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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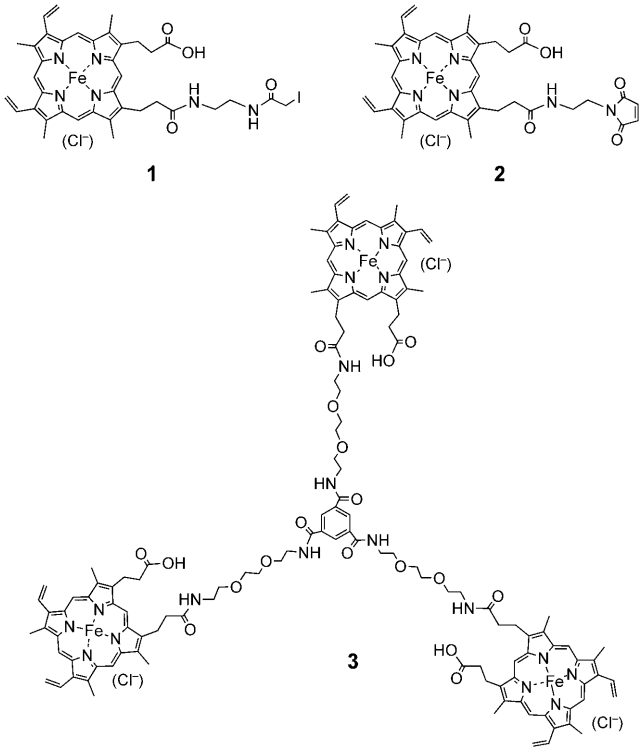
The construction of supramolecular polymers has recently become one of the principal challenges in nanoscience based on chemistry and biology.[1–5] For biomedical applications such as drug delivery[6,7] and tissue engineering[8] biomolecules have great potential as structural units in supramolecular 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. These compounds have regioisomers with respect to the substitution position in the two heme propionate side chains of protoheme IX.

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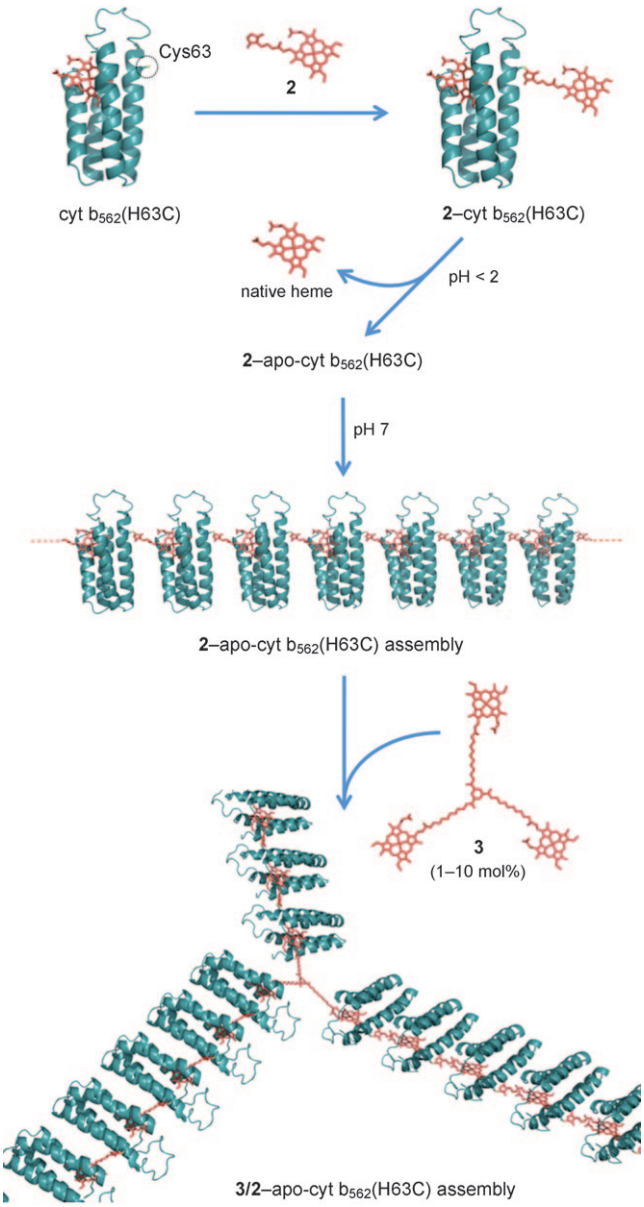


Scheme 2. Formation of the supramolecular assembly **3**/**2**-apo-cyt b_{562}-(H63C).

incorporated into the heme pocket within the protein matrix in a manner similar to that observed for **1**-apo-cyt b_{562}-(H63C).[22] The native-PAGE analysis of **2**-apo-cyt b_{562}(H63C) exhibits a protein band distributed over a wide range of molecular weights, while the cyt b_{562}(H63C) mutant without the external heme has a single protein band at the expected position (Figure 1 a). In contrast, the **2**-apo-cyt b_{562}(H63C) protein exhibits a single band by SDS-PAGE analysis (Figure 1 b), indicating that dissociation of the self-assembled protein cluster can be achieved by incubation with SDS. These results suggest that **2**-apo-cyt b_{562}(H63C) forms a large supramolecular protein assembly through interprotein heme–heme pocket interactions. The formation of the supramolecular polymer is also indicated by size-exclusion chromatography (SEC) (see the Supporting Information).

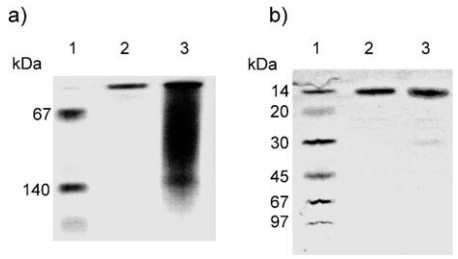


Figure 1. a) Native- and b) SDS-PAGE of cyt b_{562}(H63C) and **2**-apo-cyt b_{562}(H63C). Lane 1: marker proteins; lane 2: cyt b_{562}(H63C); lane 3: **2**-apo-cyt b_{562}(H63C).

The size and morphology of the hemoprotein assemblies were characterized by tapping-mode atomic force microscopy (TM AFM) measurements.[23,24] Samples were prepared for measurements by immersing a freshly cleaved, highly oriented pyrolytic graphite (HOPG) substrate in the protein solution for a few seconds; the substrate was then washed with deionized water to remove buffer salts and subsequently dried.[25] The AFM image of immobilized **2**-apo-cyt b_{562}-(H63C) on the substrate shows uniformly dispersed linear objects (Figure 2 a). The height of the objects ranges from 2.5 to 5.0 nm (see the Supporting Information), which is in good agreement with the size of the cylindrically shaped cyt b_{562} protein (height 5.0 nm; diameter 2.5 nm).[12,26] The length of the observed protein fiber in Figure 2 b is approximately 200 nm, which corresponds to an approximately 80-mer protein chain. Remarkably, a cyclic protein assembly structure is also detected (Figure 2 c). This structure could be formed by self-termination of linear polymerization. The doughnut-shaped polymer, which has a height of 5 nm, should comprise over 100 protein units, as estimated from its diameter (ca. 80 nm).

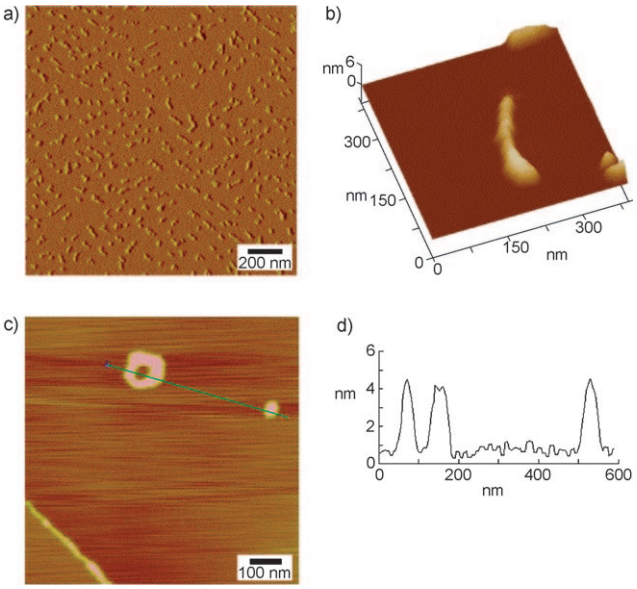


Figure 2. a)–c) AFM images of **2**-apo-cyt b_{562}(H63C) assemblies on a HOPG substrate. d) The height profile along the green line in (c).

Next, to introduce a branching point into the linear protein fiber, we have designed and synthesized the heme triad **3** with a 1,3,5-trisubstituted benzene core (Scheme 1).[27] The addition of a small amount of the heme triad **3** to the linear **2**-apo-cyt b_{562}(H63C) assembly resulted in a dramatic change in the AFM images. Surprisingly, a premixed solution of **3** and **2**-apo-cyt b_{562}(H63C) (molar ratio 1:40) gives rise to highly detailed network structures on the HOPG substrate (Figure 3a). The uniform height of the network (Figure 3b,c)

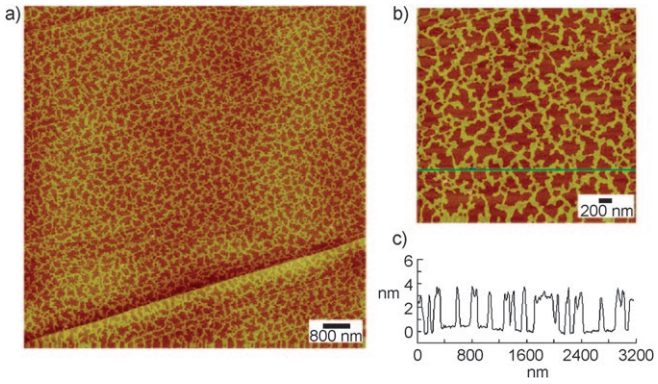


Figure 3. a),b) AFM images of **3**/**2**-apo-cyt b_{562}(H63C) assemblies at a molar ratio of 1:40 on a HOPG substrate. c) The height profile along the green line in (b).

is clearly indicative of protein monolayer assemblies in the absence of multiple deposits or unselective aggregations of protein. Figure 4 shows the morphological changes in the assembly as a function of the molar ratio of **3** and **2**-apo-cyt b_{562}(H63C). In the absence of **2**-apo-cyt b_{562}(H63C) (Figure 4a) only point particles were observed resulting from the aggregation of **3**. At a **3**/**2**-apo-cyt b_{562}(H63C) molar ratio of 1:1 (Figure 4b), a network was not formed, although several rodlike objects were evident. At a molar ratio of 1:10 (Figure 4c), a localized protein network structure was obtained which had many junctions relative to the structure obtained from a molar ratio of 1:40 (Figure 3). As expected, at a molar ratio of 1:100, a low-density network was observed (Figure 4d). An enlarged image of this network is shown in Figure 4e. The height profile (Figure 4f) of the structure formed at the 1:100 molar ratio clearly shows the branched protein chain structures that have a uniform height corresponding to the monolayer protein assembly. These morphological changes further indicate that formation of the network from the **2**-apo-cyt b_{562}(H63C) protein assembly is induced by addition of the heme triad **3**.

A plausible mechanism for the formation of the network is shown in Scheme 3. When the heme triad **3** is incorporated into the **2**-apo-cyt b_{562}(H63C) linear assemblies, a trident hemoprotein assembly is first formed. The termini of the assembly should be the externally attached hemes on the protein surfaces. The exposed hemes in aqueous solution associate with each other.[30] Therefore, the protein assembly further extends and diverges through the interprotein heme–heme interactions, thereby providing the unique two-dimensional hemoprotein networks.

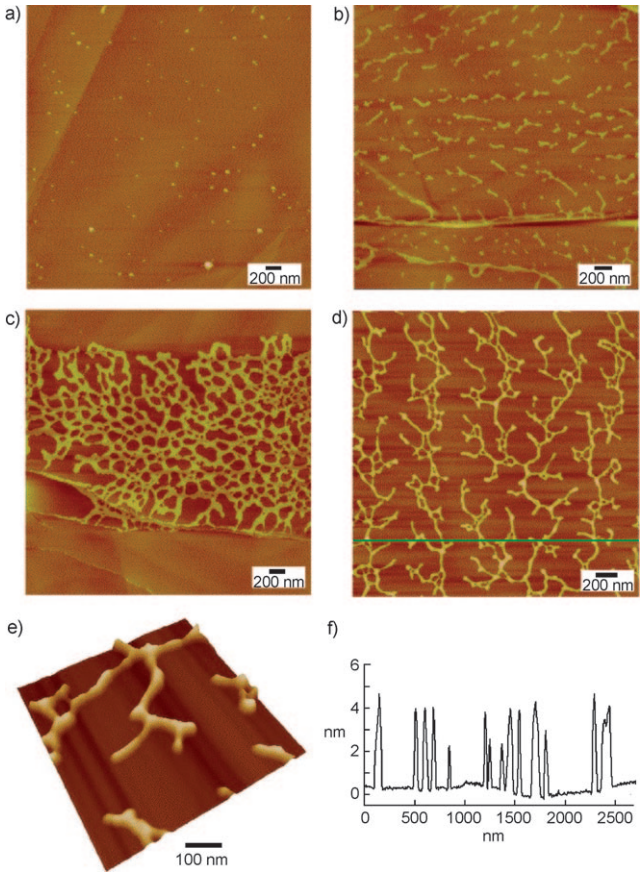
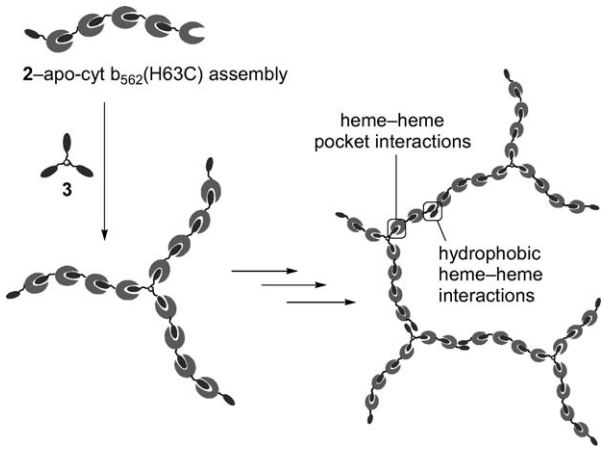


Figure 4. AFM images of **3**/**2**-apo-cyt b_{562}(H63C) assemblies at different molar ratios: a) 1:0, b) 1:1, c) 1:10, and d) 1:100 on a HOPG substrate. e) An enlarged image of (d). f) The height profile along the green line in (d).



Scheme 3. A plausible mechanism for the formation of the hemoprotein network assembly.

In conclusion, the present methodology for the construction of a supramolecular hemoprotein polymer using successive heme–heme pocket interactions should serve as a new method for creating not only linear protein fibers but also multidimensional protein networks in the form of uniform-

height monolayers on HOPG substrates. One series of our AFM studies suggests that the two-dimensional morphology of protein networks is controlled by the molar ratio of the protein and the pivot molecule. We believe that such advanced supramolecular architectures hold promise in the development of protein-based nanobiomaterials. Further investigations on the detailed structure and function of supramolecular hemoprotein polymers in solution and on substrates are in progress.

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[25] It was found that the **2**-apo-cyt b_{562}(H63C) protein was well-immobilized on the hydrophobic HOPG substrate, but it does not form fibers on hydrophilic mica under the same conditions.
[26] The size was estimated from crystal structure of wild-type cyt b_{562} (PDB ID: 1QPU).
[27] We first evaluated the affinity of **3** with apo-myoglobin.[28,29] Stepwise addition of up to 0.33 equivalents of a stock solution of **3** (in DMSO/0.1 M KOH 1:1) into an apo-myoglobin solution in 0.1 M phosphate buffer at pH 7 gave a mixture of the reconstituted myoglobin monomer, dimer, and trimer. The trimer was identified as a major product (54.3 kDa) and was isolated by SEC with an elution volume of 14.8 mL (see the Supporting Information).
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