Ring-Opening Polymerization of Hemoglobin

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ABSTRACT

Hemoglobin (Hb), an oxygen-carrying protein, has an $\alpha_2\beta_2$ tetrameric structure that dissociates reversibly into two $\alpha\beta$ dimers ($\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$). We synthesized a cyclic Hb-ring monomer with two β subunits bound through a 10 kDa PEG chain. The monomer induced ring-opening polymerization to produce a supramolecular polymer via inter-subunit interaction of $\alpha\beta$ dimers of an Hb molecule at the PEG terminals. Both the ring-closed monomer and the ring-opened supramolecular polymer were then fixed covalently by intramolecular crosslinking of two β subunits. Quantification of fixed products at various monomer concentrations revealed the equilibrium constant (K), a ratio of propagation and depropagation rate constants, as 5.68 mM^{-1}. The average degree of polymerization ($\overline{\mathrm{DP}}$) increased proportionally, concomitantly with the initial monomer concentration. Hb polymer with $\overline{\mathrm{DP}} = 13.2$ (M_n = ca. 1 MDa) was obtained by crosslinking at 2.33 mM. Our novel strategy of ring-opening polymerization of Hb will eventually realize a highly aligned and efficiently polymerized Hb for creating artificial oxygen carriers for a clinical use.

1. INTRODUCTION

A supramolecular polymer is a class of polymeric arrays of monomers bound thorough non-covalent bonding such as hydrogen bonds, metal coordination bonds, host–guest interactions, and aromatic donor–acceptor interactions.[1] Specific interactions between proteins such as coiled-coil interaction, heme–heme pocket interaction, and hapten–antibody complex formation are also targeted to produce supramolecular protein polymers.[2] We regard such supramolecular polymerization techniques as applicable to create a hemoglobin (Hb) polymer that will eventually be available as an artificial oxygen carrier for clinical use.

Hb is an oxygen-carrying protein compartmentalized in red blood cells (RBC). Many attempts have been undertaken to create artificial oxygen carriers using isolated Hb from RBC. Such Hb-based oxygen carriers (HBOCs) present several important benefits: they are pathogen free, have no blood type, and have longer shelf life than RBC. For those reasons, they are anticipated for use as RBC substitutes.[3] Nevertheless, some difficulties arise when the isolated Hb molecules are injected into circulation. An Hb molecule, which consists of $\alpha_2\beta_2$ tetramer, dissociates into two $\alpha\beta$ subunits. The dimers are caught by haptoglobin and are removed from circulation immediately, thereby causing nephrotoxicity or adverse cardiovascular effects.[4] Severe adverse effects such as vasoconstriction, hypertension, and oxidative stress are ascribed to the scavenging of the endothelium-derived relaxing factor, nitric oxide, by extravasation of small Hb molecules through the endothelial layer.[5]

Various approaches for chemical modification of Hb have been developed to resolve these difficulties. For instance, intramolecular $\alpha\alpha$- or $\beta\beta$-crosslinked Hb[6–8] inhibits dissociation of $\alpha_2\beta_2$ tetrameric structure while preventing biomolecular association with haptoglobin.[9] Non-

immunogenic polymers such as polyethylene glycol (PEG)[10,11] or albumin[12] are conjugated to Hb molecules to lower antigenicity by surface covering.

The intermolecular conjugation of multiple Hbs represents a promising approach to prevent extravasation by enlarging the particle size of HBOCs. Several kinds of oligomerized or polymerized Hbs are produced by crosslinking Hbs with glutaraldehyde,[13] polyamide linker,[14,15] oligo- or poly-saccharides,[16,17] site-specific dimerizing reagents,[18,19] and bi-functional PEGs.[20] In fact, glutaraldehyde-polymerized Hbs (poly-Hbs) entered clinical trials for patients, although some have already been discontinued.[21] These poly-Hbs contain monomeric Hb or low molecular weight Hb oligomers. Adverse effects of vasoconstriction are expected to result from permeation of such small molecules across endothelial layer and extravasation.[22] Therefore, development for realizing highly efficient polymerization of Hbs is now sought to improve existing poly-Hbs. Polymerized Hbs with molecular weight of tens of MDa was produced by zero-linking of Hb molecules.[23] Other approaches to creating HBOCs without using chemical modification have also been developed. Encapsulation of Hb molecules in polymer membranes[24] or in liposomes[25] is conducted as a shield against toxicity of Hb molecules.

For this work, we developed an efficient polymerization procedure for creating Hb polymer using a novel approach: ring-opening polymerization. We earlier evaluated the exchange reaction of αβ subunits between two-PEG-chains conjugated Hb and native Hb in a physiological condition.[26] Based on results of that work, we designed an intramolecularly PEG-bridged Hb as a cyclic Hb-ring monomer (Hb-rPEG, **Figure 1**). For this study, Hb-rPEG was synthesized by reacting native Hb with a bi-functional PEG with maleimide terminals (mal$_2$-PEG) known to conjugate selectively to β93Cys.[27] During the intermolecular subunit exchange of αβ dimers, Hb-rPEG is expected to induce ring-opening polymerization to produce

poly(Hb−PEG). It is a supramolecular polymerization using non-covalent interactions of $\alpha_2\beta_2$ tetrameric structures of Hb molecules as attachments. This study evaluates the existence of ring-opening polymerization by colloid osmotic pressure (COP) and dynamic light scattering (DLS) measurements.

Ring-closed and ring-opened structures are fixed by reaction with bis-(3,5-dibromosalicyl) fumarate (DBBF), which is known to bind two β82Lys through fumarate (DBBF crosslinking).[28] Hb-rPEG was DBBF crosslinked to produce a crosslinked monomer (XL-Hb-rPEG) and a polymer (XL-poly(Hb−PEG)). Those products were subsequently quantified using size-exclusion chromatography (SEC). The fixed polymer, XL-poly(Hb−PEG), was characterized using SEC, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), atomic force microscopy (AFM), and DLS measurements.
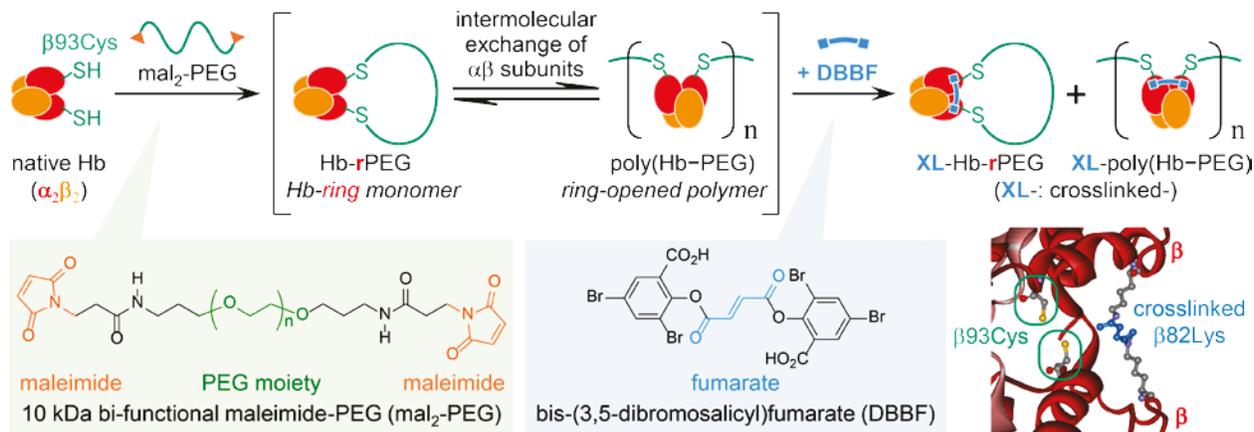
Figure 1. Ring-opening polymerization of intramolecularly PEG-bridged Hb as a cyclic Hb-ring monomer (Hb-rPEG). Hb-rPEG is synthesized by reacting native Hb with bi-functional maleimide-PEG (mal$_2$-PEG). Hb-rPEG induces ring-opening polymerization by exchanging $\alpha\beta$ subunits intermolecularly to produce poly(Hb−PEG). Ring-closed and ring-opened forms are fixed by site-selective crosslinking of two β subunits in $\alpha_2\beta_2$ tetrameric structures using bis-(3,5-

dibromosalicyl) fumarate (DBBF) to produce a corresponding fixed monomer and a polymer: XL-Hb-rPEG and XL-poly(Hb−PEG). The X-ray crystal structure (PDB ID: 1BIJ) of the Hb crosslinked by DBBF (XLHb) is shown, particularly displaying the binding sites for maleimide (β93Cys) and for DBBF (β82Lys).[28]

2. MATERIALS AND METHODS

2.1. Materials

Human Hb used for this work was purified from RBCs provided by the Japanese Red Cross. As reported elsewhere in the literature,[29] a highly purified carbonyl Hb (native Hb) solution was obtained through pasteurization and nanofiltration. DBBF crosslinked Hb (XLHb) was also prepared as reported in the literature.[26,30] The Hb derivative concentrations were determined using Hemoglobin B-test Wako (Fujifilm Wako Pure Chemical Corp.).

Sodium chloride, potassium chloride, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), polyethylene glycol 20,000 (20 kDa PEG), ammonium sulfate, Tris(hydroxymethyl) aminomethane (Tris), sucrose, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, bromophenol blue, and Coomassie brilliant blue (Quick CBB) were purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). Saline (Otsuka normal saline) was purchased from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). Albumin (25% human serum albumin) was purchased from Japan Blood Products Organization (Tokyo, Japan). Bi-functional maleimide PEG, mal_2-PEG (**Figure 1**; SUNBRIGHT DE-100MA, Lot M153536, M_n 10456, $M_w/M_n = 1.02$, substitution 90.6%) was purchased from NOF Corp. (Tokyo, Japan). Bis-(3,5-dibromosalicyl) fumarate (**Figure 1**; DBBF) was purchased from Abcam plc. (Cambridge, U.K.).

2.2. Synthesis of Hb-ring monomer (Hb-rPEG)

To a 31.0 mL of native Hb solution (1.55 mM, 10.0 g/dL, 48.0 μmol) in phosphate-buffered saline (PBS, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) was added 0.83 molar equivalent of powdered mal$_2$-PEG (418.2 mg, 40.0 μmol, M_n 10456), followed by stirring under a CO atmosphere for 20 h at 4 °C. Crude Hb-rPEG was purified five times by salting out using 3 M ammonium sulfate aqueous solution for native Hb removal. After the precipitate was dissolved in PBS, the solution was dialyzed for 20 h using a dialysis membrane (Size 27; Fujifilm Wako Chemicals U.S.A. Corp., Richmond, U.S.A.) against 400 mL PBS to remove ammonium sulfate. Then the solution was dialyzed for 4 h against a highly concentrated 20 kDa PEG aqueous solution. In this procedure, water and other small molecules were withdrawn for concentration. Finally, it was dialyzed against 400 mL PBS for 2 h. Consequently, 10.84 mL Hb-rPEG solution was obtained (2.84 mM, [Hb] = 18.3 g/dL, yield 76.7%).

2.3. DBBF crosslinking of Hb-rPEG at different concentrations

Concentrated Hb-rPEG solutions (2.84 mM, [Hb] = 18.3 g/dL) with 13.2–52.9 μL volume were diluted with PBS to prepare solutions having seven respective concentrations. Each solution was stirred for 8 h at 4 °C. Subsequently, 2.0 molar equivalent DBBF was added as a suspension (10 or 20 mg/mL suspension in PBS). The final Hb concentrations were then adjusted to 0.04, 0.08, 0.16, 0.31, 0.62, 1.55, and 2.33 mM ([Hb] = 0.25, 0.5, 1.0, 2.0, 4.0, 10.0, and 15.0 g/dL). The mixtures were then stirred for 3 h at room temperature. Each solution was diluted with PBS to the same concentration of 0.04 mM ([Hb] = 0.25 g/dL) for SEC and SDS-PAGE analyses.

2.4. Synthesis of XL-poly(Hb−PEG)

To a 5 mL PBS solution of Hb-rPEG (2.33 mM, [Hb] = 15.0 g/dL, 11.6 μmol) was added 1.6 molar equivalent of powdered DBBF (12.50 mg, 18.6 μmol). It was then stirred under a CO atmosphere for 3 h at room temperature. The reaction mixture was filtered through a syringe-driven membrane filter (DISMIC-25CS 0.45 μm cellulose acetate filter; Toyo Roshi Kaisha Ltd., Tokyo, Japan). A higher molecular weight component was fractionated using size exclusion chromatography (2 cm diameter and 30 cm height open column, Sepharose CL4B; Sigma-Aldrich Corp., St. Louis, U.S.A.). The fraction containing a higher molecular weight component was concentrated using a centrifugal filter unit (Amicon Ultra-15, 100 kDa cutoff; Merck Millipore, Merck KGaA, U.S.A.) to obtain 0.87 mL of XL-poly(Hb−PEG) solution (0.58 mM, [Hb] = 3.74 g/dL, yield 4.3%).

2.5. Exchange of αβ subunits between Hb-rPEG and native Hb, and subsequent DBBF crosslinking

After Hb-rPEG was mixed with native Hb in 0.10 mL PBS, it was stirred for 24 h at 4 °C. Then, 2.0 molar equivalent DBBF to total Hb was reacted. The final concentration of Hb-rPEG was fixed to 1.55 mM ([Hb] = 10.0 g/dL). That of native Hb was adjusted to 1.55, 0.62, and 0.31 mM (10.0, 4.0, and 2.0 g/dL). For SEC analysis, each resulting solution was diluted 50 times with PBS to adjust the Hb-rPEG concentration to 0.031 mM ([Hb] = 0.20 g/dL). For comparison,

1.55 mM Hb-rPEG solution ([Hb] = 10.0 g/dL) without adding native Hb was also reacted with DBBF.

2.6. Intermolecular conjugation of XLHbs by mal$_2$-PEG

Preformed 0.10 mL XLHb solution (3.78 mM, 24.4 g/dL, 0.38 μmol) was mixed with 0.10 mL mal$_2$-PEG (M_n 10456) solution in PBS and was stirred for 24 h at 4 °C. The molar ratios of XLHb to mal$_2$-PEG were adjusted to 1.0 and 0.50. Each reaction mixture contained conjugated XLHbs through PEG chains (polymerized XLHb (poly(XLHb)) and/or oligomerized XLHb (oligo(XLHb)). Oligo(XLHb) was fractionated using a high-performance liquid chromatography system (HPLC, Chromaster; Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a SEC column (Shodex Protein KW-804; Showa Denko K.K., Tokyo, Japan) to obtain solutions of a dimer, a trimer, a tetramer, and a pentamer.

The dumbbell-type XLHb dimer, XLHb−PEG−XLHb, was synthesized for use as a reference. To a 240 mL of vigorously stirred XLHb solution (1.00 mM, 6.45 g/dL, 0.24 mmol) in PBS was added 0.083 molar equivalent mal$_2$-PEG solution (M_n 10456, 209.12 mg, 0.0200 mmol in 4.2 mL PBS) dropwise. Then, the mixture was stirred for 2 h under a CO atmosphere at room temperature, and subsequently at 4 °C for 10 h. Excess XLHb was removed four times by salting out using 3 M ammonium sulfate solution. After the precipitate was dissolved in PBS, the solution was dialyzed for 24 h using a dialysis membrane (Size 27; Fujifilm Wako Chemicals U.S.A. Corp.) against 400 mL saline to remove ammonium sulfate. Then it was dialyzed for 4 h against a highly concentrated 20 kDa PEG aqueous solution. Finally, it was dialyzed against 400

mL PBS for 2 h. Consequently, 8.71 mL XLHb−PEG−XLHb solution was obtained (2.57 mM, [Hb] = 16.6 g/dL, yield 56.1%).

2.7. Size exclusion chromatography (SEC)

SEC was conducted using an HPLC system (Chromaster; Hitachi High-Technologies Corp.) equipped with an SEC column (Shodex Protein KW-804; Showa Denko K.K.) at 20−29 °C. Effluent was monitored using a diode array detector (5430; Hitachi High-Technologies Corp.) with absorbance at 419 nm, which corresponds to λ_{max} of the Soret band of carbonyl Hb. The modified Hbs solutions were filtered through a syringe-driven filter unit (Millex 0.22 μm PVDF filter; Merck Millipore). Then 20 μL of analyte was injected into the SEC column through a sampler. PBS was used as an eluent with a 1.0 mL/min flow rate. The molecular weight of Hb-rPEG was estimated using a calibration curve obtained using three marker kits (MW-GF200 and MW-GF1000; Sigma-Aldrich Corp., and MW-Marker (HPLC); Oriental Yeast Co., Ltd., Tokyo, Japan). The molecular weight of the ring-opened polymer was estimated by extrapolation of the calibration curve obtained from small linear oligomers, up to pentamer, prepared by the reaction of preformed XLHb and mal$_2$-PEG, as described in the *Results* section.

2.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All solutions of Hb derivative were diluted with PBS to the Hb concentration of 0.012 mM ([Hb] = 0.075 g/dL). Then, each resulting solution was mixed with a half volume of the

denaturing buffer[31] (0.19 M Tris-HCl pH 6.8, 6.0% (wt/v) SDS, 15% (v/v) 2-mercaptoethanol, 30% (wt/v) sucrose, and 0.006% (wt/v) bromophenol blue) and was incubated at 80 °C for 15 min.

Electrophoresis was performed on a 13% polyacrylamide mini-slab gel using a mini-slab electrophoresis system (NA-1010; Nihon Eido Corp., Tokyo, Japan), or on a gradient gel (NuPAGE 3-8% Tris-Acetate Protein Gels; Invitrogen Corp., Thermo Fisher Scientific Inc., Waltham, U.S.A.) using a mini-slab electrophoresis system (XCell SureLock Mini-Cell; Invitrogen Corp., Thermo Fisher Scientific Inc.). An Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare Japan Ltd., Tokyo, Japan) and a HiMark Pre-Stained Protein Standard (Invitrogen Corp., Thermo Fisher Scientific Inc.) were used for molecular weight markers. After the gels were stained with Coomassie brilliant blue, images of the stained gels were obtained using transmission scanning (GT-F650; Seiko Epson Corp., Nagano, Japan).

2.9. Colloid osmotic pressure (COP)

COP of the Hb-rPEG was measured using a colloid osmometer (Osmomat 050; Gonotec GmbH, Berlin, Germany) with several protein concentrations at 21−24 °C. Albumin, native Hb, and XLHb were used as exemplary water-soluble proteins. COP (π) is defined as shown in **Eq. 1** using number-average molecular weights (M_n), the concentration of Hb units (C), the gas constant (R), the absolute temperature (T), and the second and the third virial coefficients (A_2, A_3).[32]

$$\pi = RT\left(\frac{C}{M_n} + A_2 C^2 + A_3 C^3 + \cdots\right) \qquad \cdots (1)$$

Eq. 1 is converted to Eq. 2.

$$\frac{\pi}{C} = RT\left(\frac{1}{M_n} + A_2 C + A_3 C^2 + \cdots\right) \qquad \cdots (2)$$

From the intercept $(\pi/C)_{C \to 0}$ of curve fitting on the plots of π/C versus C, $1/M_n$ is obtained according to Eq. 3.

$$\frac{1}{M_n} = \frac{1}{RT}\left(\frac{\pi}{C}\right)_{C \to 0} \qquad \cdots (3)$$

2.10. Dynamic light scattering (DLS)

The Hb-rPEG particle size distribution was measured using a particle analyzer (ELSZ-2000ZS; Otsuka Electronics Co. Ltd., Osaka, Japan) equipped with a semiconductor laser ($\lambda =$ 660 nm) at 25 °C. The light-scattering signal was detected at 165° (backscatter detection). The particle sizes of native Hb and purified XL-poly(Hb−PEG) were measured using a nanoparticle size analyzer (SZ-100-S; Horiba, Ltd., Tokyo, Japan) equipped with a 10 mW laser ($\lambda = 532$ nm) at 25 °C. The light-scattering signal was detected at 173° (backscatter detection). The Hb concentration of the Hb-rPEG solution was adjusted to 0.10, 0.50, 1.0, 2.0, and 4.0 g/dL (0.015−0.62 mM), and those of native Hb and purified XL-poly(Hb−PEG) solutions were adjusted to 0.73 and 0.12 g/dL (0.11 and 0.019 mM), respectively, in PBS (pH 7.4). Before DLS measurement, each solution was filtered through a syringe-driven filter unit (Millex 0.22 μm PVDF filter; Merck Millipore) to remove dust.

2.11. Atomic Force Microscopy (AFM)

AFM measurement was conducted in liquid following the method reported by Tomita et al.[33] with some modification (UBE Scientific Analysis Laboratory, Inc., Ube, Japan). A native Hb and a XL-poly(Hb−PEG) solutions in PBS were desalted using a centrifugal filter unit (100 kDa cutoff, Amicon Ultra-15; Merck Millipore) to obtain ca. 0.1 g/dL solution in distilled water. Each solution was diluted 10^3 times with ultrapure water. Then the native Hb and the XL-poly(Hb−PEG) molecules were fixed on a mica plate. Microscopic images were obtained using atomic force microscopy (MultiMode 8; Bruker Corp., Santa Barbara, U.S.A.) with a control system (Nanoscope V; Bruker Corp.) using a Si_3N_4 cantilever. The measurement was conducted in ultrapure water at room temperature. The spring constant was 0.7 N/m, and the scanning resolution was 512 pixels versus 512 lines.

3. RESULTS

3.1. Synthesis and characterization of Hb-rPEG

Hb-rPEG was identified using SEC and SDS-PAGE. In the SEC profile, Hb-rPEG was observed as a narrow peak at 10.89 min, indicating larger size than native Hb (**Figure 2A**). The molecular weight of Hb-rPEG was found to be 98 kDa by comparison with molecular weight marker proteins for SEC.

SDS-PAGE analysis clarified that free α subunits remained in Hb-rPEG (**Figure 2B**). The band of free β subunit disappeared at Hb-rPEG. Instead, a band *a* ascribed to two β subunits linked with PEG (β−PEG−β) appeared. Band *a* was observed near the 45 kDa marker, which coincided to the expected molecular weight (42.2 kDa). These results indicate site-selective conjugation between mal$_2$-PEG and two β subunits (β93Cys) of an Hb molecule.[34]
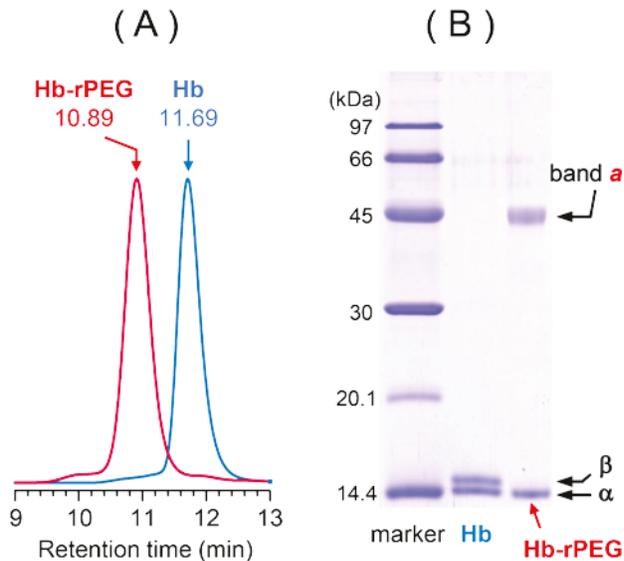
Figure 2. (A) SEC profiles of Hb-rPEG and native Hb. Both Hb-rPEG and native Hb are observed as narrow peaks. (B) SDS-PAGE analysis of native Hb and Hb-rPEG. One Hb

molecule consists of two α (15.1 kDa) and two β (15.9 kDa) subunits. One Hb-rPEG molecule consists of two α subunits and one PEG-linked two-β subunit (band **a**, β−PEG−β, 42.2 kDa).

3.2. Concentration-dependence of Hb-rPEG size by COP and DLS measurements

The colloid osmotic pressure (COP, π) of Hb-rPEG solution increases with the concentration of Hb units (C), similarly to native Hb (**Figure 3A**). Plots of π/C versus C of Hb-rPEG showed a parabolic curve (red line), whereas those of some representative water soluble proteins, such as human serum albumin (green line), XLHb (purple line), and native Hb (blue line), showed linear relations (**Figure 3B**). The number average molecular weight (M_n) of Hb-rPEG was found to be 104 kDa from the intercept of the quadric curve fitting (Supporting Information **Table S1**). The value is regarded as reflecting the molecular size in an infinitely diluted condition. It almost coincides with that ascertained from SEC analysis (98 kDa). Dynamic light scattering (DLS) of Hb-rPEG showed an inconsistent size distribution depending on the concentration (**Figure 4**). The particle diameter of Hb-rPEG was 10.6±3.6 nm at [Hb] = 0.10 g/dL. It increased with the concentration to reach 20.4±13.7 nm at [Hb] = 4.0 g/dL. The polydispersity index also increased with the concentration, indicating that the size distribution tends to become broader at a higher concentration (Supporting information **Table S2**).
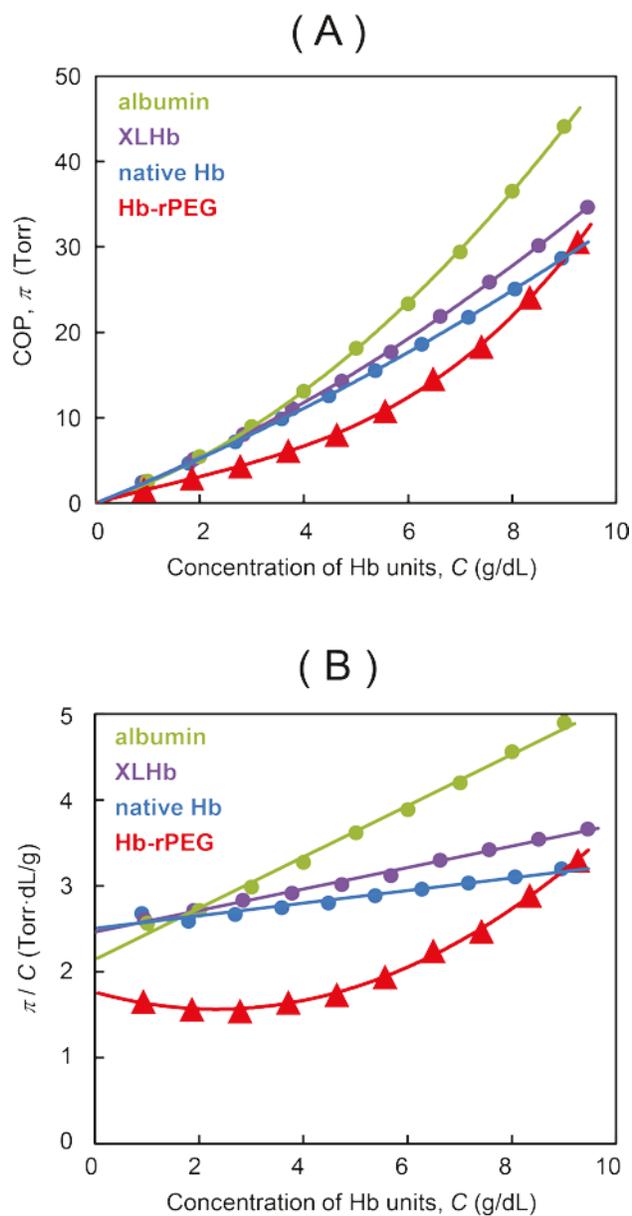
Figure 3. (A) Colloid osmotic pressure (COP, π) of Hb-rPEG (red line) as a function of the concentration of Hb units C, with representative water soluble proteins of human serum albumin (green line), XLHb (purple line), and native Hb (blue line). (B) Plots of π/C versus C. Only Hb-rPEG showed a parabolic curve, whereas others showed linear relations.
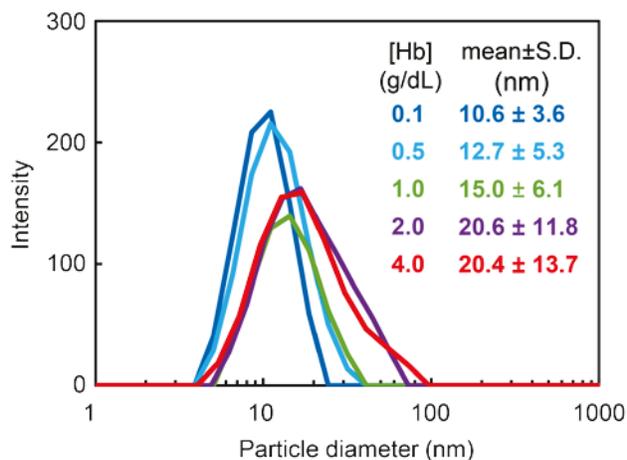
Figure 4. Particle size distribution of Hb-rPEG obtained using dynamic light scattering (DLS) measurements for different concentrations of Hb units of 0.10–4.0 g/dL. The average size and the standard deviation (S.D.) at each concentration are shown in the graph.

3.3. Fixing $\alpha_2\beta_2$ tetrameric structures of ring-opened and ring-closed Hb-rPEGs by DBBF crosslinking

DBBF crosslinking was used to fix $\alpha_2\beta_2$ tetrameric structures that reflect the equilibrated state of ring-opening and ring-closing reactions for Hb-rPEG (**Figure 1**). After the Hb-rPEG concentration was adjusted to various concentrations of 0.04–2.33 mM ([Hb] = 0.25–15 g/dL), the $\alpha_2\beta_2$ structures were fixed by DBBF crosslinking. The crosslinked products were analyzed using SEC (**Figure 5**). When reacting with DBBF at a low concentration of 0.04 mM (blue line), the cyclic monomer (Hb-rPEG) remained as the corresponding ring-closed monomeric XLHb (XL-Hb-rPEG) at 10.91 min, showing almost equal retention time of non-crosslinked Hb-rPEG (10.89 min). The ratio of monomeric XL-Hb-rPEG decreased concomitantly with increasing

concentration of DBBF crosslinking. As inferred from the decrease of the peak area of XL-Hb-rPEG, nearly half of the cyclic monomers turned to the opened form at 0.31 mM (purple line). A broad peak ascribed to the fixed ring-opened polymers (XL-poly(Hb−PEG)) was observed at 7.51 min. The retention time is much shorter than that of 669 kDa thyroglobulin (8.78 min). The peak of XL-poly(Hb−PEG) shifted to shorter retention times with increasing the monomer concentration. The high molecular weight component (6.45 min) increased when crosslinked at a high Hb concentration of 2.33 mM (red line).
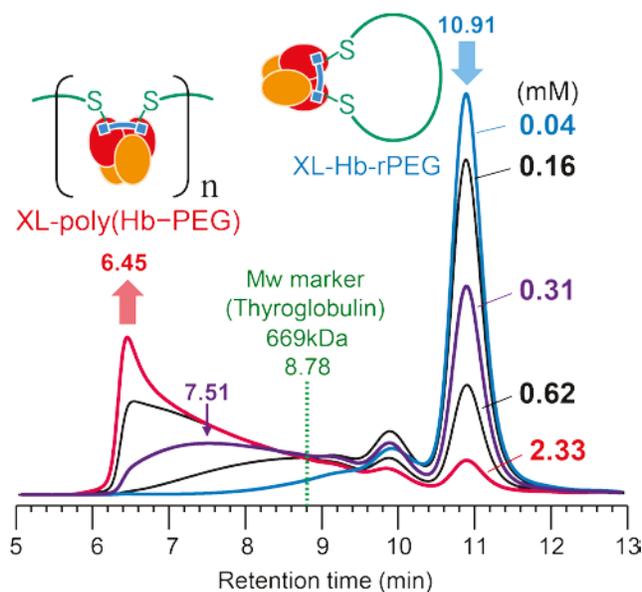
Figure 5. SEC profiles obtained for products of reacting Hb-rPEG with DBBF at different monomer concentrations of 0.04, 0.16, 0.31, 0.62, and 2.33 mM ([Hb] = 0.25, 1.0, 2.0, 4.0, and 15 g/dL). After DBBF crosslinking, each reaction mixture was diluted to the same concentration of 0.04 mM ([Hb] = 0.25 g/dL). Each was then analyzed.

Products obtained by reacting Hb-rPEG with DBBF at different concentrations of 0.04, 0.31, and 2.33 mM ([Hb] = 0.25, 2.0, and 15 g/dL) were analyzed using SDS-PAGE (**Figure 6**). Band **a** of β−PEG−β, one component of Hb-rPEG, disappeared by DBBF crosslinking at 0.04 mM. Alternatively, a new band **b** with a lower mobility than band **a** appeared. The new band **b** was assigned as the PEGylated β−β component with a closed ring structure (β−β−ringPEG), which is one component of XL-Hb-rPEG. Both β−PEG−β and β−β−ringPEG are expected to have equal molecular weight (42.2 kDa), but because of their different morphologies, they showed different mobility in the polyacrylamide gel. In contrast to the diluted condition, a polymeric component (poly(β−β−PEG)) was observed as band **c** by DBBF crosslinking of Hb-rPEG at a higher concentration of 2.33 mM. When crosslinked at 0.31 mM, both bands **b** and **c** were observed at almost equal intensity, which supported the SEC result (**Figure 5**). Free α subunits remained after crosslinking at every concentration, indicating that DBBF crosslinks PEGylated β subunits selectively in the same manner as that of native Hb.[26]
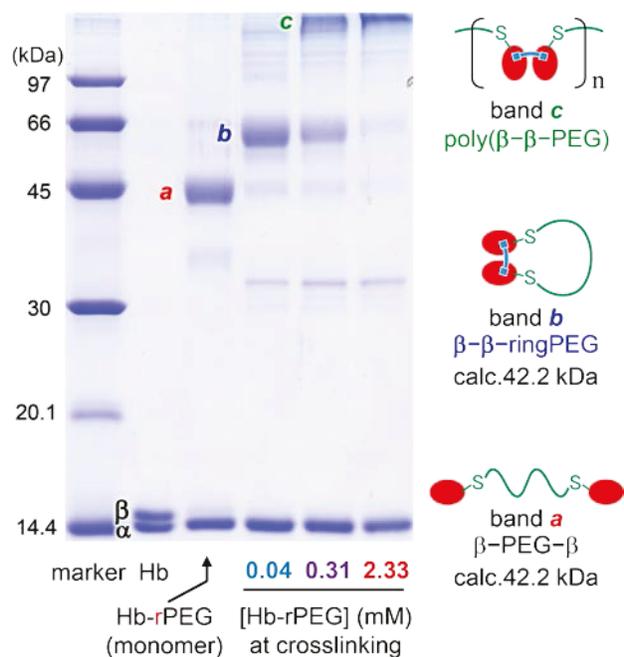
Figure 6. SDS-PAGE of the DBBF crosslinked Hb-rPEG at different concentrations of 0.04, 0.31, and 2.33 mM ([Hb] = 0.25, 2.0, and 15.0 g/dL). A band ***a*** ascribed to β−PEG−β observed in Hb-rPEG disappeared by DBBF crosslinking. Instead, a band ***b*** of the PEGylated β−β component with a closed ring structure (β−β−ringPEG), and a band ***c*** of the polymeric component (poly(β−β−PEG)) were observed. Band ***b*** was observed preferentially at a lower crosslinking concentration of 0.04 mM. Band ***c*** was observed preferentially at a higher crosslinking concentration of 2.33 mM. Both bands ***b*** and ***c*** were observed at a crosslinking concentration of 0.31 mM.

3.4. Ring-opening polymerization of Hb-rPEG versus stepwise XLHb conjugation by mal$_2$-PEG

The SEC profile of the reaction products of Hb-rPEG and DBBF at Hb concentration of 10 g/dL (1.55 mM) is shown in **Figure 7A**. As ring-opening polymerization proceeded, the fixed polymer, XL-poly(Hb−PEG), was observed at 6.47 min with 10.9% monomeric XL-Hb-rPEG remaining at 10.94 min. The size of the ring-opened product gradually decreased concomitantly with increasing concentration of native Hb from 2.0 to 10.0 g/dL (**Figures 7B, 7C, and 7D**). In these chromatograms, discontinuous peaks of a crosslinked dimer, a trimer, a tetramer, and a pentamer were observed respectively at ca. 10.0, 9.1, 8.5, and 8.1 min. Monomeric Hbs (Hb-rPEG and native Hb) were also DBBF crosslinked to generate XL-Hb-rPEG and XLHb, and were observed respectively at 10.9 and 11.6 min. No oligomers were found before reacting with DBBF (Supporting information **Figure S1**), indicating that the production of such oligomers depends strongly on the concentration, and it is difficult to detect them by SEC without crosslinking.

For comparison, preformed XLHb molecules were conjugated by 1.0 or 0.50 equivalent molar mal$_2$-PEG (**Figures 7E** and **7F**). In the case of the 1.0 equivalent molar reaction, polymerized XLHb (poly(XLHb)) was observed at 6.45 min, with smaller oligomers (9.20 and 9.99 min) and unreacted XLHb (11.65 min). In the case of reaction of XLHb with 0.50 equivalent molar mal$_2$-PEG, the SEC profile of the products (**Figure 7F**) showed a similar profile to that shown in **Figure 7D**. This result demonstrates that the products of oligomeric XLHb obtained by reacting with bi-functional PEG were identical to those of subunit exchange between native Hb and Hb-rPEG, followed by DBBF crosslinking.

Each product observed in **Figure 7F** at 10.05, 9.18, 8.59, and 8.06 min was fractionated and then analyzed again using SEC (**Figure 7G**). As a reference, the XLHb dimer (XLHb−PEG−XLHb) was synthesized by reacting excess XLHb with mal$_2$-PEG (**Figure 7H**).

The profiles of blue chromatograms of **Figures 7G** and **7H** coincided, indicating that the peak at 10.05 min is ascribable to a dumbbell dimer: XLHb−PEG−XLHb. SDS-PAGE analysis of the fractions in **Figure 7G** clarified that the peak of 9.17 min is a trimer, and those of 8.53 and 8.11 min are inferred to be a tetramer and a pentamer, respectively (Supporting Information **Figure S2**).

The relation between the retention time and the molecular weights of these oligomers and XLHb is shown as a blue line on a logarithmic scale in **Figure 8**. They are on a linear fit ($r^2 > 0.999$), and are applicable to produce a calibration curve for XL-poly(Hb−PEG). The green line in the figure represents a calibration curve obtained from globular proteins. The slope of the green line is steeper than that of the blue line, indicating that PEG moiety provides large hydrodynamic volume to the globular proteins. The molecular weights of the ring-opened products, XL-poly(Hb−PEG), observed in **Figure 5** were estimated by extrapolation of this blue line. The molecular weight of XL-poly(Hb−PEG) was controlled in the wide range of 218−812 kDa by regulating the initial monomer concentration of 0.16−2.33 mM (Supporting information **Table S3**). The molecular weight of XL-poly(Hb−PEG) observed in **Figure 7A** (6.47 min) was estimated as 804 kDa, which corresponded to an undecamer (11 mer). **Figures 5**, **7A**, **and 7E** show that the peak of the polymer appeared abruptly at 6.2 min. The polymer size almost reaches the void volume, and it is the detection limit of the column (protein KW804). Therefore, the actual molecular weight of XL-poly(Hb−PEG) might be larger than that estimated from the calibration curve.
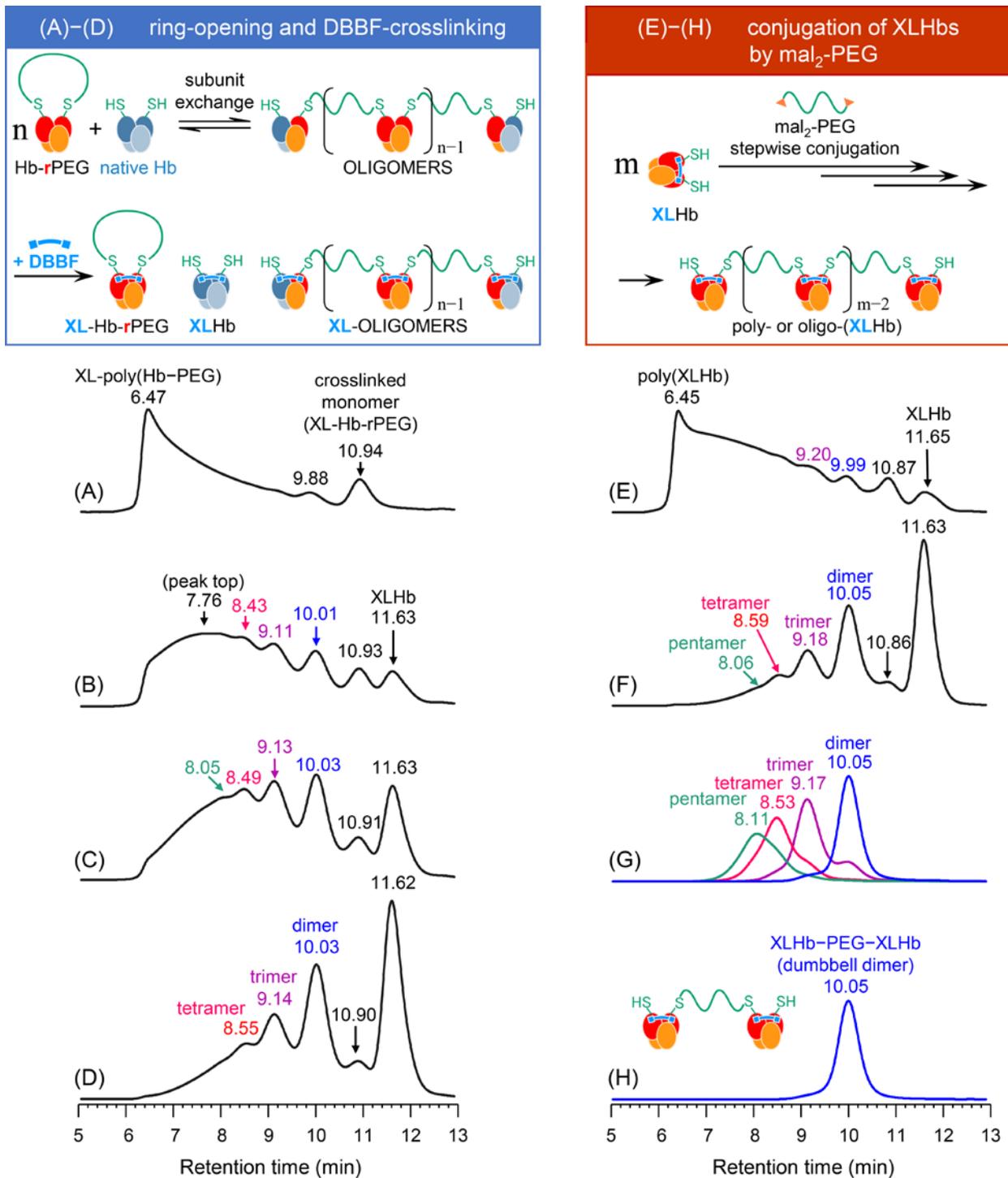
Figure 7. Comparison of SEC profiles of ring-opening and subsequent DBBF crosslinked products in the presence of different amount of native Hb (left panels, (A)−(D)), and intermolecularly conjugated XLHbs by mal$_2$-PEG (right panels, (E)−(H)). (A) 0 g/dL (no native

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Hb), (B) 2.0 g/dL, (C) 4.0 g/dL, and (D) 10.0 g/dL native Hb was mixed with 10.0 g/dL Hb-rPEG, and then DBBF was reacted to each mixture. The preformed XLHb was reacted (E) with equimolar mal₂-PEG and (F) with 0.50 equimolar mal₂-PEG. (G) Fractionated products of XLHb and 0.50 equimolar mal₂-PEG: a dimer (blue), a trimer (purple), a tetramer (pink), and a pentamer (green). (H) Dumbbell-type XLHb dimer, XLHb−PEG−XLHb, for reference.
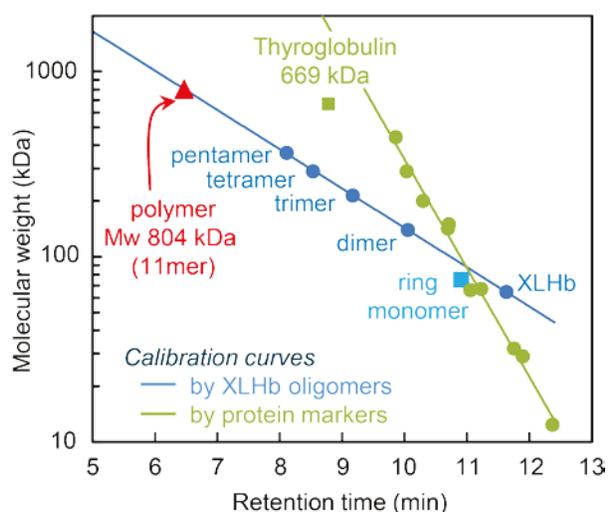
Figure 8. Calibration curves obtained by retention times (t) of fractionated XLHb oligomers as shown in **Figure 7F** (blue line, $\log M_n = -0.2124\,t + 4.2794$, $r^2 = 0.9998$), and that obtained using protein markers except for thyroglobulin (green line, $\log M_n = -0.5875\,t + 8.4000$, $r^2 = 0.9889$). The molecular weight of the ring-opened and subsequently fixed polymer, as shown in **Figure 7A**, was estimated by extrapolation as 804 kDa.

3.5. Kinetic analysis of ring-opening polymerization equilibrium of Hb-rPEG

The ring-opening polymerization mechanism of Hb-rPEG is regarded as similar to that of low molecular weight organic compounds such as lactone, lactam, cycloalkene, and cyclic ether.[35] Reactions of propagation and depropagation proceed simultaneously. They are expected to be in an equilibrated state (**Scheme 1**).
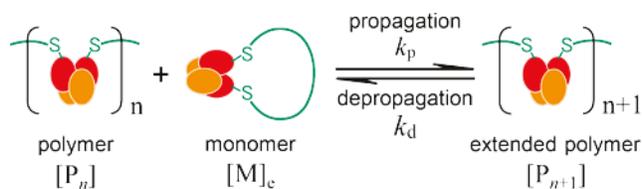
Scheme 1. Ring-opening polymerization equilibrium of Hb-rPEG.

The equilibrium in **Scheme 1** can be described as **Eq. 4**.

$$P_n \ + \ M \ \rightleftarrows \ P_{n+1} \qquad \qquad …(4)$$
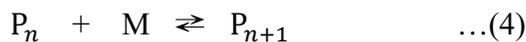
The equilibrium constant, K, which is the ratio of polymerization (k_p) and depolymerization (k_d) rate constants, is defined by **Eq. 5** using the concentrations of a polymer and a one unit extended polymer [P_n] and [P_{n+1}], and the equilibrium monomer concentration [M]$_e$.

$$K = \frac{k_p}{k_d} = \frac{[P_{n+1}]}{[P_n][M]_e} \qquad \qquad ⋯(5)$$

When the polymer is sufficiently long, [P_n] and [P_{n+1}] become nearly identical. Therefore, K is obtained as a reciprocal of [M]$_e$ as shown in **Eq. 6**.[35]

$$K \approx \frac{1}{[M]_e} \quad {}_{(n\gg1)} \qquad \cdots (6)$$

Hb-rPEG was reacted with DBBF at various initial monomer concentrations ($[M]_0 = 0.04$, 0.08, 0.16, 0.31, 0.62, 1.55, and 2.33 mM). Then, each solution was diluted to the same concentration (0.04 mM). The products were analyzed using SEC as shown in **Figure 5**. The amount of XL-Hb-rPEG was quantified from the peak area at 12.91 min (Supporting information **Table S4**). The remaining ratio of cyclic monomer at the equilibrium state is regarded to correspond to that of DBBF crosslinked one: XL-Hb-rPEG. Actually, the equilibrium monomer concentration $[M]_e$, was obtained from the product of the $[M]_0$ and the remaining ratio of the cyclic monomer.

The relation between $1/[M]_e$ and $[M]_0$ is presented in **Figure 9**. The value of $1/[M]_e$ declines at a lower $[M]_0$; it becomes constant at a higher $[M]_0$. From the average of $1/[M]_e$ at higher concentrations ($[M]_0 = 0.31$, 0.62, 1.55, and 2.33 mM), the K value was found to be 5.68 mM^{-1}. The corresponding $[M]_e$ was 0.176 mM ($[Hb] = 1.14$ g/dL). From $[M]_e$, the ratio of the monomer reacted (P) is defined as **Eq. 7**.

$$P = \frac{[M]_0 - [M]_e}{[M]_0} \qquad \cdots (7)$$

The number average degree of polymerization ($\overline{DP}$) defined using P is converted to the ratio of $[M]_0$ to $[M]_e$ as **Eq. 8**.[36]

$$\overline{DP} = \frac{1}{1-P} = \frac{[M]_0}{[M]_e} \qquad \cdots (8)$$

The relation between estimated $\overline{DP}$ and $[M]_0$ is shown in the inset of **Figure 9**. As shown there, $\overline{DP}$ increases proportionally with $[M]_0$. The estimated molecular weight reaches nearly 1 MDa

(990 kDa) at $[M]_0 = 2.33$ mM ($[Hb] = 15$ g/dL). That value roughly matches the result obtained from SEC (>800 kDa).
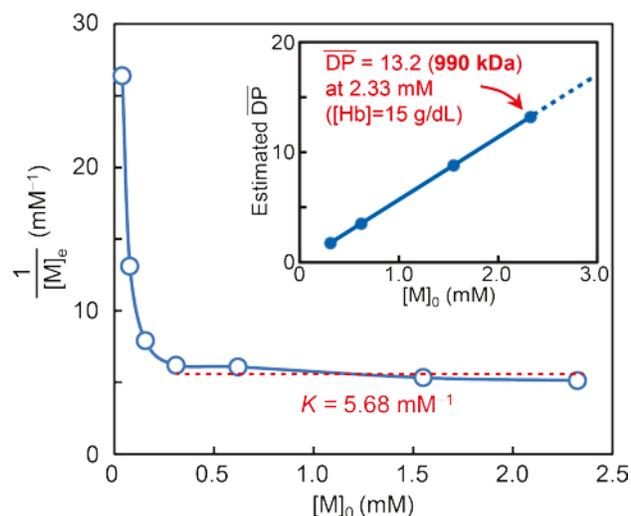
Figure 9. Relation between $1/[M]_e$ and $[M]_0$, where $[M]_e$ is a monomer concentration at the equilibrium state and $[M]_0$ is the initial monomer concentration. $[M]_e$ was quantified from the peak area of XL-Hb-rPEG in SEC analysis (Supporting Information **Table S4**). Using **Eq. 6**, the K value was found to be 5.68 mM^{-1} from the average of $1/[M]_e$ at $[M]_0 = 0.31$, 0.62, 1.55, and 2.33 mM. The inset graph shows the relation between estimated $\overline{DP}$ and $[M]_0$, where $\overline{DP}$ is the number average degree of polymerization obtained from **Eq. 8**.

3.6. Purification and characterization of ring-opened and fixed polymer XL-poly(Hb−PEG)

The Hb concentration of Hb-rPEG was adjusted to 2.33 mM ($[Hb] = 15.0$ g/dL). Then DBBF was reacted. At this concentration, Hb-rPEG forms the corresponding supramolecular

polymer (poly(Hb−PEG)) conjugated through non-covalent intermolecular binding. The $\alpha_2\beta_2$ tetrameric structures in poly(Hb−PEG) were fixed using DBBF crosslinking. Then the fixed polymer XL-poly(Hb−PEG) with the higher molecular weight was fractionated using an open column SEC. The SEC profiles and SDS-PAGE images before and after purification are presented for comparison in **Figure 10**. Monomeric structure was observed after DBBF crosslinking as a peak at 10.91 min (XL-Hb-rPEG) in chromatogram **I**, and as bands **b** (β−β−ringPEG) in lanes **I**. They disappeared after purification (chromatogram **II** and lane **II**). The retention time of the peak top in **Figure 10A** becomes shorter by purification from 6.45 to 6.37 min, indicating the higher molecular weight distribution after purification. The molecular weights of XL-poly(Hb−PEG) before and after purification were estimated respectively as 812 and 844 kDa by extrapolation of the calibration curve in **Figure 8**.

The result of 13% SDS-PAGE (**Figure 10B**, left, lane **II**) shows that purified XL-poly(Hb−PEG) consists of free α subunits and polymeric β−β components (poly(β−β−PEG), bands **d**). When we specifically examine the higher molecular weight region in SDS-PAGE using a 3−8% gradient gel (**Figure 10B**, right), bands **c** and bands **d** ascribed to poly(β−β−PEG) are observed as ladder bands, indicating the discontinuous distribution of molecular weight for XL-poly(Hb−PEG). The molecular weight distribution of poly(β−β−PEG) observed as bands **d** in lane **II** is higher than that of bands **c** in lane **I**, which coincides with the SEC result. Bands **d** are observed mainly around the 460 kDa marker, indicating that the molecular weight of the corresponding XL-poly(Hb−PEG) is nearly 800 kDa.

The structure of purified XL-poly(Hb−PEG) was confirmed directly using atomic force microscopy (AFM). Hb molecules constituting XL-poly(Hb−PEG) were localized, whereas native Hb molecules were dispersed uniformly on the mica plate (**Figure 11A**). This result

indicates the existence of the conjugated Hb polymer. The height of each Hb molecule consisting XL-poly(Hb−PEG) was ca. 2−3 nm, which is similar to that of native Hb (Supporting information **Figure S3**). Around 10 Hb molecules were conjugated to form 50−80 nm diameter particles. The particle diameter of the purified XL-poly(Hb−PEG) was also estimated as 97±45 nm by DLS measurement (**Figure 11B**). That value roughly coincides with the size observed in AFM image.
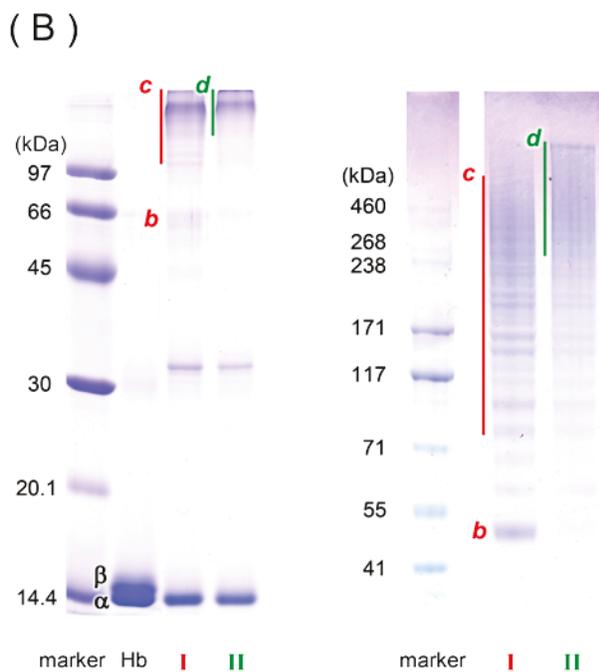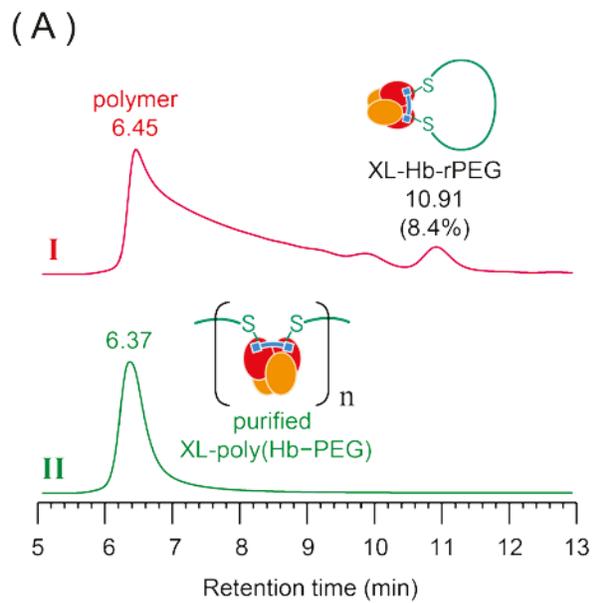
Figure 10. (A) SEC profiles and (B) SDS-PAGE of ring-opened and fixed polymer, XL-poly(Hb−PEG), before (chromatogram **I** and lane **I**) and after (chromatogram **II** and lane **II**) purification using SEC. In (B), the left panel shows the electrophoretic image conducted in a

15% acrylamide gel. The right panel shows that conducted in a 3−8% gradient gel. Band **b** is assigned as β−β−ringPEG; bands **c** and bands **d** are assigned as poly(β−β−PEG).

(A)
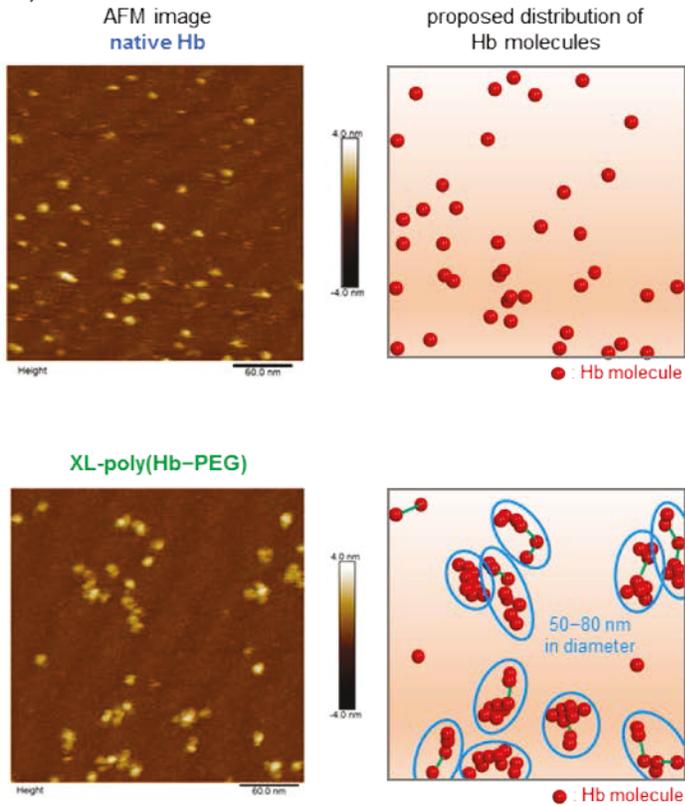
(B)
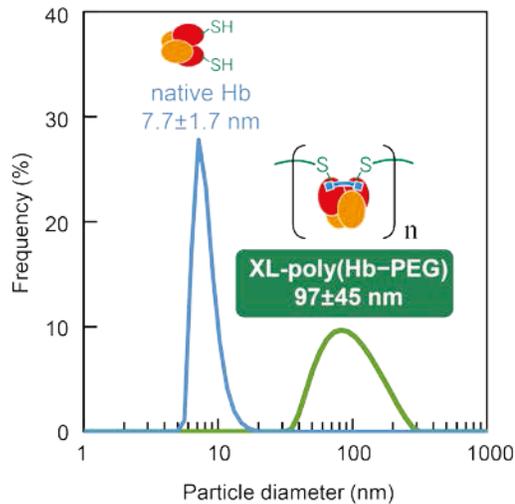
Figure 11. (A) AFM images of native Hb and the purified XL-poly(Hb−PEG) on mica surfaces in distilled water, with schematic illustrations of Hb molecules inferred from the AFM images. Hb molecules constituting XL-poly(Hb−PEG) were conjugated to form particles of 50−80 nm diameter. (B) Particle size distributions of native Hb (blue) and purified XL-poly(Hb-PEG) (green) obtained from DLS measurements.

4. DISCUSSION

As described herein, we developed an efficient polymerization procedure for creating polymerized Hb using a novel approach: ring-opening polymerization. We synthesized a cyclic Hb-ring monomer with two β subunits bound through 10 kDa PEG chain (Hb-rPEG, **Figure 1**). During the intermolecular subunit exchange of αβ dimers, Hb-rPEG induces ring-opening polymerization to produce a supramolecular Hb polymer: poly(Hb–PEG). The ring-closed monomer and the ring-opened polymer were fixed respectively by covalent bonding using DBBF crosslinking to obtain XL-Hb-rPEG and XL-poly(Hb–PEG). This is the first example of an Hb polymer obtained by ring-opening polymerization and succeeding covalent bonding using site-specific crosslinking.

The Hb-ring monomer, Hb-rPEG, was synthesized as shown in **Figure 1**. A similar modification of Hb was reported by Manjula et al. using a shorter 2 kDa bi-functional maleimide-PEG (2 kDa mal_2-PEG).[37] Their purpose was to create an intramolecularly ββ-PEG-crosslinked-Hb for maintaining allosteric effects of the Hb tetramer.[38] As described in their report, two equimolar native Hb was reacted to 2 kDa mal_2-PEG. Once a dumbbell type Hb dimer (Hb–PEG–Hb) is generated, however, it readily transforms to monomeric ββ-PEG-crosslinked-Hb (with similar structure as Hb-rPEG) and native Hb. Consequently, only a trace amount of Hb–PEG–Hb appeared. Acharya et al. reported that a shorter 600 Da mal_2-PEG tends to generate Hb–PEG–Hb and oligomers through a stepwise reaction, and that of a 2 kDa mal_2-PEG preferentially generates a thermodynamically stable monomeric Hb with a ring structure because it has suitable length only for intramolecular binding of two β93Cys thiol groups.[39]

For the work described in this report, a longer 10 kDa mal$_2$-PEG was reacted to a slightly excessive amount of native Hb. In the reaction mixture, both Hb-rPEG and dimeric Hb–PEG–Hb, and their corresponding oligomers were generated because a longer PEG chain enhances the exchange of αβ subunits between PEGylated and native Hbs.[26] The existence of such oligomers was ascertained by DBBF crosslinking of the mixture of Hb-rPEG and native Hb (**Figures 7B, 7C,** and **7D**). We reported recently that PEGylated Hb is allowed to exchange αβ subunits with native Hb.[26] Based on this insight, Hb–PEG–Hb is transformed continuously to Hb-rPEG through equilibrium by removing native Hb using a salting out technique. The synthesis of Hb-rPEG and the site-selective PEGylation of two β subunits were confirmed using SEC and SDS-PAGE (**Figure 2**).

The molecular weights of Hb-rPEG determined using SEC (98 kDa) and COP (104 kDa) are much larger than expected (75 kDa). Reportedly, conjugated hydrophilic PEG chains expand in an aqueous solution and increase the hydrodynamic volume of PEGylated Hb.[40] COP (π) is described as a function of the concentration of Hb units (C), as shown in **Eq. 1**. It is converted to the equation of π/C as **Eq. 2**. In general, water-soluble protein shows a linear relation between π/C and C, as shown in **Figure 3B** because the third-order and higher-order virial coefficients in **Eq. 2** are sufficiently small as to be negligible.[32] It is noteworthy that the plots of Hb-rPEG show an incontestable parabolic curve (**Figure 3B**, red line). This result indicates that Hb-rPEG shows inconsistent size at every concentration, and indicates that the third virial coefficient A_3 is not negligible. Actually, the particle diameter increase and dispersity increase depending on the concentration were observed in the DLS analysis of Hb-rPEG (**Figure 4**). These results suggest the intermolecular association ascribed to the ring-opening polymerization as shown in **Figure 1** at a higher concentration. Actually, Hb-rPEG can be regarded as the two αβ dimers connected by

PEG, induced by dissociation of $\alpha_2\beta_2$ tetrameric structure. The terminal $\alpha\beta$ subunits interact strongly to form a stable $\alpha_2\beta_2$ tetramer. The interaction functions intramolecularly at a lower concentration, and intermolecularly at a higher concentration. Reportedly, the third virial coefficient is ascribed to interaction among three molecules.[41,42] The intermolecular association of two $\alpha\beta$ dimers at PEG terminals is inferred to the indispensable third virial coefficient in COP measurements of Hb-rPEG.

Accordingly, Hb-rPEG is regarded as forming a monomeric ring structure by itself. In actuality, it also forms a ring-opened polymeric structure (poly(Hb−PEG)), especially at a higher concentration. Such behavior is regarded as a ring-chain mechanism of supramolecular polymerization.[43] Reportedly, some supramolecular protein polymers are constructed using specific interactions of proteins such as heme-heme pocket interaction,[44] coiled−coil interaction,[45] helix bundle folding,[46] and inter-subunit interaction.[47] Our ring-opening polymerization technique is the first reported use of the $\alpha_2\beta_2$ tetrameric structure of Hb molecule to construct a supramolecular protein polymer.

In the supramolecular polymers, interactions between monomers are reversible. The molecular weight of supramolecular polymer depends on the combination of the monomer concentration and the binding constant.[1] A few fixation methods have been reported for supramolecular polymers of organic compounds using covalent bonding[48,49] or mechanical interlocking.[50] For the present work, the ring-opened and ring-closed structures were fixed by DBBF crosslinking, a site-specific covalent bonding of β subunits at a cavity of Hb molecule (**Figure 1**). No example of such a fixing strategy of supramolecular protein polymer employing

site-specific covalent bonding of protein subunits has been described in any report of the relevant literature.

By adding DBBF, Hb-rPEG and poly(Hb−PEG) became the corresponding XL-Hb-rPEG and XL-poly(Hb−PEG) (**Figures 5** and **6**). The ratios of crosslinked monomer XL-Hb-rPEG, as determined by the peak area in SEC (**Figure 5**), are regarded as reflecting the ratios of the equilibrated state immediately before adding DBBF. Quantitative analysis was done based on the ratios of monomeric XL-Hb-rPEG at various initial monomer concentrations $[M]_0$ before DBBF crosslinking. The equilibrium monomer concentration $[M]_e$ becomes constant at higher $[M]_0$, which is the same behavior as the traditional ring-opening polymerization equilibrium of small cyclic monomers, as represented by lactones.[51]

Equilibrium constant K for **Eq. 4** was determined as 5.68 mM^{-1}. Moreover, $[M]_e$ was 0.176 mM ($[Hb]$ = 1.14 g/dL) at a higher polymerization rate (**Figure 9**). When $[M]_0$ is lower than asymptotic $[M]_e$, polymerization is thermodynamically forbidden.[52] Hb-rPEG cannot be converted into linear macromolecules at a concentration below $[M]_e$ (0.176 mM, $[Hb]$ = 1.14 g/dL), and it starts polymerization above this concentration. Estimated $\overline{DP}$ increased proportionally with $[M]_0$. The molecular weight ideally reached nearly 1 MDa (990 kDa) at $[M]_0$ = 2.33 mM ($[Hb]$ = 15.0 g/dL). This result suggests that the higher concentration of Hb-rPEG realizes the higher molecular weight of the ring-opened polymer. However, a limitation of solubility exists, as ascribed to the self-volumes of Hbs and PEG chains. Regarding Hb-rPEG using 10 kDa PEG, it was difficult to raise the Hb concentration beyond 20 g/dL.

The higher molecular weight component of XL-poly(Hb−PEG) was purified and characterized. The molecular weight of purified XL-poly(Hb−PEG) obtained by SEC (844 kDa, **Figure 10A**) and that of consisting of polymerized β−β subunits by SDS-PAGE (around 460 kDa, **Figure 10B**) show that 11 or more Hb molecules are conjugated by PEG chains. The number of conjugated Hb molecules was confirmed directly from the AFM image (**Figure 11A**).

In the case of glutaraldehyde-polymerized Hbs (poly-Hbs), which are clinically tested HBOCs, the $\overline{DP}$ is almost 2−4 and the maximum number of conjugated Hb units is 6−8.[53] Reportedly, the molecular size of HBOCs is an important factor preventing adverse effects; small HBOCs would cause vasoconstriction by extravasation and permeation across the endothelial layer.[22] Our novel strategy of ring-opening polymerization of Hb realizes higher $\overline{DP}$ compared with the poly-Hbs, with notably larger particle diameter (97 nm, **Figure 11B**) than PEG-conjugated Hb (13 or 22 nm),[10,17] glutaraldehyde-polymerized Hbs (47 nm),[17] zero-linked Hbs (48 nm),[54] and polysaccharide-polymerized Hbs (68 nm).[17]

To confirm the polymerization mechanism of Hb-rPEG, native Hb was added as an inhibitor before DBBF crosslinking. Results demonstrate that the products become gradually smaller with increasing amounts of native Hb. Additionally, discontinuous peaks of small oligomers were observed in SEC (**Figures 7A−7D**), which were identical to oligomeric XLHbs conjugated by mal$_2$-PEG (**Figures 7F−7H**). This result indicates the mechanism clearly. The exchange of αβ subunits is the trigger of ring-opening polymerization of Hb-rPEG. Moreover, this result indicates that using a chemically modified Hb bearing a functional group as an inhibitor makes it possible to provide functional groups at the terminals of XL-poly(Hb−PEG). Such terminal-targeted modification would be difficult if using stepwise conjugation of XLHb.

Equimolar reaction of XLHb and mal$_2$-PEG produces linear polymer poly(XLHb) by alternative conjugation of PEGs and XLHbs (**Figure 7E**). The poly(XLHb) structure is similar or identical to XL-poly(Hb−PEG). Poly(XLHb) is observed at 6.45 min in SEC, which corresponds to molecular weight greater than 800 kDa. However, poly(XLHb) contains unreacted XLHb. Moreover, the amounts of small oligomers were greater than in XL-poly(Hb−PEG) (**Figure 7A**).

The supramolecular polymer (poly(Hb−PEG)) prepared here is regarded as inapplicable for blood substitutes because it would decompose to the monomeric structures by dilution in blood through the dissociation equilibrium of $\alpha_2\beta_2$ subunits. DBBF crosslinked polymer, XL-poly(Hb−PEG), is expected to retain the $\alpha_2\beta_2$ structure, which makes it as an injectable fluid. Visible light absorption spectrometry of XL-poly(Hb−PEG) showed reversible oxygen binding. Its oxygen dissociation curve clarified a higher oxygen affinity (lower P_{50} value, 5.2 Torr) and a smaller Hill number ($n = 1.1$) than those of native Hb ($P_{50} = 8.2$ Torr, $n = 2.2$) (Supporting information **Figure S4**). Both PEGylation at β93Cys and DBBF crosslinking at β82Lys are expected to affect the left shift of the oxygen equilibrium curve.[55] Reportedly, HBOCs with a high oxygen affinity are effective for targeted oxygen delivery to a hypoxic tissue.[56,57] Consequently, XL-poly(Hb−PEG) will eventually be applicable as other HBOCs with high oxygen affinity for such clinical use.

For other supramolecular protein polymers, multi-level architectures such as microtube,[58] nanolayer,[59] nanoparticle, and other three-dimensional structures[60] are constructed by the combination of self-assembling proteins and artificial scaffolds. Our novel strategy is expected to be applicable not only for providing a simple linear Hb polymer but also for constructing other highly ordered Hb architectures.

5. CONCLUSION

In this work, we achieved ring-opening polymerization of Hb triggered by intermolecular subunit exchange of αβ dimers. The ring-opened polymer was fixed by covalent bonding with intramolecular crosslinking of two β subunits. Analyses of the obtained polymer clarified that ring-opening polymerization provides greater benefits for efficiency ($\overline{DP}$ = 13.2 on the average, M_n = 990 kDa) compared with random polymerization of Hb using glutaraldehyde. Additionally, the amount of lower molecular weight components was found to be smaller than the stepwise conjugation of XLHb. This is the first example of ring-opening polymerization using the association–dissociation equilibrium of $\alpha_2\beta_2$ subunits in an Hb molecule ($\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$), and of covalent bonding of the supramolecular Hb polymer using site-specific Hb modification. The technique is expected to be applicable not only for providing a simple linear Hb polymer but also for constructing other highly ordered Hb architectures, which are expected to realize highly aligned and efficiently polymerized Hbs.

6. ASSOCIATED CONTENTS:

Supporting Information

The supporting information contains the detailed information of COP, DLS, SEC, and AFM; Oxygen equilibrium curve measurement (PDF).

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Author Contributions

T.M., K.Y., and H.S. conceived the study and designed the experiments. T.M. performed experiments and data analyses. T.M. and H.S. wrote the manuscript. All authors have given their approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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