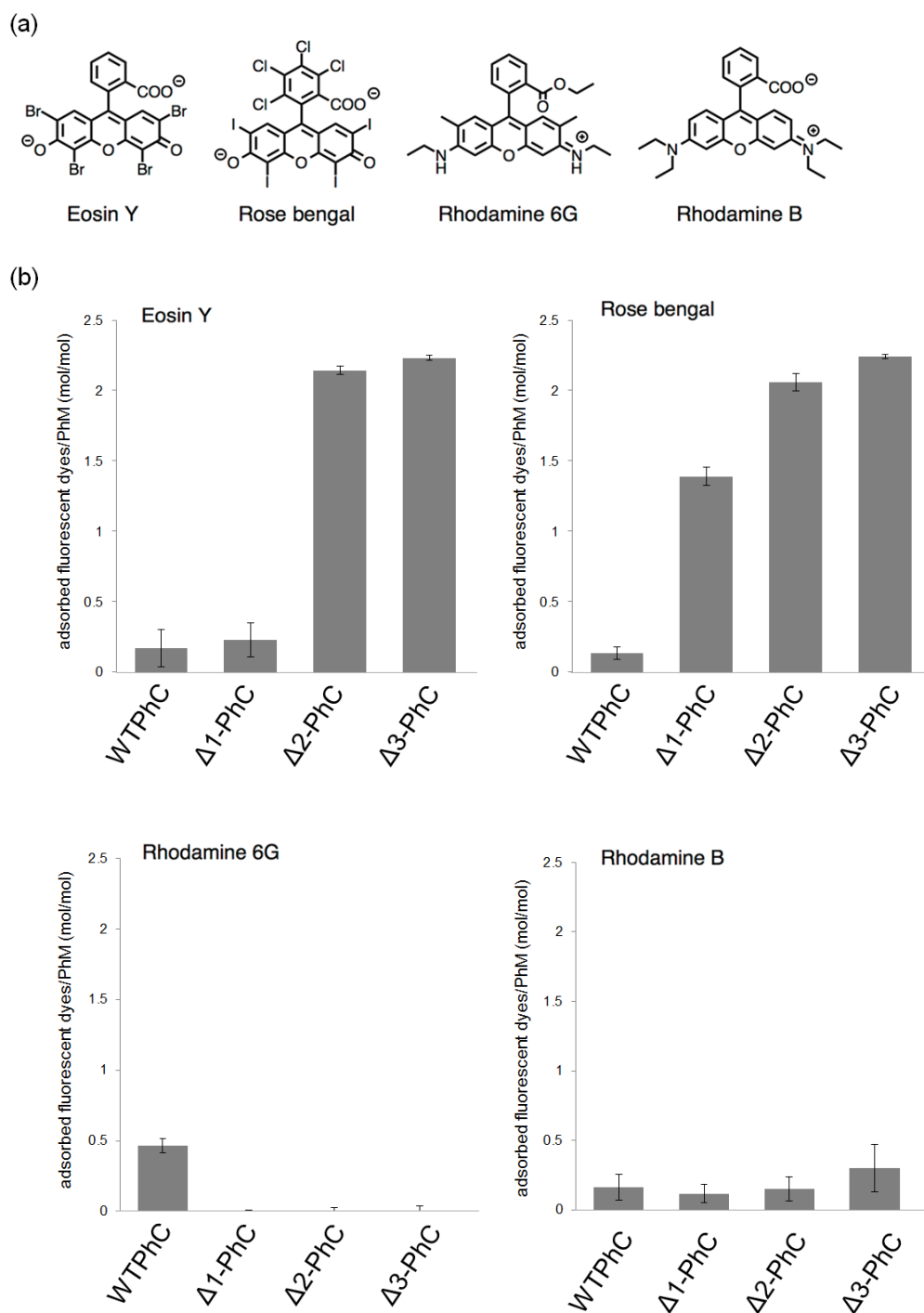


Figure 5. (a) Chemical structure of fluorescent dyes used in the adsorption study. (b) Adsorption efficiency of fluorescent dyes in PhC crystals. The value represents the number of fluorescent dyes per PhM. The experiments were performed three times under each set of conditions. The data represent mean $\pm$ SEM.

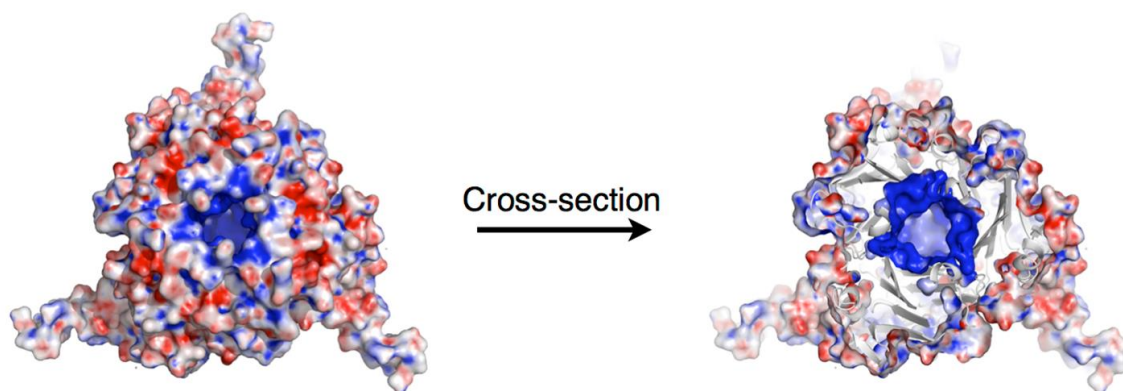


Figure 6. The trimer structure of WTPhC with the surface electrostatic potential. The electrostatic potential is represented by a color spectrum extending from red to blue indicating negative to positive charges for each residue.

Conclusion

In conclusion, the author demonstrated the enlargement of solvent channels in PhC by the replacement and deletion of amino acid residues of contact interfaces of PhT. The genetically engineered PhCs increase their solvent contents from 19% to 24% with retaining the original lattice structure. These engineered PhCs can adsorb maximally 16-fold large amounts of fluorescent dye because the amino acid residues of contact interfaces of PhT have high flexibility due to decreasing non-covalent intra- and inter-molecular interactions. Moreover, they selectively adsorbed the anionic dyes probably due to the electrostatic interactions between amino acid residues newly exposed on solvent channels. This result provides simple method to reconstitute different porous environments without using different crystal systems of protein crystals. The engineered solvent channels can be obtained from various protein crystals with/without porous structures. They have a potential for further chemical reactions such as selective catalysis and separation of exogenous molecules. The author believes that this method will provide attractive bionanomaterials having unnatural reaction spaces.

Experimental Section

Materials. Reagents were purchased from TCI, Wako, Nacalai Tesque, and Sigma–Aldrich and were used without further purification. The *Spodoptera frugiperda* cell line IPLB–Sf21–AE (Sf21) was maintained in tissue culture flasks in Grace’s medium (Gibco–BRL) at 27°C with 10% fetal bovine serum (MP Biomedicals. Inc.), 2.6 mg/mL tryptose broth, 100 U/mL penicillin, and 100 µg/mL streptomycin. Primers for site–directed mutagenesis were obtained from Gene Design Inc. PCR was performed using the QuickChange® Site–Directed Mutagenesis Kit (Stratagene). The LR reaction was performed using the Gateway® LR Clonase® Enzyme mix (Life Technologies).

Preparation of WTPhC and Mutant PhC. Expression and purification of recombinant wild-type *Bombyx mori* CPV polyhedra (WTPhC) in insect cells were performed according to a previous literature.²⁵ The plasmid DNAs of $\Delta 1$ -PhC (–V₁₉₁GGH₁₉₅–), $\Delta 2$ -PhC (–V₁₉₁GH₁₉₅–) and $\Delta 3$ -PhC (–V₁₉₁H₁₉₅–) were prepared using the Quick Change Site–Directed Mutagenesis Kit (Stratagene). To produce transfer vectors encoding mutant PhC, the open reading frame cloned between attL1 and attL2 sites in the entry vectors was transferred to destination vectors (pDEST) via LR Clonase™ reactions. The resulting transfer vectors were co-transfected into Sf21 cells with linearized Baculogold Baculovirus DNA (BD Pharmingen), and replication–competent baculovirus was rescued by recombination between the transfer vector and the linearized viral DNA and ultimately harvested from the supernatant. Expression and purification of mutants were performed by the same procedures with WTPhC.²⁵

X-ray Crystal Structure Analysis. Prior to the data collection, $\Delta 1$ -PhC, $\Delta 2$ -PhC and $\Delta 3$ -PhC were immersed in a buffer solution containing 50 % (w/w) ethylene glycol and were spread on MicroMesh and subsequently frozen in liquid nitrogen. X-ray diffraction data of $\Delta 1$ -PhC, $\Delta 2$ -PhC and $\Delta 3$ -PhC were collected at 100 K at beamline BL32XU at SPring-8 using X-ray wavelength of 1.00 Å. The data were processed with the program HKL2000.²⁶ The crystal parameters and the data collection statistic are summarized in Table 1. The structures were solved by molecular replacement with MOLREP using polyhedrin structure (PDB ID : 2OH6).²⁷ Refinement of the protein structure was performed at resolutions of 1.93, 2.15, and 2.18 Å for $\Delta 1$ -PhC, $\Delta 2$ -PhC and $\Delta 3$ -PhC, respectively, using REFMAC5²⁸ in the CCP4 suite.²⁹ Rebuilding was

performed using COOT³⁰ based on sigma weighted ($2Fo-Fc$) and ($Fo-Fc$) electron density maps. Residues ACE1-Asn9, Leu68-Asn103, Asn128-Asp134, and Ser187-Tyr189 for $\Delta 2$ -PhC, and ACE1-Ser8, Leu68-Asn103, Ala129-Asp134, and Pro186-Asn196 for $\Delta 3$ -PhC were decided model because electron densities corresponding to these residues are missing. The models were subjected to quality analysis during the various refinement stages with omit maps and RAMPAGE.³¹ The refinement statistics are summarized in Table 2.

Adsorption of exogenous organic compounds. PhCs (1.0×10^6 crystals) were soaked in a 100 μ M HEPES buffer (pH 7.0) solution containing fluorescent dyes (10 μ M) at room temperature for 24 hours to adsorb the fluorescent dyes into PhCs. Eosin Y, rose bengal, rhodamine B, and rhodamine 6G were used as fluorescent dyes. The amounts of adsorbed dyes in the PhCs were determined by supernatant fluorescence intensities using HITACHI F-7000 fluorescence spectrometer. The experiments were performed three times under each set of conditions. The data represent mean $\pm$ SEM. The amounts were also determined by elemental analysis using X-ray fluorescence.

References

1. T. Ueno and Y. Watanabe, *Coordination Chemistry in Protein Cages: Principles, Design, and Applications*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2013.
2. N. J. M. Sanghamitra and T. Ueno, *Chem. Commun.*, 2013, **49**, 4114-4126.
3. S. Howorka, *Curr. Opin. Biotechnol.*, 2011, **22**, 485-491.
4. M. Uchida, M. T. Klem, M. Allen, P. Suci, M. Flenniken, E. Gillitzer, Z. Varpness, L. O. Liepold, M. Young and T. Douglas, *Adv. Mater.*, 2007, **19**, 1025-1042.
5. J. Zhang, F. Zheng and G. Grigoryan, *Curr. Opin. Struct. Biol.*, 2014, **27**, 79-86.
6. P. A. Sontz, W. J. Song and F. A. Tezcan, *Curr. Opin. Chem. Biol.*, 2014, **19**, 42-49.
7. J. C. Sinclair, *Curr. Opin. Chem. Biol.*, 2013, **17**, 946-951.
8. S. Bozic, T. Doles, H. Gradisar and R. Jerala, *Curr. Opin. Chem. Biol.*, 2013, **17**, 940-945.
9. Q. Luo, Z. Y. Dong, C. X. Hou and J. Q. Liu, *Chem. Commun.*, 2014, **50**, 9997-10007.
10. T. Ueno, *Chem. Eur. J.*, 2013, **19**, 9096-9102.
11. A. L. Margolin and M. A. Navia, *Angew. Chem. Int. Ed.*, 2001, **40**, 2205-2222.
12. J. C. Falkner, M. E. Turner, J. K. Bosworth, T. J. Trentler, J. E. Johnson, T. W. Lin and V. L. Colvin, *J. Am. Chem. Soc.*, 2005, **127**, 5274-5275.
13. M. Guli, E. M. Lambert, M. Li and S. Mann, *Angew. Chem. Int. Ed.*, 2010, **49**, 520-523.
14. H. Wei, Z. D. Wang, J. O. Zhang, S. House, Y. G. Gao, L. M. Yang, H. Robinson, L. H. Tan, H. Xing, C. J. Hou, I. M. Robertson, J. M. Zuo and Y. Lu, *Nat. Nanotechnol.*, 2011, **6**, 92-96.
15. S. Abe, M. Tsujimoto, K. Yoneda, M. Ohba, T. Hikage, M. Takano, S. Kitagawa and T. Ueno, *Small*, 2012, **8**, 1314-1319.
16. A. Cvetkovic, C. Picoreanu, A. J. J. Straathof, R. Krishna and L. A. M. van der Wiel, *J. Am. Chem. Soc.*, 2005, **127**, 875-879.
17. A. Cvetkovic, M. Zomerdijk, A. J. J. Straathof, R. Krishna and L. A. M. van der Wielen, *Biotechnol. Bioeng.*, 2004, **87**, 658-668.
18. Z. Q. Hu and J. W. Jiang, *Langmuir*, 2008, **24**, 4215-4223.

19. J. P. K. Doye and W. C. K. Poon, *Curr. Opin. Colloid Interface Sci.*, 2006, **11**, 40-46.
20. L. Redecke, K. Nass, D. P. DePonte, T. A. White, D. Rehders, A. Barty, F. Stellato, M. N. Liang, T. R. M. Barends, S. Boutet, G. J. Williams, M. Messerschmidt, M. M. Seibert, A. Aquila, D. Arnlund, S. Bajt, T. Barth, M. J. Bogan, C. Caleman, T. C. Chao, R. B. Doak, H. Fleckenstein, M. Frank, R. Fromme, L. Galli, I. Grotjohann, M. S. Hunter, L. C. Johansson, S. Kassemeyer, G. Katona, R. A. Kirian, R. Koopmann, C. Kupitz, L. Lomb, A. V. Martin, S. Mogk, R. Neutze, R. L. Shoeman, J. Steinbrener, N. Timneanu, D. J. Wang, U. Weierstall, N. A. Zatsepin, J. C. H. Spence, P. Fromme, I. Schlichting, M. Duszynko, C. Betzel and H. N. Chapman, *Science*, 2013, **339**, 227-230.
21. H. Aruga and Y. Tanada, *Cytoplasmic Polyhedrosis Virus of the Silkworm*, University of Tokyo Press, Tokyo, 1974.
22. F. Coulibaly, E. Chiu, K. Ikeda, S. Gutmann, P. Haebel, C. Schulze-Bries, H. Mori and P. Metcalf, *Nature*, 2007, **446**, 97-101.
23. H. Ijiri, F. Coulibaly, G. Nishimura, D. Nakai, E. Chiu, C. Takenaka, K. Ikeda, H. Nakazawa, N. Hamada, E. Kotani, P. Metcalf, S. Kawamata and H. Mori, *Biomaterials*, 2009, **30**, 4297-4308.
24. B. W. Matthews, *J. Mol. Biol.*, 1968, **33**, 491-497.
25. H. Mori, R. Ito, H. Nakazawa, M. Sumida, F. Matsubara and Y. Minobe, *J. Gen. Virol.*, 1993, **74**, 99-102.
26. Z. Otwinowski and W. Minor, *Macromol. Crystallogr. A*, 1997, **276**, 307-326.
27. A. Vagin and A. Teplyakov, *J. Appl. Crystallogr.*, 1997, **30**, 1022-1025.
28. G. N. Murshudov, A. A. Vagin and E. J. Dodson, *Acta Crystallogr. D*, 1997, **53**, 240-255.
29. E. Potterton, P. Briggs, M. Turkenburg and E. Dodson, *Acta Crystallogr. D*, 2003, **59**, 1131-1137.
30. P. Emsley and K. Cowtan, *Acta Crystallogr. D*, 2004, **60**, 2126-2132.
31. S. C. Lovell, I. W. Davis, W. B. Adrendall, P. I. W. de Bakker, J. M. Word, M. G. Prisant, J. S. Richardson and D. C. Richardson, *Proteins Struct. Funct. Genet.*, 2003, **50**, 437-450.

List of Publications

General Introduction

Artificial Metalloenzymes Constructed From Hierarchically-Assembled Proteins

Takafumi Ueno, Hiroyasu Tabe and Yuya Tanaka

Chemistry an Asian Journal, 2013, 8, 1646-1660.

Chapter 1

Porous Protein Crystals as Catalytic Vessels for Organometallic Complexes

Hiroyasu Tabe, Satoshi Abe, Tatsuo Hikage, Susumu Kitagawa and Takafumi Ueno

Chemistry an Asian Journal, 2014, 9, 1373-1378.

Chapter 2

Preparation of a Cross-linked Porous Protein Crystal Containing Ru Carbonyl

Complexes as a CO-releasing Material

Hiroyasu Tabe, Kenta Fujita, Satoshi Abe, Masahiko Tsujimoto, Takahiro Kuchimaru, Shinae Kizana-Kondoh, M. Takano, Susumu Kitagawa and Takafumi Ueno

Inorganic Chemistry, 2015, 54, 1, 215-220.

Chapter 3

Design of a CO-releasing Extracellular Scaffold using *in-vivo* Protein Crystals

Hiroyasu Tabe, Takuya Shimoi, Kenta Fujita, Satoshi Abe, Hiroshi Ijiri, Masahiko Tsujimoto, Takahiro Kuchimaru, Shinae Kizaka-Kondoh, Hajime Mori, Susumu Kitagawa and Takafumi Ueno

Chemistry Letters, accepted.

Chapter 4

Light-triggered CO Releasing from Extracellular Scaffold Constructed by *in-vivo* Protein Crystals

Hiroyasu Tabe, Takuya Shimoi, Kenta Fujita, Satoshi Abe, Hiroshi Ijiri, Masahiko Tsujimoto, Takahiro Kuchimaru, Shinae Kizaka-Kondoh, Hajime Mori, Susumu Kitagawa and Takafumi Ueno

to be submitted.

Chapter 5

In-vivo Crystal Engineering for Preparing Porous Protein Architecture

Hiroyasu Tabe, Satoshi Abe, Masaki Akai, Hiroshi Ijiri, Kunio Hirata, Hajime Mori, Susumu Kitagawa and Takafumi Ueno
to be submitted.

Other Publications

Post-Crystal Engineering of Zinc-Substituted Myoglobin to Construct a Long-Lived Photoinduced Charge-Separation System

Tomomi Koshiyama, Masanobu Shirai, Tatsuo Hikage, Hiroyasu Tabe, Koichiro Tanaka, Susumu Kitagawa and Takafumi Ueno
***Angewandte Chemie International Edition*, 2011, 21, 4849-4852.**

Surface Functionalization of Protein Crystals with Carbohydrate Using Site-selective Bioconjugation

Satoshi Abe, Yu Tokura, Rita Pal, Naoko Komura, Akihiro Imamura; Kaori Matsumoto, Hiroshi Ijiri, Nusrat J. M. Sanghamitra, Hiroyasu Tabe, Hiromune Ando, Makoto Kiso, Hajime Mori, Susumu Kitagawa and Takafumi Ueno
***Chemistry Letters*, 2015, 44, 29-31.**