Communication

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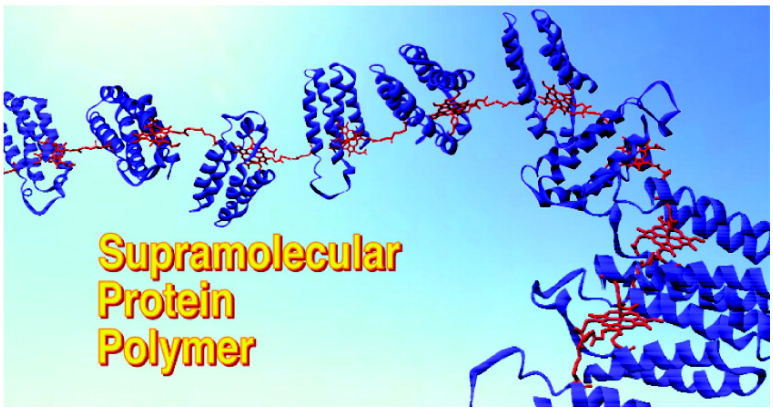

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Supramolecular Hemoprotein Linear Assembly by Successive Interprotein Heme−Heme Pocket Interactions

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Supramolecular polymers are defined as thermally equilibrated polymers formed by spontaneous connections of the monomer components through noncovalent intermolecular interactions.[1] Because of their thermodynamically reversible association/dissociation characteristics, supramolecular polymers of well-designed structures are of great interest as a new class of functional and responsive polymeric materials.[2] Since Lehn et al. prepared the first synthetic supramolecular polymer linked by triple hydrogen-bondings,[3] many efforts have been devoted into preparing various types of self-assembled clusters by accumulation of the functionalized small molecules.[1,4] A primary requirement for the construction of the supramolecular polymer is to design a monomeric component bearing two or more complementary interaction/recognition groups. Especially, in the case of a homopolymer, donor (host) and acceptor (guest) parts have to be incorporated into a single molecule (self-complementary monomer).[5]

We now extend this basic concept of supramolecular polymer chemistry to "supramolecular protein engineering". To construct the programmed protein that is self-assembled into a supramolecular homopolymer, we have designed and prepared the monomeric hemoprotein unit having an external cofactor moiety (a heme analogue) at the back of the heme pocket of the protein. As a result, a unique supramolecular linear assembly of hemoprotein was obtained via successive interprotein heme−heme pocket interactions (interprotein reconstitutions).[6] To the best of our knowledge, this is the first example of the supramolecular hemoprotein homopolymer, although several artificially controlled protein assemblies have been reported to create new nanobiomaterials.[7] The self-assembly process in this study is schematically outlined in Figure 1.

We have chosen a four-helix bundle hemoprotein, *Escherichia coli* cytochrome b_{562} (cyt b_{562}), as a scaffold for a hemoprotein self-assembling system. This protein is a soluble cytochrome having a single replaceable heme,[8] which is bound by Met7 and His102 ligations. For a site-selective modification on the protein surface, the His63 residue of cyt b_{562}, that is located on the protein surface opposite to the heme pocket, was first replaced with Cys to yield the H63C single mutant. Next, to introduce a heme moiety onto the surface Cys63 residue, modified hemes (**1a**−**c**) having an iodoacetamidated group at the terminal of the one of the heme-propionate side chains were synthesized. The coupling of **1** with the H63C mutant was carried out in an aqueous solution at pH 9.0. After gently stirring the mixture of the H63C mutant and excess **1** at room temperature for 7 h, the solution was acidified to pH 1.9 by the addition of an aqueous HCl solution to undergo acid-denaturation of the protein. The acidic aqueous solution was extracted with 2-butanone to remove the excess **1** and the intrinsic native heme, and then the aqueous phase was neutralized by successive dialysis with Tris-HCl buffer (50 mM, pH 7.3) to give the heme-attached apoH63C mutant (**1**-H63C).

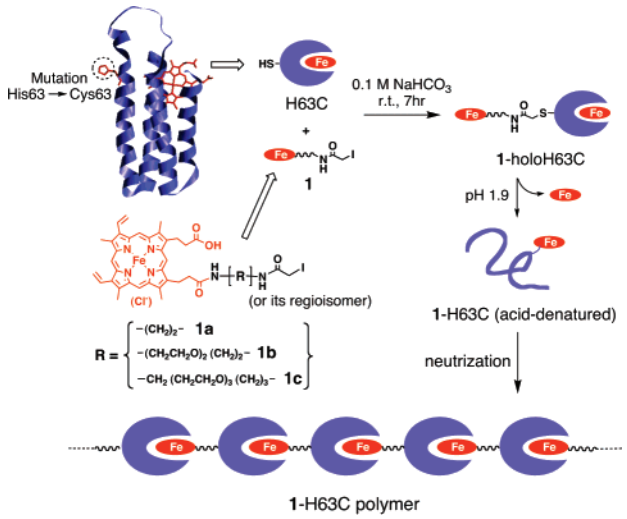

Figure 1. Schematic representation of the supramolecular hemoprotein polymerization. The structure of the wild-type cyt b_{562} was obtained from the protein data bank (1QPU).

The deconvoluted ESI-TOF-MS spectra of **1**-H63C gave the expected mass numbers of the corresponding monomer proteins; 12444.9 for **1a**-H63C (calcd = 12444.8), 12533.2 for **1b**-H63C (cacld = 12532.9), and 12606.1 for **1c**-H63C (calcd = 12606.0), whereas no peaks assigned as the unreacted H63C mutant and **1** were observed. The DTNB assay also supported the fact that the thiol group of the H63C mutant quantitatively reacted with the iodoacetamide of **1**.[9,10] The UV−vis spectra of the **1**-H63C solutions showed characteristic Soret (418 nm) and Q (530 and 564 nm) bands, which were consistent to those of the wild-type ferric cyt b_{562}.[11] The finding indicates that the external heme on the **1**-H63C surface revealed the 6-coordinated low spin species, suggesting that the heme was incorporated into the heme pocket of the protein matrix. The circular dichroism (CD) spectra of **1**-H63C showed characteristic 222 and 208 nm band minima, which are the typical patterns of the wild-type cyt b_{562}.[12] There were no differences in the molar ellipticities between the H63C mutant and **1**-H63C, indicating that the secondary structure of cyt b_{562} was conserved. Therefore, the UV−vis and CD spectra of **1**-H63C support the fact that the **1**-H63C monomer proteins preferably form the interprotein complex as shown in Figure 1.

To evaluate the degree of the polymerization, the solutions of **1**-H63C (50 mM Tris-HCl buffer, pH 7.3, 0.15 M NaCl) were

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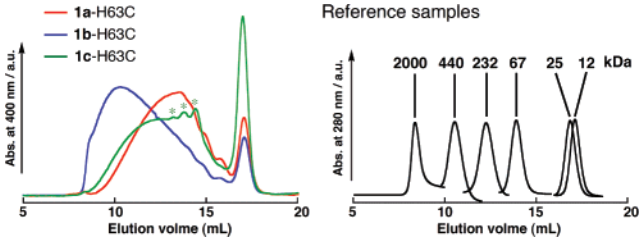

Figure 2. Comparison of the SEC traces of the **1**-H63C polymers (left) with the standard proteins as reference samples (right). The peaks with an asterisk correspond to the 4-, 5-, and 6-mers of **1c**-H63C.

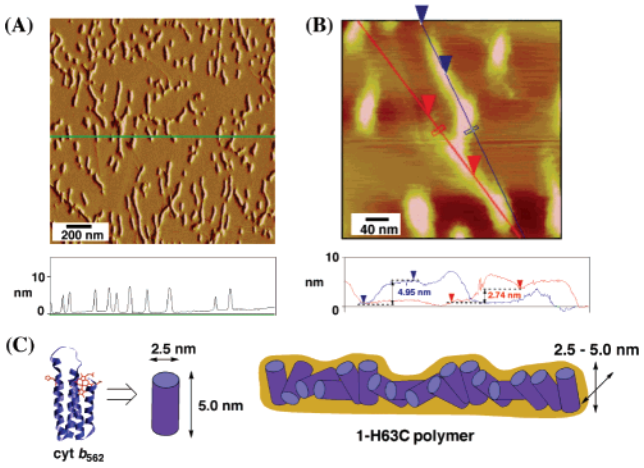

Figure 3. (A) AFM image of the **1a**-H63C polymer on the HOPG substrate with a height profile along the green line; (B) the enlarged image of panel A. Surface contours are monitored by the height analyses along the blue and red lines. (C) Schematic illustration of cyt b_{562} and the **1**-H63C polymer.

analyzed by size exclusion chromatography (SEC) using a Superdex 200 column (exclusion limits; 1.3×10^6 Da). The H63C mutant (12 kDa) was eluted at 17.1 mL, while the **1**-H63C assemblies were eluted quite earlier from the column (Figure 2). As compared with the elution peaks of several standard proteins in Figure 2, it is obvious that the **1**-H63C proteins convert to stable supramolecular clusters with large molecular sizes.[13] The cluster size is dependent on the linker length between the heme and the protein surface. The **1b**-H63C protein with the middle linker length has the largest cluster size, while the **1a**-H63C protein with the short linker shows a smaller cluster size than **1b**-H63C, probably because of the loss of the flexibility of the **1a**-H63C polymer chain. On the other hand, the linker of **1c**-H63C is so long that oligomeric cyclization may occur under these conditions. In fact, the SEC trace of **1c**-H63C showed the distinguishable oligomeric peaks corresponding to the 4-, 5-, and 6-mers of the protein-ring formation (asterisks in Figure 2). All of the elution profiles showed monomeric peaks at 17.1 mL. The origin of the monomeric peaks could be the denatured or misfolded **1**-H63C proteins, which cannot be incorporated into the polymer chains.

The **1**-H63C assemblies were directly visualized by tapping mode atomic force microscopy (AFM).[14] Samples were prepared by immersing a freshly cleaved highly oriented pyrolytic graphite (HOPG) substrate in the **1**-H63C aqueous buffer solution for a few seconds, and then the substrate was rinsed well with deionized water to remove the buffer salts before drying. As shown in Figure 3A, many linear assemblies with various lengths were observed. The uniform height (5.4 ± 0.6 nm) of the fibers corresponds to the single molecular height of the **1a**-H63C polymer (ca. 5.0 nm), and so each object is regarded as a single protein chain. The shape of each object suggested the flexible nature of the protein assembly. One of the linear objects is shown in Figure 3B, whose length reached ca. 350 nm, corresponding to over 100-mer of the protein. The surface contour of this object also indicated a feature of the hemoprotein linear assembly; the overall height of this object is ca. 5 nm, while the height of the concave site is ca. 2.7 nm. The height profile is quite reasonable for the expected polymeric structures of the cylindrical cyt b_{562} proteins, as illustrated in Figure 3C.

In conclusion, our preliminary work has established unique submicrometer-sized structures using hemoprotein as a building block. The protein supramolecular structures showed the feasibility of "soft materials"; the structures were thermodynamically equilibrated and regulated by the pH value. In the future, this methodology will be used to provide well-ordered multidimensional hemoprotein arrays with various biological functions, such as, for instance, electron-transfer, O_2-binding, and/or enzymatic property. The study of such functional hemoprotein assemblies is now in progress.

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Supporting Information Available: Experimental procedures, ESI-TOF-MS, UV−vis and CD spectra of **1**-H63C, inhibition assay using the native heme, AFM images, and the table of the mean molecular weight of the supramolecular polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) The term "heme" is generally an Fe(II) complex of a prosthetic porphyrin as in a narrow sense. In this paper, however, the heme also includes the Fe(III) porphyrin complex.
(9) The reactive thiol content of the protein surface was determined by incubating protein solutions with excess 5,5′-dithiobis-2-nitrobenzoate (DTNB). The UV-vis spectrum was then measured and the formation of 5-thio-2-nitrobenzoate was monitored. As expected, the H63C mutant was found to possess a single reactive thiol, whereas **1**-H63C contained no free thiol.
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