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Authors	Basudev Maity, Mohd Taher, Shyamalava Mazumdar, Takafumi Ueno
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Artificial metalloenzymes based on protein assembly

Basudev Maity,[1†] Mohd Taher,[2†] Shyamalava Mazumdar,[2,3*] Takafumi Ueno[1,3*]

[1] Schoool of Life Science and Technology, Tokyo Institute of Technology, Nagatsuta-cho 4259, Midori-ku, Yokohama 226 8501, Japan.

[2] Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai 400005, India

[3] World Research Hub Initiative (WRHI), Tokyo Institute of Technology, Nagatsuta-cho 4259-B55, Midori-ku, Yokohama 226-8501, Japan

[†] These two authors contributed equally.

Correspondence: T. Ueno, email; tueno@bio.titech.ac.jp

Abstract

Metalloenzymes play essential roles in biology, whereas artificial metalloenzymes use synthetic metal cofactors for promoting non-natural reactions. In the past decades, tremendous advances have been made in manipulating artificial metalloenzymes for various organic transformation reactions, including C-H activation, C-C coupling, transfer hydrogenation, etc. Advanced methods like "Directed evolution," "high throughput screening," "rational design" have stimulated artificial metalloenzyme research. Applications of artificial metalloenzymes have been extended to cells for controlling functions like prodrug activation. Usually, for more complicated processes like multistep reactions or isolation of reaction environments, nature uses sophisticated strategies, such as positional assembly and compartmentalization of catalysts. However, artificial metalloenzyme research in this direction is relatively less. Several researchers have designed and constructed various protein assembly structures through metal coordination. However, only a few of them have been tested for catalytic activities. Assembled metalloenzymes have multiple advantages like promoting multistep reactions, stabilizing the catalyst, cooperativity in the reaction, higher-order complexity, sophisticated structures, confinement of reaction, etc. Therefore, systematic investigations on their design, structure, and activity are necessary to represent them as next-generation biocatalysts. In this context, the current review highlights the importance of self-assembled metalloenzymes, available design strategies, current developments, catalytic activities, and the future direction of the research.

Keywords: Artificial metalloenzyme; Protein self-assembly; Catalysis; Metalloprotein assembly design

Contents

1. Introduction

Metalloenzymes catalyze fundamental reactions in life processes, whereas artificial metalloenzymes (ArMs) use synthetic metal cofactors into protein scaffolds for promoting non-natural reactions (Figure 1a).[1] Design and development of ArMs have been improved significantly from past decades with a wide range of metal-catalyzed organic transformation reactions, such as C-C coupling, C-H bond activation, metathesis, C-N bond functionalization, transfer hydrogenation etc.[2] For example, cytochrome P450 (P450) is one of the most studied metalloenzymes for non-natural reactions, including N-H bond functionalization[3], olefin cyclopropanation[4], carbene transfer reaction,[5] etc. Similarly, Mn^V-porphycene has been reconstituted into myoglobin matrix for hydroxylation of inert C–H bonds.[6] ArMs can now be evolved as highly active and selective biocatalysts through "directed evolution"[7], "high-throughput screening,"[8] and "rational design,"[9] etc. Such evolved enzymes are not only efficient but also show diverse reactions.[10] Recently, ArMs are used in-vivo applications for controlling cell functions, prodrug activation, etc.[11-13] Considering all such tremendous development, several articles have been dedicated to rational design, current challenges, and future perspective of ArMs.[14, 15] [2]

So far, most of the reports on ArMs are based on single catalytic sites. As the development of "next-generation" metalloenzymes, researchers are now focusing more on constructing sophisticated protein structures with multiple active sites not only for high catalytic efficiency but also for promoting cascade processes and cell functions.[9, 16] Introducing multiple active sites in a single enzyme is not a good idea as it raises concerns about the stability and folding of the enzyme. Nature uses various strategies like programmed protein assembly, homo or hetero-oligomerization, compartmentalization for complicated reaction processes, including the isolation of reaction environments. To achieve such processes artificially, suitable design is necessary to avail unique structures. Designing protein self-assembly structures is an interesting choice for having controllable sophisticated structures with multiple active sites, which cannot be achieved by a single protein (Figure 1b).[17] Thus, protein assembly-based ArMs can have the advantages of unique controllable structures, confined environment, multiple catalytic metal centers. However, current ArMs research focusing in this direction is relatively less. Recently, several researchers have been interested in designing various protein assembly structures through metal coordination, electrostatic interaction, covalent bond formation, etc., which has been nicely reviewed by Tezcan et al.[17] Very few of them have been tested for catalysis. For example, surface modified Zn coordinated tetrameric assembly of cyt cb562 can show enzymatic activity in vivo.[18] Similarly, Roefles et al. have developed Cu induced supramolecular lactococcal multidrug resistance regulator (LmrR) assembly for promoting various reactions, including Friedel-crafts alkylation, Diels-Alder reaction, etc.[19] These examples suggest that protein monomers can be tuned into unique assembly structures through metal coordination which can be used as artificial metalloenzymes. Such strategies are promising as we can get controllable structures with protein homomer or heteromer with multiple active sites together.

This review focused on highlighting the importance of self-assembly-based ArMs, available design strategy, recent significant developments in catalysis, and the future direction of such research (Figure 1). Thus, the current review is expected to be a valuable guide for new design and expand the research development of artificial self-assembled metalloenzymes.

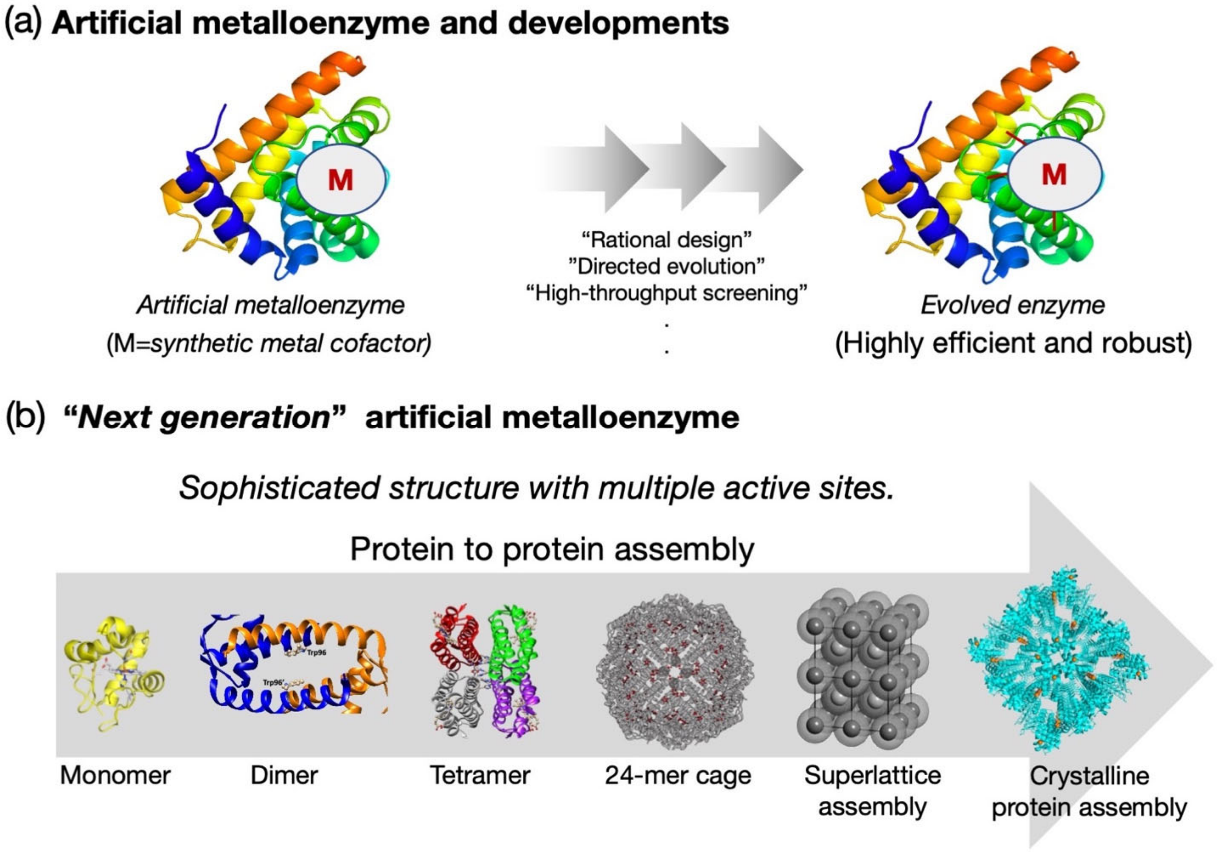

Figure 1: Trends in developing artificial metalloenzymes. (a) A scheme for conventional artificial metalloenzyme and developments towards a highly efficient, evolved, and robust enzyme. (b) Representation of the current trend in developing ArMs starting from monomeric protein to protein assemblies with multiple active sites and sophisticated structures. We termed such trends in development as "second generation" metalloenzymes. Figures are drawn in pymol.

2. Supramolecular protein self-assembly.

Protein assemblies are highly ordered and complex supramolecular machineries that control many functions in cells. Nature uses such assembly structures over their monomeric counterparts due to advantages, including unique structures with higher stability, higher-order complexity, cooperativity in functions, etc. Depending on the source and functions, there are various protein assemblies, such as cage, ring, tube, and each of them has specific biological functions (Figure 2).[13] The majority of the natural protein assemblies are formed by symmetric homo-oligomers. Compared to such homo-assembly, hetero-oligomerized protein assemblies are more complex in structures and functions as they consist of different protein subunits. Each monomeric form in the hetero-oligomerized protein assembly has unique and distinct functions that cooperate to execute the whole process within the cell. Hemoglobin is one of the simplest and mostly studied heteromeric protein assembly, whereas photosystem is an example of a complex system with up to 20 different protein monomers. Due to complexity in structure and cooperative function, it is difficult for scientists to understand the detail of such protein assembly. In addition to such complex protein assemblies, nature uses another unique protein assembly with a confined cage structure to isolate metabolic reactions in cells.[20] This is also special as it can separate the toxic intermediates formed during the metabolic process. Thus, protein assembly provides distinct functions from monomeric proteins or enzymes.

Considering the advantages of protein assembly, there are growing interests on artificial design of protein assembly structures using various methods such as introducing covalent, noncovalent, or metal-coordination bonding, etc.[17] Usually, the surface of the proteins are mutated with suitable residues and allowed for self-assembly which gave structures with up to several hundred nanometers. It is also possible to construct hetero-oligomerized assembly structures using such methods. Thus, the diversity of artificially designed protein assembly opens new possibilities for design structures in catalysis, biomedical and electronic applications. For example, enzyme clusters are constructed inside the coat protein assembly of bacteriophage P22 for chiral alcohol production.[21] More recently, Douglas and coworkers have introduced multiple enzymes such as CelB, GALK, and GLUK into a virus-like particle assembly from bacteriophage P22 to create a synthetic metabolon.[22] They successfully demonstrated the enzymatic cascade reactions and the confinement effect of the protein cage. Therefore, there is a great scope for designing ArMs based on protein assembly for new functions as they can provide unique structures with multiple active sites arranged in an ordered fashion.

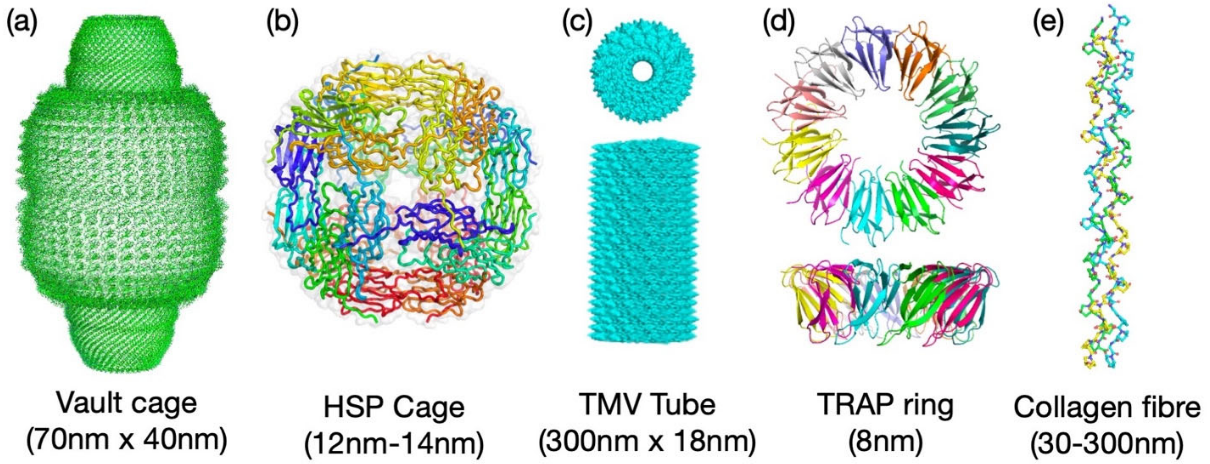

Figure 2: Selected examples of natural protein assembly structures. (a) Giant vault protein cage (pdb:4v60). (b) Small protein cage: Heat shock protein (pdb: 1shs). (c) Tubular assembly: Tobacco mosaic virus (TMV) (pdb: 3j06). (d) Ring assembly: Thrombospondin-related anonymous protein (TRAP) (pdb:1qaw). (e) Fibrillar assembly: Collagen fiber (pdb: 3b0s). Images are prepared in pymol.

3. Artificial metalloenzymes based on protein assembly.

Although ArMs are familiar from the past few decades, catalysis using self-assembly structures are relatively new and thus, has lesser reports. Other than the protein active site cavity in a conventional metalloenzyme, protein assemblies provide unique reaction environments beyond the second coordination sphere and isolate the whole reaction and trapping of intermediate as a substrate for subsequent reactions like metabolic processes in cells.[20, 23] In addition, a sophisticated tunable structure in protein assembly is an essential feature while considering complex reactions by metal complex catalysts. Multiple actives in the assembled enzyme can increase efficiency and enhance product selectivity.[24] Heteromeric protein assemblies can give multiple active sites for the cascade process. Naturally occurring protein assemblies like ferritin, heat chock protein, DNA binding protein from starved cells (DPS) can be directly used in apo-form for catalysis after functionalization with synthetic catalysts. Synthetic protein assembly structures can also be used for such purposes. The advantage of designed protein assembly is that their structures can be tuned, and the catalytic active sites can be varied. So far, the naturally occurring protein assemblies like 24-mer ferritin cage assembly, dimeric Lactococcal multidrug resistance Regulator (LmrR), streptavidin, etc., have been studied for catalytic reactions.[25, 26] Such protein assemblies were functionalized with various synthetic metal catalysts to promote catalytic reactions. Table 1 listed various protein assemblies, including artificially designed ones with their metal cofactors and the respective enzymatic reactions discussed in this review.

Table 1: List of selected protein assemblies used for catalytic reactions.

Protein	Nature of assembly	Metal cofactor	Reaction	Reference
Ferritin	Natural 24-mer cage	Pt NP	Peroxidase/catalase	[27]
		Pd, Au/Pd NP	Hydrogenation	[28-30]
		Pd(allyl)	C-C coupling	[31, 32]
		Rh(nbd)[a]	Polymerization	[26]
		IrCp*	Hydrogenation	[33]
		IrCp*/Pd(allyl)	Cascade hydrogenation and C-C coupling	[34]
		Fe-S cluster	H_2 evolution	[35]
		Artifical hydrogenase	Transfer hydrogenation	[23]
		Hemin	Peroxidase	[36]
LmrR (Lactococcal multidrug resistance Regultor)	Dimer assembly	Cu(phen)	Friedel-Crafts alkylation Michael addition, Diels-Alder reaction, Hydration of alkene, Metallohydratase	[19, 37-40]
		Heme	Cyclopropanation	38[40]
		Zn	Hydrolysis (Esterase)	
Streptavidine	Dimer of dimer assembly (Tetramer)	Ir	Transfer hydrogenation	[41]
		Rh	C-H activation	[42]
		Pd	C-C coupling	[43]
		Au	hydroaminase	[24]
		Ru	Allylcarbamate cleavage, Metathesis	[12, 44]
Catalysis by artificial protein assembly				
Cyt cb$_{562}$	Tetramer assembly (Surface modification by Zn coordination)	Zn	Hydrolase activity	[18, 45, 46]
Protein needle	Dimer of trimer assembly	Ru/Re	Photocatalytic CO_2 reduction	[47]
		Sc	Ring opening	[48]
		Cu	Azide-alkyne click reaction	[49]
Cyt c	Domain swapping dimer	Heme	Peroxidase	[50]
Crystalline protein assemblies				
HEWL (Hen egg white lysozyme)	Crystal	Rh(benzene)	Hydrogenation	[51]

		Pt NP	H_2 evolution	[52]
		Au NP	NO_2 reduction	[53]
Polyhedrin	Crystal	Enzymes (Alcohol dehydrogenase)	Dehydrogenation	[54]
Ferritin	Superlattice	FeO, CeO NP	Oxidase/Peroxidase	[55]
Others				
Peptide assembly	Beta-sheet assembly	Zn	Hydrolysis (Esterase)	[56]
		IrCp*	Transfer hydrogenation	[57]
		Hemin	Cyclopropanation	[58]
Phenyl alanine	Tubular assembly	Zn	Hydrolysis	[59, 60]
Coiled coils peptide	Trimeric α-helical assembly	Cu	Nitrite reductase	[61]

3.1. **Metalloenzyme catalysis using ferritin cage assembly**.

Ferritin is a naturally occurring iron storage protein found in nearly all living organisms. The protein is organized by the self-assembly of 24 monomeric subunits, which gave a 12 nm diameter spherical cage structure (Figure 3a). The ferritin cage has a hollow cavity diameter of 8 nm (Figure 3b). Interestingly, the cage contains eight symmetric 3-fold pores through which metal ions can pass into the cage (Figure 3c). Depending on the source, approximately 4500 iron atoms can be stored in the cage. Considering the unique cage structure and ability to store a large number of metal ions, current ferritin research has attracted attention for various applications in chemistry, biology, and medicine.[62, 63] Major applications include sensors, metal nanoparticle synthesis, drug delivery systems, contrast agents in MRI and catalysis.[64] Along with the direct use of naturally occurring ferritin, the cage can be modified with synthetic metal complexes, metal ions, and organic molecules that widen the scope of applications of ferritin.[26],[63, 65] In this section, we limited our scope to highlight the recent and significant catalytic reactions promoted by using modified or unmodified ferritin cage assembly.

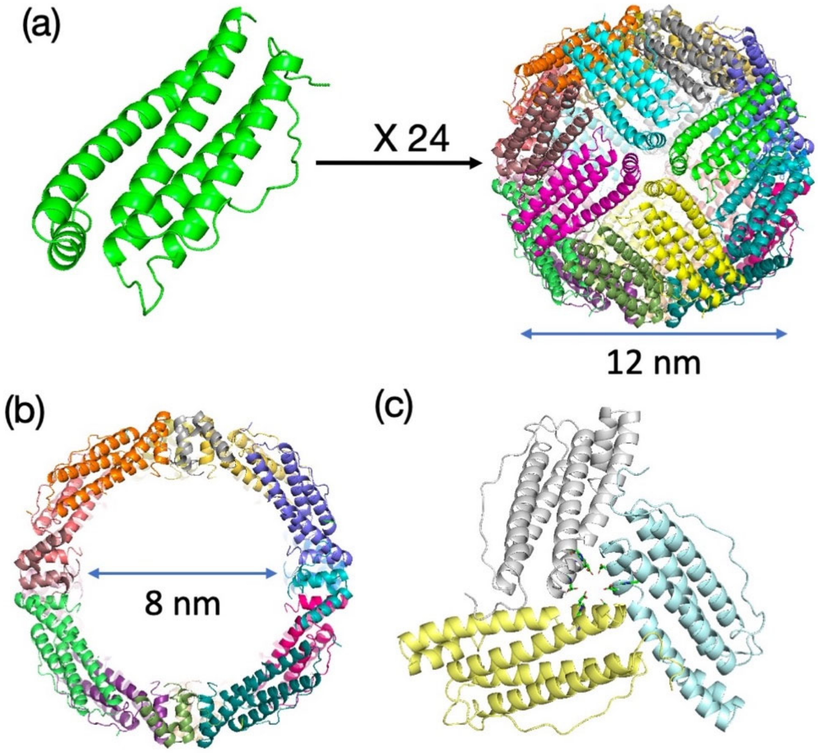

Figure 3: Basic structure of ferritin cage. (a) Ferritin cage assembly formation from a monomer subunit. (b) Interior cage structure of ferritin. (c) Interface of three ferritin subunits forming a 3-fold symmetric pore which is known for metal ions transport. The negatively charged residues like Asp and Glu are directed towards the channel. Images are prepared in pymol using pdb ID: 1dat.

The core of the ferritin cage contains iron oxide in ferrihydrite form as revealed by the high-resolution transmission electron microscopy (HRTEM), which showed a crystalline lattice structure with d-spacings of 2.5–2.6 Å.[66],[67] Such naturally occurring iron core was found to show various catalytic reactions including selective oxidation of organic compounds, water oxidation, etc.[65, 68] Such examples motivated researchers to prepare metal nanoparticles utilizing ferritin cage assembly as a template. S. Mann and coworkers have initiated the preparation of synthetic nanoparticles such as iron sulfide, manganese, and uranium oxide nanoparticles inside an empty ferritin cage.[69] After that, many nanoparticles were synthesized using ferritin cages template and applied for catalysis, biomedical and materials technology.[27-29, 70] In an earlier report, Ueno et al. have synthesized small Pd nanoclusters using the restricted environment of ferritin cage.[29] They showed that such artificial preparation of metal nanoclusters inside the cage did not affect the 24-mer ferritin cage structure. They introduced Pd^{2+} ions into the cage, followed by the addition of sodium borohydride to form tiny Pd nanoclusters (~2nm) inside the cage, which can catalyze the hydrogenation of olefins. High TOF up to 33000 per hour per cage was observed for the hydrogenation of acrylamide in an aqueous medium. Various acrylamide derivatives were used for more precisely understanding the reaction. It was found that the substrates passed into the channel through 3-fold pores as less TOF was observed for anionic substrates. Inspired by this, bimetallic Au/Pd nanoparticles were prepared inside the ferritin cage.[28] By controlling the entry sequence of metal ions, various types such as core-shell or alloy nanoparticles were prepared. Thus, prepared fine-tuned bimetallic nanoparticle showed improved catalytic activities compared to only Pd^0 nanoclusters inside the cage. Arends et al. have synthesized 5nm Pd^0 nanoclusters using the confined environment of hyperthermophilic ferritin cage (*from Pyrococcus furiosus*), which showed selective aerobic oxidation of alcohols in an aqueous medium.[30] Similarly, Pt^0 and ferrihydrite nanoparticles were also prepared inside the ferritin cage for enzyme mimetic activities like peroxidase and catalase.[27, 71]

Besides nanoparticle synthesis and subsequent catalysis, various synthetic metal complex catalysts were also incorporated into the ferritin cage. Ueno and coworkers have modified the interior cage surface of ferritin by immobilizing various synthetic metal catalysts such as Pd(allyl), IrCP*, and Rh(nbd), to promote various organic transformations like Suzuki-Miyuara coupling, transfer hydrogenation, polymerization reactions. [26]'[29]'[72]'[31]'[34] Since small metal complexes can easily transport into the channel through the 3-fold pore, synthetic metal catalysts can be quickly immobilized on the surface of the ferritin cage just by stirring at RT or elevated temperatures such as 50°C. Usually, no apparent changes were observed after metal immobilization, suggesting that the ferritin cage is a versatile protein assembly for developing artificial metalloenzymes or metalloproteins.

The organometallic Pd(allyl) complex was immobilized into the ferritin cage assembly (Figure 4). Various mutational studies were done to control the metal coordination structures.[31, 32, 73] Interestingly, no apparent changes were observed due to mutagenesis rather than unique Pd coordination structures inside the cage.[32] Pd(allyl) complex was predominantly bounded by the Cys residues and supported by the surrounding Glu and His residues. Thus, by controlling these three residues, coordination structures of Pd(allyl) can be controlled. The ferritin-Pd(allyl) composites were active towards the Suzuki-Miyuara cross-coupling reaction between Phenyl boronic acid and 4-iodo aniline in an aqueous medium. A maximum of 4300 TOF per hour per cage was achieved for the mutant apo-E45C/R52H.[32] TOF was used as a measure to explore the catalytic site and the entry mechanism of Pd(allyl) into the cage. Mutations at His, Glu, and Cys located at the 3-fold channel by Ala led to a decrease in both metal accumulation and TOF.[31]

This clarified that the Pd(allyl) entered the cage through the 3-fold symmetric channel of the cage. This also signifies the importance of protein assembly structure in which monomeric subunits are assembled in such a way to form unique pores, which play crucial roles in the transportation of small substrates into the cage during catalysis. After successful demonstration of immobilization of Pd(allyl) complex and catalysis by Ferritin-Pd(allyl) composites, an organometallic Rh(nbd) (nbd: norbornadiene) complex was fixed onto the inner surface of the ferritin nanocage to promote the catalytic polymerization of phenylacetylene and its derivatives (Figure 4).[26] The idea of using such polymerization reactions inside the confined space of the ferritin cage was to restrict the narrow molecular weight distribution of the polymer product. The X-ray crystal structure revealed the binding site of Rh(nbd) at Cys48. In the presence of phenylacetylene, the ferritin-Rh catalyst showed a yellow-colored solution confirming the polymerization reaction inside the cage and thus became water-soluble. A control reaction with a free Rh(nbd) catalyst showed only water-insoluble polymer formation suggesting that the confinement protein environment played a significant role in catalysis. Various phenylacetylene derivatives were used and found that the activity was low for anionic derivatives due to restricted entry through the negatively charged 3-fold pores. To better understand the reaction mechanism, QM/MM (quantum mechanics/molecular mechanics) studies were done, which revealed that all the binding sites were not equally active in catalysis.[74] Thus, the results support the fact that confined protein assembly environments can play important roles in the product selection of the artificial metalloenzyme catalysis.

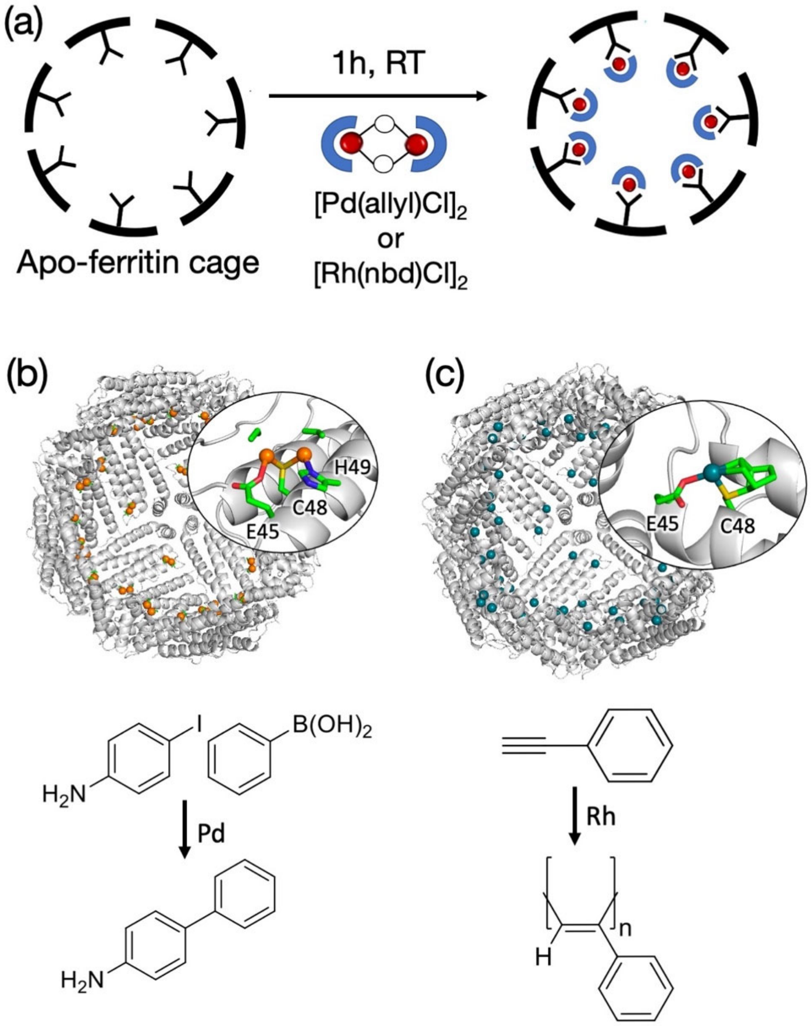

Figure 4: (a) Reaction scheme for the preparation of Pd(allyl) and Rh(nbd) complex into ferritin cage. (b) X-ray crystal structure showing the immobilization of Pd(allyl) on the interior surface of the ferritin cage (pdb: 2zg7) and the corresponding Suzuki-Miyuara coupling reaction. The inset shows the catalytic active site containing Pd(allyl) binding by Cys48, Glu45 and His49. (c) X-ray crystal structure showing the immobilization of Rh(nbd) on the interior surface of the ferritin cage (pdb: 2zur) and the corresponding polymerization reaction of phenyl acetylene. The inset shows the catalytic active site containing Rh(nbd) binding by Cys48 and Glu45.

A recent report shows that the IrCp* can be immobilized inside the inner cavity of ferritin cage assembly to promote transfer hydrogenation of substituted acetophenones (Figure 5a-d).[33] Ferritin protein architecture provided the asymmetric environment for the catalysis to control the diffusion of substrates depending on their polarity and size. Two residues viz. Asp38 and Arg52 near the metal-binding site at Cys48 inside the ferritin cavity were replaced with the histidine residues for controlled uptake of an organometallic $[IrCp^*Cl_2]_2$ complex (Figure 5b-d). Position of mutation was important as the replacement of Arg52 by His gave a 1.5-fold increase in the metal uptake compared to other mutant and wild-type ferritins. The hybrid nanocage showed efficient catalysis to produce chiral alcohols from acetophenone derivatives giving up to 88% conversion. Aromatic interactions with the side chains near the catalytic site at Cys48 were found to control the substrate's approach and thus, gave higher enantioselectivity.

For understanding more details of the reaction, a modified Hammett equation was used for analysis[75],[76],[77]. It was found that the catalytic reaction rate was influenced not only by the electronic property of the substrates but also by the steric property of the substituents. Since the Ferritin-Ir composite contained multiple Ir binding sites, they controlled the Ir uptake to have a single Ir site binding site, which revealed better TOF than the composite having multiple active sites. This suggested that all the Ir binding sites were not equally active in catalysis. In an another report, two different organometallic catalysts, IrCp* and Pd(allyl), were incorporated into a single ferritin cage assembly to promote catalytic cascade reactions (Figure 5f).[34] This is interesting while considering nature's choice of utilizing microcompartments to isolate a metabolic process.[20] Both metal catalysts were found to have different binding preferences in the cage. Ir preferred to bind to His while Pd preferred Cys. The dual metal catalysts inside the cage successfully promoted a catalytic cascade reaction of C-C coupling (Pd) and hydrogenation (Ir) reactions inside the confined cage. Although the catalytic efficiency was poor, the work demonstrated the potential of establishing multiple reactions within a single cage.

Recently, Feng and coworkers have developed a novel ferritin nanocage containing iron-sulfur clusters for photocatalytic H_2 evolution reaction (HER) in acidic aqueous solutions.[35] They used a synthetic iron-sulfur cluster containing $Fe_2(\mu\text{-}SC_2H_4)$ $(\mu\text{-}SCH)$ $(CO)_6$ as a building block which was incorporated into the ferritin cage at pH7.4 simply by stirring at 4°C. Up to 312 moieties were incorporated into a single cage nearly 25 wt% of the cage. The water-soluble homogeneous ferritin cage catalyst showed visible-light-induced H_2 evolution with a turnover number (TON) up to 31 per single catalyst moiety, nearly 8.5-fold higher than the free catalyst. The core-shell assembled structure of the ferritin cage allowed higher iron uptake, water solubility, and long-distance electron transportation from photosensitizer, which were considered the enhanced activity. Thus, the investigations on ferritin-based HER catalyst signifies the uniqueness of the self-assembled structure, which can make a free catalyst more efficient within protein environments.

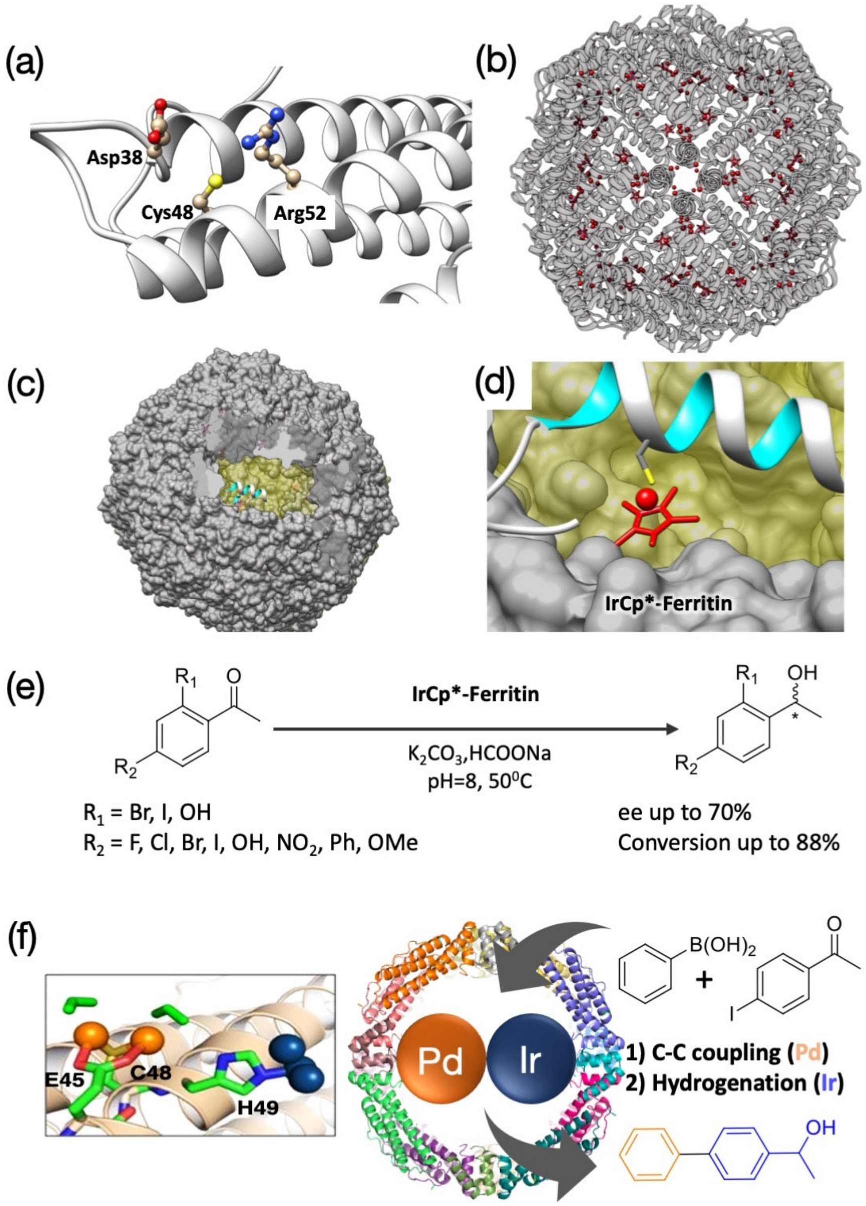

Figure 5: (a) Ferritin monomer unit showing a typical metal binding site and the position of Asp38 and Arg52 residues subjected to mutation. (b) IrCp* bound form of 24-mer self-assembly of ferritin (pdb: 7eml). IrCp* units are shown in red sphere. (c) Surface view of the IrCp* bound ferritin cage showing hollow cavity of the self-assembly. (d) Enlarged view of showing the Ir catalytic center. (e) Schematic representation of the transfer hydrogenation reaction catalyzed by IrCp* bound ferritin nanocages. (f) Immobilization of two metal complexes (Pd(allyl) and IrCp*) into a single ferritin cage and promotion of catalytic cascade reactions.

Not only synthetic metal catalysts or nanoparticles but also larger molecules and enzymes can also be incorporated inside the ferritin cage assembly.[23, 78, 79] As mentioned before, small molecules can easily enter the cage through narrow 3-fold pores (< 1nm diameter), while larger molecules usually can not pass through this (Figure 3c). Ferritin has a unique disassembly-reassembly mechanism induced by pH (Figure 6a). At pH2 or pH13, when the ferritin cage is disassembled, large molecules can be added, and while reassembly at neutral pH, the foreign molecules can be encapsulated by the cage.[23, 36] Using such methodology, large molecules, including enzymes, can be encapsulated into the cage.[23, 36] For example, T. Ward et al. have incorporated an artificial transfer hydrogenase (ATHase) based on the streptavidin-biotin technology into the confined environment of the ferritin cage via re-assembly/disassembly mechanism. An organometallic IrCp* catalyst was anchored into the streptavidin to construct the artificial hydrogenase. Interestingly, encapsulation of enzymes (4.5 nm × 5.5 nm × 5.1 nm) did not have any apparent effect on the 24-mer cage structure (8 nm inner diameter). The restricted space of the ferritin cage behaved as the third coordination sphere to the catalytic site and significantly influenced the catalytic reduction of cyclic imines with >3800 TON. The enhanced activity compared to free enzyme suggested that the ferritin cage protected the Sav catalyst. It is interesting to note that the unique protein assembly structure protected another protein assembly-based artificial metalloenzyme. In another approach, ferritin cage disassembly/reassembly methodology was used to incorporate Hemin, a larger porphyrin compound that can not enter the cage through narrow channels.[36] Interestingly, the Hemin-containing ferritin cage showed high catalytic peroxidase activity under harsh conditions such as high temperatures and in the presence of denaturants (Figure 6c). The reaction is selective and can be promoted in tumor cells as a colorimetric probe.

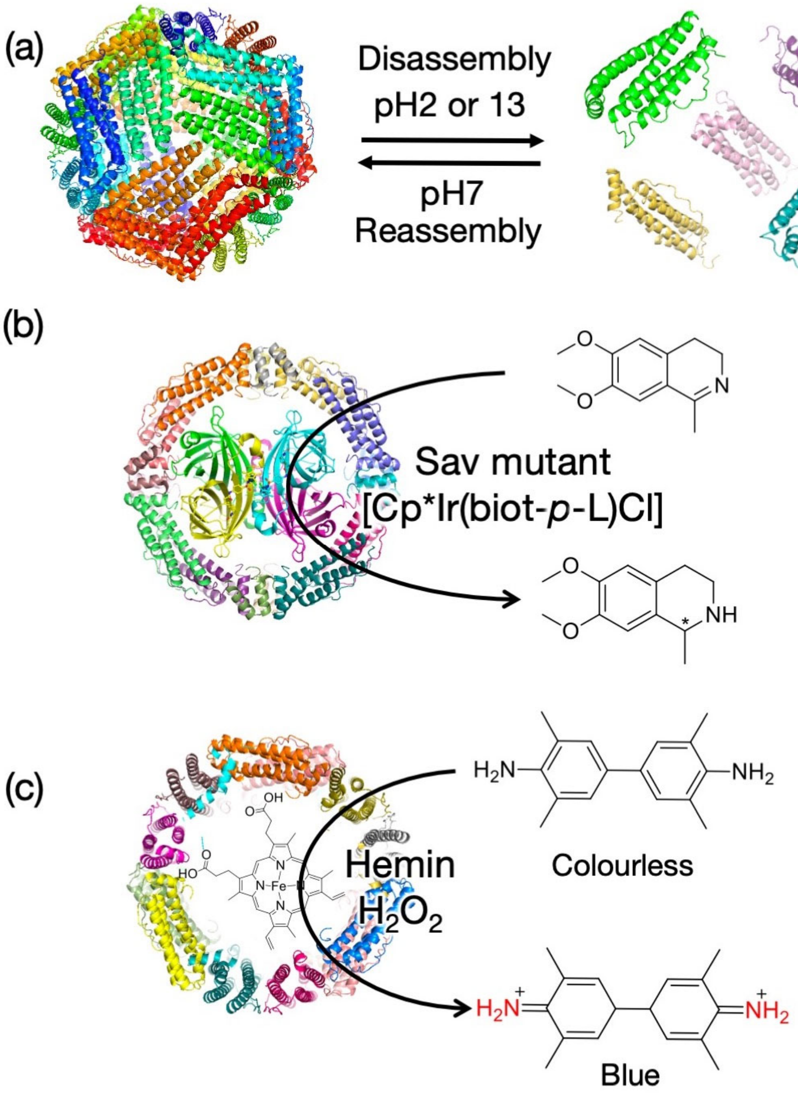

Figure 6: Ferritin cage disassembly/reassembly induced guest encapsulation. (a) Schematic representation of the process of pH induced ferritin cage disassembly/reassembly. (b) Encapsulation of tetrameric streptavidin based artificial transfer hydrogenases into a ferritin cage and the corresponding catalytic hydrogenation reaction. Ferritin cage was disassembled at pH2 and reassembled at neutral pH. (c) Encapsulation of Hemin into a ferritin cage and the corresponding peroxidase activity. Ferritin cage was disassembled at pH13 and reassembled at neutral pH. Figures are prepared in pymol and chemdraw.

3.2. Dimeric Lactococcal multidrug resistance Regulator (LmrR) assembly for catalysis

LmrR is a transcriptional repressor associated with antibiotic resistance of Lactococcus lactis bacterium.[80] The protein LmrR is a dimer formed by the self-assembly of two typical β-winged helix-turn-helix domains with an additional C-terminal helix (Figure 7a-b). Interestingly dimeric interface contains a large hydrophobic pocket that can accommodate planer aromatic organic molecules.[81] Drug molecule stacks inside the hydrophobic pocket between two Trp residues through π- π stacking interactions (Figure 7c). It has been reported that the dimeric LmrR assembly structure exhibit a broad and shallow conformation energy landscape which helps to accommodate the structurally unrelated compounds with protein conformational change.[82] Taking advantage of such proximity of the hydrophobic pocket, Gerard Roelfes and co-workers have designed various artificial metalloenzymes mostly by incorporating Cu(Phen) metal catalysts.[19]'[83]'[25]'[84]'[85] When Cu(II) complex was grafted into the hydrophobic cavity, it showed various catalytic reactions like Friedel-Crafts alkylation, Dield-Alder reactions, etc. which are listed in Table1 (Figure 7e-f)). For the Diels-Alder reaction, they achieved exceptionally high enantioselectivity of up to 97%.[86] For bioconjugation, they replaced Asn19 or Met89 with Cys residue (Figure 7d). Aromatic ligands like phenanthroline- or bipyridine-based functionalized with bromo acetamide group were used for selective conjugation to cysteine for covalent attachment to the LmrR. Phenanthroline- and bipyridine-based ligand have good binding affinity with Cu(II). The quaternary structure of the dimeric LmrR had no apparent changes upon functionalization and thus developed assembly remains dimeric and catalytically active. The results showed that the protein scaffold provides the enantioselectivity for the reaction and accelerates the rate of reaction. They reported several examples of LmrR ArMs to catalyze Friedel craft alkylation reaction[25], Diels Alder reaction[19], Cyclopropanation[40], Tandem Friedel craft alkylation-enantioselective protonation reaction[83], tandem Michael addition-enantioselective protonation[37] and hydratase reaction[85]. They recently used applied this LmrR based ArM for catalysis within live cells.[19] The designed ArM was active in both Friedel-Crafts and Diels-Alder reaction, and the enzymes can also be evolved for higher efficiency and enantioselectivity.

Other than the usual Cu(II) complex, they introduced a Hemin group into the hydrophobic pocket of the LmrR assembly. The encapsulated Hemin showed efficient catalysis in cyclopropanation reactions. This example suggests that the hydrophobic cavity at the dimer interface can be expanded to other planar aromatic compounds. After establishing various reactions by LmrR based artificial metalloenzymes containing a single catalytic site, they expanded the strategy by introducing two abiological units in a single scaffold to have synergistic catalysts.[87] To achieve this, they combined a genetically encoded unnatural *p*-aminophenylalanine residue (which activates an enal through iminium ion formation) and a Cu(II) complex as a Lewis acid. they accelerated the Michael addition reaction synergistically with high activity and enantioselectivity up to 99% using such a strategy. This example showed the importance of protein assembly structure could not be availed from the monomeric counterpart.

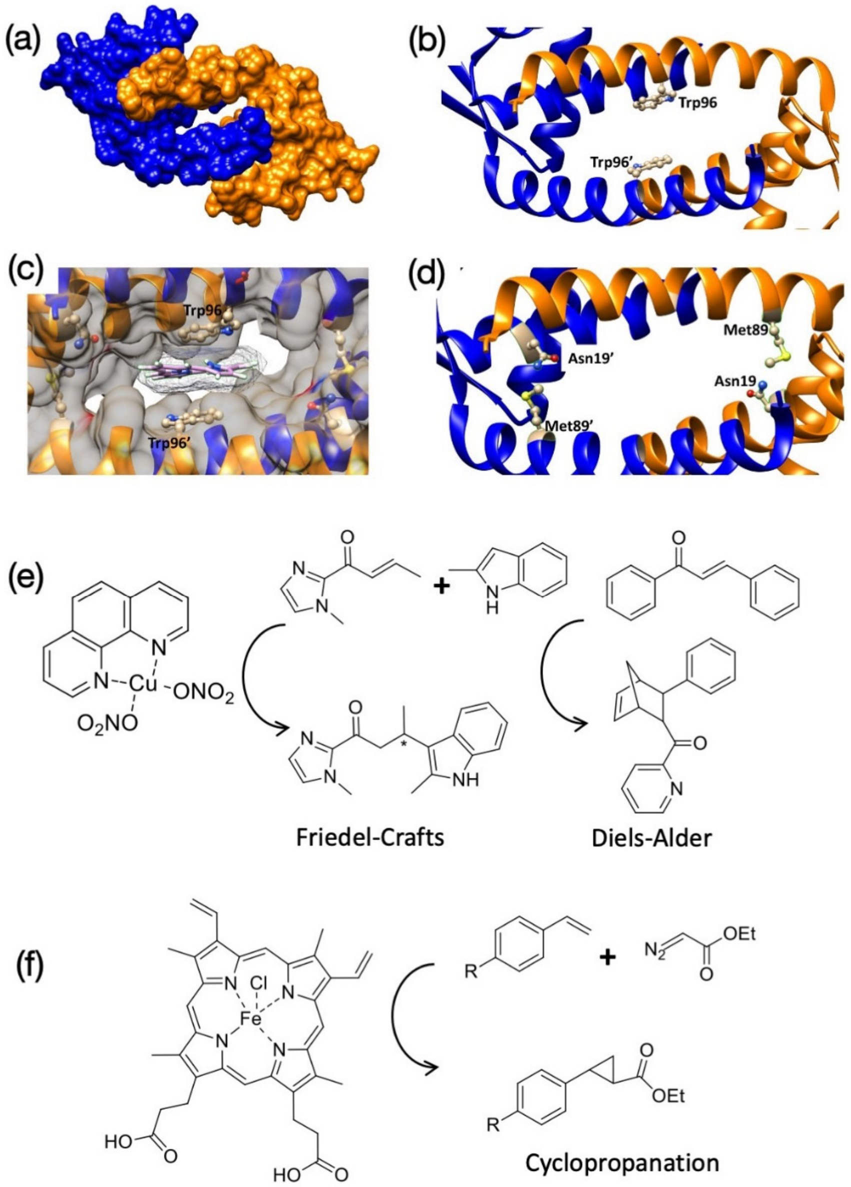

Figure 7: Dimeric self-assembly of LmrR in space filling model (a), Position of Trp96 residues in the hydrophobic cavity of the LmrR (b), Stacking of planer aromatic molecules inside the cavity (c) and the residues Asn19 and/or Met89 for the mutagenesis and/or for the covalent attachment of the metal binding ligand. Figures were made in chimera using the crystal structure of LmrR (PDB ID.3f8b). (e) Structure of the encapsulating Cu(Phen) complex and the corresponding catalytic reactions of Friedel-Crafts alkylation and Diels-Alder reaction with cyclopentadiene. (f) Structure of the encapsulated Hemin and the corresponding cyclopropanation reaction.

3.3. Artificial metalloenzyme catalysis based on tetrameric streptavidin assembly.

Another promising system for developing protein assembly-based artificial metalloenzyme is the biotin-streptavidin complex.[88] Streptavidin (SAV) is a homo-tetrameric (dimer of dimer) self-assembly that has exceptionally high binding affinity to biotin.[89] Using such specific biotin-SAV interaction, various artificial metalloenzymes have been developed to promote organic transformations.[90],[91] A unique methodology was used in which biotin was functionalized with synthetic catalysts and then allowed to assemble with SAV (Figure 8a). The high binding affinity of the biotin molecule to SAV brings the synthetic catalysts into the desired protein cavity. The secondary coordination sphere in the protein matrix can trigger the reaction rate and enantioselectivity during catalysis. Using such anchoring technology, many artificial metalloenzymes were constructed to mimic the naturally occurring enzyme functions.

T. Ward and coworkers have extensively utilized this technology to develop various ArMs for about 12 different reactions with high turnover rates and enantioselectivity.[88] For example, a biotinylated Rh(III) catalyst was anchored into an engineered SAV to promote asymmetric catalytic C-H activation.[42] The constructed ArM gave nearly 100-fold higher activity than isolated Rh(III) complex, and the enantioselectivity reached up to 93%. Similarly, they have developed artificial Suzukiase and transfer hydrogenase by anchoring biotinylated monophosphine Pd and IrCp* complex, respectively, into the streptavidin scaffold.[43, 92] Biotinylated Co complex was also anchored into streptavidin to promote water reduction reaction in basic solution which gave over a 12-fold increase in the activity compared to free biotinylated catalyst.[93]

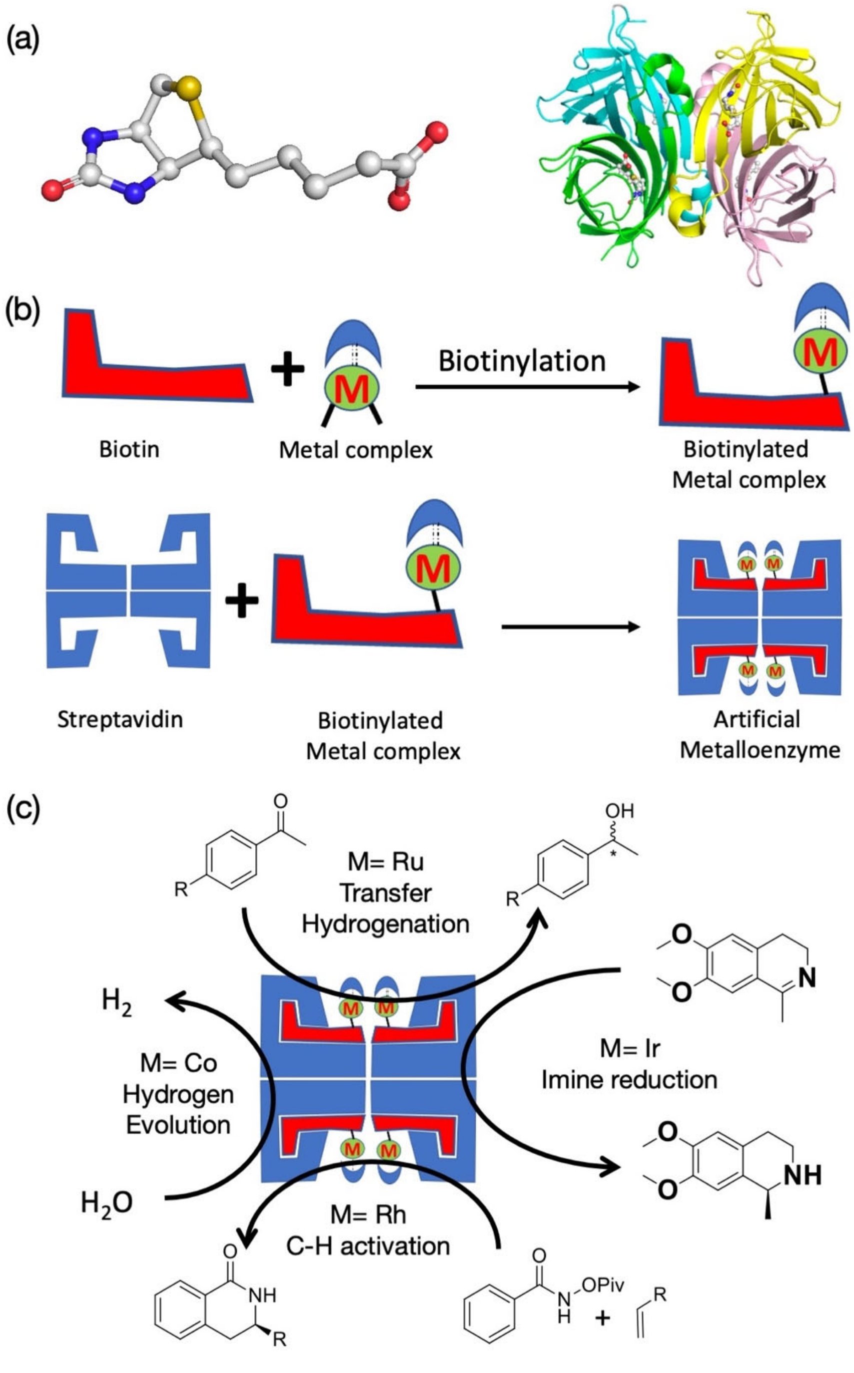

Figure 8: Construction of streptavidin (SAV)-based ArMs and reactions. a) Structure of biotin and tetrameric assembly of SAV (pdb:1df8). (b) Schematic representation showing the biotinylation of metal catalyst and preparation of SAV-based ARMs. (c) Schematic representation showing various catalytic reactions promoted by SAV based ArMs.

After many successes, streptavidin-based ArM was focused on applying in vivo catalysis (Figure 9a).[44] To avoid inhibition of catalysis by cellular glutathione (GSH), periplasm-localized ArM was constructed because periplasm has relatively less glutathione concentration than cytoplasm.[94] Biotinylated Ru catalyst (Hoveyda−Grubbs second-generation catalyst) was employed for ring-closing olefin metathesis using a diolefin umbelliferone precursor as a substrate. It was found that the biotinylated Ru catalyst can diffuse through the outer membrane, accumulate in the periplasm, and form assembly with periplasmic SAV. After adding diolefin substrate, the E. coli bearing periplasmic SAV yielded fluorescent umbelliferon confirming the catalytic ring-closing metathesis reaction within cells. The control experiment with free biotinylated Ru catalyst without periplasmic SAV showed minimal catalysis. After successfully demonstrating of such periplasmic assembly and olefin metathesis, the SAV ArM was used to evolve for higher catalytic efficiency. The work demonstrated that the SAV ArM is suitable for application in vivo and is expected to have a promising future particularly for cell function control.

Advantages of the self-assembled SAV structure were successfully utilized by Ward and coworkers.[12, 24] In contrast to conventional ArMs by a single type of biotinylated catalyst, they developed unique and multifunctional ArMs by anchoring various types of conjugated biotins into a single tetrameric SAV matrix (Figure 9b). Using this method, they introduced three different features into a single ArM: the cell-penetrating module, fluorescence imaging to track inside cells, and biorthogonal catalysis in HEK-293T cells.[12] To construct such multifunctional ArM, they used two different types of biotinylated conjugates with an organometallic Ru complex for catalytic uncaging of allyl carbamate substrate and a cell-penetrating poly(disulfide) (CPD) bearing a fluorescent moiety like Atto565. For uncaging, amine-protected anti-thyroid hormone, triiodothyronine (T_3),was used because after uncaging, it is known to cause the upregulation of a gene circuit, which leads to the expression of a NanoLuc-luciferase. The in vivo studies successfully demonstrated that the multifunctional ArM could promote catalytic uncaging of triiodothyronine inside the mammalian cell with TON over 25 in various mutants. Such biorthogonal reaction upregulated the activity of a genetically encoded sec-Nluc. Overall, the demonstration established that metalloenzyme assembly could be functionalized with multiple features to control cell functions which is difficult to achieve using a single monomeric counterpart. Based on this concept, they further expand the work by positioning two metal cofactors into a single ArM to activate a single substrate.[24] The developed an artificial hydroaminase to activate a single alkyne compound by dual gold activation within SAV (Figure 9c). Using the tetrameric self-assembling features of SAV, two synergistic biotinylated gold cofactors were placed into the hydrophobic pockets. The dual gold catalysts selectively convert an ethynylphenylurea substrate to selective anti-Markovnikov product, indole carboxamide with 96% selectivity. The same reaction in the presence of a single gold catalyst gave selective Markovnikov product, phenyl-dihydroquinazolinone, with 99% selectivity. Such design of ArM can be evolved to enhance the activity and selectivity. Further, with the help of computational study, they evolved the system to favor either σ, π-activation or π-activation of the alkyne resulting in either substituted indole or quinazolinone.

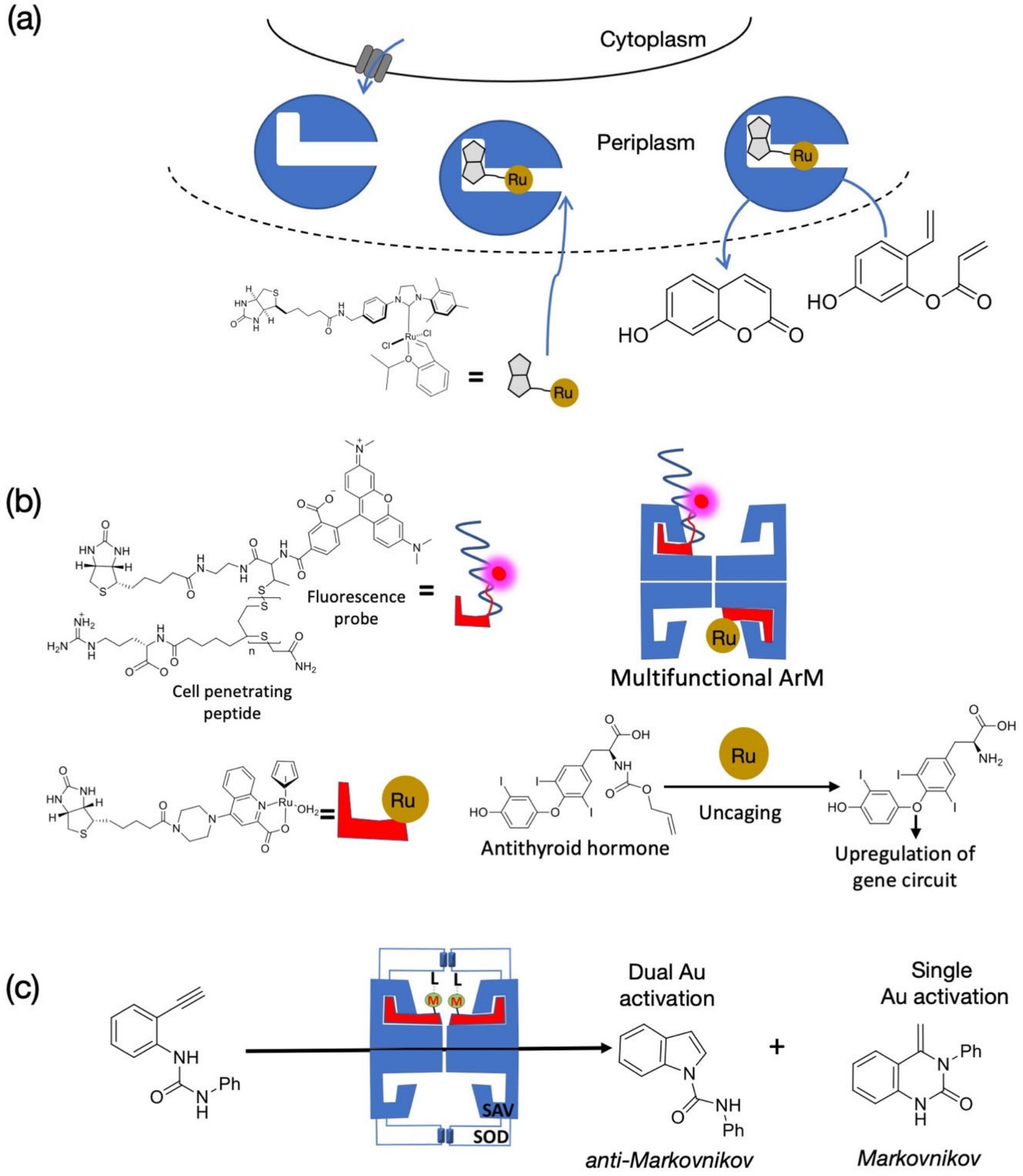

Figure 9: (a) In vivo metathesis by a Ru based SAV ArM. After expression, the periplasmic SAV comes to periplasm. The biotinylated substrate diffused into periplasm and binds to SAV to construct ArM which catalyzes the diolefin substrate to coumarin. (b) Construction of a multifunctional SAV ArM containing a cell penetrating peptide, a fluorescence tag, and an uncaging Ru-arene catalyst. Catalytic uncaging of antithyroid hormone leads to upregulation of genetically encoded sec-nluc. (c) Selective activation of an ethynylphenylurea substrate by single and dual gold catalysis.

4. Design of artificial metalloprotein assembly structures.

Recently, there has been growing interest in designing and constructing artificial protein assembly structures considering the potential future applications as bionanomaterials.[17] Various strategies like electrostatic interaction, non-covalent interaction, covalent bond formation, metal coordination, etc., were employed. Among various strategies, metal coordination is one of the most interesting approaches considering their usefulness as artificial metalloenzymes. The immobilized metal cofactors can act as an inducer of self-assembly and catalytic site. Since metal coordination is very specific, the coordination bond formation brings two or more protein monomers together to form unique assembly structures. Tezcan and coworkers have first introduced such metal-mediated protein assembly in 2007.[95] Several histidine residues have been introduced on the surface of cytochrome cyt cb562 self-assemble into a 16-helix quaternary structure in the presence of Zn ions (Figure 10a).[95] In a similar way, they also prepared cryptand-like tetrameric protein assembly via disulfide bond formation.[96] Based on this concept, various oligomeric structures were developed by changing the metal ions, coordination positions, interface modifications, etc. They used a new engineering strategy called “reverse metal-templated interface redesign”[97] in which the 2-fold symmetric interface of the ferritin cage was mutated with several histidine residues which existed as monomers in the inclusion bodies (Figure 10b). In the presence of Cu^{2+}, the ferritin monomers selectively interact to form the 24-mer cage. Such re-engineering on the surface of a natural protein cage to lead metal-inducible self-assembly is useful for developing responsive biomaterials. More recently, Zhao et al. introduced Histidine residues at the symmetric surface of the ferritin cage to develop various supramolecular structures.[98, 99] In addition to such metal-mediated protein assemblies, Tezcan and coworkers have developed protein metal-organic frameworks (PMOF) in which protein-protein assembly occurred through metal-organic linker directed interactions (Figure 10c).[100, 101] They used a ferritin cage and mutated Histidine at the 3-fold symmetric channel. In the presence of Zn^{2+} ions and organic linker with terminal hydroxamic acid functionalities, ferritin molecules assembled and formed a porous network. By varying the organic linker containing hydroxamic acid functionalities, the PMOF structure and their mechanical properties can be tuned.[101] Other than the common Histidine mediated protein assembly, recently, Song et al. have genetically introduced an unnatural amino acid called bipyridine-alanine into the D3 homohexamer of acetyltransferase protein.[102] Addition of Ni ions leads to giving unique 1D or 3D assemble structures depending on the position of mutations. The chelating bipyridine-alanine was the driving force for diverse protein assembly.

Besides such metal-mediated protein assemblies formation, Hayashi and coworkers have used a unique strategy in which various protein assembly structures such as filament can be constructed using modified heme reconstruction in heme protein matrixes.[103-107] They introduced a Cys at the opposite side of the Heme pocket and removed heme at lower pH. Then they introduced a maleimide functionalized Heme which gave a fibrillar assembly structure through covalent conjugation through Cys and heme reconstitution (Figure 10d). In another approach, they developed a hexameric Zn chlorin e_6 protein assembly reconstituted with Zn porphyrinoid molecules (Figure 10e).[107] Studying fluorescence quenching in the presence of methyl viologen showed energy migration within the porphyrinoid photosensitizers. Since the Heme groups are familiar with many metalloenzyme reactions, such protein assembly structures might be useful for developing artificial metalloenzymes. Recently, the Heddle group has reported an ultra-stable Au inducible protein cage assembly by precise manipulation of protein-protein interfaces (Figure 10f).[108] They introduced eleven Cysteine residues in the surface of tryptophan RNA-binding attenuation protein (TRAP). The addition of Au(I)-triphenylphosphine compound leads to forming a 24-mer assembly of uniform rings containing 120 S-Au-S bonds, which were confirmed by cryo-electron microscopy. The assembly and disassembly of such a giant cage can be reversibly tuned by metal coordination. Such a higher order metal-induced supramolecular structure open up a new direction to construct bionanocage materials.

After much success in constructing artificial protein assemblies, researchers now focus on designing such structures by computation design considering the energy functions.[109-112] Such design widens the scope and possibilities through screening before in vivo expression.[112] David Baker and coworkers have reported a computational design of 12-mer and 24-mer protein assembly with tetrahedral and octahedral symmetry, respectively.[110] They validated their design experimentally in which a closely matched model protein assembly was formed in solution. This suggests that we can design various protein assembly structures using the computational method. L Millar et al have computationally designed the symmetric interface of imidazole glycerol phosphate synthase to form a homodimer validated by the X-ray crystal structure analysis.[113] Many computational tools are available for design, out of which Rosetta is one of the commonly used software to design protein dimers or small oligomers. Computational protein assembly design is mainly considered weak electrostatic interactions compared to metal coordination which is difficult as it is too specific.

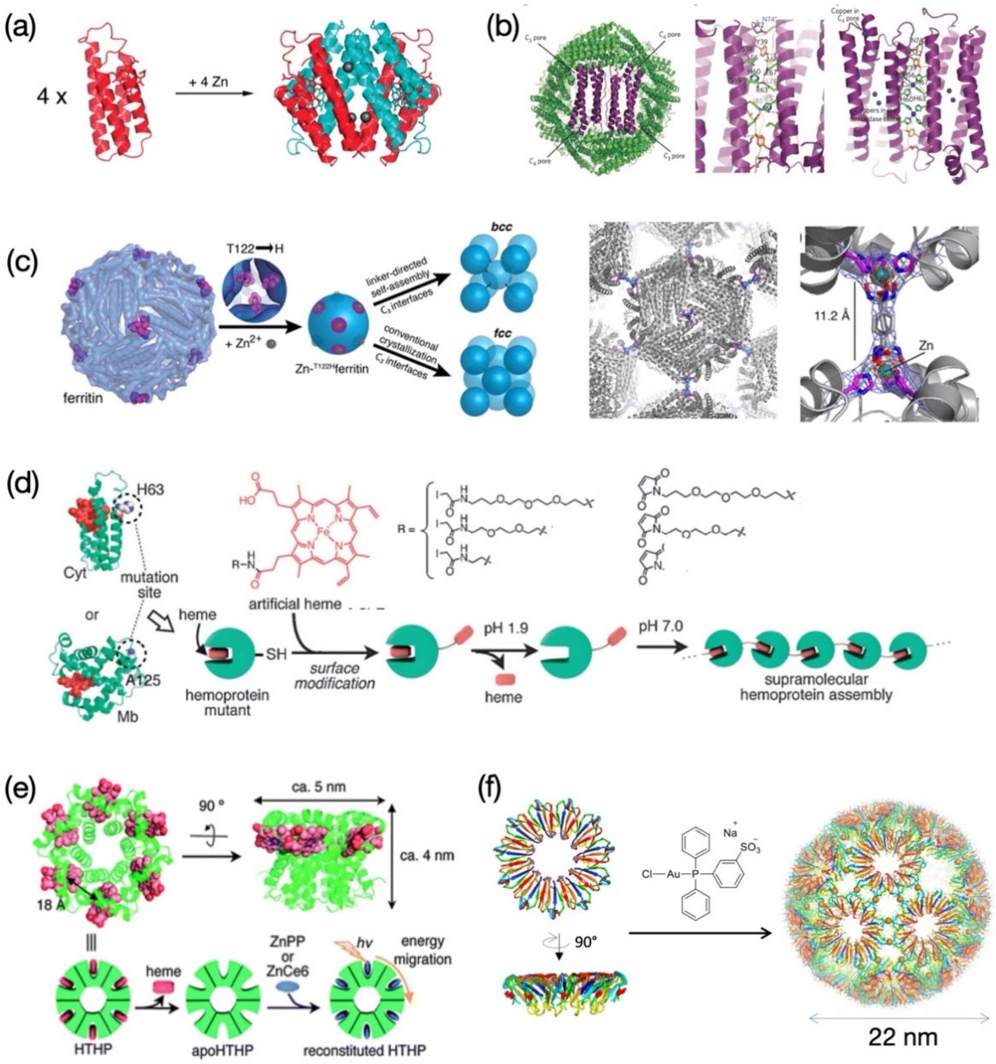

Figure 10: Selected examples of artificially designed metalloprotein assemblies. (a) Zn mediated tetrameric assembly structure formation by cyt cb562 after four His mutations. (b) Re-engineering of the 2-fold symmetric interface of ferritin cage with His mutation for construction of Cu^{2+} inducible responsive protein cage assembly. (c) Ferritin MOF formation in presence of Zn and organic linker. (c) Fibrillar myoglobin assembly formation through heme reconstruction and modification. (d) Heme reconstitution in HTHP to construct a nanoassembly for energy migration. (f) Construction of a giant protein cage assembly of 22 nm diameter from TRAP in presence of $ClAu(Ph)_2(Ph\text{-}SO^{3-})$. The structure was drawn using pdb:6rvw in pymol. The metal ions are shown in red spheres. Figures (a-e) are reproduced with permission from ACS, RSC and Springer nature [95] [97] [100, 103] [107].

5. Catalysis by artificially designed protein assembly.

As described in the previous section, protein assembly can be designed artificially by computational and experimental methods. Their structures can also be tuned by suitable mutations. In addition, suitable mutation can also introduce catalytic metal center into the designed protein assembly either by direct metal coordination or through bioconjugation. When metal coordination is used for self-assembly, it can also act as a catalytic site. Thus, artificial metalloprotein assembly is a promising scaffold for developing metalloenzymes. Here we describe a few examples of artificially designed protein assembly utilized for catalysis.

5.1. Catalysis by tetrameric cyt cb562 assembly: Tezcan et have developed various protein assembly structures using the cyt cb562 scaffold, including a dimer, trimer, and tetramer.[18, 95] Recently, they have demonstrated the development of a novel tetrameric cyt cb562 assembly as an artificial β-lactamase.[18] The ArM was constructed via the self-assembly of a four-helix-bundle redox protein cyt cb_{562} which does not bear any similarity with metallo-β-lactamase or hydrolytic enzyme (Figure 11). [114],[115] Using the perfect combination of His and Glu mutations at the surface and in the presence of Zn ions, they constructed a tetrameric protein assembly. Out of a total of 8 Zn ions per assembly, four Zn sites were considered to play a structural role, and the other four Zn ions to act as potential catalytic sites (Figure 11b).

Various mutants were prepared and tested for β-lactamase activity in vitro using ampicillin as a β-lactam antibiotic substrate. Zn8:^{A104}AB3_4 mutant was found to hydrolyze ampicillin at pH9. They further tested the activity within E. coli. Since the β-lactamase activity is directly linked to the survival of bacteria, higher activity was measured based on the antibiotic resistance by the bacteria. For in vivo studies, they grew the E. Coli cells on agar/Luria-Bertani (LB) plates containing various amounts of ampicillin. E. Coli expressing the Zn8:^{A104}AB3_4 mutant survived with ampicillin concentration up to 1.1 mg/lit suggesting that the artificially designed protein assembly catalyst maintained β-lactamase activity in living cells. They also studied the scaffold flexibility and rigidity of the artificial metallo-β-lactamase activity.[45] A balance between these two is the key in designing enzymes artificially. Such supramolecular protein assembly enzymes can be evolved to enhance their activity and stability within the cell. Directed evolution needs high-throughput screening. Song et al. have recently established that residues at the symmetric region are the promising hotspots for evolving de novo designed oligomeric enzymes.[46] They found a drastic effect in the β-lactamase activity when the symmetry-related residues at the rotational axes were randomized. This is because mutations at this location altered the structure and functions of the protein assembly. Such a novel strategy could be used to prepare libraries of symmetric artificial metalloenzymes.

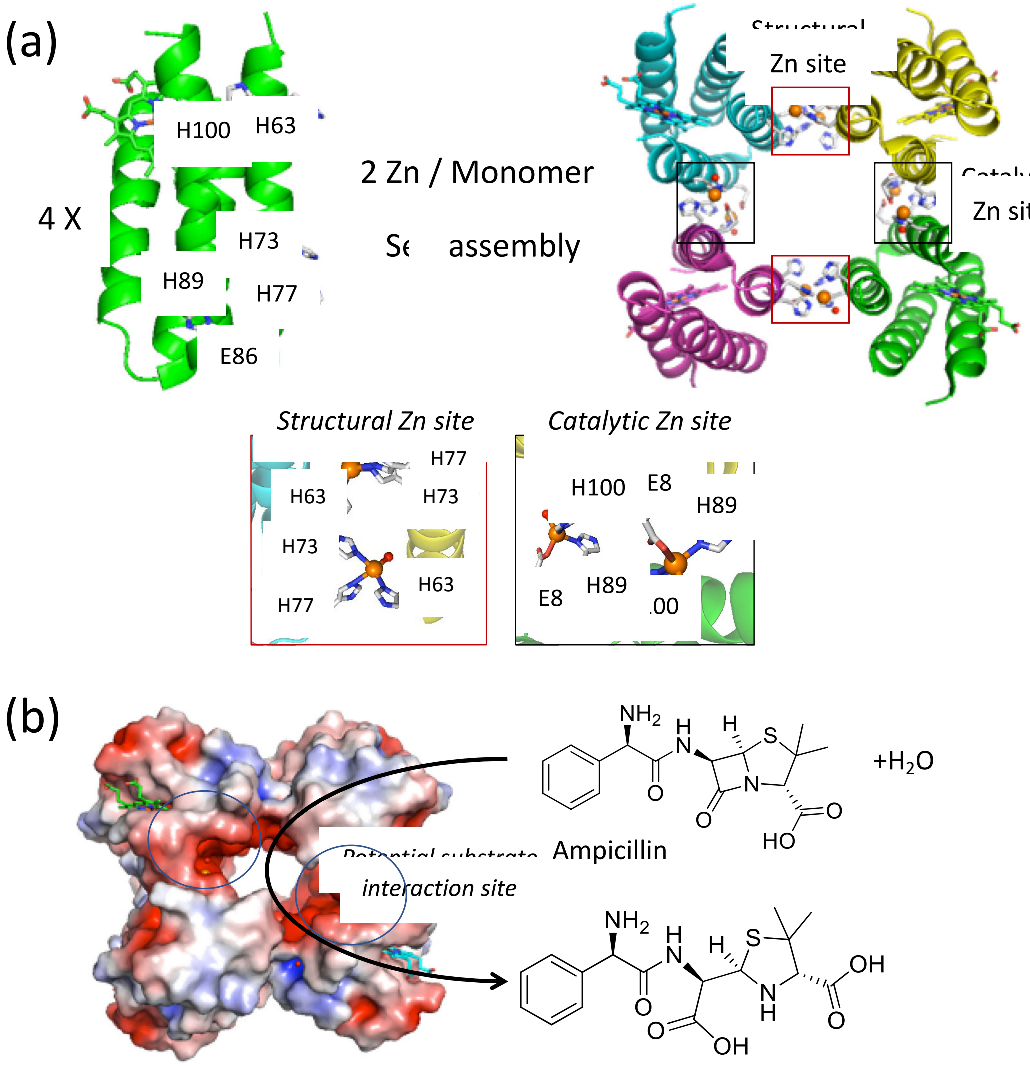

Figure 11: Design and construction of an artificial metalloenzyme assembly ($\mathrm{Zn_8}$:$^{A104/G57A}$B3$_4$.) and catalysis. (a) Structure of the cyt cb562 monomer and formation of tetrameric assembly in presence of Zn^{2+} ions (pdb: 4u9e). The coordination structure of the catalytic and structural Zn sites are shown in the bottom. (b) A surface model of the artificial metalloenzyme showing the β-lactamase activity: Hydrolysis of ampicillin.

5.2. Catalysis by artificially constructed protein needle.

Ueno and coworkers have developed a unique needle-type protein assembly called "protein needle (PN)" from the gene product from bateriophage T4.[49] Such PN gave a long 17nm β-helix assembly structure consisting of a dimer of trimer $[(gp5\beta f)_3]_2$ in solution (Figure 12a). The PN assembly is thermostable and tolerant to high organic solvent content, making PN as a good candidate of developing artificially metalloenzyme. The PN surface can be functionalized with synthetic metal cofactor to promote catalytic reactions. For example, a photocatalytic reaction system was constructed by immobilizing a Ru and Re complex on the PN surface to promote the CO_2 reduction reaction (Figure 12b-c).[47] Alignment of the dual metals on the PN surface can be tuned by introducing or deleting residues like Lys and Cys for functionalization. Such alignment accelerated the electron transfer rate of light reaction. The TOF per hour for CO production from CO_2 was 100, which is 3.3 times higher than the mixtures of free metal complexes and PN. The proximity of two catalytic (Re) and light-harvesting (Ru) centers was found to be responsible for enhanced activity. The unique self-assembled structure of the PN allowed the manipulations of the positions of the Ru^{II} and Re^{I} centers.

An artificial Sc(III) enzyme was constructed by a semi-synthetic approach in which three Sc(bpy) complexes were conjugated to the surface of β-helical bionanotube via maleimide-thiol(Cys) coupling reaction.[48] Tetradentate coordination of $Sc[bpy\text{-}(ROH)_2]$ is known for the epoxide ring-opening reaction.[116] In the PN assembly, the two -ROH can be exchanged with the nearer Thr and Ser side chains. Several mutants were prepared to adjust the distance. The Sc bounded PN acted as a Lewis acid catalyst for an epoxide ring-opening reaction, and the activity was 20-times higher than the protein and ligands. The cooperative effect of Sc^{3+} stabilization and substrate binding on the PN surface was found to be the reason for enhanced reaction. Such a semi-synthetic approach is a useful method to construct artificial metalloenzyme. In a similar way, a Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition was achieved using flavin derivatives of PN.[49]

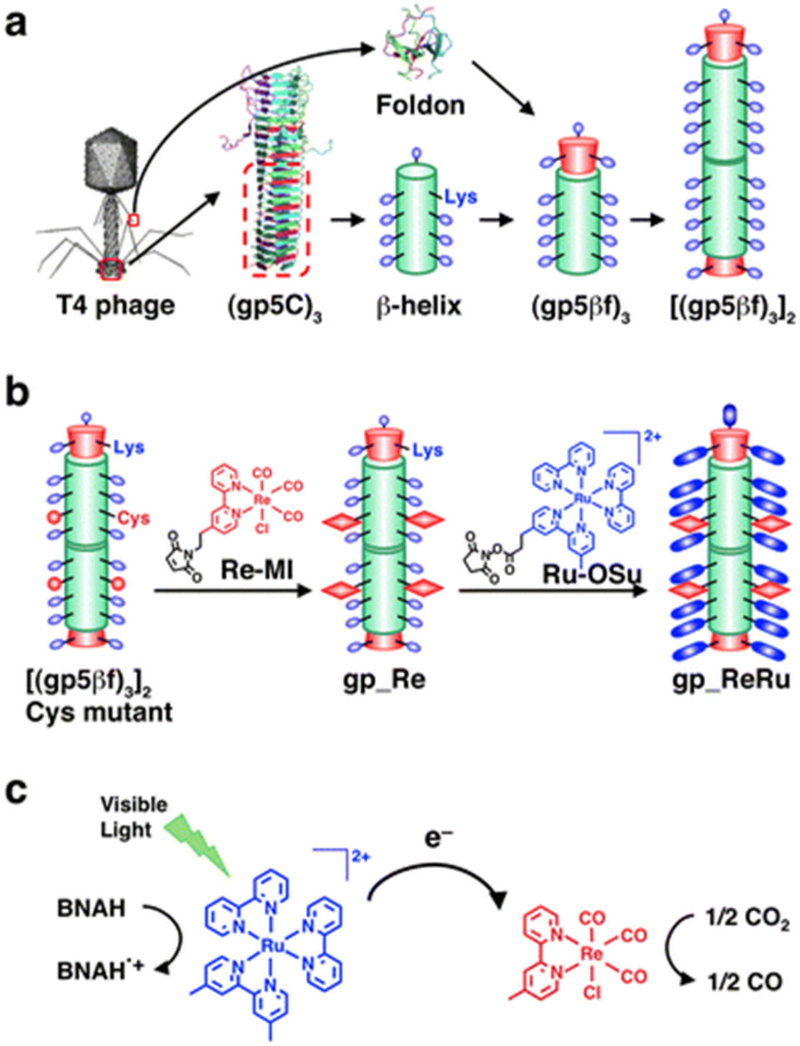

Figure 12. (a) Construction of an artificial hexameric protein needle (PN) assembly, $[(gp5\beta f)_3]_2$. from T4 bacteriophage. (b) Design of a bimetallic metalloenzyme based on PN. (c) Schematic representation of the photocatalytic reduction of CO_2. Figures are reproduced from [47] with permission from Willey.

5.3. Peroxidase activity of cyt c oligomers formed by 3D domain swapping.

Hirota and coworkers have constructed several hemoprotein swapping assembly which are capable of peroxidase reactions.[117] The cyt c, a heme containing electron transfer protein, exchange the C-terminal α-helix region between molecules and the process is known as three-dimensionally domain swapping (3D-DS) (Figure 13a). Such 3D-DS leads to formation of various oligomers and polymers.[118] Apart from the original electron transfer activity, it can promote oxidation of substrate with H_2O_2 (peroxidase activity) if the heme coordinating Met80 residue dissociates from the heme iron.[50] When the 3D-DS cyt c dimer, trimer etc. are formed, the Met80 dissociates from the heme iron, which creates a binding site for substrate and oxidant and thus, the peroxidase activity gets enhanced than that of monomer (Figure 13b-c). This provides a unique methodology to develop protein-assembly based artificial metalloenzyme.

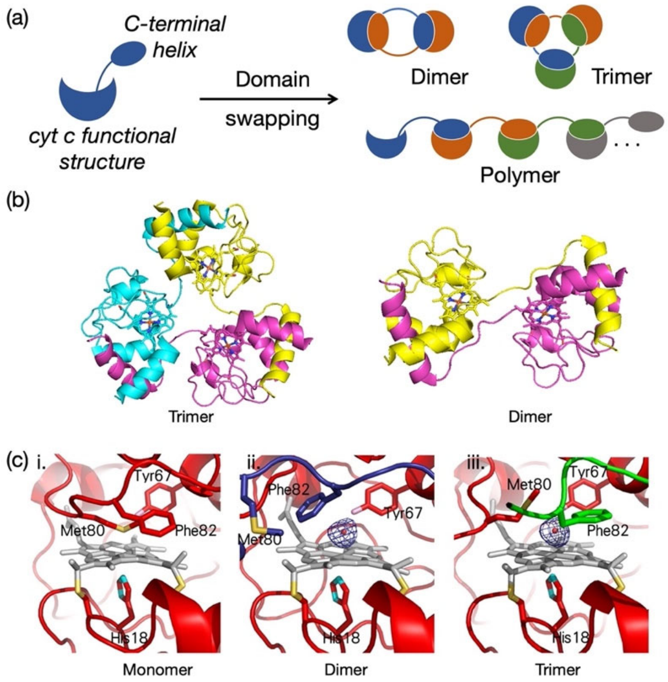

Figure 13. Cyt c assembly structure (oligomer) formation by domain swapping. (a) Schematic representation showing the process of domain swapping and assembly formation. (b) Crystal structure of a trimer and dimer assembly of cyt c. (c) Active site structures (oxidized form) of a cyt c monomer (pdb: 1hrc) (i), dimer (pdb: 3nbs) (ii) and trimer (pdb: 3nbt) (iii).

6. Catalysis by self-assembled peptides with metal cofactors.

Considering many advantages and success of protein assembly-based ArMs, several peptide-based self-assembled nanostructures are now developing for various catalytic applications. Korendovych and coworkers have developed many catalytic short-peptide nanoassemblies with metal cofactors which showed various catalytic reactions such as ester hydrolysis, cyclopropanation, transfer hydrogenation etc. For example, a series of short seven residues (i.e. LILHLFL) amyloid-forming peptide was assembled in presence of Zn^{2+} ions to a fibrillar structure which showed acyl ester hydrolysis activity which is greater than that of the natural carbonic anhydrase by weight (Figure 14a).[56] The Zn^{2+} ions played dual roles here. It stabilized the fibril structure and acted as a cofactor to catalyze the ester hydrolysis reaction. The coordination of Zn^{2+} by Histidine on the fibril surface was crucial for catalysis. Replacement of His by Ala decreased the activity by 60-fold. Single Zn^{2+} binding was found per two peptides (IHIHIQI). Such demonstration showed the potential of a self-assembling nanostructure to develop artificial enzyme-like catalysis. They further extended the work by changing metal cofactors for different reactions. A semi-rationally short peptide assemblies were designed to construct hemin-binding catalytic amyloid beta fibrils.[58] Such unique peptide assembly showed enantioselective cyclopropanation reaction with efficiency similar to that of reported heme-containing proteins.[119] In an another report, , they designed short peptides with C-terminal functionalized with long alkyl tail which spontaneously self-assembled into vesicle-like nanoassembly in presence of an organometallic IrCp* complex (Fugure 14b).[57] The presence of metal catalyst made the nanoassembly catalytically active towards transfer hydrogenation reaction of acetophenone derivatives to chiral alcohols. Similarly, catalytic amyloids were also developed for Cu^{2+} induced oxidation of of 2,6-dimethyloxyphenol in presence of oxygen via C-H bond activation and C-C bond formation.[120] All such examples suggest that self-assembly played key role to construct unique structures which in presence of metal cofactors showed enzyme-like catalytic reactions. More recently, Ding and coworkers have constructed a series of histidine-rich peptides self-assembled into a planar β-sheet nanostructures via charges interaction, hydrogen bonding, and pi-pi stacking interactions.[121] Such a unique planar nanostructure catalyzed the H_2O_2 reduction and thus acted as a cofactor-free oxidase mimetic nanomaterials. In another report, Zn^{2+} induced tubular phenylalanine self-assembly was constructed for enzymatic activity. The single amino acid assembly showed catalytic ester hydrolysis reaction, and the activity was found better than the natural enzyme.[59, 60] Such catalytic esterase activities represent a novel strategy for creating self-assembling nanostructured metalloenzyme.

Pecoraro and coworkers have constructed a de novo designed hydrolytic metalloenzyme based on coiled coils.[122, 123] They introduced two different metal binding sites in the assembly for dual functionality. Considering the active site structure of carbonic anhydrase, Histidine residues were introduced for a catalytically active mononuclear Zn site and at the same time, Cys residues were introduced at the hydrophobic region for Hg binding to stabilize the three-helix bundle structure (Figure 14c). Thus, designed de novo enzyme $[Hg(II)_S][Zn(II)(OH^-)]_N(TRIL9CL23H)_3$ was found high stability due to duel metal binding. The construct showed high catalytic efficiency of 1398±18M^{-1} min^{-1} towards p-nitrophenyl acetate hydrolysis which is just 100-fold lesser than the human carbonic anhydrase and ~550-fold higher than the comparable synthetic catalysts. In addition, $[Hg(II)_S][Zn(II)(OH^-)]_N(TRIL9CL23H)_3$ construct was also CO_2 hydration reaction which showed ~500-fold higher activity than carbonic anhydrase. Such demonstration revealed important structural features to design artificial metalloenzyme. The coordination spheres of the trimeric coiled coils can be changed to other metals for new functions such as Cu(II) for redox chemistry. A functional model of the catalytic Cu_{T2} center of copper nitrite reductase was constructed using $(TRIL23H)_3$ in presence of Cu(I/II). [61, 124] The triple stranded assembly showed higher affinity towards Cu(II) than Cu(I). The coordination structure Cu(His)$_3$ was found to be similar with the Cu$_{T2}$ center. The Cu(II) catalytic center in the triple-stranded coiled coils can be reduced by ascorbate in an aqueous medium (pH5.8) and reoxidized by nitrate, producing nitric oxide. Such catalytic redox activities were facilitated by the tight binding of the active site metal ion in both oxidation states. The reaction rate can be controlled by methylating the histidine for altering the tautomeric preferences of Cu coordination.[122, 124]

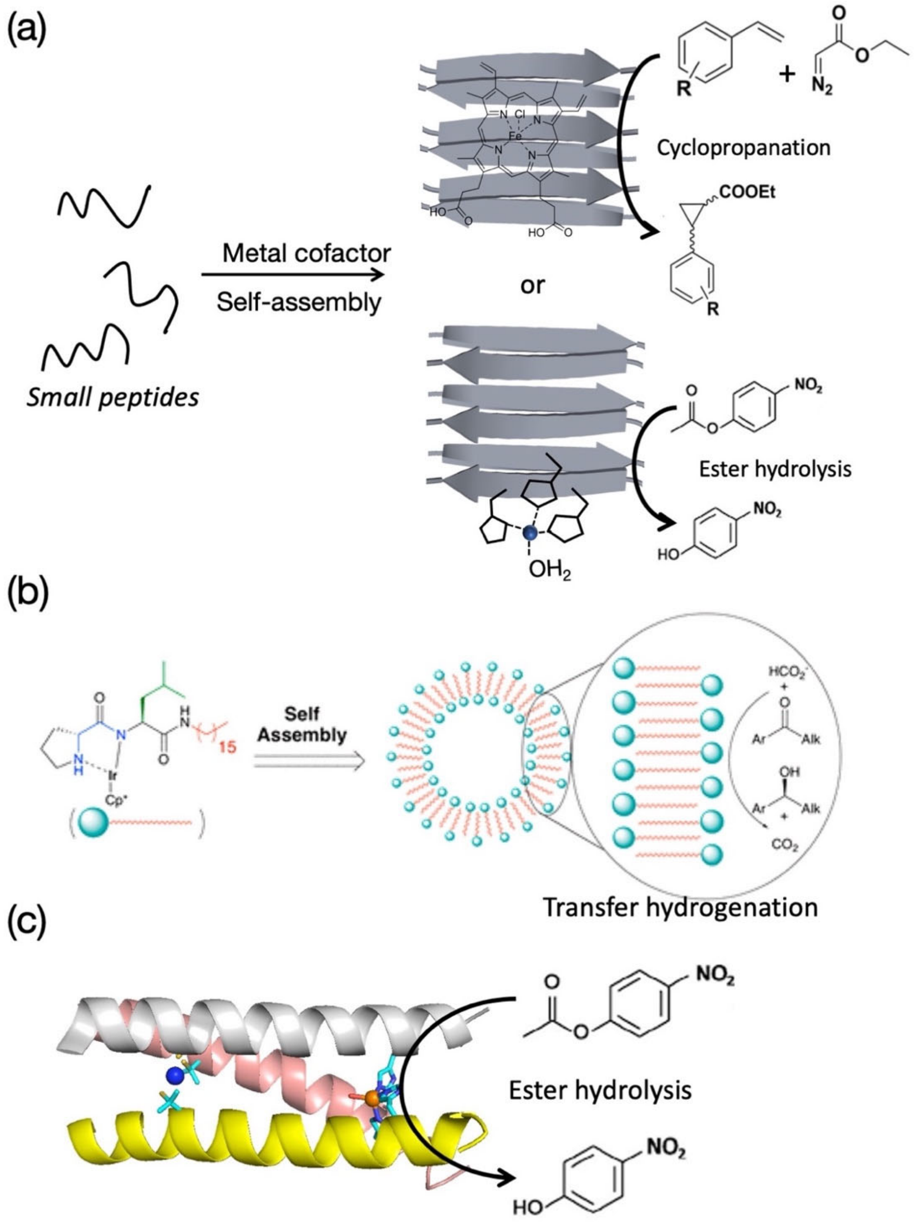

Figure 14: Catalysis by self-assembled peptides with metal cofactors. (a) Construction of catalytic amyloid peptide assembly and the corresponding catalytic reactions. (b) Vesicle like slef-assembly of an organometallic IrCp* functionalized dipeptide with an alkyl chain and the corresponding catalytic transfer hydrogenation reaction. Image reproduced from [57] with permission from ACS. (c) A catalytic three helix bundle containing Hg(II) and Zn(II) coordination with schematic representation of Zn mediated hydrolysis of p-nitrophenyl acetate to p-nitrophenol.

7. **Heterogeneous metalloenzymes based on crystalline protein assembly**.

In contrast to the commonly used artificial metalloenzymes, protein crystals can be considered an ordered three-dimensional assembly of protein monomers functionalized with metal catalysts to develop heterogeneous artificial metalloenzymes. Typical protein crystals contain ~30-60% of solvent. Therefore, they have a large solvent pore to accommodate various synthetic metal catalysts for catalysis.[125, 126] There are several advantages of using porous protein crystals as artificial metalloenzymes, which includes (i) Recyclability and easy separation of products due to heterogeneous nature, (ii) Cross-link treatment enabling their use under harsh conditions such as variable pH, temperature and high percentages of organic solvent, (iii) Easy functionalization via soaking of a variety of desired metal catalysts, (iii) Genetic manipulations in both catalyst binding site and surrounding environment for controlling the reaction and (iv) Since it is a crystal, there is a chance to capture the reaction intermediates via X-ray structure determination. Hen egg white lysozyme (HEWL) crystal is one of the mostly studied matrix for metal complex immobilization. Ueno group have functionalized organometallic Ru(benzene) catalyst into the porous HEWL crystal via soaking, which showed enantioselective transfer hydrogenation of acetophenone derivatives to the corresponding alcohols (Figure 15a).[51] Asymmetric protein environment controlled the approach of the substrates towards the catalytic center. Since HEWL forms different polymorph lattice structures, the catalytic activity of the Ru(Benzene) catalyst also varied accordingly due to changes in the reaction environments and cavity size. Recently, Pt nanoparticles were also grown inside the HEWL crystals, which in the presence of light-harvesting dye, methyl viologen showed efficient photocatalytic H_2 production reaction.[52] Similarly, gold nanoparticles inside the HEWL single crystals were capable of reducing nitroaromatics to the corresponding amines.[53] Such examples demonstrated the potential of protein crystals as heterogeneous artificial metalloenzymes.

In contrast to such conventional soaking of metal catalyst into porous protein crystals, Beck et al. have incorporated metal oxide nanoparticles into an engineered ferritin container. The surface of the protein cages was engineered with positively and negatively charged residues. Due to electrostatic interactions, ferritin caged formed a three-dimensional superlattice structure and was accessible for a range of substrates.[55] Such nanoparticle superlattices showed oxidase- and peroxide-like activity, which can be recycled several times (Figure 15b). Recently, Chakraborty and Heddle et al. have developed a three-dimensional superlattice structure of ferritin protein cage with gold nanoparticles (AuNP) for catalysis.[79] Before the superlattice formation, the ferritin cage was filled with lysozyme via the self-assembly reaction. Such enzyme encapsulated ferritin cages formed superlattice structures in the presence of gold nanoparticles. The encapsulated enzymes were found to retain the original activity in the crystalline lattice, although the efficiency was found less possibly due to low accessibility of the substrate to catalyst. Such examples were demonstrated as alternate methods to develop heterogeneous artificial metalloenzymes.

In addition to synthetic metal catalyst immobilization, proteins and enzymes were recently incorporated into porous protein crystals for various functions, including catalytic reactions.[127] It was found that the encapsulated enzymes inside the protein crystals maintained their stability and activity even under harsh conditions like drying or elevated temperature.[128] Porous solvent channels facilitate the diffusion of substrates and products. Proteins and enzymes can be immobilized into the protein crystals by three common methods, which include (i) Genetic fusion,[129, 130] (ii) Soaking,[127] and (iii) co-expression of enzymes during crystal formation in vivo.[54] For example, human ubiquitin was fused with R1EN, an endonuclease domain of silkworms, via genetic fusion to determine the structure. R1EN crystallizes to give a hexagonal honeycomb lattice structure.[130] The crystal structure of ubiquitin fused R1EN showed the presence of fused protein in the porous channel. Soaking proteins and enzymes into large pore crystals is an alternate method of encapsulation.[127] Snow and coworker have constructed highly porous crystals (~200-600μm) of putative periplasmic polyisoprenoid-binding protein from *Campylobacter jejuni* (CJ), which has large (13 nm) axial pores and small (~3 nm) perpendicular pores arranged via the P622 space group.[131] They incorporated multiple enzymes into the porous crystals via soaking, which showed catalytic cascade reactions.[127] Such hybrid solid catalyst showed long-term stability with high thermal tolerance. In addition to the method of genetic fusion and soaking, foreign proteins and enzymes can also be incorporated into protein crystals via in vivo grown crystals by co-expression .[54, 132] Recently, Ueno et al. have reported the in vivo encapsulation dual enzymes H1-tagged *Candida Antarctica* lipase B (H1-CALB) and *Lactobacillus kefir* alcohol dehydrogenase (H1-ADH) into a hollow polyhedral crystal to promote catalytic cascade reactions.[54] The solid hybrid catalyst successfully promoted the catalytic cascade reactions of hydrolysis followed by dehydrogenation of 7-(3,4-diacetoxybutyloxy)-4-methyl-2H-chromen-2-one with higher activity than the mixture of free enzymes and wild type crystals (Figure 15c). This implies that (i) enzymes in porous protein environments have higher activity than free form in solution, and (ii) substrate and products can quickly diffuse into the porous solvent channel. Such studies demonstrate the great scope of developing a wide range of solid catalysts.

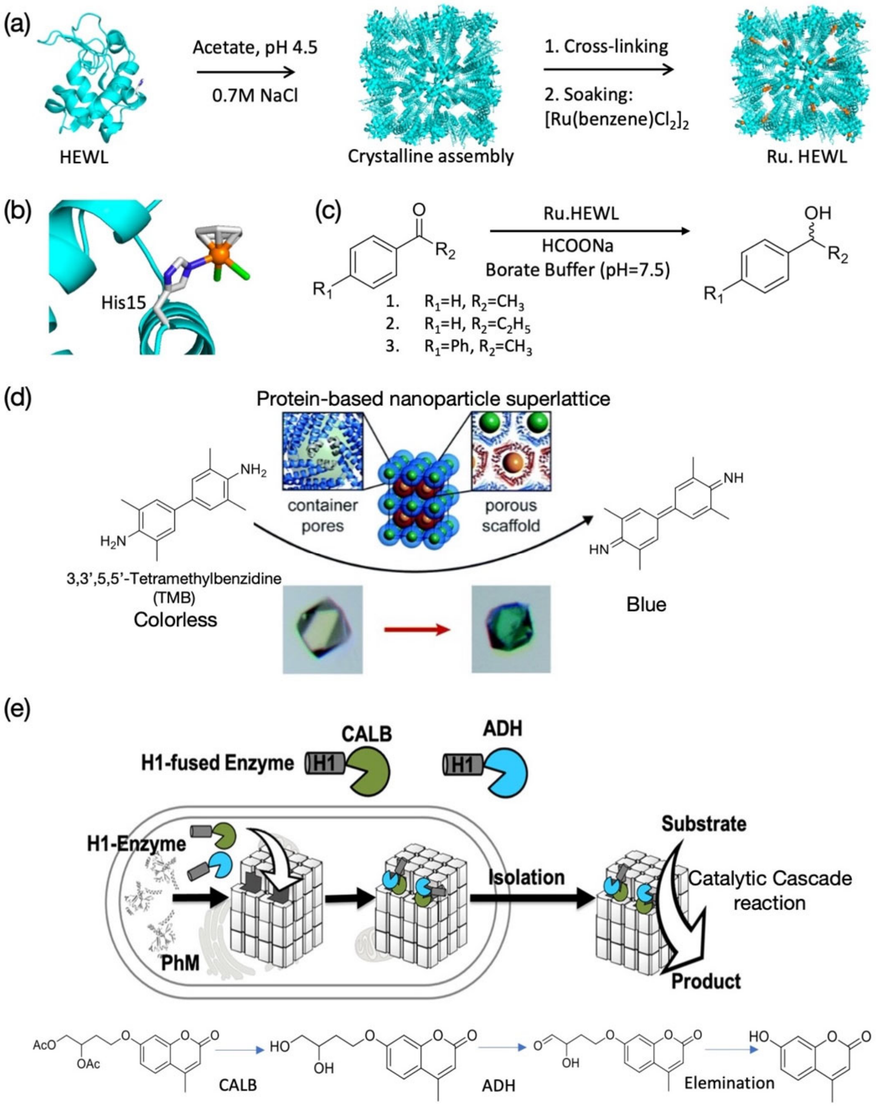

Figure 15: Catalysis by Protein crystal assembly as heterogeneous metalloenzymes. (a-c) Construction of porous HEWL crystal, metal immobilization, crystal structure of the active site and transfer hydrogenation of various acetophenone derivatives. (d) Binary superlattice structure formation from two different types of ferritin cages loaded with CeO_2 and FeO_x nanoparticles and the corresponding peroxidase activity. (e) In-cell enzyme encapsulation into a designed porous polyhedral crystal. The encapsulated metalloenzyme, alcohol dehydrogenase can promote Zn mediated dehydrogenation reaction. Figure d and a part of the figure e are reproduced from [55] and [54] with permission from Willy and ACS .

8. Conclusion and Future perspectives.

In conclusion, we highlighted various recent significant examples of catalysis by protein assembly-based artificial metalloenzymes. The primary designing strategy of metalloprotein assembly was also discussed. Being multimeric, protein assembly offers multiple features in a single assembly enzyme which was nicely demonstrated.[12] This is the beauty of protein assembly-based metalloenzymes compared to their monomeric counterpart. Such assembled enzymes were also successfully demonstrated to promote catalytic reactions inside living cells for more advancement. These are significant achievements as they mimic the natural enzymes in cells. Interestingly, such assembled enzymes can also be evolved for higher activity and selectivity. Despite many promising features and activities, the number of reports in which the unique features of protein assembly structures are more minor to date.

Proteins are such an amazing fundamental building block owned by nature for the synthesis of biomaterials that researchers from almost every discipline of science have tried their hand at designing protein assemblies with the structural and functional complexity of naturally occurring self-assemblies. Considering the current success and promising future, it is essential to develop metalloprotein assembly. Computational design is an excellent option for screening and designing protein assembly. With the advent of the high-performance computing field of protein, the design has evolved tremendously. There have been many reports on the ab initio and de novo design of enzymes or proteins for novel applications in recent years. AlphaFold,[133]’[134] Rosetta,[135]’[136] and i-tasser[137]’[138] are some of the leading names in protein structure prediction and the synthesis of novel protein architectures. Methods for designing novel protein-protein interfaces[139]’[140] and self-assembling proteins have progressed slowly, yet the functional and physical properties make them attractive as building blocks for developing advanced functional biomaterials. Computational design of the novel protein-protein interfaces[141] has also shown the application in the synthesis of the new protein self-assemblies[142] and protein nanomaterials[143]. In a report, Baker et. At.[110] has described a general computational method for designing self-assembling protein materials. With their method, they successfully designed cage-like protein nanomaterials with either tetrahedral (T) or octahedral (O) point group symmetry. Their results establish a method for designing self-assembling protein materials with high accuracy. In addition to the finite, cage-like materials described in the report, their method opens up the possibility to be applied for the synthesis of unbounded materials in one, two, or three dimensions (i.e., fibers, layers, or crystals) by choosing an appropriate target symmetric architecture.

All these reports are exciting, but to the best of our knowledge, there is no report on the computational design of the self-assembly protein architectures with catalytic properties. In the past, it has been observed that the field of protein engineering and nanotechnology has taken a leap from structure-building to function-building, i.e., now the main focus of protein engineering research is on the development of novel protein architectures for chemical and biomedical applications, such as in sensor design, medicine, and catalysis. The protein assembly design field has greatly benefited from technological advances in all the other fields of science such as new protein biochemistry and molecular biology tool and the development of new computational methodologies for protein self-assembly. Still, there is a long way to go. There is still room for improvement. We are still far from the natural protein architectures in sophisticated design and wide applications. Photosystem II[144] and cytoskeletal filaments[145] are a few such examples and inspirations for us. One important point to note here is that all the protein self-assembly described in this article were symmetric and more or less very rigid and stable. To expand the applicability of the protein self-assembly for more novel applications either in catalysis or in the biomedical area, one has to think of designing reversible, dynamic protein self-assembly having a responsive behavior in the presence of external stimuli. To achieve that, one has to think beyond the one-pot synthesis of protein self-assembly and involve the cooperative effect of other responsive elements, and interactive systems as nature does, such as using metal coordination, DNA assistance, hydrophilic/hydrophobic forces, and chaperons. Looking at the pace with which the field of protein self-assembly is growing, it is not surprising to imagine the development of the protein self-assembly with better design and more real-life applications, e.g., drug development, drug delivery, artificial photosynthesis, and a catalyst of industrial-scale synthesis of fine chemicals, compared to the naturally occurring marvelous protein materials such as photosystem II and filaments.

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10. Declaration of conflicts of interests

There is no conflicts of interests.

11. Abbreviations.

ArMs	Artificial metalloenzymes
Allyl	Structural formulae of $H_2C{=}CH{-}CH_2^-$.
Bpy	Bipyridine
CPV	cell-penetrating poly(disulfide)
Cp*	Pentamethyl cyclopentadienyl
CJ	*Campylobacter jejuni*
DNA	Deoxyribonucleic acid
DPS	DNA binding protein from starved cell
3D-DS	Three-dimensionally domain swapping
GSH	Glutathione
HEWL	Hen egg white lysozyme
H1-CALB	H1-tagged *Candida Antarctica* lipase B
H1-ADH	H1-tagged *Lactobacillus kefir* alcohol dehydrogenase
HER	Hydrogen evolution reaction
HRTEM	High resolution transmission electron microscopy
HSP	Heat shock protein
LmrR	Lactococcal multidrug resistance Regulator
MRI	Magnetic resonance imaging
nbd	Norbornadiene
PDB	Protein data bank
PMOF	Protein metal organic frameworks
PN	Protein needle
PhC	Polyhedra crystal
PhM	Polyhedra monomer.
Phen	Phenanthroline
QM/MM	Quantum mechanics/Molecular mechanics
SAV	Streptavidin
TMV	Tobacco mosaic virus
TRAP	Tryptophan RNA-binding attenuation protein
TOF	Turn over frequency
TON	Turn over number

12. References:

[1] L. Alonso-Cotchico, J. Rodríguez-Guerra, A. Lledós, J.-D. Maréchal, Accounts of Chemical Research, 53 (2020) 896-905.

[2] F. Schwizer, Y. Okamoto, T. Heinisch, Y. Gu, M.M. Pellizzoni, V. Lebrun, R. Reuter, V. Köhler, J.C. Lewis, T.R. Ward, Chemical Reviews, 118 (2018) 142-231.

[3] Z.J. Wang, N.E. Peck, H. Renata, F.H. Arnold, Chemical Science, 5 (2014) 598-601.

[4] P.S. Coelho, E.M. Brustad, A. Kannan, F.H. Arnold, Science, 339 (2013) 307-310.

[5] O.F. Brandenberg, K. Chen, F.H. Arnold, Journal of the American Chemical Society, 141 (2019) 8989-8995.

[6] K. Oohora, H. Meichin, Y. Kihira, H. Sugimoto, Y. Shiro, T. Hayashi, Journal of the American Chemical Society, 139 (2017) 18460-18463.

[7] M.T. Reetz, Accounts of Chemical Research, 52 (2019) 336-344.

[8] H. Xiao, Z. Bao, H. Zhao, Industrial & Engineering Chemistry Research, 54 (2015) 4011-4020.

[9] Y.-W. Lin, Coordination Chemistry Reviews, 336 (2017) 1-27.

[10] F.H. Arnold, Angewandte Chemie International Edition, 57 (2018) 4143-4148.

[11] T.-C. Chang, K. Vong, T. Yamamoto, K. Tanaka, Angewandte Chemie International Edition, 60 (2021) 12446-12454.

[12] Y. Okamoto, R. Kojima, F. Schwizer, E. Bartolami, T. Heinisch, S. Matile, M. Fussenegger, T.R. Ward, Nature Communications, 9 (2018) 1943.

[13] B.J.G.E. Pieters, M.B. van Eldijk, R.J.M. Nolte, J. Mecinović, Chemical Society Reviews, 45 (2016) 24-39.

[14] H.J. Davis, T.R. Ward, ACS Central Science, 5 (2019) 1120-1136.

[15] O. Pàmies, M. Diéguez, J.-E. Bäckvall, Advanced Synthesis & Catalysis, 357 (2015) 1567-1586.

[16] P. Hosseinzadeh, E.N. Mirts, T.D. Pfister, Y.-G. Gao, C. Mayne, H. Robinson, E. Tajkhorshid, Y. Lu, Biochemistry, 55 (2016) 1494-1502.

[17] J. Zhu, N. Avakyan, A. Kakkis, A.M. Hoffnagle, K. Han, Y. Li, Z. Zhang, T.S. Choi, Y. Na, C.-J. Yu, F.A. Tezcan, Chemical Reviews, 121 (2021) 13701-13796.

[18] W.J. Song, F.A. Tezcan, Science, 346 (2014) 1525-1528.

[19] S. Chordia, S. Narasimhan, A. Lucini Paioni, M. Baldus, G. Roelfes, Angewandte Chemie International Edition, 60 (2021) 5913-5920.

[20] C.A. Kerfeld, C. Aussignargues, J. Zarzycki, F. Cai, M. Sutter, Nature Reviews Microbiology, 16 (2018) 277-290.

[21] Y.-Q. Zhang, T.-T. Feng, Y.-F. Cao, X.-Y. Zhang, T. Wang, M.R. Huanca Nina, L.-C. Wang, H.-L. Yu, J.-H. Xu, J. Ge, Y.-P. Bai, ACS Catalysis, 11 (2021) 10487-10493.

[22] D.P. Patterson, B. Schwarz, R.S. Waters, T. Gedeon, T. Douglas, ACS Chemical Biology, 9 (2014) 359-365.

[23] M. Hestericová, T. Heinisch, M. Lenz, T.R. Ward, Dalton Transactions, 47 (2018) 10837-10841.

[24] F. Christoffel, N.V. Igareta, M.M. Pellizzoni, L. Tiessler-Sala, B. Lozhkin, D.C. Spiess, A. Lledós, J.-D. Maréchal, R.L. Peterson, T.R. Ward, Nature Catalysis, 4 (2021) 643-653.

[25] J. Bos, W.R. Browne, A.J.M. Driessen, G. Roelfes, Journal of the American Chemical Society, 137 (2015) 9796-9799.

[26] S. Abe, K. Hirata, T. Ueno, K. Morino, N. Shimizu, M. Yamamoto, M. Takata, E. Yashima, Y. Watanabe, Journal of the American Chemical Society, 131 (2009) 6958-6960.

[27] J. Fan, J.-J. Yin, B. Ning, X. Wu, Y. Hu, M. Ferrari, G.J. Anderson, J. Wei, Y. Zhao, G. Nie, Biomaterials, 32 (2011) 1611-1618.

[28] M. Suzuki, M. Abe, T. Ueno, S. Abe, T. Goto, Y. Toda, T. Akita, Y. Yamada, Y. Watanabe, Chemical Communications, (2009) 4871-4873.

[29] T. Ueno, M. Suzuki, T. Goto, T. Matsumoto, K. Nagayama, Y. Watanabe, Angewandte Chemie International Edition, 43 (2004) 2527-2530.

[30] S. Kanbak-Aksu, M. Nahid Hasan, W.R. Hagen, F. Hollmann, D. Sordi, R.A. Sheldon, I.W.C.E. Arends, Chemical Communications, 48 (2012) 5745-5747.

[31] S. Abe, J. Niemeyer, M. Abe, Y. Takezawa, T. Ueno, T. Hikage, G. Erker, Y. Watanabe, Journal of the American Chemical Society, 130 (2008) 10512-10514.

[32] Z. Wang, Y. Takezawa, H. Aoyagi, S. Abe, T. Hikage, Y. Watanabe, S. Kitagawa, T. Ueno, Chemical Communications, 47 (2011) 170-172.

[33] M. Taher, B. Maity, T. Nakane, S. Abe, T. Ueno, S. Mazumdar, Angewandte Chemie International Edition, n/a.

[34] B. Maity, K. Fukumori, S. Abe, T. Ueno, Chemical Communications, 52 (2016) 5463-5466.

[35] W. Chen, S. Li, X. Li, C. Zhang, X. Hu, F. Zhu, G. Shen, F. Feng, Chemical Science, 10 (2019) 2179-2185.

[36] M. Liu, Y. Zhu, D. Jin, L. Li, J. Cheng, Y. Liu, Inorganic Chemistry, 60 (2021) 14515-14519.

[37] Z. Zhou, G. Roelfes, ACS Catalysis, 11 (2021) 9366-9369.

[38] G. Roelfes, Journal of Inorganic Biochemistry, 222 (2021) 111523.

[39] L. Alonso-Cotchico, G. Sciortino, P. Vidossich, J. Rodríguez-Guerra Pedregal, I. Drienovská, G. Roelfes, A. Lledós, J.-D. Maréchal, ACS Catalysis, 9 (2019) 4616-4626.

[40] L. Villarino, K.E. Splan, E. Reddem, L. Alonso-Cotchico, C. Gutiérrez de Souza, A. Lledós, J.-D. Maréchal, A.-M.W.H. Thunnissen, G. Roelfes, Angewandte Chemie International Edition, 57 (2018) 7785-7789.

[41] A. Stein, D. Chen, N.V. Igareta, Y. Cotelle, J.G. Rebelein, T.R. Ward, ACS Central Science, 7 (2021) 1874-1884.

[42] T.K. Hyster, L. Knörr, T.R. Ward, T. Rovis, Science, 338 (2012) 500-503.

[43] A. Chatterjee, H. Mallin, J. Klehr, J. Vallapurackal, A.D. Finke, L. Vera, M. Marsh, T.R. Ward, Chemical Science, 7 (2016) 673-677.

[44] M. Jeschek, R. Reuter, T. Heinisch, C. Trindler, J. Klehr, S. Panke, T.R. Ward, Nature, 537 (2016) 661-665.

[45] W.J. Song, J. Yu, F.A. Tezcan, Journal of the American Chemical Society, 139 (2017) 16772-16779.

[46] J. Yu, J. Yang, C. Seok, W.J. Song, Chemical Science, 12 (2021) 5091-5101.

[47] N. Yokoi, Y. Miura, C.-Y. Huang, N. Takatani, H. Inaba, T. Koshiyama, S. Kanamaru, F. Arisaka, Y. Watanabe, S. Kitagawa, T. Ueno, Chemical Communications, 47 (2011) 2074-2076.

[48] H. Inaba, S. Kanamaru, F. Arisaka, S. Kitagawa, T. Ueno, Dalton Transactions, 41 (2012) 11424-11427.

[49] N. Yokoi, H. Inaba, M. Terauchi, A.Z. Stieg, N.J.M. Sanghamitra, T. Koshiyama, K. Yutani, S. Kanamaru, F. Arisaka, T. Hikage, A. Suzuki, T. Yamane, J.K. Gimzewski, Y. Watanabe, S. Kitagawa, T. Ueno, Small, 6 (2010) 1873-1879.

[50] Z. Wang, T. Matsuo, S. Nagao, S. Hirota, Organic & Biomolecular Chemistry, 9 (2011) 4766-4769.

[51] H. Tabe, S. Abe, T. Hikage, S. Kitagawa, T. Ueno, Chemistry – An Asian Journal, 9 (2014) 1373-1378.

[52] H. Tabe, H. Takahashi, T. Shimoi, S. Abe, T. Ueno, Y. Yamada, Applied Catalysis B: Environmental, 237 (2018) 1124-1129.

[53] H. Wei, Y. Lu, Chemistry – An Asian Journal, 7 (2012) 680-683.

[54] T.K. Nguyen, S. Abe, M. Kasamatsu, B. Maity, K. Yamashita, K. Hirata, M. Kojima, T. Ueno, ACS Applied Nano Materials, 4 (2021) 1672-1681.

[55] M. Lach, M. Künzle, T. Beck, Chemistry – A European Journal, 23 (2017) 17482-17486.

[56] C.M. Rufo, Y.S. Moroz, O.V. Moroz, J. Stöhr, T.A. Smith, X. Hu, W.F. DeGrado, I.V. Korendovych, Nature Chemistry, 6 (2014) 303-309.

[57] M.A. Dolan, P.N. Basa, O. Zozulia, Z. Lengyel, R. Lebl, E.M. Kohn, S. Bhattacharya, I.V. Korendovych, ACS Nano, 13 (2019) 9292-9297.

[58] O. Zozulia, I.V. Korendovych, Angewandte Chemie International Edition, 59 (2020) 8108-8112.

[59] P. Makam, S.S.R.K.C. Yamijala, K. Tao, L.J.W. Shimon, D.S. Eisenberg, M.R. Sawaya, B.M. Wong, E. Gazit, Nature Catalysis, 2 (2019) 977-985.

[60] O.V. Makhlynets, I.V. Korendovych, Nature Catalysis, 2 (2019) 949-950.

[61] M. Tegoni, F. Yu, M. Bersellini, J.E. Penner-Hahn, V.L. Pecoraro, Proceedings of the National Academy of Sciences, 109 (2012) 21234-21239.

[62] F. Carmona, Ò. Palacios, N. Gálvez, R. Cuesta, S. Atrian, M. Capdevila, J.M. Domínguez-Vera, Coordination Chemistry Reviews, 257 (2013) 2752-2764.

[63] G. Jutz, P. van Rijn, B. Santos Miranda, A. Böker, Chemical Reviews, 115 (2015) 1653-1701.

[64] D. He, J. Marles-Wright, New Biotechnology, 32 (2015) 651-657.

[65] N. Zhang, F. Li, Q.j. Fu, S.C. Tsang, Reaction Kinetics and Catalysis Letters, 71 (2000) 393-404.

[66] N. Jian, M. Dowle, R.D. Horniblow, C. Tselepis, R.E. Palmer, Nanotechnology, 27 (2016) 46LT02.

[67] V. Drits, B. Sakharov, A. Salyn, A. Manceau, Clay Minerals, 28 (1993) 185-207.

[68] M. Ji, J. Cai, Y. Ma, L. Qi, ACS Applied Materials & Interfaces, 8 (2016) 3651-3660.

[69] F.C. Meldrum, V.J. Wade, D.L. Nimmo, B.R. Heywood, S. Mann, Nature, 349 (1991) 684-687.

[70] K. Fan, C. Cao, Y. Pan, D. Lu, D. Yang, J. Feng, L. Song, M. Liang, X. Yan, Nature Nanotechnology, 7 (2012) 459-464.

[71] H. Chen, L. Ma, Y. Zhang, RSC Advances, 11 (2021) 26211-26217.

[72] J. Niemeyer, S. Abe, T. Hikage, T. Ueno, G. Erker, Y. Watanabe, Chemical Communications, (2008) 6519-6521.

[73] S. Abe, T. Hikage, Y. Watanabe, S. Kitagawa, T. Ueno, Inorganic Chemistry, 49 (2010) 6967-6973.

[74] Z. Ke, S. Abe, T. Ueno, K. Morokuma, Journal of the American Chemical Society, 134 (2012) 15418-15429.

[75] C. Hansch, S.D. Rockwell, P.Y.C. Jow, A. Leo, E.E. Steller, Journal of Medicinal Chemistry, 20 (1977) 304-306.

[76] T. Fujita, J. Iwasa, C. Hansch, Journal of the American Chemical Society, 86 (1964) 5175-5180.

[77] C. Hansch, A. Leo, S.H. Unger, K.H. Kim, D. Nikaitani, E.J. Lien, Journal of Medicinal Chemistry, 16 (1973) 1207-1216.

[78] S. Tetter, D. Hilvert, Angewandte Chemie International Edition, 56 (2017) 14933-14936.

[79] S. Chakraborti, A. Korpi, M. Kumar, P. Stępień, M.A. Kostiainen, J.G. Heddle, Nano Letters, 19 (2019) 3918-3924.

[80] H. Agustiandari, E. Peeters, J.G. de Wit, D. Charlier, A.J.M. Driessen, Microbiology, 157 (2011) 1519-1530.

[81] P.K. Madoori, H. Agustiandari, A.J.M. Driessen, A.-M.W.H. Thunnissen, The EMBO Journal, 28 (2009) 156-166.

[82] K. Takeuchi, Y. Tokunaga, M. Imai, H. Takahashi, I. Shimada, Scientific Reports, 4 (2014) 6922.

[83] L. Villarino, S. Chordia, L. Alonso-Cotchico, E. Reddem, Z. Zhou, A.M.W.H. Thunnissen, J.-D. Maréchal, G. Roelfes, ACS Catalysis, 10 (2020) 11783-11790.

[84] I. Drienovská, A. Rioz-Martínez, A. Draksharapu, G. Roelfes, Chemical Science, 6 (2015) 770-776.

[85] J. Bos, A. García-Herraiz, G. Roelfes, Chemical Science, 4 (2013) 3578-3582.

[86] J. Bos, F. Fusetti, A.J.M. Driessen, G. Roelfes, Angewandte Chemie International Edition, 51 (2012) 7472-7475.

[87] Z. Zhou, G. Roelfes, Nature Catalysis, 3 (2020) 289-294.

[88] A.D. Liang, J. Serrano-Plana, R.L. Peterson, T.R. Ward, Accounts of Chemical Research, 52 (2019) 585-595.

[89] P.C. Weber, D.H. Ohlendorf, J.J. Wendoloski, F.R. Salemme, Science, 243 (1989) 85-88.

[90] C. Letondor, N. Humbert, T.R. Ward, Proceedings of the National Academy of Sciences of the United States of America, 102 (2005) 4683.

[91] M. Creus, T.R. Ward, Organic & Biomolecular Chemistry, 5 (2007) 1835-1844.

[92] Y. Okamoto, V. Köhler, T.R. Ward, Journal of the American Chemical Society, 138 (2016) 5781-5784.

[93] A. Call, C. Casadevall, A. Romero-Rivera, V. Martin-Diaconescu, D.J. Sommer, S. Osuna, G. Ghirlanda, J. Lloret-Fillol, ACS Catalysis, 9 (2019) 5837-5846.

[94] G.V. Smirnova, O.N. Oktyabrsky, Biochemistry (Moscow), 70 (2005) 1199-1211.

[95] E.N. Salgado, J. Faraone-Mennella, F.A. Tezcan, Journal of the American Chemical Society, 129 (2007) 13374-13375.

[96] A. Medina-Morales, A. Perez, J.D. Brodin, F.A. Tezcan, Journal of the American Chemical Society, 135 (2013) 12013-12022.

[97] D.J.E. Huard, K.M. Kane, F.A. Tezcan, Nature Chemical Biology, 9 (2013) 169-176.

[98] X. Zhang, Y. Liu, B. Zheng, J. Zang, C. Lv, T. Zhang, H. Wang, G. Zhao, Nature Communications, 12 (2021) 4849.

[99] X. Tan, H. Chen, C. Gu, J. Zang, T. Zhang, H. Wang, G. Zhao, Communications Chemistry, 3 (2020) 151.

[100] P.A. Sontz, J.B. Bailey, S. Ahn, F.A. Tezcan, Journal of the American Chemical Society, 137 (2015) 11598-11601.

[101] J.B. Bailey, F.A. Tezcan, Journal of the American Chemical Society, 142 (2020) 17265-17270.

[102] M. Yang, W.J. Song, Nature Communications, 10 (2019) 5545.

[103] K. Oohora, A. Onoda, T. Hayashi, Chemical Communications, 48 (2012) 11714-11726.

[104] K. Oohora, A. Onoda, H. Kitagishi, H. Yamaguchi, A. Harada, T. Hayashi, Chemical Science, 2 (2011) 1033-1038.

[105] K. Oohora, R. Kajihara, N. Fujimaki, T. Uchihashi, T. Hayashi, Chemical Communications, 55 (2019) 1544-1547.

[106] S. Hirayama, K. Oohora, T. Uchihashi, T. Hayashi, Journal of the American Chemical Society, 142 (2020) 1822-1831.

[107] K. Oohora, T. Mashima, K. Ohkubo, S. Fukuzumi, T. Hayashi, Chemical Communications, 51 (2015) 11138-11140.

[108] A.D. Malay, N. Miyazaki, A. Biela, S. Chakraborti, K. Majsterkiewicz, I. Stupka, C.S. Kaplan, A. Kowalczyk, B.M.A.G. Piette, G.K.A. Hochberg, D. Wu, T.P. Wrobel, A. Fineberg, M.S.

Kushwah, M. Kelemen, P. Vavpetič, P. Pelicon, P. Kukura, J.L.P. Benesch, K. Iwasaki, J.G. Heddle, Nature, 569 (2019) 438-442.

[109] G. Grigoryan, Y.H. Kim, R. Acharya, K. Axelrod, R.M. Jain, L. Willis, M. Drndic, J.M. Kikkawa, W.F. DeGrado, Science, 332 (2011) 1071-1076.

[110] N.P. King, W. Sheffler, M.R. Sawaya, B.S. Vollmar, J.P. Sumida, I. André, T. Gonen, T.O. Yeates, D. Baker, Science, 336 (2012) 1171-1174.

[111] A. Courbet, J. Hansen, Y. Hsia, N. Bethel, Y. Park, C. Xu, A. Moyer, S.E. Boyken, G. Ueda, U. Nattermann, D. Nagarajan, D. Silva, W. Sheffler, J. Quispe, N. King, P. Bradley, D. Veesler, J. Kollman, D. Baker, bioRxiv, (2021) 2021.2011.2011.468255.

[112] C.H. Norn, I. André, Current Opinion in Structural Biology, 39 (2016) 39-45.

[113] C. Fortenberry, E.A. Bowman, W. Proffitt, B. Dorr, S. Combs, J. Harp, L. Mizoue, J. Meiler, Journal of the American Chemical Society, 133 (2011) 18026-18029.

[114] J. Faraone-Mennella, F.A. Tezcan, H.B. Gray, J.R. Winkler, Biochemistry, 45 (2006) 10504-10511.

[115] E.N. Salgado, X.I. Ambroggio, J.D. Brodin, R.A. Lewis, B. Kuhlman, F.A. Tezcan, Proceedings of the National Academy of Sciences, 107 (2010) 1827-1832.

[116] C. Schneider, A.R. Sreekanth, E. Mai, Angewandte Chemie International Edition, 43 (2004) 5691-5694.

[117] S. Hirota, S. Nagao, Bulletin of the Chemical Society of Japan, 94 (2020) 170-182.

[118] S. Hirota, Y. Hattori, S. Nagao, M. Taketa, H. Komori, H. Kamikubo, Z. Wang, I. Takahashi, S. Negi, Y. Sugiura, M. Kataoka, Y. Higuchi, Proceedings of the National Academy of Sciences, 107 (2010) 12854-12859.

[119] O.F. Brandenberg, R. Fasan, F.H. Arnold, Current Opinion in Biotechnology, 47 (2017) 102-111.

[120] O.V. Makhlynets, P.M. Gosavi, I.V. Korendovych, Angewandte Chemie International Edition, 55 (2016) 9017-9020.

[121] Q. Liu, K. Wan, Y. Shang, Z.-G. Wang, Y. Zhang, L. Dai, C. Wang, H. Wang, X. Shi, D. Liu, B. Ding, Nature Materials, 20 (2021) 395-402.

[122] T.B.J. Pinter, E.C. Manickas, A.E. Tolbert, K.J. Koebke, A. Deb, J.E. Penner-Hahn, V.L. Pecoraro, Angewandte Chemie International Edition, 59 (2020) 20445-20449.

[123] M.L. Zastrow, A.F.A. Peacock, J.A. Stuckey, V.L. Pecoraro, Nature Chemistry, 4 (2012) 118-123.

[124] K.J. Koebke, F. Yu, C. Van Stappen, T.B.J. Pinter, A. Deb, J.E. Penner-Hahn, V.L. Pecoraro, Journal of the American Chemical Society, 141 (2019) 7765-7775.

[125] S. Abe, B. Maity, T. Ueno, Current Opinion in Chemical Biology, 43 (2018) 68-76.

[126] T. Ueno, Chemistry – A European Journal, 19 (2013) 9096-9102.

[127] A.E. Kowalski, L.B. Johnson, H.K. Dierl, S. Park, T.R. Huber, C.D. Snow, Biomaterials Science, 7 (2019) 1898-1904.

[128] S. Abe, H. Ijiri, H. Negishi, H. Yamanaka, K. Sasaki, K. Hirata, H. Mori, T. Ueno, Advanced Materials, 27 (2015) 7951-7956.

[129] P. Ernst, A. Plückthun, P.R.E. Mittl, Scientific Reports, 9 (2019) 15199.

[130] N. Maita, Journal of the American Chemical Society, 140 (2018) 13546-13549.

[131] T.R. Huber, L.F. Hartje, E.C. McPherson, A.E. Kowalski, C.D. Snow, Small, 13 (2017) 1602703.

[132] B.S. Heater, Z. Yang, M.M. Lee, M.K. Chan, Journal of the American Chemical Society, 142 (2020) 9879-9883.

[133] M. AlQuraishi, Bioinformatics, 35 (2019) 4862-4865.

[134] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S.A.A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, Nature, 596 (2021) 583-589.

[135] P. Löffler, S. Schmitz, E. Hupfeld, R. Sterner, R. Merkl, PLOS Computational Biology, 13 (2017) e1005600.

[136] A.L. Loshbaugh, T. Kortemme, Proteins: Structure, Function, and Bioinformatics, 88 (2020) 206-226.

[137] J. Yang, R. Yan, A. Roy, D. Xu, J. Poisson, Y. Zhang, Nature Methods, 12 (2015) 7-8.

[138] A. Roy, A. Kucukural, Y. Zhang, Nature Protocols, 5 (2010) 725-738.

[139] P.-S. Huang, J.J. Love, S.L. Mayo, Protein Science, 16 (2007) 2770-2774.

[140] S.D. Khare, S.J. Fleishman, FEBS Letters, 587 (2013) 1147-1154.

[141] J. Karanicolas, B. Kuhlman, Current Opinion in Structural Biology, 19 (2009) 458-463.

[142] Y. Mou, P.-S. Huang, F.-C. Hsu, S.-J. Huang, S.L. Mayo, Proceedings of the National Academy of Sciences, 112 (2015) 10714.

[143] T. Kortemme, D. Baker, Current Opinion in Chemical Biology, 8 (2004) 91-97.

[144] J.P. McEvoy, G.W. Brudvig, Chemical Reviews, 106 (2006) 4455-4483.

[145] A.J. Maniotis, C.S. Chen, D.E. Ingber, Proceedings of the National Academy of Sciences, 94 (1997) 849-854.