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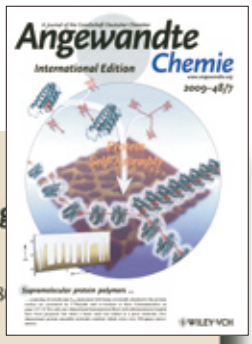

Protein Self-Assembly

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Self-Assembly of One- and Two-Dimensional Hemoprotein Systems by Polymerization through Heme–Heme Pocket Interactions**

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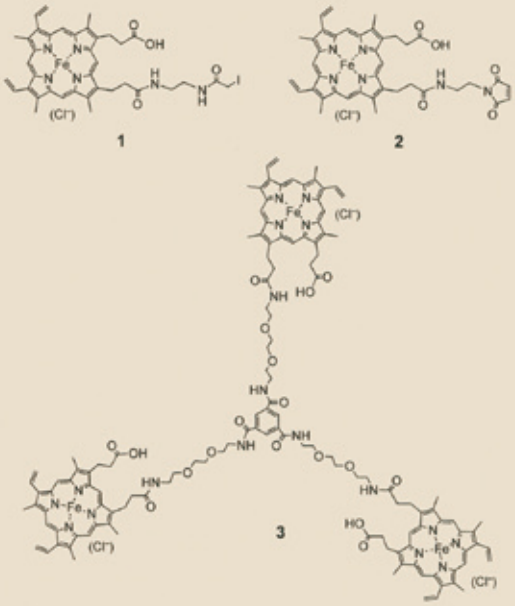
The construction of supramolecular polymers has recently become one of the principal challenges in nanoscience based on chemistry and biology.[1–5] For biomedical applications such as drug delivery[6,7] and tissue engineering[8] biomolecules have great potential as structural units in supramolecular polymers because of their biocompatibility and biodegradability. In addition, the design of highly ordered structures of biomacromolecules by self-assembly should shed light on the principles required for the development of "bottom-up" nanobiotechnology.[9–11] We have recently reported a system for the self-assembly of linear hemoproteins composed of subunits in which an externally introduced heme moiety is attached to the surface of a H63C single mutant of cytochrome b_{562} (cyt b_{562}(H63C)). This system displays an interprotein heme–heme pocket interaction after the native heme is removed from the modified protein.[12,13] The next attractive target for the development of further applications would be a supramolecular polymer with more highly ordered and/or higher-dimensional structures.[14–16] We demonstrate herein the construction of a supramolecular polymer composed of the genetically modified cyt b_{562}(H63C) protein and the synthetic heme analogue **2** (Scheme 1). The morphology of this polymer was investigated by atomic force microscopy (AFM). This linear hemoprotein self-assembly system has now been developed further into two-dimensional network structures by the introduction of the novel heme triad **3** as a pivot molecule. The strategy of this approach is illustrated in Scheme 2.

In our previous work, the synthetic heme **1**, which has an iodoacetamide-derivatized thiol-reactive group at the terminus of one of the heme propionate side chains, was prepared and used to obtain the heme-appended protein **1**-cyt b_{562}(H63C).[12] However, for completion of the conjugation between **1** and cyt b_{562}(H63C), alkaline conditions (pH 9.0) and long reaction times (over 7 h) were required because of the low reactivity of **1** to the thiol group of Cys63. Therefore, we prepared the synthetic heme **2**; its maleimide group is more reactive than iodoacetamide towards thiols.[17–19] The surface modification of cyt b_{562}(H63C) with **2** was carried out in aqueous 0.05 M Tris-HCl buffer at pH 7.3. After a mixture of cyt b_{562}(H63C) was gently stirred with an excess of **2** for 1.5 h at room temperature, the solution was acidified to pH 1.9 and the native heme was removed from cyt b_{562}(H63C) by using conventional extraction with 2-butanone.[20] The aqueous phase was neutralized by dialysis against a 0.05 M Tris-HCl buffer solution at pH 7.3. The UV/Vis spectrum of the resulting **2**-apo-cyt b_{562}(H63C) exhibits characteristic Soret (418 nm) and Q (530 and 564 nm) bands consistent with those of wild-type cyt b_{562}.[21] This finding indicates that the heme externally attached on the cyt b_{562}(H63C) surface is



Scheme 1. Structures of synthetic heme analogues. The compounds have regioisomers with respect to the substitution position in the two heme propionate side chains of protoheme IX.

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The following is a comment on the published paper shown on the preceding page.

Artificially-Created One- and Two-Dimensional Hemoprotein Self-Assembly Systems Formed by Supramolecular Polymerization via Heme–Heme Pocket Interactions

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Introduction

Thousands of molecular assembly composites exist in nature. The formation of these natural composites is triggered by multiple weak interactions of proteins, nucleotides, carbohydrates and lipids. The elegant and intriguing structures of these composites provide various biologically critical functions and chemists have attempted to mimic such structures by synthesizing highly ordered molecular clusters based on spontaneous intermolecular interactions. Over the past decade, many research groups have attempted to form polymeric supramolecular species by association of two complementary ditopic components (···A···B···A···B···A···) or by association of a self-complementary component (···C···C···C···C···C···) via hydrogen bonding interactions, hydrophobic contacts and/or by metal–ligand coordination [1]. The directed manipulation of intermolecular interactions has led to the formation of a nano-sized molecular array or cluster. However, the formation of a functionalized self-assembly system using a simple small molecular unit has not yet been achieved. We thus sought to create a "supramolecular polymer" using a chemically-modified protein.

Various hemoproteins are self-assembled by molecular recognition-induced association between the heme molecule and the heme pocket of the protein with large affinity constants ranging between of 10^8 to 10^{15} M^{-1}. Our research group has recently focused on the modification of hemoproteins by replacing the native heme prosthetic group with an artificial metal complex in the interior of the protein [2,3]. Our results have indicated that hemoproteins may act as building blocks for the development of supramolecular polymers. To design and construct self-assembled supramolecular composites, we prepared a modified cytochrome b_{562} protein (cyt b_{562}) with an external heme moiety covalently bound to the protein surface as shown in Figure 1. Construction and characterization of hemoprotein polymers with fiber and mesh structures are described in this report [4,5].

One-Dimensional Structure – Hemoprotein Fiber

Cyt b_{562}, a stable four-helix bundle hemoprotein, has a labile heme which is ligated to Met7 and His102. This protein does not have any cysteine residues within the interior of the protein. Thus, to introduce a heme moiety to the exterior of the protein on the opposite side of the heme pocket, the His63 residue on the protein surface was replaced with a Cys residue to provide a reaction site. An exogenous heme derivative with a reactive maleimide group linked to one of the heme-propionate side chains was then coupled to the thiol group of the surface Cys63 residue as shown in Figure 1. The Cyt b_{562} native heme was then removed under acidic conditions and the hemoprotein polymer was generated upon neutralization of the solution. The degree of polymerization was evaluated by size exclusion chromatography using a Superdex 200 column (exclusion limits: 1.3 x 10^6 Da) and electrophoresis with native-PAGE which indicated that the mutant of Cyt b_{562} modified with the external heme acts as a monomer for production of stable supramolecular clusters with large molecular sizes. In addition, the concentration of the protein affects the self-assembly process because the system is driven by the thermodynamically controlled heme–heme pocket interaction [6]. The sizes and morphologies of the hemoprotein assemblies were characterized using tapping-mode atomic force microscopy (AFM). For

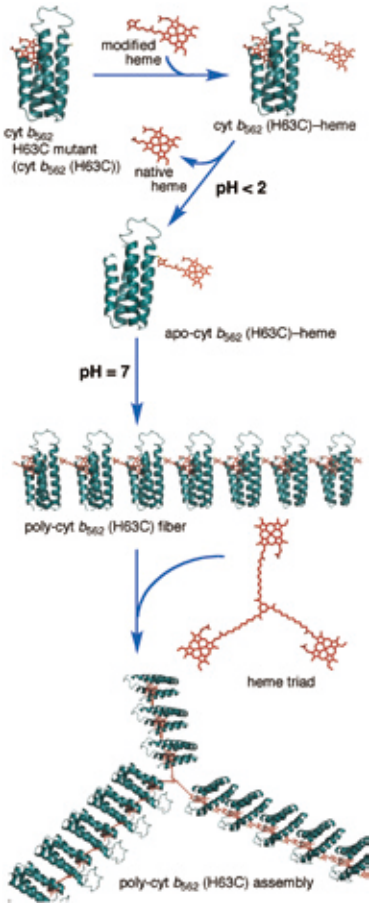

Fig.1 Schematic representation of the formation of supramolecular hemoprotein polymers using cyt b_{562} as a monomer unit.

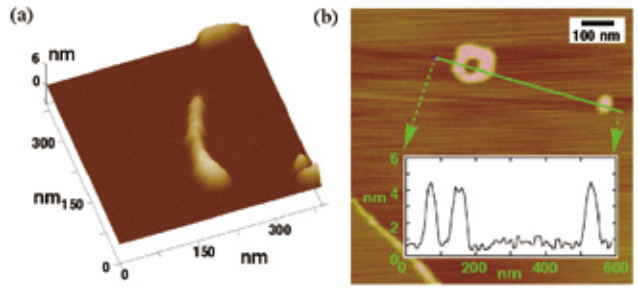

Fig.2 AFM images of the supramolecular hemoprotein polymers; (a) a cyt b_{562} fiber and (b) a cyt b_{562} ring on the HOPG substrate (Inset: the height profile of the ring structure).

example, an elegant protein fiber with a length of 250 nm was observed on a freshly cleaved highly oriented pyrolytic graphite (HOPG) substrate (Figure 2(a)). The AFM images indicate that the typical fiber includes more than 100 protein molecules. The height of the fiber is essentially consistent with the height of the cylindrically shaped monomeric cyt b_{562} protein (height 5.0 nm x diameter 2.5 nm). Interestingly, a cyclic protein assembly structure is also detectable. The doughnut-shaped polymer comprises over 100 protein units as shown in Figure 2(b).

Two-Dimensional Structure – Hemoprotein Network

To spread the protein fiber on the HOPG substrate, we prepared a heme triad with a 1,3,5-trisubstituted benzene core as a pivot molecule as shown in Figures 1 and 3. The addition of the pivot molecule into the solution of the hemoprotein fibers clearly produces a branch point in the linear fiber on the substrate (Figure 4(a)). Furthermore, a mixture of the modified protein with the external heme and the heme triad in a molecular ratio of 40 : 1 produced an elaborate hemoprotein network with an area of 100 μm^2 as measured by AFM (Figure 4(b)). The height profile of the AFM image indicates that the network is formed by a monolayer assembly with a height of 5 nm (Figure 4(c)). It was also determined that the mesh size may be controlled by the ratio of the protein and the triad. As expected, at a molar ratio of 100 : 1, a low-density network appears on the HOPG substrate. A plausible mechanism for the formation of this

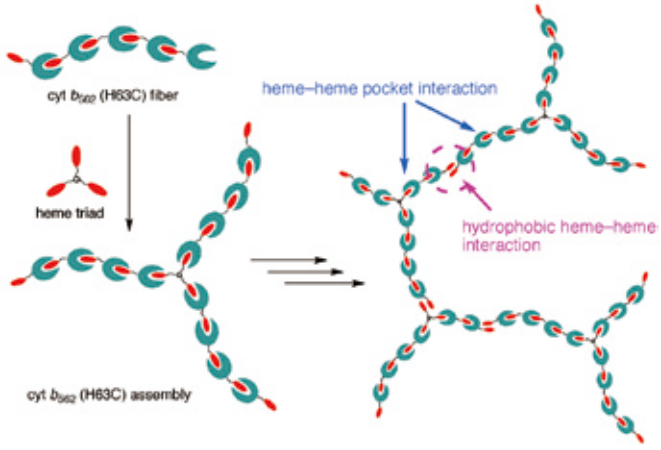

Fig.3 A structure of the heme triad (pivot molecule) and a plausible mechanism for the formation of the hemoprotein network assembly.

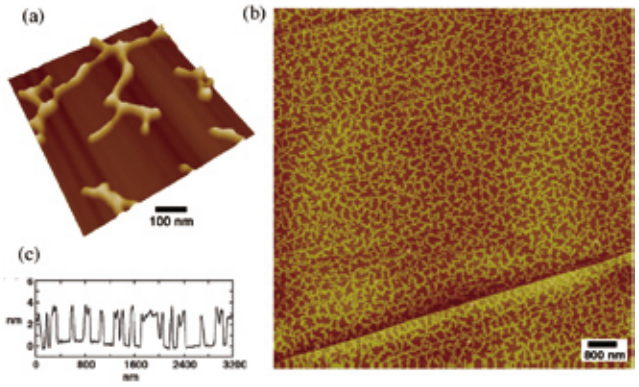

Fig.4 AFM images of the 2D network. (a) An enlarged image of the hemoprotein polymer in the presence of the pivot moleculae. (b) The polymeric cyt b_{562} network at a molar ratio of 40 : 1 (protein : pivot molecule). (c) The height profile of the network on the HOPG substrate.

attractive network is depicted in Figure 3. After the incorporation of the heme triad into the hemoprotein fiber, the termini of the fibers will associate with the exposed hemes on the protein surfaces. This causes fiber–fiber linkage to yield the two-dimensional hemoprotein network.

Summary and Scope

We have demonstrated a new strategy for the construction of supramolecular hemoprotein polymers via a spontaneous intermolecular heme–heme pocket interaction. The AMF images of the self-assembled fibers suggest that the polymers consist of 50–500 proteins with lengths ranging between 200 to 2000 nm. The addition of a heme triad pivot molecule generates a two dimensional protein network in which the mesh size is readily controlled by the molar ratio of the protein and pivot molecule. It is expected that the present supramolecular self-assembly method will serve as a new process for creation of highly ordered bionanomaterials. In addition, a series of nano- or micro-sized hemoprotein polymers will potentially be interesting subjects of further study because the iron porphyrin is a functional chromophore with gas binding affinity, catalytic reactivity and redox activity.

References

[1] Lehn, J.-M., *Chem. Soc. Rev.*, **36**, 151–160 (2007).

[2] Hayashi, T., and Hisaeda, Y., *Acc. Chem. Res.*, **35**, 35–43 (2002).

[3] Our recent papers: Hayashi, T., Harada, K., Sakurai, K., Shimada, H., and Hirota, S., *J. Am. Chem. Soc.*, **131**, 1398–1340 (2009), Harada, K., Sakurai, K., Ikemura, K., Ogura, T., Hirota, S., Shimada, H., and Hayashi, T., *J. Am. Chem. Soc.*, **130**, 432–433 (2008) and Matsuo, T., Murata, D., Hisaeda, Y., Hori, H., and Hayashi, T., *J. Am. Chem. Soc.*, **129**, 12906–12907 (2007).

[4] Kitagishi, H., Oohora, K., Yamaguchi, H., Sato, H., Matsuo, T., Harada, A., and Hayashi, T., *J. Am. Chem. Soc.*, **129**, 10326–10327 (2007).

[5] Kitagishi, H., Kakikura, Y., Yamaguchi, H., Oohora, K., Harada, A., and Hayashi, T., *Angew. Chem. Int. Ed.*, **48**, 1271–1274 (2009).

[6] Kitagishi, H., Oohora, K., and Hayashi, T., *Biopolymers*, **91**, 194–200 (2009).