SUPPLEMENTARY INFORMATION

A chemically-controlled supramolecular protein polymer formed by a myoglobin-based self-assembly system

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Supplementary Methods

Instruments. The ESI-MS data were obtained using a TOF mass spectrometer coupled to a Mariner API-TOF Workstation (Applied Biosystems). FAB-MS data were measured using a JEOL JMS-700 spectrometer with positive ion mode. UV-vis spectra were collected on a SHIMADZU UV-3150 or UV-2550 spectrometer equipped with a thermostated cell holder. The pH measurements were conducted on an F-52 Horiba pH meter. SEC analyses were performed at 4 °C using an $ÄKTA_{FPLC}$ system equipped with a Superdex 200 10/300 GL column and a fraction collector Frac-920 (GE Healthcare). SDS-PAGE analyses were carried out using an automated electrophoresis system (PhastSystem, GE Healthcare) with Precast gel (PhastGel Homogeneous 12.5 and 20). Proteins on the gel plates were detected by Coomassie blue staining. The AFM and SEM measurements were obtained using a Digital Instruments Nanoscope V and a Hitachi High-Technologies S-5500, respectively. Kinetic measurements of O_2 binding were carried out using a stopped-flow/laser flash photolysis system constructed by Unisoku, CO., Ltd (Osaka, Japan). A Xe arc lamp was employed as the source of the probe light to follow the spectral changes. For laser flash photolysis, a sample was excited with 5 ns pulses (532 nm) from a Q-switched Nd: YAG laser (Surelite I, Continuum).

Materials. Native sperm whale Mb was purchased from Biozyme Laboratories, Ltd. The proteins were purified by column chromatography through a CM-52 (Whatman). Distilled water was deionized with a Barnstead NANOpure Diamond^{TM} apparatus. The other reagents and chemicals were purchased and used as received.

Expression and purification of recombinant sperm whale Mb mutant (A125C). The recombinant sperm whale Mb mutant was overexpressed in the *E. coli* TB-1 cells. Metmyoglobin mutant was purified according the previous report with a few modifications.[S1] After purification by CM-52, the collected protein solution was concentrated and passed through Sephadex G-50 gel filtration column. The fraction of dimerized protein through the Cys125 on its surface was collected and concentrated. This A125C dimer was reduced to monomer with dithiothreitol (DTT) before

being used for chemical modification. Excess DTT was added to A125C, and then DTT was separated from the protein monomers by the Sephadex G-25 column equilibrated with degassed 100 mM L-histidine aqueous solution. A125C monomer was prepared and reacted with chemicals in a glove box.

Preparation of modified heme 1. Heme **1** was synthesized as shown in Scheme S1. Protoporphyrin IX mono *t*-butylester (**4**),[S2] *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine[S3] and *N*-methoxycarbonylmaleimide[S4] were prepared according to the procedures reported in the literatures. Synthetic method of the protoporphyrin IX iron (III) complex having an amino chain (**7**) was described previously.[S5]

1: In a 100-mL round bottom flask, protohemin IX monodiamine amide **7** (50 mg) was dissolved in a mixed solvent of acetone/sat. $NaHCO_3$ aq. at a ratio of 1/2 (10 mL) and *N*-methoxycarbonyl maleimide (120 mg) was added in the solution at 0 ºC. After stirring at 0 ºC in the dark for 2 h, 50 mL of water was added in the reaction solvent and stirred at room temperature in the dark for 1 h. The suspended aqueous solution was added in 0.1 M HCl (150 mL) and the product was extracted by $CHCl_3$ (30 mL x 3). The organic phase was dried over Na_2SO_4 and evaporated. The residue was passed through a short column chromatography (SiO_2, $CHCl_3$/MeOH = 5/1). After the eluent was collected and concentrated, the residue was dissolved in a minimum amount of $CHCl_3$. Hexane was added to the solution and the resulting precipitate was collected and dried to give **1** (23 mg, 46%): MS (FAB) *m*/*z* 898.3348 $(M - Cl^-)^+$, calcd for $C_{48}H_{54}FeN_6O_8$ 898.3353; UV-vis ($CHCl_3$/MeOH = 1/1, v/v) λ_{max} / nm (absorbance) 596 (0.064) 483 (0.087) 398 (0.78).

1 was reduced with equivalent sodium dithionite before being used for reaction with the thiol residue on the protein surface in a glove box.

Dissociation of poly-1-Mb upon addition of native heme. For the SEC analyses of poly-**1**-Mb in the presence of the native heme, the samples were prepared as described below. Native heme (protohemin IX chloride, 2.6 mg, 4.0 μmol) was dissolved in 10 mL of 100 mM potassium phosphate buffer, pH 7.0, containing pyridine (6%, v/v). To 15 μL of the poly-**1**-Mb solution (4.0×10^{-4} M) in 100 mM potassium phosphate buffer, pH 7.0, were added the native heme solution (0, 4.5,

9.0 and 15.0 μL) and 100 mM potassium phosphate buffer, pH 7.0, containing pyridine (15.0, 10.5, 6.0 and 0 μL, 6%, v/v) for samples provided with 0, 0.3, 0.6 and 1.0 eq. of heme, respectively. The resulting protein solutions were incubated at 4 ºC over 12 h.

References

S1. S. Hirota, K. Azuma, M. Fukuba, S. Kuroiwa, and N. Funasaki, *Biochemistry*, 2005, **44**, 10322–10327.

S2. T. Matsuo, T. Hayashi and Y. Hisaeda, *J. Am. Chem. Soc.*, 2002, **124**, 11234–11235.

S3. R. Schneider, F. Schmitt, C. Frochot, Y. Fort, N. Lourette, F. Guillemin, J.-F. Muller and M. Barberi-Heyob, *Bioorg. Med. Chem.*, 2005, **13**, 2799–2808.

S4. O. Keller and J. Rudinger, *Helv. Chim. Acta*, 1975, **58**, 531–541.

S5. H. Kitagishi, K. Oohora, H. Yamaguchi, H. Sato, T. Matsuo, A. Harada and T. Hayashi, *J. Am. Chem. Soc.*, 2007, **129**, 10326–10327.

Supplementary Figures

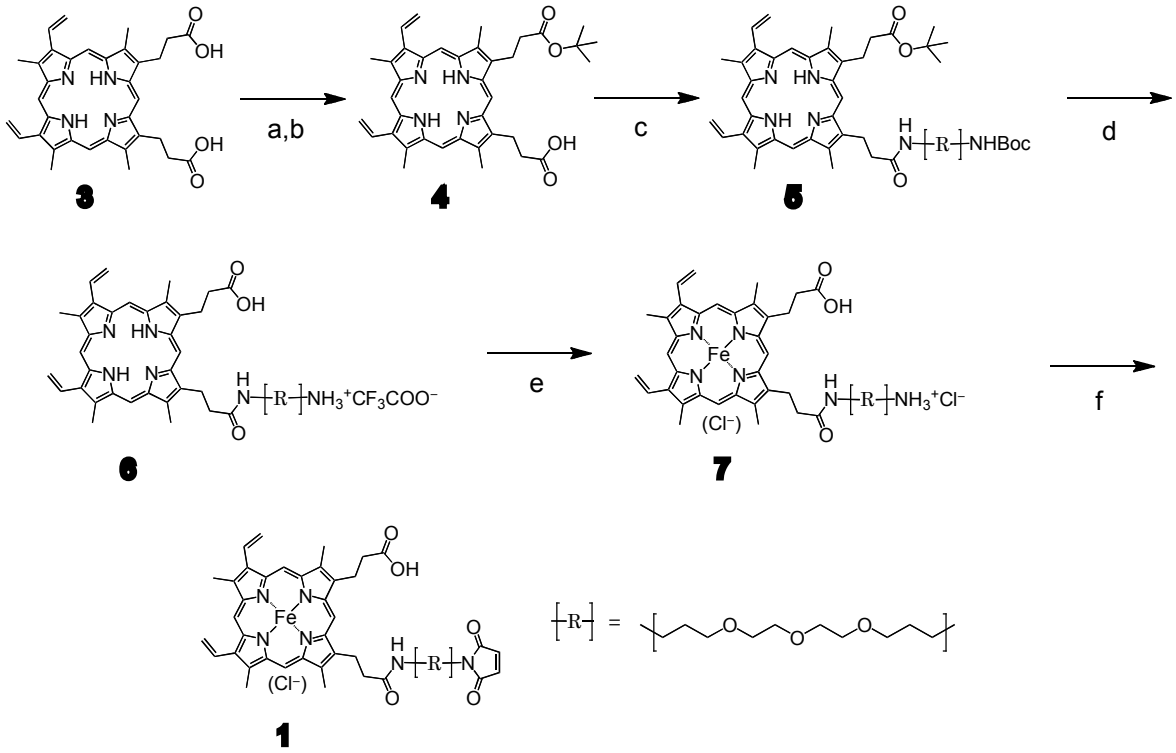

Fig. S1 Synthesis of modified heme **1**. Reaction conditions: (a) $(Boc)_2O$, DMAP, pyridine; (b) *t*-BuOH, room temp, 64% yield; (c) *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine, DPPA, Et_3N, DMF, 0 ºC, 49 % yield; (d) TFA, CH_2Cl_2, 0 ºC, quant; (e) $FeCl_2$, $NaHCO_3$, $CHCl_3/CH_3OH$, reflux, 80% yield; (f) *N*-methoxycarbonylmaleimide, 0.1 M $NaHCO_3$/acetone, 0 ºC, 46% yield. In **1**, **3**–**7,** the regioisomers of the propionate-modified porphyrins were also obtained because each heme-propionate side chain was introduced to the asymmetric heme framework.

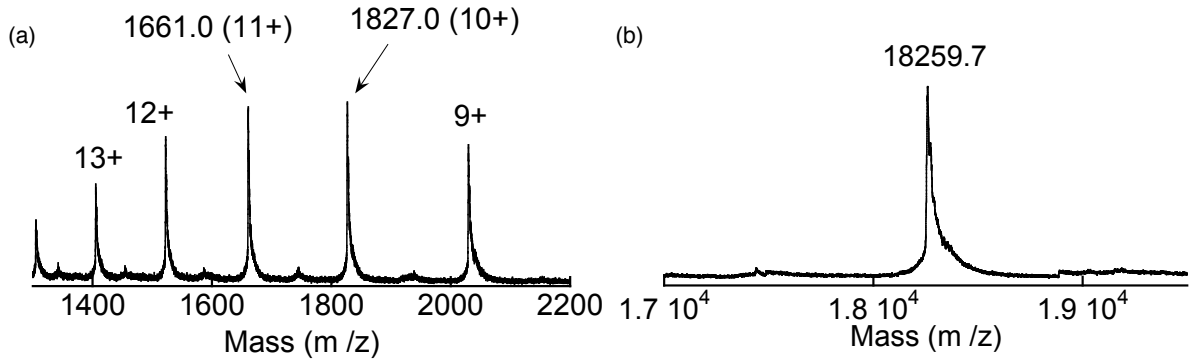

Fig. S2 ESI-TOF-MS spectra of **1**-Mb(A125C). (a) The raw spectrum of **1**-Mb(A125C). (b) The deconvoluted spectrum of **1**-Mb(A125C), the calculated mass is 18260.9.

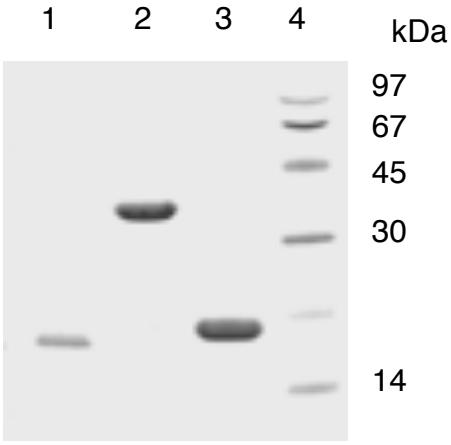

Fig. S3 SDS-PAGE analysis for Poly-**1**-Mb (18 kDa, lane 1), disulfide dimmer of Mb(A125C) (34 kDa, lane 2), nMb (17 kDa, lane 3), marker (lane 4). Samples were denatured using usual SDS buffer without 2-mercaptoethanol and analyzed by 12.5% polyacrylamide gel.

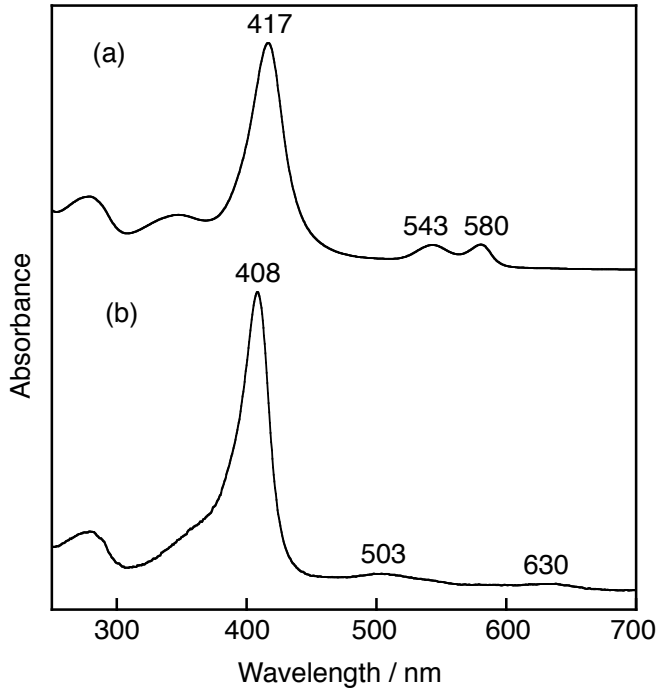

Fig. S4 UV-vis spectra of poly-**1**-Mb. (a) Oxy-form. (b) Met-form.

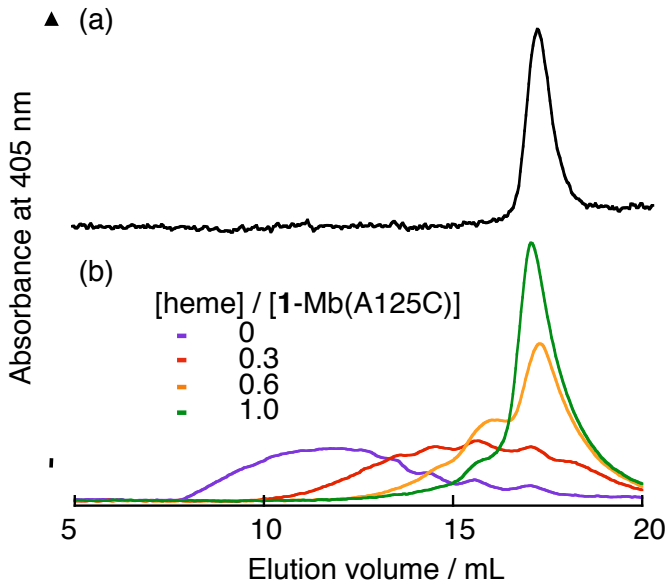

Fig. S5 Dissociation of poly-**1**-Mb. (a) SEC trace of poly-**1**-Mb under acidic conditions (pH 4.8). (b) Changes in the SEC traces of poly-**1**-Mb upon the addition of native heme.

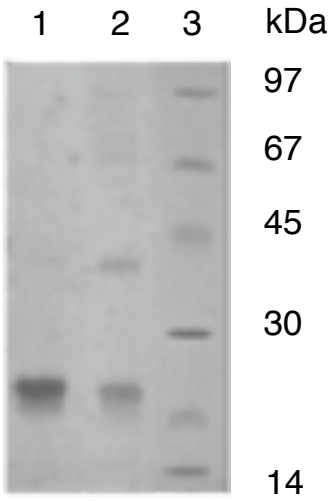

Fig. S6 SDS-PAGE analysis to evaluate covalent cross-linking. Poly-**1**-Mb (lane 1), cross-linked poly-**1**-Mb (lane 2) and marker (lane 3). Samples were analyzed by 20% polyacrylamide gel.

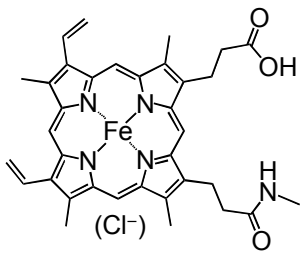

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Fig. S7 Structure of model heme **2**. This compound has regioisomers with respect to the substitution position in the two heme propionate side chains of protoheme IX.

Supplementary Tables

Table S1 Data obtained from the concentration-dependent SEC analysis.

[**1**-Mb(A125C)] / mM	V_e[a] / mL	M_p[b] / kDa	N[c]
1000	8.6	440	25
500	8.8	380	21
100	9.4	310	17
10	10.8	180	10

[a]Elution volume at the SEC peak maxima of the assembly. [b]Molecular weight at the corresponding peak. [c]Degree of polymerization. Since the apparent molecular weights of a nonspherical protein monitored by SEC are larger than their relative true value due to their asymmetric shapes, the degree of polymerization of poly-**1**-Mb was determined using a calibration curve obtained from the peaks tentatively assigned as the monomer, dimer, trimer, tetramer and pentamer in the chromatogram of poly-**1**-Mb.

Table S2 O_2 binding parameters for Mbs[a].

protein	k^{O2}_{on} ($\mu M^{-1}s^{-1}$) [b]	k^{O2}_{off} (s^{-1}) [c]	K^{O2} (M^{-1}) [d]	k_{auto} (h^{-1}) [e]
poly-**1**-Mb	11 ± 0.1	22 ± 2.0	5.0×10^5	0.12 ± 0.01
cross-linked poly-**1**-Mb	11 ± 0.2	23 ± 0.4	4.8×10^5	0.15 ± 0.01
nMb	18 ± 1.1	21 ± 0.6	8.6×10^5	0.10 ± 0.01
reconstituted Mb(**2**)	8.9 ± 0.2	17 ± 0.1	5.2×10^5	0.12 ± 0.01

[a]pH 7.0 (100 mM potassium phosphate buffer) and 25 ºC. [b]Rate constants of O_2 association. [c]Rate constants of O_2 dissociation. [d]O_2 binding affinities are determined by the ratio of k^{O2}_{on} and k^{O2}_{off}. [e]Rate constants for autoxidation from the oxy-form to the met-form at pH 7.0 (100 mM potassium phosphate buffer) and 37 ºC.