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REVIEW ARTICLE

Supramolecular assembling systems of hemoproteins using chemical modifications

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Abstract

Inspired by protein assemblies in biological systems, various artificial protein assemblies have been constructed in these decades. Hemoprotein containing porphyrin iron complex, heme, is a unique building block of the artificial protein assemblies due to the structures, physicochemical properties and functions. In the case of hemoprotein containing *b*-type heme, the heme cofactor is non-covalently bound to the heme-binding site, heme pocket, in the protein matrix. This review summaries our efforts to utilize heme–heme pocket interactions toward supramolecular hemoprotein assembling systems with various structures and/or functions. Simple monomeric hemoprotein, mainly cytochrome b_{562}, was employed as a useful building block and synthetic heme was attached to the cysteine-introduced variant to form a building block showing self-assembling behavior by interprotein heme–heme pocket interactions. The modulations of linker between synthetic heme and protein surface and/or protein modification site contribute to provide various structures such as fiber, ring, branched shape and micelles. Furthermore, hexameric hemoprotein was utilized for another building block with supramolecular approach toward light harvesting system by replacement of heme cofactors with porphyrinoid photosensitizers. A series of artificial hemoprotein assembling systems will contribute to new-type of functional biomaterials.

Keywords Heme · Hemoprotein · Porphyrin · Supramolecular assembly · Light harvesting system

Introduction

A lot of supramolecular protein assembling systems are found to demonstrate sophisticated structures and functions in biological systems [1, 2]. These assemblies play essential roles to maintain vital activities. By replication of this type of self-assembling behavior, supramolecular chemistry has been evolved to achieve much more unique supramolecular systems of synthetic molecules during this half a century [3, 4]. In recent two decades, protein itself has been utilized as a building block of artificial supramolecular system [5–9]. This development is possibly caused by the innovations of analytical tools of proteins: mass spectrometry X-ray crystallography, electron transmission microscopy and so on. The approaches to construct the artificial protein assemblies are categorized by biological one and chemical one. Biological approach utilizes the intrinsic interprotein interactions such as hydrogen bonding, hydrophobic and electrostatic interactions between amino acid residues [10–14]. These are also employed in native protein assemblies but the controls are usually difficult due to the non-specific features. To overcome this problem, genetic fusion of dimer, trimer and/or tetramer has been often used to prepare sophisticated structures and computer-guided design recently demonstrates the improved accuracy to afford highly symmetric protein assemblies [10, 15]. The chemical approach employs the relatively strong and specific interprotein interactions such as coordination bonding, protein–ligand, protein–cofactor and host–guest interactions [16–25]. To introduce these interactions, synthetic molecules such as ligands and cofactors are often modified onto the protein surface, whereas some systems use amino acid residues as metal-coordination ligands or host molecules. These approaches have been investigated by several groups individually to form a wide variety of supramolecular protein assemblies with various structures [5, 6]. In contrast to various structures, generation of new function by assembling states is limited to catalysis, drug delivery and light harvesting systems [26–32].

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Hemoprotein is a promising building block of functional protein assemblies because heme, porphyrin iron complex, and its derivatives are unique metal complex showing useful reactivity and physicochemical properties [33]. The functions of hemoproteins in biological systems are varied to dioxygen storage/carrier, gas sensing, electron transfer and catalysis for metabolism. Hemoprotein containing heme b, protoporphyrin IX iron complex, can be chemically modified by replacement of a heme cofactor with a synthetic cofactor as well as mutagenesis, a common strategy to modify protein [34, 35]. Thus, modified hemoproteins further broaden its useful properties. Actually, hemoprotein is employed as a building block of hemoprotein by several research groups [21]. Tezcan and his coworkers reported a series of hemoprotein assemblies using coordination bonding interactions by introducing the metal binding motif on the protein surface (Fig. 1) [36]. First generation of coordination-driven assembly was designed by quite simple strategy using natural amino acid residues as metal ligands but the sophisticated tetrameric structures were shown as crystal structures [37]. Further design of interface of the protein assemblies enables the stabilization of tetramer in solution state and expand to higher ordered structures such as sheet and tube [38–44]. In contrast to the metal ligand by natural amino acid residues, non-natural metal ligands such as phenanthroline, quinolate and hydroxamate are also introduced by chemical modification with cysteine residues [45–47]. Especially, hydroxamate -introduction achieved favorable 3-axis fold channel like ferritin to form a sophisticated cage. Tezcan's group also demonstrates the application of protein assemblies toward unique functional biomaterials: photo-reduction, catalysis, selective metal chelator and elastic materials [48–59]. Hirota and his coworkers demonstrated the hemoprotein assemblies using 3D domain swapping, where a few of helices in two or more hemoprotein units are exchanged each other under the specific conditions to form kinetically trapped oligomers (Fig. 2) [60]. The dimerization, trimerization and oligomerization of hemoprotein were achieved by addition of ethanol or transient heating and cooling process [61–70]. The ring-shaped dimer and trimers were characterized by crystal structures. The strategy is evolved to heterodimerization of different mutants of the same hemoprotein [71, 72]. Further detailed design of domain-swapped oligomerization enables to construct new metal binding sites in the interface of the swapped region [73]. The CO-responsive materials based on hemoprotein domain swapping are also reported [74, 75]. This review shows the effort of our group to create supramolecular hemoprotein assembling systems by chemical modifications toward the functional biomaterials. Figure 3 summarizes the representative assemblies in this review.

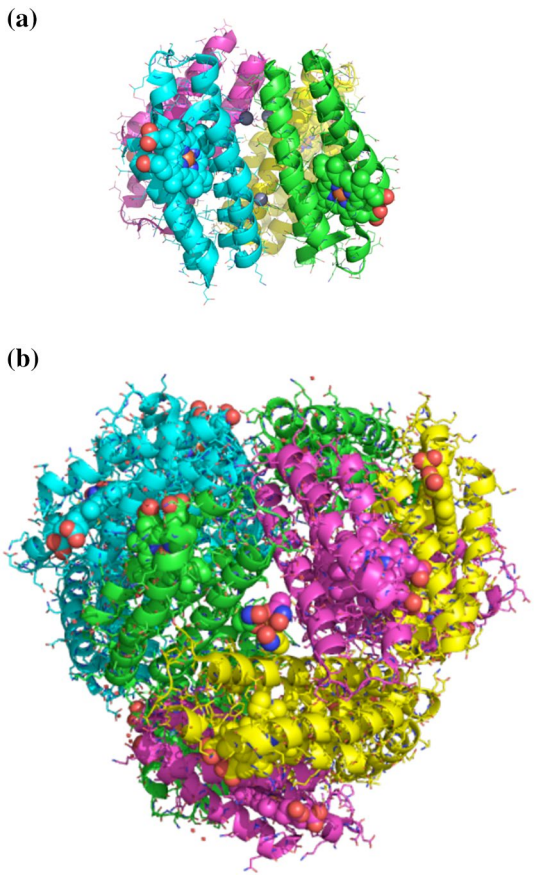

Fig. 1 Crystal structures of representative coordination-driven hemoprotein assemblies. **a** Tetramer (PDB ID: 2QLA). **b** Caged dodecamer (PDB ID: 6OT9).

Hemoprotein assemblies formed by interprotein heme–heme pocket interactions

Heme binding for heme-binding site, heme pocket in hemoprotein is strong and specific, inspiring us suitable interprotein interaction for protein assemblies. Successive interprotein heme–heme pocket interactions have been employed to drive the formation of supramolecular hemoprotein assemblies. In the first example, cytochrome b_{562} (Cyt*b*), a simple electron transfer hemoprotein, was selected as a building protein unit [8, 76]. The Cyt*b* mutant, Cytb^{H63C}, which includes a single cysteine residue on the surface, was designed and prepared. A thiol group on the side chain of the Cys63 was found to selectively react with iodoacetamide- or maleimide-tethering artificial heme via a relatively hydrophilic flexible linker to produce a covalently heme-attached protein. The native heme cofactor was removed by extraction using 2-butanone under acidic conditions and the followed neutralization induced successive heme–heme pocket interactions for the modified hemoprotein to form the fibrous assemblies

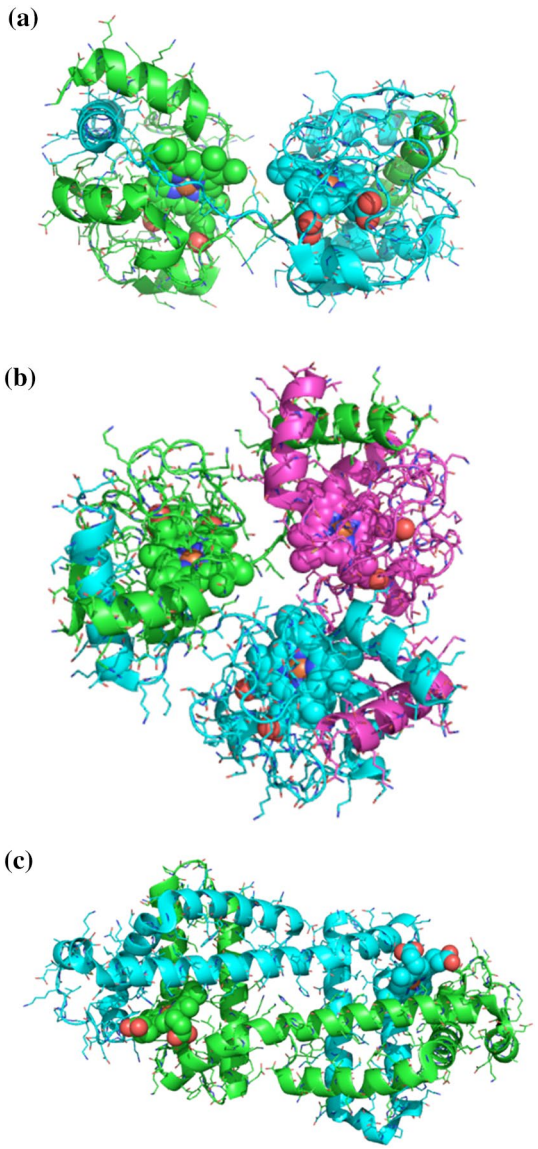

Fig. 2 Crystal structures of representative domain swapped-hemoprotein assemblies. **a** Dimer (PDB ID: 3NBS). **b** Trimer (PDB ID: 3NBT). **c** Heterodimer (PDB ID: 3WYO).

of hemoprotein. Size exclusion chromatography (SEC) and atomic force microscopy (AFM) measurements were carried out on the assembling system to evaluate structures and thermodynamic behavior. Figure 4 shows representative AFM image of the fibrous structure. The results indicate thermodynamic reversibility of the hemoprotein assembly formation and the degree of polymerization depends on the concentration of monomer, pH value and the additional heme or apoprotein units [8]. UV-vis and CD spectra indicate the successful incorporation of heme into the apoprotein matrix, suggesting that each protein unit in the supramolecular assembly is correctly folded. Further addition of a synthetic trimer of heme into the fibrous Cytb^{H63C} hemoprotein assembly led to the conversion into two-dimensional network structures, which were characterized on the graphite substrates by AFM [8].

The above approach using the interprotein interaction heme and heme pocket was adopted for myoglobin (Mb), an oxygen-binding hemoprotein, to generate unique functions (Fig. 5a). The mutant, Mb^{A125C}, was designed where a cysteine residue was introduced at the position 125 [77]. A supramolecular Mb assembly was prepared using the same procedure used for Cyt*b*. The system was found to form longer fibrous assemblies relative to that of Cyt*b*. This could be caused by the higher affinity of heme for apoMb than that for apo-from of Cyt*b*. Formation of the assembly of Mb is thermodynamically controlled by the heme–heme pocket interaction similar to the assembling system of Cyt*b*. Coordination of CO or CN^- to iron center of the heme moiety as a sixth ligand stabilizes the Mb assembly against the unfavorable effect of dilution. It is known that native apoMb has greater affinity for heme in the presence of these sixth ligand [78]. The inherent reactivity of Mb also provides the interesting behavior to this assembly. Reaction of the supramolecular Mb assembly with H_2O_2 yielded a nanogel-like cross-linked network because of coupling reaction between free radicals formed at a tyrosine residue on surface of each protein unit. The network structure was directly visualized by scanning electron microscopy (SEM) as shown in Fig. 5b. The dioxygen affinity was found to be maintained even after H_2O_2-tiriggered cross-linking, proposing that the Mb assembly does not have a major negative effect on the intrinsic functions and properties of the hemoprotein units.

Another strategy for preparing a supramolecular hemoprotein assembly was demonstrated using two building blocks: a heme dimer and apoMb dimer [8]. This system also yields submicrometer-sized fibrous assemblies and the polymerization degree of the assemblies is strongly influenced by the molecular design of the heme dimer and charges on the protein surface. Only small oligomers were produced whrn there is electrostatic repulsion between the building blocks. Furthermore, the strategy using multiple building blocks was utilized to prepare an alternating aligned assembly of heterotropic proteins, where apoMb dimer, streptavidin and a biotin-linked heme conjugate were employed as building blocks [8]. A co-assembly produced from the synthetic molecule and two protein building blocks was characterized by AFM and SEC, showing that the assembly has a sub-micrometer-sized fiber. Further AFM measurements indicate the alternating alignment of Mb dimer and streptavidin. These findings support the fact that the specific heme–heme pocket interaction is also useful for generating a heterotropic co-assembly.

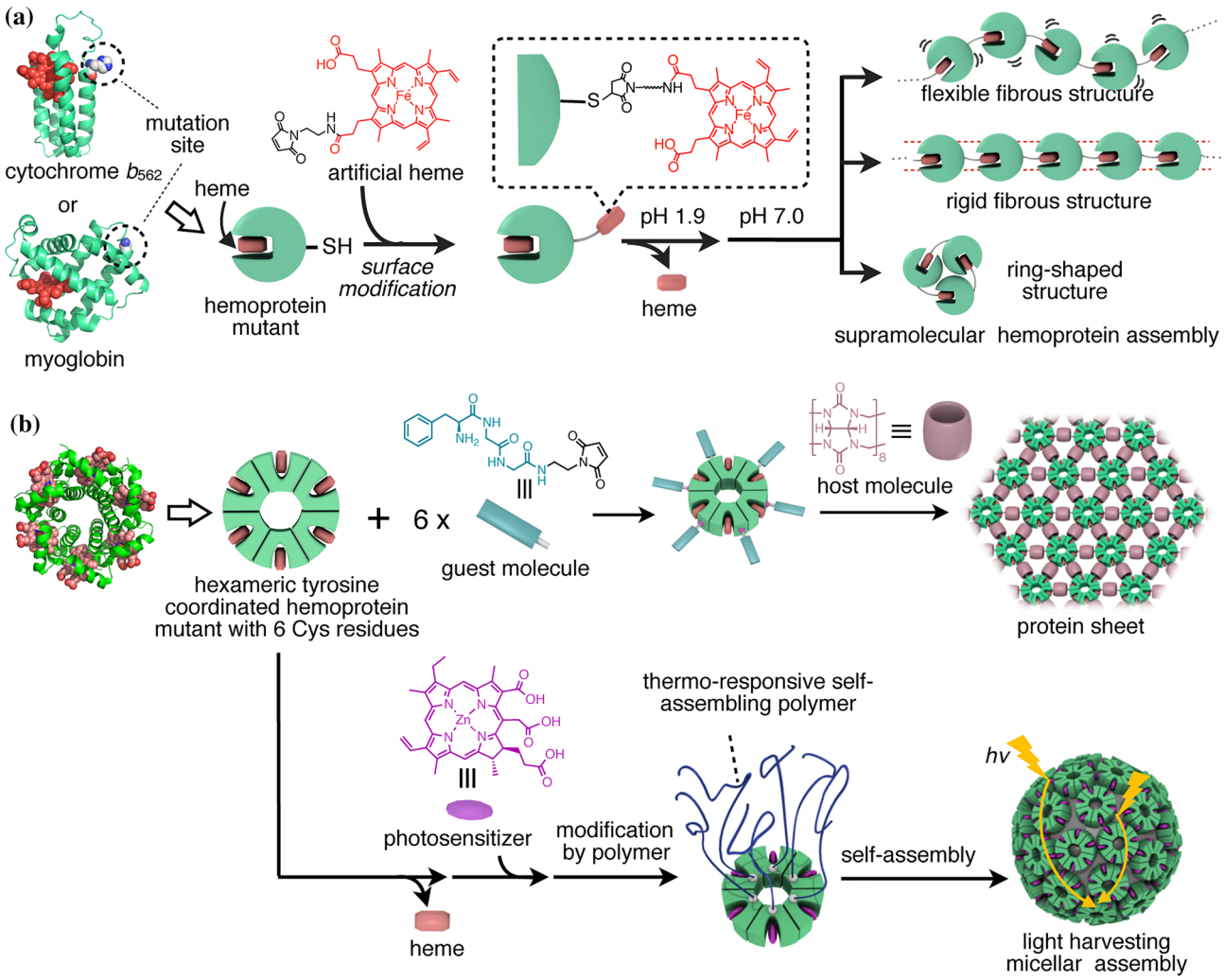

Fig. 3 Schematic representation of supramolecular hemoprotein assemblies. **a** Assemblies of monomeric hemoprotein via heme–heme pocket interactions. **b** Assemblies of hexameric hemoprotein by chemical modification of self-assembling molecules

Micelle-type assembly of covalently heme-attached hemoprotein

In the supramolecular assembling system of Cytb^{H63C}, a linker moiety between protein and attached heme moiety is found to be one of essential factors to determine the assembled structure and its stability. This point motivated us investigation of the flexibility and hydrophobicity of a linker in the heme-attached protein for supramolecular assembling behavior [79]. An azobenzene or stilbene moiety was employed as a rigid and hydrophobic linker and an octyl moiety was chosen as a flexible and hydrophobic linker, whereas a flexible and hydrophilic linker was used in former work. In the original attempt, assembling behavior change by trans–cis isomerization was expected for azo-benzene or stilbene linker but it did not work effectively. However, quite unique assembling behavior with transitions between fiber- and micelle-like structures was confirmed by supramolecular assembling system containing only a rigid and hydrophobic linker (Fig. 6a). Transient thermal stimuli provided to the fibrously assembled protein was found to induce formation of the spherical structure, which was maintained after cooling with slow conversion to the fibrous structure for 3 or 4 days. The micelle-type assembly appears to be metastable or kinetically trapped state. The TEM image supports this anomalous transition (Fig. 6b). These findings indicate that the heme-mediated supramolecular assembling systems based on hemoproteins are strongly modulated by the chemical structure of the linker moiety.

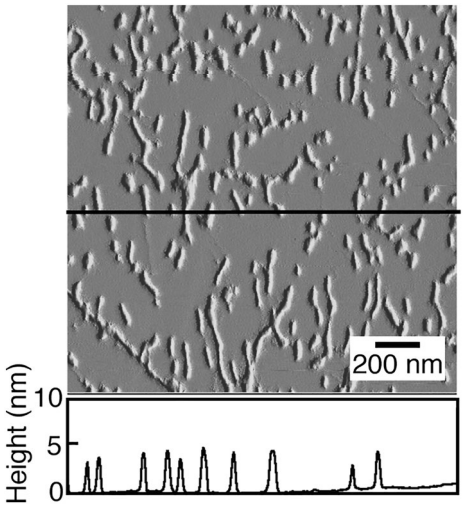

Fig. 4 AFM image of Cytb^{H63C}-based supramolecular assembly on a mica substrate under air

Hemoprotein assembly with periodic and rigid structure showing exciton coupling

His63 position was selected as a heme-attached site in first trial of supramolecular Cyt*b* assembly according to the simple design by just opposite site of the heme-binding site. The modulation of heme-attached site should show higher impact relative to the changes of linker moiety [80]. Newly employed Asn80 position has complementary charges around the residue against surrounding charges of heme-binding site (Fig. 7a). Thus, additional interprotein interaction was expected to be induced by proximity effect in the protein assembly. The N80C mutant, Cyt-b^{N80C}, was prepared and modification of the protein with maleimide-tethered heme was successfully carried out. Cytb^{N80C}-based supramolecular assembly showed larger molecular weight relative to Cytb^{H63C}-based supramolecular assembly indicating that the secondary interactions effectively was induced. Significant difference in two systems was observed in CD spectrum. In the case of Cytb^{H63C}-based supramolecular assembly, only negative Cotton effect in Soret band region at 418 nm induced by a protein matrix was shown. This is almost same as the wild-type protein. In contrast, the split type Cotton effect was clearly observed in Cytb^{N80C}-based supramolecular assembly. This finding supports the fact that the heme-heme exciton coupling in rigid protein assemblies with defined conformation of chromophores. This experimental data is helpful to suggest the plausible structure. Molecular dynamics (MD) simulations of dimer model proposed the several candidates and the calculated structure was screened by comparison of simulated CD spectra with experimental one. In the model replicating the CD spectra, two interprotein hydrogen bonding interactions were found and mutation of related residue with alanine convert the split-type Cotton effect in CD spectra into that with negative Cotton effect. This mutation experiment is supportive for the model structure. Furthermore, the structure was evaluated by high-speed AFM measurement (Fig. 7b), which enables the clear visualization of protein weakly adsorbed onto the substrate despite its dynamics in solution state. The fibrous objects were clearly imaged and analysis indicates the 3-nm pitch. This pitch length is consistent with the estimated pitch in the oligomer structure based on plausible dimer model (Fig. 7c). Therefore, rigid and periodic supramolecular hemoprotein assembly was obtained only by change of heme-attached site.

The rigid and periodic structures contribute to the construction of unique functional assembling systems (Fig. 8).

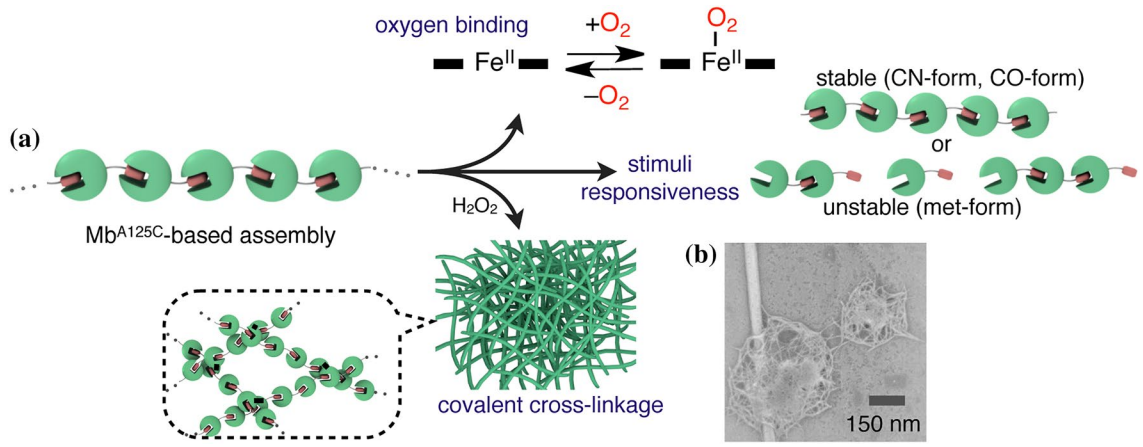

Fig. 5 **a** Characteristics of Mb^{A125C}-based assembly: maintained oxygen binding affinity, stimuli responsive stability and covalent cross-linkage upon addition of H_2O_2. **b** SEM image of cross-linked Mb^{A125C}-based assembly

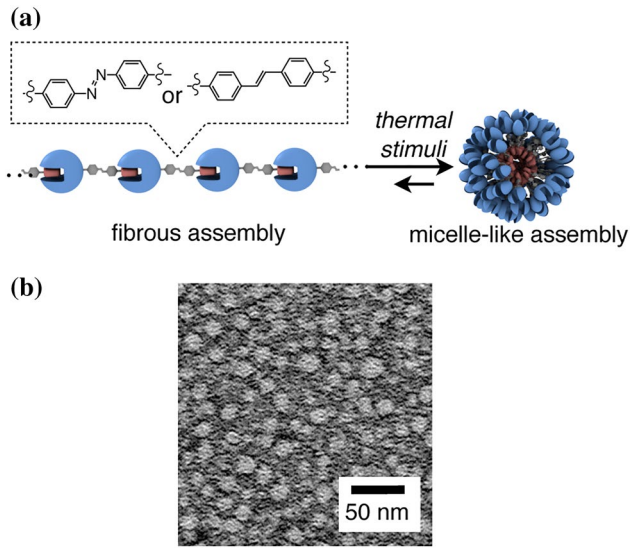

Fig. 6 **a** Transition between fiber and micelle-like structure. **b** Negatively stained TEM image of micelle-like assembly

The hydrogen bonding network at the interface of protein units inspires us the interprotein electron transfer pathway. Here, photo-induced electron transfer from excited triplet state to ferric heme was utilized. Co-assemblies consisting of heme-attached Cytb^{N80C} and Zn porphyrin-attached Cytb^{N80C} were prepared and laser flash photolysis indicate the expected charge separation and recombination events, whereas the co-assembly using mutant providing the flexible structure did not show the events [81]. The rigid and periodic structures were also utilized to generate the array of molecules showing cluster effect [82]. Oligo arginine is known to be a cell-penetrating tag and especially arginine octamer is often used. Although arginine tetramer does not have enough cell-penetrating ability, clustered tetramers on the C-terminals of units in rigid assembly generate the efficient incorporation function into the cell. The flexible protein assemblies with arginine tetramer tags decrease the cell-penetration efficiency. The findings indicate that the efficient cluster effects can be generated by rigid protein structure. These two examples are future possibility to create functional biomaterials based on supramolecular hemoprotein assembly.

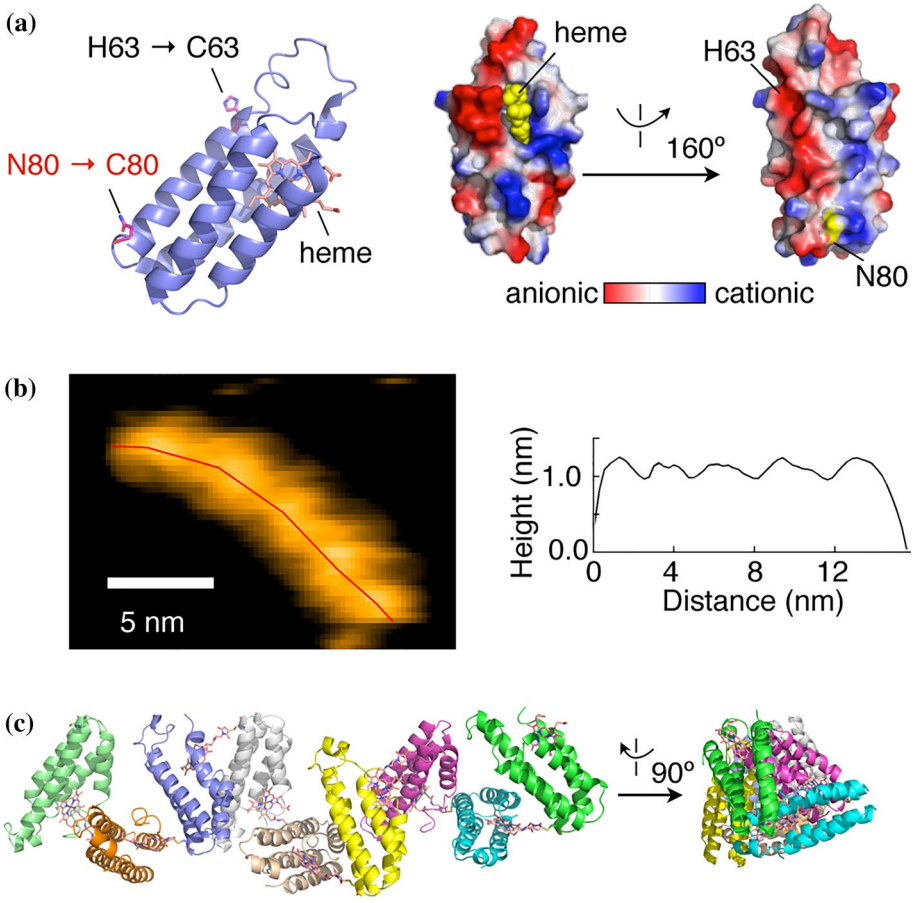

Fig. 7 **a** Cysteine introduced sites for Cyt*b* with surface potential map. **b** representative snapshot in high-speed AFM images of Cytb^{N80C}-based supramolecular assembly on a mica substrate in solution with a height profile along a red line. **c** Plausible model of nonamer which is supported by MD simulation and mutation experiments

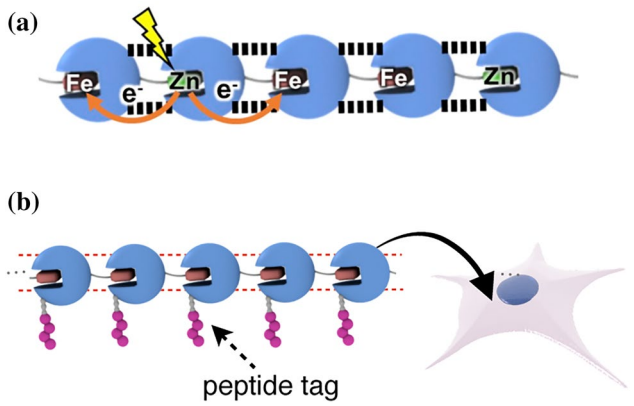

Fig. 8 Schematic representations of electron transfer (**a**) and cellular-uptake systems (**b**) based on Cytb^{N80C}-based supramolecular assembly

Ring-shaped hemoprotein assembly

In the case of supramolecular Cyt*b* assemblies, fibrous structures are mainly obtained whereas ring structure should also be formed. Dominant formation of fibrous structure may be caused by strong affinity of heme cofactor for the protein matrix. However, a ring-shaped structure is accidentally afforded in the optimization process of a linker length for the supramolecular Cytb^{N80C} assembling system [83]. Although an ethylene diamine-derived short linker demonstrates periodic and rigid structures, moderately long linker provides the ring-shaped trimer under a diluted condition. The obtained structure is clearly characterized by high-speed AFM and mass analysis (Fig. 9). The transition between ring-shaped trimer and fibrous oligomers was clearly observed by SEC. This equilibrium is well investigated by supramolecular polymer of synthetic small molecules [3, 4]. Purified ring-shaped trimer by SEC is durable for concentration indicating that the ring-shaped structure appears to be a kinetically trapped state. This feature implies favorable interprotein interaction is possibly induced to form the ring-shaped structure.

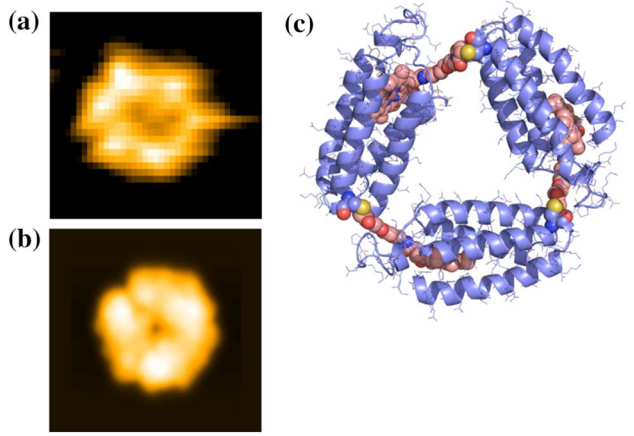

Fig. 9 **a** Snapshot in high-speed AFM image of ring-shaped trimer on a mica substrate in solution. **b** MD-optimized model of ring-shaped trimer. **c** Simulated AFM image based on the model structure in b

Supramolecular assemblies of hexameric hemoprotein toward light harvesting systems

Hexameric tyrosine coordinated hemoprotein, HTHP, is also a unique building block of supramolecular assembly because of relatively small molecular weight, thermal stability and C_6-symmetric structure [84, 85]. The heme moieties in HTHP are replaceable with other metal porphyrinoid. The toroidal hexameric structure containing chromophores motivated us to construct light harvesting system model in photosynthesis (Fig. 10). Actually, reconstituted HTHP with Zn porphyrin, a useful photosensitizer, was prepared and exciton coupling was observed in CD spectrum [86]. The fluorescence quenching experiments and quencher binding analysis indicate the energy migration, which is successive rapid energy transfer among same chromophores. This energy migration event is observed in native light harvesting system to be responsible to collect sun-light energy to

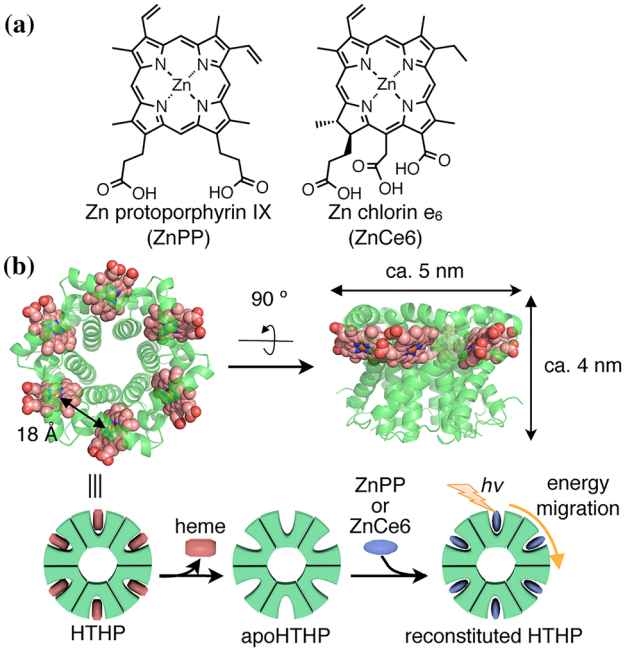

Fig. 10 **a** Zn porphyrin and Zn chlorin e_6 as porphyrinoid photosensitizers. **b** Crystal structure of HTHP and schematic representation for reconstitution of HTHP.

reaction center. Binding of Zn chlorin e_6 is also capable to apo-form of HTHP and also energy migration property was found in the reconstituted HTHP. This finding clearly indicates that HTHP is useful and versatile scaffold to generate artificial porphyrin-based light harvesting systems. Furthermore, chemical modification of donor and/or acceptor of Zn porphyrin was carried out for reconstituted HTHP [87]. The successive energy transfer in multiple chromophores was demonstrated in the system. Apo-form of HTHP as a branched unit is also combined with supramolecular Cyt-b^{N80C} assembling system to form a star-shaped structure [88].

To achieve the efficient use of sun light with low energy density, light harvesting systems are employed in natural system. For this purpose, large assembling structure is required to collect the excited energy to reaction center from hundreds of chromophores. In this context, HTHP assemblies by chemical modifications have been investigated. First, a pyrene derivative with maleimide group was attached to HTHP mutant with cysteine residue at a V44 position and the multiple hydrophobic interaction between pyrene units interestingly yield supramolecular dimer of the pyrene-attached HTHP [89]. The formation of dimer was confirmed by SEC. Removal of heme triggers disassembly of supramolecular dimer of the pyrene-attached HTHP into monomer. This is caused by the insertion of pyrene moieties into the vacant heme binding sites because pyrene-attached site is quite close to heme binding site. Further, reinsertion of heme molecules into apo-form of pyrene-attached HTHP to produces the supramolecular dimer. Next, host-guest interactions were employed to generate the huge HTHP assembly [90]. FGG tripeptide as a host moiety was attahed onto Cys-introduced HTHP mutant using thiol-maleimide coupling and subsequent addition of cucurbituril as a guest moiety provides the sheet-type assembly (Fig. 3b). Although the formation of sheet is strongly dependent on the Cys-introduced site, optimized mutant affords the several hundreds micrometer-sized sheet. Detailed structures were evaluated by high-speed AFM. The image proves the formation of monolayer which have reasonable height consistent with the height in HTHP crystal structure. Further investigation of this system using high-speed AFM reveals the formation process of sheet assembly. Within several tens seconds, small oligomeric assemblies forms on mica substrate. Within two minutes, the oligomers are connected to produce huge sheet structure.

The micelle-type protein assemblies were designed by the combination of HTHP with thermoresponsive synthetic polymer, poly-N-isopropylacrylamide (pNIPAAM) [91]. The modified HTHP with pNIAAM was successfully obtained and forms micelle type assembly (Fig. 3b). Dynamic light scattering measurements indicate the formation of the 40–50 nm size spherical structure at 60 ºC. Cooling to 25 ºC induces the disassembly into hexameric unit. This assembling and disassembling behavior was repeatable. The further characterization was carried out by high-speed AFM. Spherical structure is clearly observed but the units on the sphere appear to move rapidly. Cross-linkage of units using glutaraldehyde shows 5 nm objects on a 40–50 nm sphere (Fig. 11). This is good evidence of micelle structure which have hydrophilic HTHP units on the surface of a spherical object and hydrophobic polymer chains make core. Micelle-type HTHP assembly containing Zn porphyrin was prepared. To confirm the energy migration ability, time-resolved fluorescence anisotropy decay was measured. Fluorescence anisotropy is generally reduced by molecular motion but energy transfer causes the rapid decay. In the case of assembly, rapid decay within several tens picoseconds was observed. Control experiments using monomeric or partially chromophore lacking assembly does not show this significant decay. Thus, the large assembled structure of HTHP containing photosensitizers serves as a promising model of light harvesting systems.

Conclusion

Since noughties, artificial protein assemblies have been developed using various strategies. Especially, recent advances are toward sophisticated structures such as helical fiber, tube, cage and sheet are quite remarkable. In contrast to the variety of structures, development of function derived from assembling of protein units is still under investigation. As shown in this review, hemoprotein building block for supramolecular assemblies potentially possesses unique characteristics for both strategies in formation of assembly and generation of function. Strong and specific interaction between heme and protein matrix in hemoprotein is enough to form the supramolecular assemblies. Hemoprotein intrinsically include a heme moiety as a functional center. The

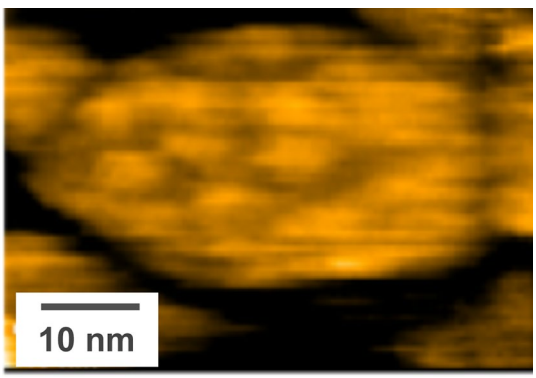

Fig. 11 AFM image of micelle-like assembly of HTHP after cross-linkage

obtained assembly shows stimuli responsive assembling behavior: the assembly is modulated by changes of pH, temperature, concentration, redox and 6th ligand as well as addition and removal of heme. Redox active heme is available for interprotein electron transfer. Generally, heme–heme pocket interaction provides the flexible fibrous assemblies but further optimization of a linker between heme and protein and/or heme-attached site affords the defined structures such as helical fiber, ring and micellar structures. These structures are useful to express the cluster effect of tethered functional groups. In addition to monomeric hemoprotein, ring-shaped hexameric hemoprotein is utilized as building block to form stacked dimer, sheet and micellar structures. The unique structure of assembling unit inspires the natural light harvesting system. Simple replacement of heme with porphyrinoid photosensitizer achieves artificial light harvesting systems. Future application of the assembled light harvesting units will enable molecular conversion including multiple electron transfer triggered by sun-light by combination with a suitable catalysis center. Supramolecular hemoprotein assemblies are promising systems to replicate biological protein assemblies as well as to contribute functional biomaterials overcoming natural functions.

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Author contributions KO wrote the main manuscript text and prepared all offigures.

Declarations

Conflict of interest There are no conflict to declare

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