Protein Assembly

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Chemically Programmed Supramolecular Assembly of Hemoprotein and Streptavidin with Alternating Alignment**

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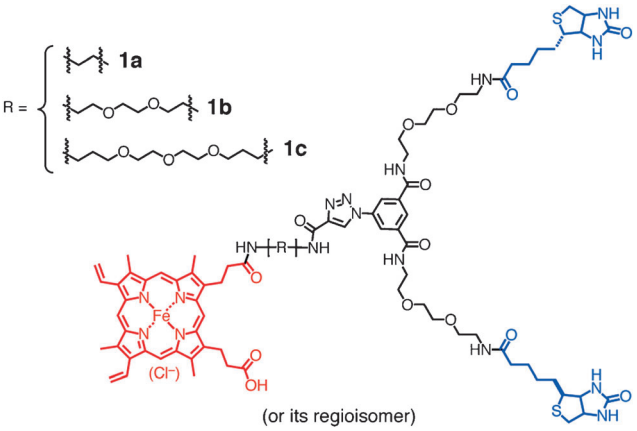
In the last decade, several artificial protein assemblies have been reported as a mimic of biological systems or as an extension of synthetic supramolecular composites. Most of the man-made protein assemblies reported to date, however, are limited to a single protein building block to generate one- and two-dimensional polymers.[1–5] In contrast to the artificial protein polymers, many natural protein assemblies are heterotropic. These assemblies possess a well-defined structure, which is a key requirement to their complex function.[6]

In a biomimetic manner and relying on a chemical approach, we set out to generate and characterize a supramolecular copolymer[7] consisting of two orthogonal protein building blocks. To create an alternating one-dimensional heterotropic protein assembly, we designed the dyad **1** bearing a high-affinity appendage for each of the proteins (Scheme 1).

First, we demonstrated the heterotropic protein–protein complexation between homotetrameric streptavidin (SAv, with four biotin binding sites) and apomyoglobin (apoMb, which binds tightly to protoheme IX[8]). To assemble these proteins, we synthesized an artificial prosthetic group **1** incorporating two biotin units tethered through a flexible linker to one heme-propionate side chain (Scheme 1).[9] The bis(biotin) unit has been shown to displace biotin from SAv (that is, $K_a > 10^{13}\,M^{-1}$).[10] In contrast to this essentially irreversible binding event to SAv, the affinity of the modified heme moiety **1** to apoMb is approximately $K_a > 10^6\,M^{-1}$.[11] The resulting heme-bis(biotin) dyad **1** was added to apoMb to yield reconstituted Mb bearing the artificial cofactor **1** (rMb(**1**); Figure 1a).[12] The resulting rMb(**1**) was characterized by MS and UV/Vis spectroscopy (Figure S1 in the Supporting Information). The UV/Vis spectrum of rMb(**1**) was fully consistent with that observed for native Mb, thus suggesting that the appended bis(biotin) moiety has no deleterious influence on the overall structure of Mb.



Scheme 1. Molecular structures of the heme-bis(biotin) dyads **1a–c**.

Next, the association of rMb(**1b**) with SAv (possessing four biotin-binding sites as determined by the biotin-4-fluorescein assay)[13] at various ratios was evaluated. The resulting assemblies were characterized by size exclusion chromatography (SEC, using a Superdex 200 column). To evaluate the effect of the SAv/Mb ratio, the SEC elution peaks were analyzed both at 408 nm (to determine the Mb concentration) and 280 nm (concentration of aromatic residues; Figure 1). The SAv and rMb(**1b**) proteins eluted at 15.4 and 17.0 mL, respectively, whereas the larger species eluted at 13.7 mL, suggesting the formation of rMb(**1b**)-SAv-rMb(**1b**) (Figure 1c). Importantly, the SEC revealed a single elution peak with an R_z value (ratio of absorbances at 408 and 280 nm) consistent with the estimated ratio of the extinction coefficients for a rMb/SAv stoichiometry of 2:1 (Figure S2 in the Supporting Information).

These results demonstrate that: 1) the heme-bis(biotin) dyads **1a–c** bind selectively to both rMb and SAv (Figure S3 in the Supporting Information), 2) the preorganized bis(biotin) moiety binds exclusively to two adjacent biotin binding sites of SAv,[10] and thus prevents the formation of undesired branched oligomers,[14] 3) the resulting supramolecular protein assembly rMb(**1b**)-SAv-rMb(**1b**) is stable

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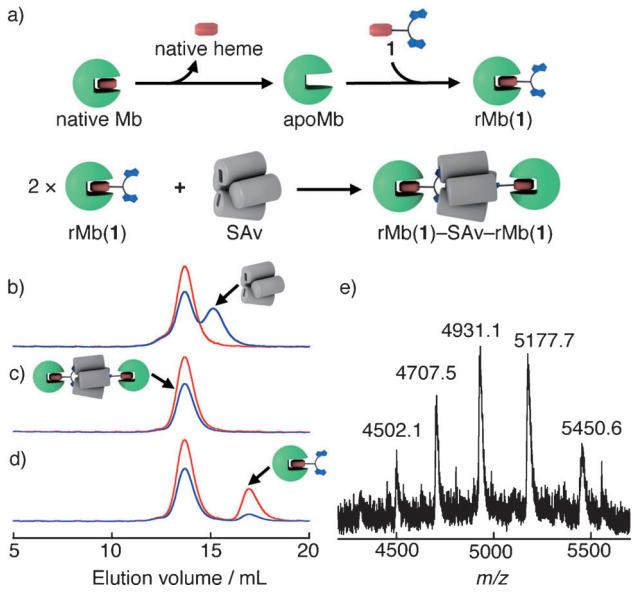
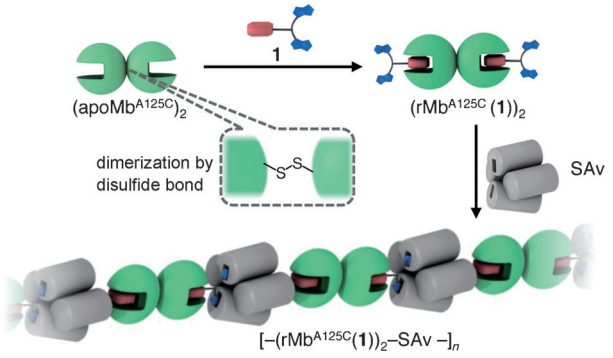


Figure 1. Assembly of rMb(**1**) and SAv. a) Schematic representation of reconstitution of Mb with **1** and formation of rMb(**1**)-SAv-rMb(**1**). SEC traces of complexation of SAv with b) one, c) two, and d) three equivalents of rMb(**1b**) compared to SAv. The red and blue traces show the absorbance at 408 and 280 nm, respectively. e) ESI-TOF MS spectrum of rMb(**1b**)-SAv-rMb(**1b**); calculated m/z (z) = 5450.4 (19+), 5177.9 (20+), 4931.4 (21+), 4707.3 (22+), and 4502.7 (23+).



Scheme 2. Schematic representation of the programmed assembly toward the alternating copolymer [-(rMb^{A125C}(**1**))$_2$-SAv-]$_n$.

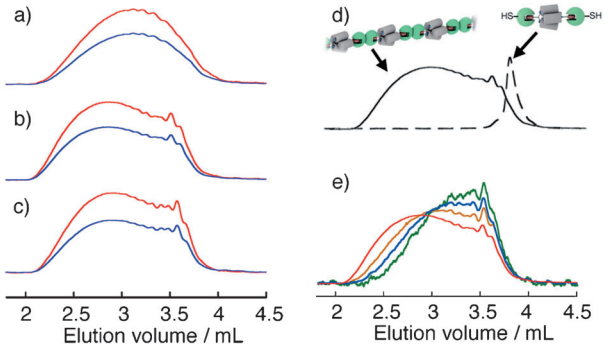


Figure 2. SEC traces for the stoichiometric copolymer of SAv and (rMb^{A125C}(**1**))$_2$ with the heme-bis(biotin) dyads a) **1a**, b) **1b**, and c) **1c**. The red and blue traces in a–c) show the absorbance at 408 and 280 nm, respectively. d) SEC traces before (solid line) and after reductive cleavage of the disulfide bond of [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$ upon the addition of TCEP (broken line). e) SEC traces of [-(rMb^{A125C}-(**1b**))$_2$-SAv-]$_n$ at various concentrations of (rMb^{A125C}(**1b**))$_2$ and SAv at a constant ratio of 1:1. The concentrations of (rMb^{A125C}(**1b**))$_2$ and SAv were 40 μM (red trace), 15 μM (brown trace), 7 μM (blue trace), and 2 μM (green trace). In parts d) and e), the normalized absorbance at 408 nm is plotted.

and can be unambiguously characterized by ESI-TOF MS (Figure 1e), and 4) the exclusive formation of a 2:1 rMb(**1b**)-SAv-rMb(**1b**) assembly occurs, even at low rMb(**1b**)/SAv ratios. This observation suggests that the binding of biotin to SAv is cooperative,[15] thus precluding the isolation of rMb-(**1b**)-SAv.

Having established the robustness of the encoding of the heme-bis(biotin) dyad **1**, we sought to create a one-dimensional protein copolymer. To ensure perpetuation of the programmed copolymer, a dimeric Mb was prepared: the Mb^{A125C} mutant was produced and homodimerized through a disulfide bridge to afford (Mb^{A125C})$_2$. The reconstituted dimer (rMb^{A125C}(**1**))$_2$ was prepared and characterized (Scheme 2 and Figure S5 in the Supporting Information). Gratifyingly, addition of SAv to the reconstituted dimeric (rMb^{A125C}(**1**))$_2$ yielded a one-dimensional copolymer [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$, which was analyzed by SEC (on a Shodex KW405-4F column). As expected, the 1:1 conjugate between (rMb^{A125C}(**1**))$_2$ and SAv was eluted faster than rMb(**1**)-SAv-rMb(**1**), the elution volume of which is approximately 3.8 mL (Figure S6 in the Supporting Information). As can be appreciated from the SEC traces, the molecular-weight distribution depends on: 1) the length of the linker between the heme and the bis(biotin) moiety; 2) the ratio of the building blocks (Figure 2 and Figure S7 in the Supporting Information); and 3) the concentration of the building blocks.

1) The copolymer of SAv and (rMb^{A125C}(**1a**))$_2$ with the shortest spacer **1a** formed smaller oligomers than [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$ or [-(rMb^{A125C}(**1c**))$_2$-SAv-]$_n$. This result suggests that the short ethylene spacer in **1a** inhibits the formation of large copolymers, which is possibly due to unfavorable Mb···SAv contacts. The SEC traces obtained for copolymers derived from (rMb^{A125C}-(**1b**))$_2$ and (rMb^{A125C}(**1c**))$_2$ are very similar and suggest the presence of higher-molecular-weight oligomers.
2) Upon varying the (rMb^{A125C}(**1b**))$_2$/SAv ratio, smaller copolymers with narrower size distribution were obtained (Figure S7 in the Supporting Information). Especially, an excess of (rMb^{A125C}(**1b**))$_2$ against SAv produced the capped oligomers, (rMb^{A125C}(**1b**))$_2$-SAv-[-(rMb^{A125C}-(**1b**))$_2$-SAv-]$_{(n-1)}$-(rMb^{A125C}(**1b**))$_2$ (n = 1–5), in which the number of repeating units n was assigned by R_z values. These resulting peaks in the SEC give a more reliable SEC calibration curve than those calibration curves based on globular protein standards, because the elution depends not only on the molecular weight but also on the shape of the protein in SEC experiments.[2,16] Based on this calibration, we suggest that the molecular weight at the maximum of the peak of the SEC trace in Figure 2b is approximately 1300 kDa (corresponding to [-(rMb^{A125C}-(**1b**))$_2$-SAv-]$_{12}$). Upon addition of excess tris(2-carboxy-

ethyl)phosphine (TCEP), a known disulfide-reducing agent, the copolymer [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$ was mostly converted into a single small component, the elution volume of which in SEC is consistent with that of rMb(**1b**)-SAv-rMb(**1b**) (Figure 2d and Figure S6 in the Supporting Information). This conversion clearly supports the formation of a programmed alternating copolymer of (rMb^{A125C}(**1**))$_2$ and SAv through the cofactor dyad **1**.

3) For a given SAv/(rMb^{A125C}(**1**))$_2$ ratio, the molecular-weight distribution of the copolymer depends on the concentration of the building blocks (Figure 2e). The results suggest that increasing the SAv and rMb concentrations leads to larger one-dimensional heterotropic assemblies. In analogy to reports on homotropic hemoprotein polymers,[2] we hypothesize that the growth and termination of this supramolecular polymerization are driven by the Le Chatelier principle (that is, thermodynamically controlled).[17]

Next, the copolymer [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$ was scrutinized by atomic force microscopy (AFM) in buffered solution (Figure 3a,b). Samples were prepared by casting the protein solution on a mica substrate modified with aminopropyltriethoxysilane (AP mica), followed by washing with a buffer solution. The solution AFM image clearly shows flexible one-dimensional fibers. The heights of the objects range between 3 and 5 nm, which are consistent with the expected sizes of SAv (ca. 5 nm) and Mb (ca. 3–4 nm). Further insight was gained by AFM images carried out on washed and dried samples (Figure 3c).[18] Along a fiber, the maxima (ca. 3 nm height) lie (16 ± 2) nm apart. This value closely matches the estimated periodicity of the copolymer (SAv = 5 nm, Mb dimer = 6–8 nm, and two spacers ca. 1.8 nm × 2 for **1b**; Figure 3d,e). Therefore, the alternating alignment of (rMb^{A125C}(**1**))$_2$ and SAv in the copolymer can be revealed by AFM. We thus propose the formation of a submicrometer-sized fibrous heterotropic protein copolymer with programmed sorting during the polymerization in the solution.

In conclusion, we have shown that an intramolecularly linked cofactor dyad enforces the association of two different proteins according to a predefined program. The present study demonstrates that the heme-bis(biotin) conjugate **1** contains the information to produce a stable 2:1 Mb–SAv complex. Propagation of this moiety into a polymer results from using a dimeric Mb building block. To our knowledge, the supramolecular composite fiber observed by AFM techniques is the first example of a chemically programmed heterotropic protein copolymer with alternating alignment. Importantly, the heme cofactor's dioxygen binding function is maintained upon incorporation within the fiber, suggesting that this approach is well-suited for the creation of functional nanobiomaterials.[17] Immobilization of the first building block will allow to: 1) study the programmed assembly by quartz microbalance analysis, 2) increase the complexity by varying the capping groups on the dyad and/or 3) introduce additional protein building blocks to afford protein polymers with a desired sequence and function. Potential applications include multifunctional catalysis, artificial photosynthesis, and smart nanomaterials for medical applications.

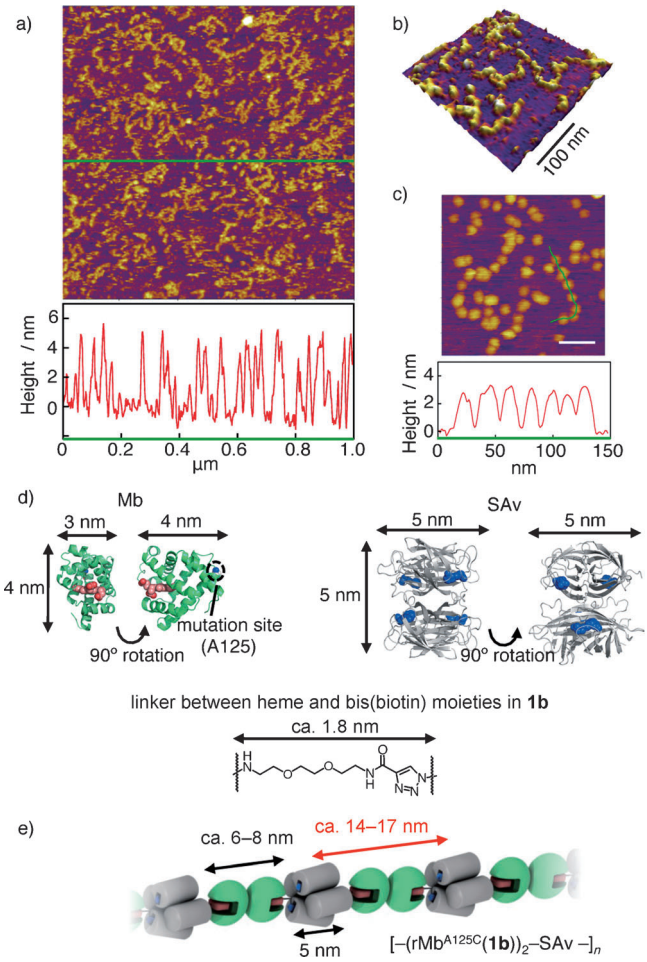


Figure 3. AFM images and heights of [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$. a) The image was taken in 100 mM potassium phosphate buffer, pH 7.0 and the height profile was measured along the green line. b) Enlarged 3D representation image of a part of (a). c) AFM image of the dry state and height profile along the green trace. Scale bar = 50 nm. d) Approximate sizes of the building blocks; Mb (green) with heme (pink), SAv (gray) with biotin (blue), and the linker in **1b**. e) Schematic representation of [-(rMb^{A125C}(**1b**))$_2$-SAv-]$_n$ to elucidate the AFM images of the dried samples. The estimated distance between tops of two nearest neighbors of SAv in the assembly ranges between 14 nm and 17 nm.

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[7] Herein, the term "copolymer" is used in the broader sense to signify that the large fibrous structure is formed by the co-assembly with two different proteins.

[8] Strictly speaking, the term "heme" stands for a ferrous porphyrin. As a matter of convenience, we will employ this term throughout this paper for an iron porphyrin regardless of its oxidation state.

[9] This compound has its regioisomer with respect to the substitution position in the two heme propionate side chains of protoheme IX.

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[14] This conclusion is further substantiated by a nondenaturing SDS-PAGE analysis of the inhibition of the binding of biotin-4-fluorosceine to SAv with varying amounts of bis(biotin) units. The results reveal the exclusive formation of SAv with two bis(biotin) units, but no formation of oligomers was observed (Figure S4 in the Supporting Information).

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[17] The absorption spectrum of $[\text{-}(\text{rMb}^{\text{A125C}}(\mathbf{1b}))_2\text{-SAv-}]_n$ is similar to that of native Mb in the visible region (Figure S8 in the Supporting Information). Furthermore, the native oxygen binding function seems to be retained even after formation of the copolymer. This is evidenced by the autoxidation rate for the oxy-form of $[\text{-}(\text{rMb}^{\text{A125C}}(\mathbf{1b}))_2\text{-SAv-}]_n$, which is comparable with that of native Mb (former; $0.12\ h^{-1}$, latter; $0.10\ h^{-1}$).

[18] For dried samples on AP mica, the heights of SAv and $(\text{rMbA125C}(\mathbf{1b}))_2$ were determined separately, and are ca. 3 and 0.5 nm, respectively (Figure S9 in the Supporting Information).

