

Analysis of dimeric  $\alpha\beta$  subunit exchange between PEGylated  
and native hemoglobins ( $\alpha_2\beta_2$  tetramer) in an equilibrated state  
by intramolecular  $\beta\beta$ -crosslinking

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## **ABSTRACT**

Various chemical modifications of hemoglobin (Hb) including PEGylation have been investigated to produce red blood cell substitutes. Some of those modifications are designed on the premise that the  $\alpha_2\beta_2$  tetrameric structure of Hb is fundamentally stable and that it rarely dissociates into two  $\alpha\beta$  dimers in a physiological condition. However, in the present work using the “clipping” method we detected and quantitatively analyzed the considerable degree of exchange reaction of  $\alpha\beta$  subunits between  $\beta 93\text{Cys-bis-PEGylated}$  and native Hbs through dissociation into  $\alpha\beta$  dimers and restructuring to  $\alpha_2\beta_2$  tetramer in a physiological condition. The equilibrium constant ( $K_{\text{eq}}$ ) of subunit exchange reactions increased from 0.82 to 2.86 with increasing molecular weight of PEG from 2 to 40 kDa, indicating that longer PEG chains enhanced such exchange reaction. The results suggest that the exchange might occur for other modified Hbs even at a practically high concentration for use as a red blood cell substitute.

**KEYWORDS:** blood substitutes, artificial oxygen carriers, hemoglobin-based oxygen carriers, erythrocytes, PEGylation, size exclusion chromatography

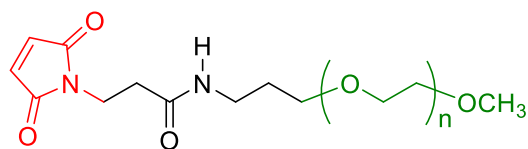
## 1. INTRODUCTION

Hemoglobin (Hb) based oxygen carriers (HBOCs) have been investigated as red blood cell substitutes to overcome transfusion-related problems such as the possibility of infectious disease, blood type mismatching, immunological responses, and shortage.<sup>1,2</sup> HBOCs are classified mainly into two categories: cellular and acellular. The former are produced by encapsulation of a native Hb solution in synthetic polymer membranes<sup>3,4</sup> or in liposomes<sup>5,6</sup> to shield the toxicity of Hb molecules. The latter are produced by chemical modification of native Hb to prevent subunit dissociation or to enlarge the molecular size by dimerization,<sup>7</sup> polymerization,<sup>8,9</sup> conjugation with gel particles,<sup>10</sup> and conjugation with non-immunogenic compounds for covering the Hb surface.<sup>11-14</sup> Polyethylene glycol (PEG) conjugation (PEGylation) of Hb is a standard method for lowering the antigenicity and for extending the circulation time of isolated Hbs.<sup>12-14</sup> Site-selective PEGylation is achieved by maleimide-terminated PEG (mal-PEG), which is known to bind to human Hb through the residue of  $\beta 93\text{Cys}$  to produce bis-PEGylated Hb (bis-PEG-Hb).<sup>13</sup> Multiple PEGylation of Hb can be achieved by combination use of 2-iminothiolane and mal-PEG.<sup>14,15</sup> Several acellular HBOCs have come into the stage of clinical trials.<sup>16-19</sup> Nevertheless, some of them retain side effects<sup>20</sup> such as vasoconstriction, hypertension, and oxidation stress, which are ascribed to the extravasation of small Hb molecules through the endothelial layer.<sup>21-24</sup>

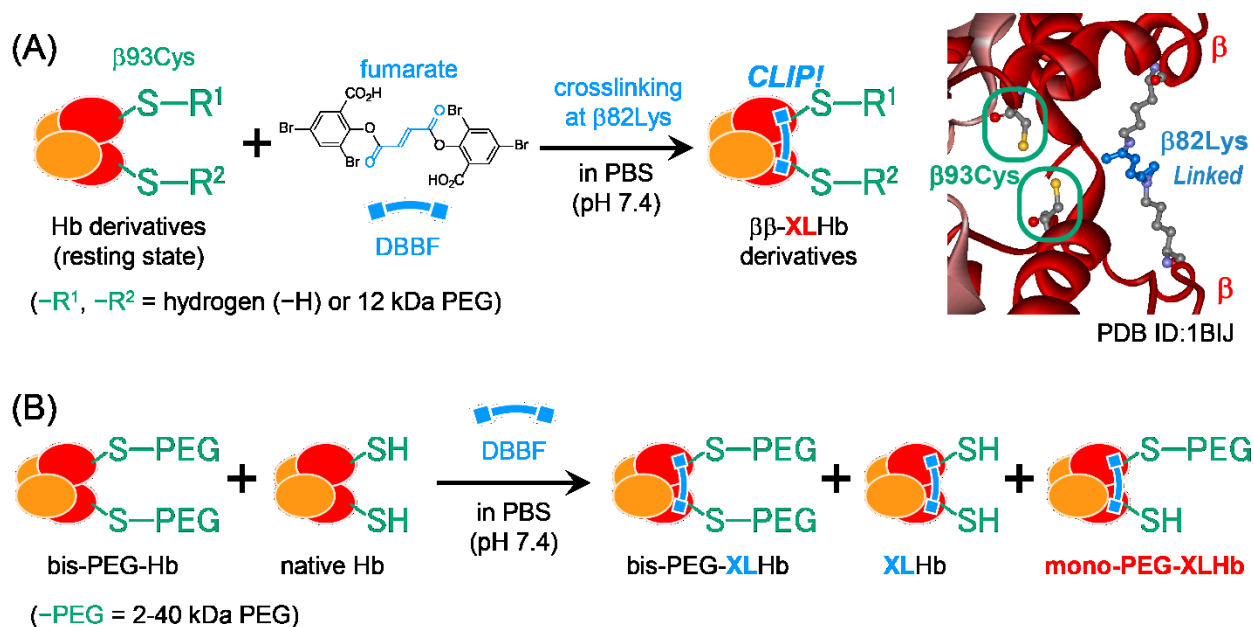
One Hb molecule consists of an  $\alpha_2\beta_2$  tetramer, with dissociation equilibrium to two  $\alpha\beta$  dimers. The dissociation constant of the tetramer to the dimers ( $K_d$ ) is reportedly of micromolar order.<sup>25</sup> The tetrameric structure of native Hb is regarded as extremely stable, and dissociation is expected to occur rarely in a physiological condition. The  $K_d$  values were also reported for

multiple PEGylated Hbs. The  $\alpha_2\beta_2$  structure showed almost equal stability to that of native Hb up to hexa-PEGylation.<sup>26</sup> However, the stability of the  $\alpha_2\beta_2$  tetramer of native and PEGylated Hbs decreases in highly acidic and basic conditions,<sup>27</sup> or in a diluted and an aerobic condition.<sup>28</sup> A question arises of whether a PEGylated Hb in the presence of native Hb shows subunit exchange reactions through dissociation and association of the  $\alpha_2\beta_2$  tetramers, even at physiological pH and high Hb concentrations.

In this work, we detected the exchange of  $\alpha\beta$  subunits between PEGylated and native Hbs. Mal-PEG (**Scheme 1**) was reacted with native Hb at different molar ratios to produce a mixture of native Hb, mono-PEGylated Hb (mono-PEG-Hb), and bis-PEGylated Hb (bis-PEG-Hb). To analyze the distribution of  $\alpha_2\beta_2$  tetrameric structures, we clipped the  $\alpha\beta$  dimers together to fix the  $\alpha_2\beta_2$  tetramer structures by reacting bis-(3,5-dibromosalicyl)fumarate (DBBF) (**Scheme 2A**). A native Hb ( $-R^1 = -R^2 = -H$ ) in a relaxed state is known to be crosslinked between two  $\beta 82\text{Lys}$  by a fumarate framework to obtain  $\beta\beta$ -crosslinked Hb (XLHb).<sup>29,30</sup> However, no reaction between PEGylated Hbs and DBBF has been reported. As described in the present report, PEGylated Hbs ( $-R^1$  and/or  $-R^2 = 12$  kDa PEG) were crosslinked in the same manner as native Hb. Moreover, mal-PEGs with different molecular weight (Mw: 2–40 kDa) were reacted to native Hb, respectively, to produce bis-PEG-Hb. The purified bis-PEG-Hb was mixed with native Hb. Then DBBF was added to clip tetrameric structures (**Scheme 2B**). We examined the generation of mono-PEGylated XLHb using size exclusion chromatography (SEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It constitutes strong evidence of exchange of  $\alpha\beta$  subunits between bis-PEG-Hb and native Hb. We strove to ascertain the kinetic parameters for the exchange equilibrium based on the ratios of clipped components.



**Scheme 1.** Chemical structures of maleimide-terminated polyethylene glycols (mal-PEGs). Number averaged Mw ( $M_n$ ) of mal-PEGs were, respectively, 2, 5, 12, 20, 30, and 40 kDa.



**Scheme 2.** (A) Intramolecular  $\beta\beta$ -crosslinking (XL) of native and PEGylated Hbs by DBBF. The X-ray crystal structure of  $\beta\beta$ XLHb is shown particularly addressing the binding site for mal-PEG (PDB ID:1BIJ).<sup>30</sup> Using DBBF, PEGylated Hbs are crosslinked by fumarate through two  $\beta 82\text{Lys}$  in the same manner as that of the native Hb. (B) Exchange of  $\alpha\beta$  subunits between bis-PEGylated and native Hbs, and subsequent crosslinking using DBBF. Mono-PEGylated XLHb was produced as a result of the subunit exchange.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of native and $\beta\beta$ -crosslinked Hbs

Human Hb was purified from red blood cells provided by the Japanese Red Cross Society, as reported previously in the literature.<sup>31,32</sup> Highly purified human carbonyl Hb (native Hb) solution was obtained through pasteurization and nanofiltration. Intramolecularly  $\beta\beta$ -crosslinked Hb (XLHb) was prepared according to the method reported by White et al.<sup>33</sup> with some modifications. To a 500 mL of native Hb solution (0.42 mM, 2.7 g/dL, 0.21 mmol) in phosphate buffered saline (PBS, pH 7.4) was added 1.6 molar equivalents of powdered bis-(3,5-dibromosalicyl)fumarate (**Scheme 2A**, DBBF, 225 mg, 0.335 mmol; Abcam plc., Cambridge, UK). They were then stirred at room temperature under a CO atmosphere for 2 h. The reaction mixture was cooled immediately in an ice bath and dialyzed continuously with PBS (pH 7.4). Then the mixture was concentrated by ultrafiltration using a lab-scale tangential flow filtration system (Merck Millipore, Merck KGaA, US) equipped with a cassette (Pellicon<sup>®</sup> XL, Biomax<sup>®</sup> 8 kDa, 50 cm<sup>2</sup> filtration area; Merck Millipore). After the effluent volume reached 1.5 L, the XLHb solution was concentrated to 57.5 mL of 23.1 g/dL XLHb solution (yield, 98.3%).

### 2.2. Reaction of native Hb and XLHb with 12 kDa mal-PEG

XLHb or native Hb (0.50 mM, 3.2 g/dL, 0.50 mL) in PBS (pH 7.4) was reacted with 12 kDa mal-PEG (**Scheme 1**,  $M_n$  12077,  $M_w/M_n = 1.02$ ; NOF Co. Ltd., Tokyo, Japan) at room temperature. Molar ratios of mal-PEG to Hb or XLHb were adjusted to 0.5, 1, 2, 3, 4, or 5. The reaction mixture was diluted by PBS to 0.050 mM (0.32 g/dL) for SEC and SDS-PAGE analyses.

### ***2.3. Intramolecular $\beta\beta$ -crosslinking for clipping PEGylated and native Hbs in an equilibrated state***

To each reaction mixture of native Hb and 12 kDa mal-PEG ([Hb] = 0.50 mM, 3.2 g/dL, 0.10 mL) described above, two molar equivalents of DBBF was added as a suspension (67.2  $\mu$ g in 0.10 mL PBS). Then, it was stirred for 2 h at room temperature. The resulting solution ([Hb] = 0.25 mM, 1.6 g/dL, 0.20 mL) was diluted further by PBS to 0.050 mM (0.32 g/dL) for analyses.

### ***2.4. Synthesis of bis-PEGylated Hbs with using various Mw of mal-PEG***

Bis-PEGylation of native Hb was conducted following the method reported by Manjula et al.<sup>12</sup> with some modifications. Native Hb was reacted with three-fold molar excess mal-PEG (**Scheme 1**; 2, 5, 12, 20, 30, or 40 kDa mal-PEG;  $M_n$  were, respectively 2278, 5255, 12077, 20137, 29858, or 40869; NOF Co. Ltd.). The initial concentrations of native Hb were 4.0 mM (26 g/dL) for 2 and 5 kDa PEGylation, 1.0 mM (6.5 g/dL) for 12 and 20 kDa PEGylation, and 0.50 mM (3.2 g/dL) for 30 and 40 kDa PEGylation, respectively, in PBS (pH 7.4). Each reaction mixture was stirred overnight at 4 °C under CO atmosphere. Then, bis-PEGylated Hb was purified three times by salting out using 3 M ammonium sulfate (ionic strength 9 M) to remove unreacted Hb and excess mal-PEG. Each precipitate was dissolved again in PBS. Then the solution was dialyzed overnight using a Dialysis Membrane (Size 27, Wako Chemicals USA, Inc., US), against 400 mL saline at 7 °C. After saline was replaced with another 400 mL PBS, it

was dialyzed again for 4 h. After dialysis, bis-PEG-Hb solution was collected. The yields were 34.1–65.3%, depending on the Mw of PEG.

### ***2.5. Exchange of $\alpha\beta$ subunits between bis-PEGylated and native Hbs, with subsequent crosslinking in an equilibrated state***

Each purified bis-PEG-Hb was mixed with native Hb in PBS (pH 7.4), and stirred for 30–120 min. The concentration of bis-PEG-Hb was fixed to 0.20 mM (1.3 g/dL), and that of native Hb was 0.20, 0.16, 0.12, 0.10, 0.08, 0.04, 0.02, and 0.01 mM (1.29–0.06 g/dL). Then, two molar equivalents of DBBF to total Hb was reacted. After crosslinking reaction was completed, each resulting solution was diluted four times by PBS for analyses. For comparison, each bis-PEG-Hb was reacted with DBBF to obtain bis-PEG-XLHb.

### ***2.6. Size exclusion chromatography (SEC)***

SEC was conducted at room temperature using a high-performance liquid chromatography system (HPLC, Chromaster<sup>®</sup>; Hitachi High-Technologies Corp., Tokyo, Japan) equipped with an SEC column (Shodex Protein KW-804; Showa Denko K.K., Tokyo, Japan) for 12, 20, 30, and 40 kDa PEGylation, and with a different SEC column (Shodex Protein KW-802.5; Showa Denko K.K.) for 2 and 5 kDa PEGylation. Effluent was monitored using a diode array detector (5430 Diode Array Detector; Hitachi High-Technologies Corp.) with absorbance at 419 nm, which corresponds to the  $\lambda_{\max}$  of Soret band of carbonyl Hb. A modified Hb solution of 0.050 mM (0.32 g/dL) was filtered through a syringe-driven filter unit (Millex<sup>®</sup> 0.22  $\mu\text{m}$



PVDF filter; Merck Millipore). Twenty  $\mu\text{L}$  of analyte was injected into the SEC column through a sampler. PBS (pH 7.4) was used as an eluent with a flow rate of 1.0 mL/min.

## ***2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)***

Tris(hydroxymethyl)aminomethane (Tris), sucrose, SDS, 2-mercaptoethanol, and bromophenol blue used to prepare Hb solutions for electrophoresis were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). A modified Hb solution (0.050 mM, 0.32 g/dL) was mixed with a half volume of a denaturing buffer<sup>34</sup> (0.19 M Tris-HCl pH 6.8, 6.0% (w/v) SDS, 15% (v/v) 2-mercaptoethanol, 30% (w/v) sucrose, and 0.006% (w/v) bromophenol blue) and was incubated at 80 °C for 15 min. Electrophoresis was conducted on a 13% polyacrylamide gel using a mini-slab electrophoresis system (NA-1010; Nihon Eido Corp., Tokyo, Japan). A kit (Amersham Low Mw Calibration Kit for SDS Electrophoresis; GE Healthcare UK Limited, Buckinghamshire, UK) was used for Mw markers. The gel was stained with Coomassie brilliant blue (Quick CBB; Wako Pure Chemical Industries Ltd.). The stained gel image was obtained by transmission scanning (GT-X970; Seiko Epson Corp., Nagano, Japan).

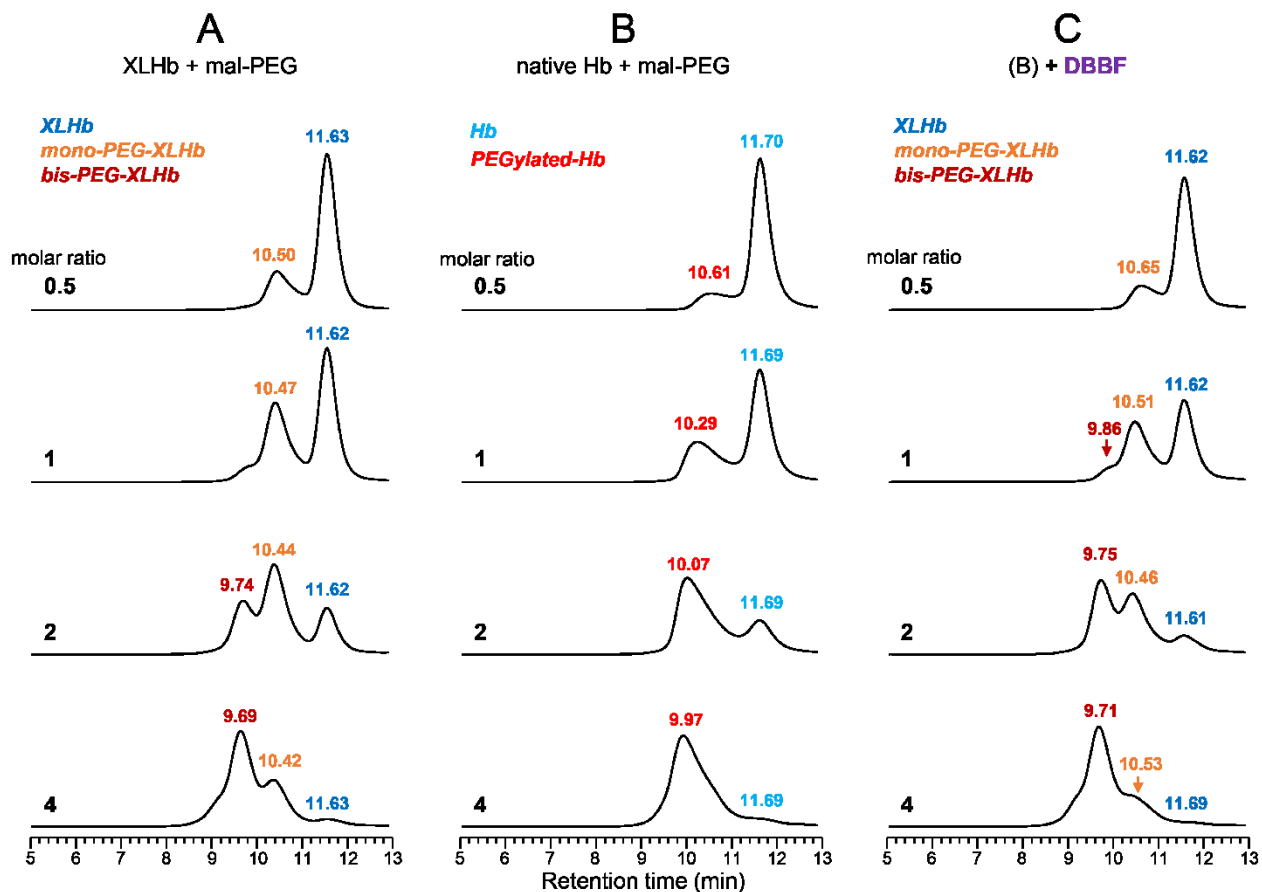
### 3. RESULTS

#### *3.1. SEC profiles of 12 kDa PEGylation and subsequent crosslinking of native Hb comparing with PEGylated XLHb*

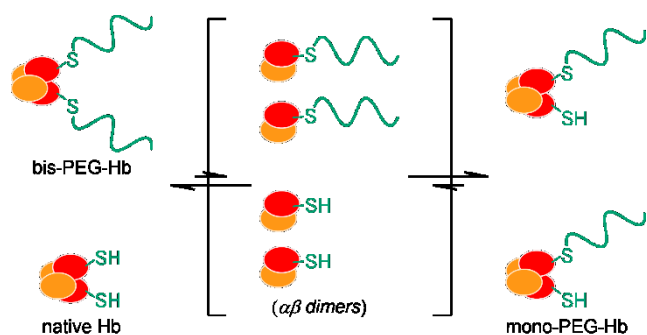
XLHb and native Hb were reacted with 12 kDa mal-PEG at different mixing molar ratios of mal-PEGs to Hb of 0.5–5. Chromatograms of the reaction mixtures of XLHb and mal-PEG with molar ratios of 0.5, 1, 2, and 4 are presented in **Figure 1A**. Those of native Hb and mal-PEG are presented in **Figure 1B**. For PEGylation of XLHb, two products with retention times (RT) of 10.42 and 9.69 min were observed in sequence, indicating step-by-step PEGylation. First, one of the two thiol groups of two  $\beta$ 93Cys residues in one Hb was conjugated to mal-PEG (mono-PEG-XLHb). Subsequently, the other residue was conjugated to produce bis-PEG-XLHb. In contrast to XLHb, PEGylation of native Hb showed a single broad peak (**Figure 1B**). RT of PEGylated Hb shifted from 10.61 to 9.97 min with the increasing molar ratio of mal-PEG. It was longer than that of bis-PEG-XLHb (9.69 min). These results imply that the reaction mixture includes mono-PEG-Hb and bis-PEG-Hb, and that their  $\alpha_2\beta_2$  tetramers dissociate and restructure along with mutual exchange of their subunits (**Scheme 3**).

We terminated this exchange reaction between mono-PEG-Hb, bis-PEG-Hb, and native Hb using DBBF (**Scheme 2A**). Chromatograms obtained after the DBBF addition toward the reaction mixture of Hb and 12 kDa mal-PEG are presented in **Figure 1C**. The single and broad peaks, ascribed to the mixture of PEGylated Hbs (**Figure 1B**), split into two individual peaks (**Figure 1C**) with RTs identical to those of mono-PEG-XLHb and bis-PEG-XLHb (**Figure 1A**), indicating that the crosslinked PEG-Hbs were identical to PEGylated XLHbs. Molar ratios of the components calculated from the relative peak areas of SEC before and after adding DBBF are

presented in **Table 1**. For all mixing ratios of mal-PEG to Hb, the molar ratio of native Hb before adding DBBF is larger than that of XLHb after adding DBBF, indicating that the molar ratio of native Hb increases during SEC.



**Figure 1.** SEC profiles of (A) the reaction mixtures of XLHb and 12 kDa mal-PEG, (B) the reaction mixtures of native Hb and mal-PEG, and (C) crosslinked native and PEGylated Hbs obtained after DBBF addition to the reaction mixtures of Hb and mal-PEG. The molar ratio of mal-PEG to Hb was fixed from 0.5 to 4.



**Scheme 3.** Schematic illustration of the exchange equilibrium between bis-PEG-Hb, native Hb, and mono-PEG-Hb by dissociation to  $\alpha\beta$  dimers and restructuring of the  $\alpha_2\beta_2$  tetramer.

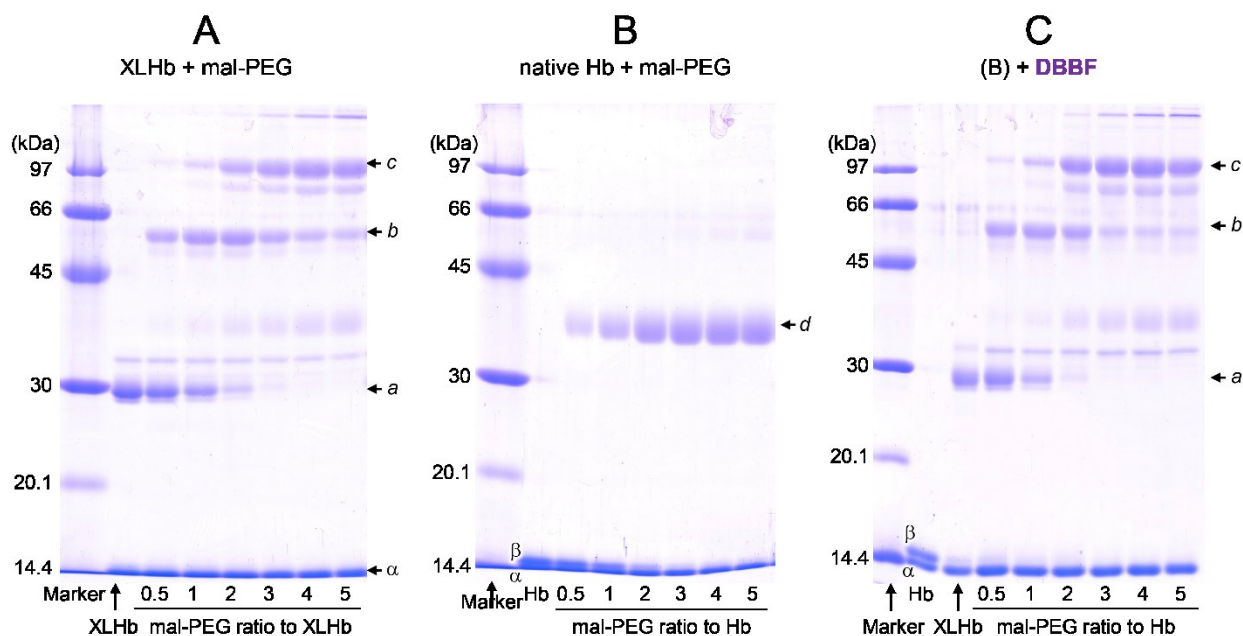
**Table 1.** Molar ratios of respective components calculated from the relative peak areas of SEC before and after addition of DBBF (Figure 1B and Figure 1C) to the reaction mixture of Hb and 12 kDa mal-PEG at different mixing ratios

molar ratio of mal-PEG to Hb	molar ratio (%)				
	before adding DBBF		after adding DBBF		
	Hb	PEG-Hbs <sup>#</sup>	XLHb	mono-PEG-XLHb	bis-PEG-XLHb
0.5	86.4	13.6	81.1	18.9	0.0
1	64.8	35.2	50.9	41.2	7.9
2	27.6	72.4	15.1	41.9	43.0
3	14.2	85.9	6.9	28.4	64.7
4	8.2	91.8	4.9	25.1	70.3
5	7.6	92.4	3.5	22.7	73.9

<sup>#</sup> mixture of mono- and bis-PEGylated Hbs

### **3.2. Confirmation of clipping modality for PEGylated Hbs by SDS-PAGE**

Band patterns of SDS-PAGE for the reaction mixtures of XLHb and 12 kDa mal-PEG with molar ratios of 0.5, 1, 2, 3, 4, and 5 are presented in **Figure 2A**. Those of native Hb and mal-PEG are presented in **Figure 2B**. For PEGylation of XLHb, the crosslinked  $\beta$  subunits ( $\beta$ - $\beta$ , band *a*) were initially single-PEGylated to generate  $\beta$ - $\beta$ -PEG (band *b*). The intensity of band *b* then decreased concomitantly with increasing molar ratio of mal-PEG. Alternatively, the band of bis-PEGylated  $\beta$ - $\beta$  subunits (band *c*,  $(\beta$ -PEG)<sub>2</sub>) appeared with the double-PEGylation. This profile is consistent with that of SEC (**Figure 1A**), indicating step-by-step PEGylation of XLHb. In the case of PEGylation of native Hb,  $\beta$  subunits were PEGylated to generate  $\beta$ -PEG subunits (band *d*, **Figure 2B**). The band patterns of **Figure 2B** changed by adding DBBF, as shown in **Figure 2C**. The band of  $\beta$ -PEG almost disappeared. The bands of  $\beta$ - $\beta$ -PEG (band *b*) and  $(\beta$ -PEG)<sub>2</sub> (band *c*) appeared. The band patterns of **Figure 2C** are identical to those of **Figure 2A**.

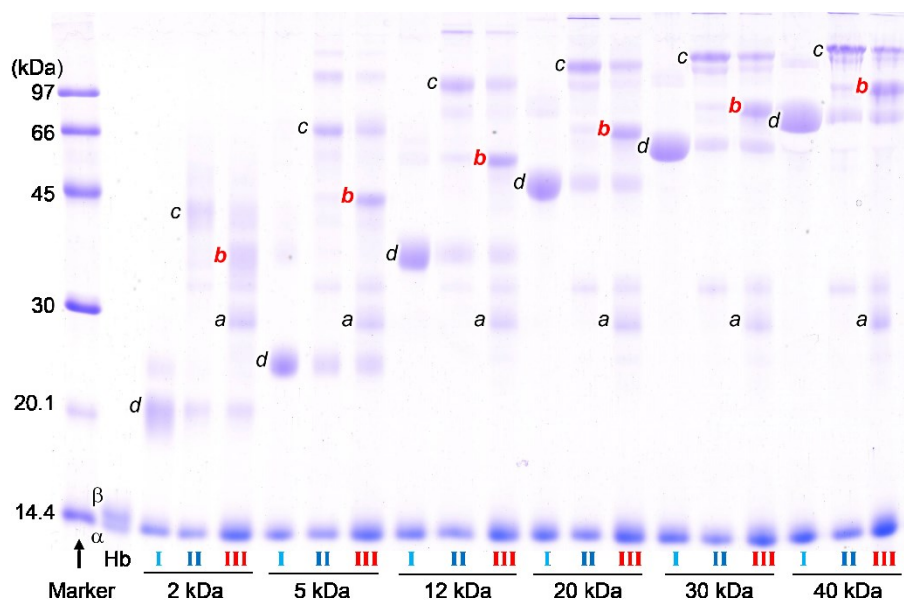


**Figure 2.** SDS-PAGEs of (A) the reaction mixtures of XLHb and 12 kDa mal-PEG, (B) the reaction mixtures of native Hb and mal-PEG, and (C) crosslinked native and PEGylated Hbs obtained after DBBF addition to the reaction mixtures of Hb and mal-PEG. The molar ratio of mal-PEG to Hb was fixed from 0.5 to 5. Bands are assigned as follows: band *a*,  $\beta$ - $\beta$  subunit of XLHb; band *b*,  $\beta$ - $\beta$ -PEG; band *c*,  $(\beta$ -PEG)<sub>2</sub>; band *d*,  $\beta$ -PEG.

### 3.3. Subunit exchange reaction confirmed by SDS-PAGE

Mal-PEGs with different Mw of 2–40 kDa were reacted to native Hb. The bis-PEGylated Hbs were purified by removing excess mal-PEG and unreacted native Hb. Each purified bis-PEG-Hbs was mixed with equimolar native Hb. Then DBBF was reacted to clip the tetrameric structures (**Scheme 2B**). After clipping, the products were analyzed using SDS-PAGE and were compared with purified bis-PEG-Hb and clipped bis-PEG-Hb (**Figure 3**). For all cases using different Mw of PEG, lanes I are purified bis-PEG-Hbs. Bands *d* observed in lanes I correspond to the PEGylated  $\beta$ -subunits ( $\beta$ -PEG). By adding DBBF to purified bis-PEG-Hb (lanes II), the  $\beta$ -

PEG subunits were crosslinked intramolecularly. Bands *c* ascribed to  $(\beta\text{-PEG})_2$  appeared as main bands. When DBBF was added to the equimolar mixture of bis-PEG-Hb and native Hb (lanes III), not only bis-PEGylated  $\beta\text{-}\beta$  subunits (bands *c*), but also mono-PEGylated ones (bands *b*,  $\beta\text{-}\beta\text{-PEG}$ ) were observed. Bands *a* observed in lanes III are ascribed to  $\beta\text{-}\beta$  subunits of unexchanged XLHb.

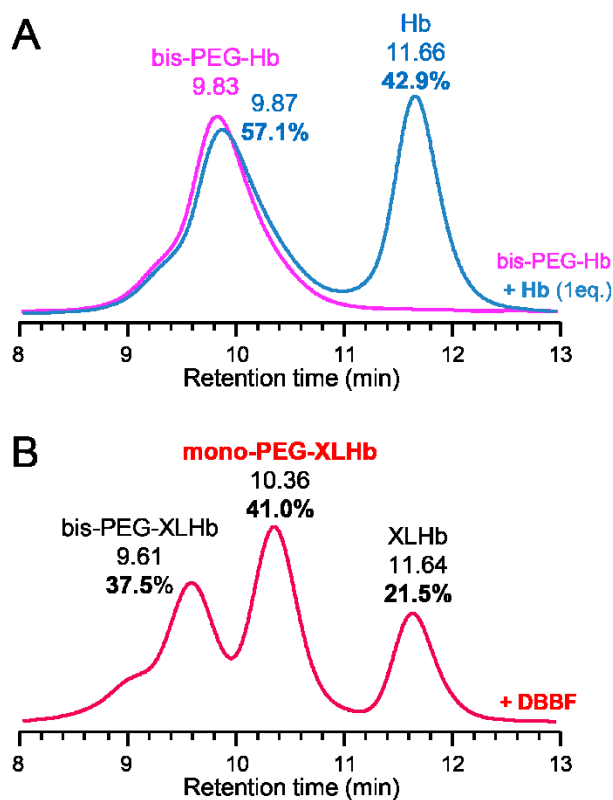


**Figure 3.** SDS-PAGE of PEGylated native and XLHbs: Lanes I, purified bis-PEG-Hb; Lanes II, after adding DBBF to the bis-PEG-Hb; Lanes III, after adding DBBF to the equimolar mixture of bis-PEG-Hb and native Hb. The Mw described below (2–40 kDa) are those of mal-PEG used for synthesis of bis-PEG-Hb. Band assignments are the same as those of Figure 2.

### 3.4. Numerical analysis of exchange reactions for different Mw of PEG

Sequential reactions of exchanging and crosslinking were also monitored by SEC. The chromatograms of 12 kDa bis-PEGylated Hb before and after mixing equimolar native Hb are presented in **Figure 4A**. RT of bis-PEG-Hb shifted slightly from 9.83 to 9.87 min. However, the

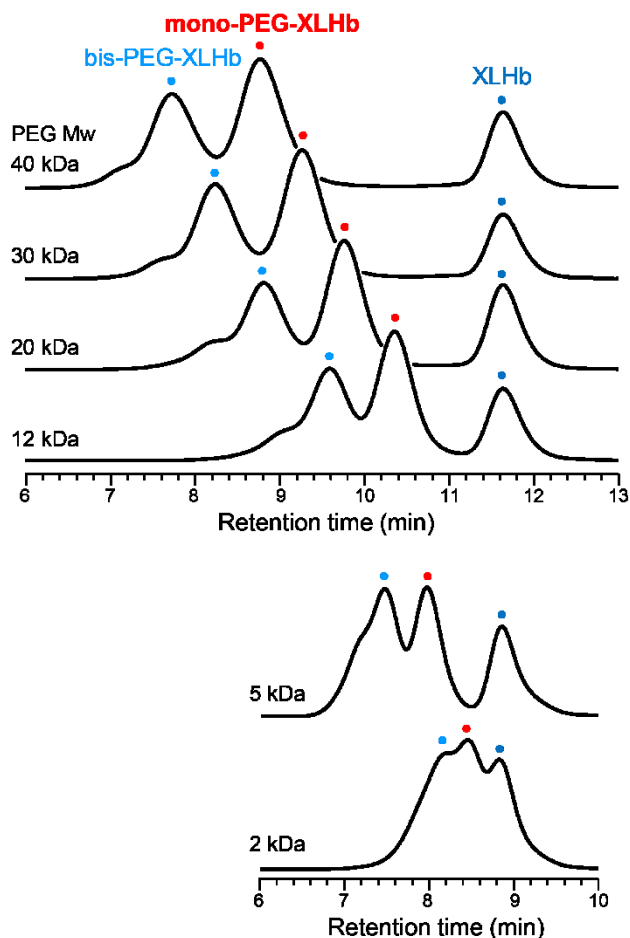
peak area of PEGylated Hb did not increase to a considerable degree. The chromatogram after addition of DBBF to the mixture is presented in **Figure 4B**. The peak of PEGylated Hb split into two individual peaks of mono- and bis-PEG-XLHb by clipping tetrameric structures. The appearance of mono-PEG-XLHb in **Figure 4B** supports the SDS-PAGE results (**Figure 3**). The relative amount of native Hb was 42.9%, and it decreased to 21.5% by clipping of the  $\alpha_2\beta_2$  tetrameric structure. That of alternatively appeared mono-PEG-XLHb was 41.0% (**Figure 4B**). The ratios after the addition of DBBF are regarded as reflecting the compositions at the equilibrated state in the solution.



**Figure 4.** SEC profiles of (A) 12 kDa bis-PEGylated Hb before and after mixing equimolar of native Hb, and (B) crosslinked products after the addition of DBBF toward the equimolar mixture of 12 kDa bis-PEGylated and native Hbs.



Chromatograms after DBBF addition to the equimolar mixture of native Hb and each purified bis-PEG-Hb with different Mw are presented in **Figure 5**. Generation of mono-PEG-XLHb (marked with a red dot) was observed for all cases. The molar ratios of bis-PEG-XLHb, mono-PEG-XLHb, and XLHb are presented in **Table 2**. The ratio of mono-PEG-XLHb increased from 31.9 to 44.4% with the increase of PEG Mw.



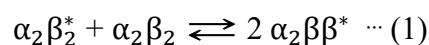
**Figure 5.** SEC profiles of bis-PEG-XLHb, mono-PEG-XLHb, and XLHb obtained by the addition of DBBF to the equimolar mixture of native Hb and bis-PEG-Hb with different Mw of PEG (2–40 kDa).

**Table 2.** Molar ratios of XLHb, mono-PEG-XLHb, and bis-PEG-XLHb calculated from the relative peak areas of SEC for different Mw of PEG (Figure 5). Equilibrium constants ( $K_{eq}$ ) were obtained from the molar ratios using Eq. (2).

PEG Mw (kDa)	molar ratio (%)			$K_{eq}$
	XLHb (crosslinked $\alpha_2\beta_2$ )	mono-PEG-XLHb (crosslinked $\alpha_2\beta\beta^*$ )	bis-PEG-XLHb (crosslinked $\alpha_2\beta_2^*$ )	
2.3	30.8	31.9	37.3	0.89
5.3	24.5	32.2	43.3	0.98
12.1	21.5	41.0	37.5	2.09
20.1	24.2	40.2	35.6	1.88
29.9	19.4	43.7	36.9	2.67
40.9	20.8	44.4	34.8	2.72

### 3.5. Kinetic analysis of exchanging equilibrium

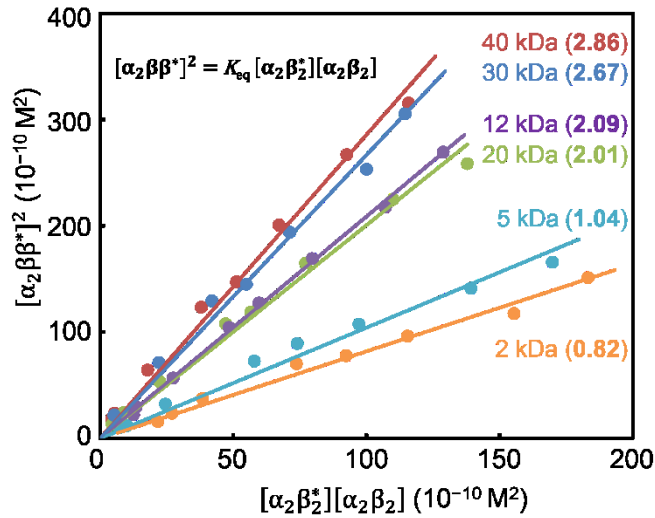
The exchange equilibrium between the PEGylated and native Hbs, as shown in **Scheme 3**, can be expressed as **Eq. (1)** using tetrameric structures consisting of  $\alpha$ ,  $\beta$ , and PEGylated  $\beta$  ( $\beta^*$ ) subunits. The equilibrium constant  $K_{eq}$  for **Eq. (1)** is defined as **Eq. (2)**.



$$K_{eq} = \frac{[\alpha_2\beta\beta^*]^2}{[\alpha_2\beta_2^*][\alpha_2\beta_2]} \quad \dots (2)$$

The ratios of crosslinked tetramers of XLHb, mono-PEG-XLHb, and bis-PEG-XLHb observed in **Figure 5** reflect those of corresponding native Hb ( $\alpha_2\beta_2$ ), mono-PEG-Hb ( $\alpha_2\beta\beta^*$ ), and bis-

PEG-Hb ( $\alpha_2\beta_2^*$ ) in the equilibrated state just before clipping with DBBF. The  $K_{eq}$  values for different Mw of PEGs are determined from the molar ratios of crosslinked tetramers observed in **Figure 5** and inserting them to **Eq. (2)** (**Table 2**). To confirm the accuracy of the  $K_{eq}$  values, the squared molar concentration of mono-PEG-Hb was plotted against the product of those of bis-PEG-Hb and Hb at various mixing ratios for each Mw of PEG (**Figure 6**). The plots showed a linear relationship, indicating the validity of the subunit exchange mechanism as **Eq (1)**. The  $K_{eq}$  values were determined from the slopes for different Mw of PEGs as shown in the Figure, which are nearly identical to those in **Table 2**. The  $K_{eq}$  value tends to increase from 0.82 to 2.86 with increasing Mw of PEG.



**Figure 6.** Relationship between the squared molar concentration of mono-PEG-Hb ( $\alpha_2\beta\beta^*$ ) and the product of the concentrations of bis-PEG-Hb ( $\alpha_2\beta_2^*$ ) and native Hb ( $\alpha_2\beta_2$ ) in the equilibrated state, according to the inset equation which is converted from **Eq. (2)**. The initial concentration of each bis-PEG-Hb was fixed to 0.20 mM, and that of native Hb was from 0.01 to 0.20 mM. The lines are obtained by least square fitting. For all the lines the correlation coefficient ( $r^2$ ) was higher than 0.97. The  $K_{eq}$  values, which are obtained as the slopes, are shown in the parentheses beside the Mw of PEG.

## 4. DISCUSSION

The most important finding of this study is that the subunit exchange between bis-PEG-Hb and native Hb is detected as generation of mono-PEG-XLHb merely by mixing them and subsequently crosslinking them. The crosslinking clips the  $\alpha_2\beta_2$  tetrameric structures of Hb derivatives and enables the direct analysis of actual ratios of mono-PEG-Hb, bis-PEG-Hb, and native Hb in a physiological condition. Using this method, the equilibrium constants  $K_{eq}$  of subunit exchange between bis-PEG-Hb and native Hb are determined for different Mw of PEG. Results reveal that the longer PEG chain enhances the subunit exchange.

PEGylation of XLHb progressed by a step-by-step process (**Figures 1A and 2A**). The PEGylated native Hb is observed as a single broad peak in SEC (**Figure 1B**). Their RTs are longer than that of bis-PEG-XLHb. Moreover, it shifts with increased mixing ratio of mal-PEG, indicating mutual exchange of  $\alpha\beta$  subunits through the dissociation equilibrium of the  $\alpha_2\beta_2$  tetramers. Then, we tried to “clip” the  $\alpha_2\beta_2$  structures of native and PEGylated Hbs by crosslinking using DBBF (**Scheme 2A**). Native Hb in a relaxed state is known to be crosslinked between two  $\beta$  subunits.<sup>30</sup> However, no report has described a study of crosslinking of PEGylated Hbs. By the addition of DBBF to the reaction mixture of native Hb and mal-PEG, mono- and bis-PEGylated Hbs were crosslinked intramolecularly to obtain products that are identical to those of the direct PEGylation of XLHb (**Figures 1C and 2C**). These results indicate that  $\beta\beta$ -crosslinking of mono- and bis-PEGylated Hbs progresses in the same manner as that of native Hb does. The exchange reaction of  $\alpha\beta$  subunits between purified bis-PEG-Hb and native Hb was examined directly (**Scheme 2B**). When equimolar native Hb was added to bis-PEG-Hb, they were observed individually without a marked peak shift or peak area change in SEC

(**Figure 4A**). However, after reacting with DBBF, mono-PEG-XLHb, produced by subunit exchange, is detected clearly (**Figures 3 and 4B**), which is strong evidence of exchanging  $\alpha\beta$  dimers between bis-PEG-Hb and native Hb.

Such exchange progresses through the dissociation to the  $\alpha\beta$  dimers (**Scheme 3**). Reportedly,  $\alpha_2\beta_2$  structures of native and PEGylated Hbs dissociate in some extreme conditions. For example, the native oxy Hb (0.1 mM) maintains a tetrameric structure between pH 7.1 and 7.4 in a phosphate buffer.<sup>27</sup> It dissociates in the presence of 0.5–1.0 M  $\text{MgCl}_2$ .<sup>35,36</sup> Generally, native Hb maintains tetrameric structures over the concentration of 0.1 mM at pH 7.4<sup>37</sup> and dissociates at lower concentrations such as 5  $\mu\text{M}$ .<sup>38</sup> Reportedly, PEGylation promotes dissociation of the  $\alpha_2\beta_2$  tetramer.<sup>26,28,38</sup> An earlier study demonstrated that the dissociation constants of  $\alpha_2\beta_2$  tetramer into dimers ( $K_d$ ) are 8.5  $\mu\text{M}$  for native Hb, 10.5  $\mu\text{M}$  for 5 kDa bis-PEGylated Hb, and 43.2  $\mu\text{M}$  for 5 kDa multiple-PEGylated Hb with 6 PEG chains in the average (hexa-PEGylated-Hb),<sup>26</sup> although it was not described how the  $K_d$  values were measured. The tetrameric structure of hexa-PEGylated Hb is stable at a higher concentration of 800  $\mu\text{M}$  in both anaerobic and aerobic conditions at pH 7. However, it dissociates in an aerobic condition at a lower concentration of 8  $\mu\text{M}$ .<sup>28</sup> By contrast, another group reported that hexa-PEGylated Hb dissociates into dimers even at 1 mM in PBS (pH 7.4).<sup>38</sup> The concentration at which tetrameric structures dissociates varies with conditions. It has not been ascertained for PEGylated Hbs.

In this work, pH was always adjusted to 7.4 in PBS. The pH and salt concentration are close to physiological conditions. Therefore, the tetrameric structure is expected to be favored. Based on the reported dissociation constant of native carbonyl Hb ( $K_d = 1.05 \mu\text{M}$ ),<sup>37</sup> the tetrameric structure is expected to be maintained as greater than 99% at 0.10 mM and as no less

than 90% at 0.050 mM at pH 7.4. The Hb concentrations for PEGylation and for subsequent clipping were 0.50 and 0.25 mM. That for clipping after subunit exchange between bis-PEG-Hb and native Hb were 0.40 mM. By contrast, SEC was conducted at a diluted concentration of 0.050 mM. From SEC analysis, the RT of purified bis-PEG-Hb was observed as between those of mono- and bis-PEG-XLHbs (**Figure 4**), indicating that the tetrameric structure of bis-PEG-Hb is fundamentally maintained, but that it is equilibrated between  $\alpha_2\beta_2$  tetramer and  $\alpha\beta$  dimers. In the solutions of higher Hb concentrations at the clipping reaction (0.25–0.40 mM), the tetrameric structures are regarded as dominant. We clarified from a clipping experiment that the subunit exchange through dissociation into  $\alpha\beta$  dimers and restructuring to  $\alpha_2\beta_2$  tetramers occurs easily between PEGylated and native Hbs. Actually, such a subunit exchange occurs not only for Hb tetrameric structure but also for other various dimeric or oligomeric proteins such as  $\alpha$ A-crystallin,<sup>39</sup> histidine kinase of *Escherichia coli*,<sup>40</sup> multimeric heat shock proteins,<sup>41</sup> and HIV-1 protease.<sup>42</sup>

Exchange reactions of  $\alpha\beta$  subunits might occur for multiple-PEGylated Hbs.<sup>43–45</sup> They presumably exchange  $\alpha\beta$  subunits with themselves and their Mw are distributed broadly because they are not crosslinked intramolecularly. Li et al.<sup>45</sup> tried to regulate the numbers of PEG chains for PEGylation (4, 6, 8, and 10). However, the separation of each PEGylated Hb by the strictly decided number of PEG seemed complicated, presumably because of the subunit exchange reactions during separations. PEGylated Hb fundamentally favors tetrameric structures in the physiological conditions. Even if the multiple-PEGylated Hb dissociates into dimers, the dimers still have non-immunogenic PEG chains with a large hydrodynamic volume. Such PEGylated dimers might not extravasate through the endothelial layer. Therefore, the absence of hypertension would not necessarily relate to the lack of dissociation.

In some pathological conditions such as hemolytic anemia, one modified Hb without intramolecular crosslinking might exchange their  $\alpha\beta$  subunits with plasma native Hbs to decrease their respective Mw, thereby decreasing their performance. The subunit exchange might occur not only for the PEGylated Hbs but also for other chemically modified Hbs. Such an exchange of the  $\alpha_2\beta_2$  tetramers has not attracted the attention of researchers. In contrast, intramolecularly  $\beta\beta$ - or  $\alpha\alpha$ -crosslinked Hbs,<sup>46</sup> intermolecularly double-crosslinked dimers,<sup>47</sup> polymerized pyridoxylated-Hb,<sup>48</sup> and *o*-raffinose-crosslinked Hb<sup>49</sup> reportedly prevent dissociation of  $\alpha_2\beta_2$  tetramer. Therefore, they are rarely caught by haptoglobin.<sup>50</sup> An exchange should not occur by the coexistence of native Hb.

The clipping method used in this work enables the SEC analysis of actual ratios of the components in a physiological concentrated condition without considering concentration changes during SEC. The relative ratios of XLHb after adding DBBF are always lower than those of native Hb before adding DBBF (**Table 1, Figure 4**). The ratios of the  $\alpha_2\beta_2$  tetrameric structures are changed during passage through the SEC column. The clipped  $\alpha_2\beta_2$  tetrameric structures are unaffected by dilution, or by separation in the SEC column. Therefore, the ratios of the respective components after the clipping reaction are regarded as reflecting the ratios in the equilibrated state in the physiological condition. The equilibrium constants ( $K_{eq}$ ) of the subunit exchange reactions increase with Mw of PEG (**Table 2, Figure 6**), indicating that bis-PEG-Hb becomes more unstable than mono-PEG-Hb for using long PEG chains. The exchange reaction proceeds through dissociation to  $\alpha\beta$  dimers (**Scheme 3**). Therefore, **Eq. (2)** can be converted to **Eq. (3)** using the concentrations of dimers.

$$K_{eq} = \frac{[\alpha\beta^*]^2}{[\alpha_2\beta_2^*]} \times \left( \frac{[\alpha_2\beta\beta^*]}{[\alpha\beta][\alpha\beta^*]} \right)^2 \times \frac{[\alpha\beta]^2}{[\alpha_2\beta_2]} \quad \dots (3)$$

This can be converted to **Eq. (4)** using dissociation constants ( $K_d$ ) from one tetramer to two dimers of native Hb ( $K_{d0}$ ), mono-PEG-Hb ( $K_{d1}$ ), and bis-PEG-Hb ( $K_{d2}$ ) as **Eq. (4)**.

$$K_{eq} = \frac{K_{d2} K_{d0}}{K_{d1}^2} \quad \dots (4)$$

Judging from the increase of  $K_{eq}$ , the dissociation constant of bis-PEG-Hb ( $K_{d2}$ ) is expected to become larger with increasing Mw of PEG. It is inferred that the tetrameric structure of bis-PEGylated Hb with longer PEG chains is destabilized by steric repulsion between two PEG chains with large hydrodynamic volumes. Also, bis-PEGylated Hb is likely to be restructured to asymmetric mono-PEGylated Hb with lower steric hindrance in the presence of native Hb.

## 5. CONCLUSION

A subunit exchange equilibrium was observed between stable  $\alpha_2\beta_2$  tetramers of native and PEGylated Hbs. In a simple and easy procedure of clipping with DBBF, the exchange reaction was terminated in an equilibrated state by intramolecular crosslinking. This clipping method enables the direct detection of  $\alpha\beta$  subunit exchange equilibrium between the native and bis-PEGylated Hb. The equilibrium constants ( $K_{eq}$ ) were found for the subunit exchange reaction between native Hb and bis-PEG-Hb using several PEGs with different Mw. Results show that the longer PEG chain enhances the dissociation of symmetric bis-PEGylated Hb in the presence of native Hb to generate asymmetric mono-PEGylated Hb. Our results suggest that the higher Hb



concentration does not inhibit exchange of  $\alpha\beta$  subunits for PEGylated Hbs, although it fundamentally favors an  $\alpha_2\beta_2$  tetrameric structure. The clipping method is applicable to other types of PEGylated Hbs using bi-functional PEG chains for evaluation of the intermolecular exchange of  $\alpha\beta$  subunits. The project is now in progress.

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

Notes

The authors declare no competing financial interest.

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## 8. REFERENCES

- (1) Chang, T. M. Therapeutic applications of polymeric artificial cells. *Nat. Rev. Drug Discov.* **2005**, *4*, 221–235.
- (2) Alayash, A. I. Hemoglobin-based blood substitutes and the treatment of sickle cell disease: More harm than help? *Biomolecules* **2017**, *7*, E2.
- (3) Chang, T. M. Recent and future developments in modified hemoglobin and microencapsulated hemoglobin as red blood cell substitutes. *Artif. Cells Blood Substit. Immobil. Biotechnol.* **1997**, *25*, 1–24.
- (4) Arifin, D. R.; Palmer, A. F. Polymersome encapsulated hemoglobin: a novel type of oxygen carrier. *Biomacromolecules* **2005**, *6*, 2172–2181.
- (5) Mobed, M.; Chang, T. M. Preparation and surface characterization of carboxymethylchitin-incorporated submicron bilayer-lipid membrane artificial cells (liposomes) encapsulating hemoglobin. *Biomater. Artif. Cells Immobil. Biotechnol.* **1991**, *19*, 731–744.

- (6) Sakai, H.; Sato, A.; Takeoka, S.; Tsuchida, E. Mechanism of flocculate formation of highly concentrated phospholipid vesicles suspended in a series of water-soluble biopolymers. *Biomacromolecules* **2009**, *10*, 2344–2350.
- (7) Kluger, R.; O'Brien, J. L.; Teytelboym, A. Connecting proteins by design. Cross-linked bis-hemoglobin. *J. Am. Chem. Soc.* **1999**, *121*, 6780–6785.
- (8) Jia, Y.; Alayash, A. I. Effects of cross-linking and zero-link polymerization on oxygen transport and redox chemistry of bovine hemoglobin. *Biochim. Biophys. Acta* **2009**, *1794*, 1234–1242.
- (9) Xiong, Y.; Steffen, A.; Andreas, K.; Müller, S.; Sternberg, N.; Georgieva, R.; Bäuml, H. Hemoglobin-based oxygen carrier microparticles: synthesis, properties, and *in vitro* and *in vivo* investigations. *Biomacromolecules* **2012**, *13*, 3292–3300.
- (10) Chen, K.; Merkel, T. J.; Pandya, A.; Napier, M. E.; Luft, J. C.; Daniel, W.; Sheiko, S.; DeSimone, J. M. Low modulus biomimetic microgel particles with high loading of hemoglobin. *Biomacromolecules* **2012**, *13*, 2748–2759.
- (11) Tomita, D.; Kimura, T.; Hosaka, H.; Daijima, Y.; Haruki, R.; Ludwig, K.; Böttcher, C.; Komatsu, T. Covalent core-shell architecture of hemoglobin and human serum albumin as an artificial O<sub>2</sub> carrier. *Biomacromolecules* **2013**, *14*, 1816–1825.
- (12) Vincent, J. L.; Privalle, C.T.; Singer, M.; Lorente, J. A.; Boehm, E.; Meier-Hellmann, A.; Darius, H.; Ferrer, R.; Sirvent, J. M.; Marx, G.; DeAngelo, J. Multicenter, randomized, placebo-controlled phase III study of pyridoxalated hemoglobin polyoxyethylene in distributive shock (PHOENIX). *Crit. Care Med.* **2015**, *43*, 57-64.

- (13) Manjula, B. N.; Tsai, A.; Upadhyaya, R.; Perumalsamy, K.; Smith, P. K.; Malavalli, A.; Vandegriff, K.; Winslow, R. M.; Intaglietta, M.; Prabhakaran, M.; Friedman, J. M.; Acharya, A. S. Site-specific PEGylation of hemoglobin at Cys-93( $\beta$ ): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjug. Chem.* **2003**, *14*, 464–472.
- (14) Vandegriff, K. D.; Malavalli, A.; Wooldridge, J.; Lohman, J.; Winslow, R. M. MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion* **2003**, *43*, 509-516.
- (15) Manjula, B. N.; Tsai, A. G.; Intaglietta, M.; Tsai, C. H.; Ho, C.; Smith, P. K.; Perumalsamy, K.; Kanika, N. D.; Friedman, J. M.; Acharya, S. A. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function. *Protein J.* **2005**, *24*, 133–146.
- (16) Modery-Pawlowski, C. L.; Tian, L. L.; Pan, V.; Sen Gupta, A. Synthetic approaches to RBC mimicry and oxygen carrier systems. *Biomacromolecules* **2013**, *14*, 939–948.
- (17) Abu Jawdeh, B. G.; Woodle, E. S.; Leino, A. D.; Brailey, P.; Tremblay, S.; Dorst, T.; Abdallah, M. H.; Govil, A.; Byczkowski, D.; Misra, H.; Abuchowski, A.; Alloway, R. R. A phase Ib, open-label, single arm study to assess the safety, pharmacokinetics, and impact on humoral sensitization of SANGUINATE infusion in patients with end-stage renal disease. *Clin. Transplant.* **2018**, *32*, e13155.
- (18) Abuchowski, A. SANGUINATE (PEGylated carboxyhemoglobin bovine): Mechanism of action and clinical update. *Artif. Organs* **2017**, *41*, 346-350.

- (19) Misra, H.; Lickliter, J.; Kazo, F.; Abuchowski, A. PEGylated carboxyhemoglobin bovine (SANGUINATE): results of a phase I clinical trial. *Artif. Organs* **2014**, *38*, 702-707.
- (20) Natanson, C.; Kern, S. J.; Lurie, P.; Banks, S. M.; Wolfe, S. M. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA* **2008**, *299*, 2304–2312.
- (21) Sakai, H.; Hara, H.; Yuasa, M.; Tsai, A. G.; Takeoka, S.; Tsuchida, E.; and Intaglietta, M. Molecular dimensions of Hb-based O<sub>2</sub> carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol.* **2000**, *279*, H908–915.
- (22) Yu, B. L.; Shahid, M.; Egorina, E. M.; Sovershaev, M. A.; Raher, M. J.; Lei, C.; Wu, M. X.; Bloch, K. D.; Zapol, W. M. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. *Anesthesiology* **2010**, *112*, 586-594.
- (23) Lui, F. E.; Yu, B. L.; Baron, D. M.; Lei, C.; Zapol, W. M.; Kluger, R. Hemodynamic responses to a hemoglobin bis-tetramer and its polyethylene glycol conjugate. *Transfusion* **2012**, *52*, 974-982.
- (24) Alayash, A. I. Mechanisms of toxicity and modulation of hemoglobin-based oxygen carriers (HBOCs). *Shock* **2017**, published online, doi: 10.1097/SHK.0000000000001044.
- (25) Yamaguchi, T.; Adachi, K. Hemoglobin equilibrium analysis by the multiangle laser light-scattering method. *Biochem. Biophys. Res. Commun.* **2002**, *290*, 1382–1387.

- (26) Acharya, S. A.; Intaglietta, M.; Tsai, A. G.; Meng, F. In *Hemoglobin-Based Oxygen Carriers as Red Cell Substitutes and Oxygen Therapeutics*; Kim, H. W., Greenburg, A. G., Eds.; Springer-Verlag: Berlin, 2013; pp 185–234.
- (27) Huang, Y. X.; Wu, Z. J.; Huang, B. T.; Luo, M. Pathway and mechanism of pH dependent human hemoglobin tetramer–dimer–monomer dissociations. *PLoS One* **2013**, *8*, e81708.
- (28) Caccia, D.; Ronda, L.; Frassi, R.; Perrella, M.; Del Favero, E.; Bruno, S.; Pioselli, B.; Abbruzzetti, S.; Viappiani, C.; Mozzarelli, A. PEGylation promotes hemoglobin tetramer dissociation. *Bioconjug. Chem.* **2009**, *20*, 1356–1366.
- (29) Walder, J. A.; Walder, R. Y.; Arnone, A. Development of antisickling compounds that chemically modify hemoglobin S specifically within the 2,3-diphosphoglycerate binding-site. *J. Mol. Biol.* **1980**, *141*, 195-216.
- (30) Fernandez, E. J.; Abad-Zapatero, C.; Olsen, K. W. Crystal structure of Lys $\beta$ (1)82-Lys $\beta$ (2)82 crosslinked hemoglobin: a possible allosteric intermediate. *J. Mol. Biol.* **2000**, *296*, 1245–1256.
- (31) Sakai, H.; Sou, K.; Tsuchida, E. Hemoglobin-vesicles as an artificial oxygen carrier. *Methods Enzymol.* **2009**, *465*, 363–384.
- (32) Sakai, H.; Hamada, K.; Takeoka, S.; Nishide, H.; Tsuchida, E. Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol. Prog.* **1996**, *12*, 119–125.
- (33) White, F. L.; Olsen, K. W. Effect of crosslinking on the thermal stability of hemoglobin. I. The use of bis-(3,5-dibromosalicyl) fumarate. *Arch. Biochem. Biophys.* **1987**, *258*, 51–57.

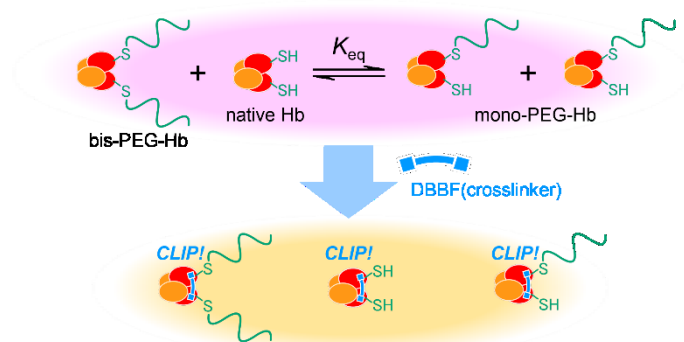
- (34)Osaki, S.; Yamamoto, K.; Matsuhira, T; Sakai, H. The effects of seasonal changes on the molecular weight of *Nephila clavata* spider silk, *Polym. J.* **2016**, *48*, 659–663.
- (35)Lui, F. E.; Dong, P.; Kluger, R. Polyethylene glycol conjugation enhances the nitrite reductase activity of native and cross-linked hemoglobin. *Biochemistry* **2008**, *47*, 10773–10780.
- (36)Macleod, R. M.; Hill, R. J. Demonstration of the hybrid hemoglobin  $\alpha_2^A\beta^A\beta^S$ . *J. Biol. Chem.* **1973**, *248*, 100–103.
- (37)Valdes, R. Jr. Modification of human haemoglobin with glucose 6-phosphate enhances tetramer–dimer subunit dissociation. *Biochem. J.* **1986**, *239*, 769–772.
- (38)Li, D.; Hu, T.; Manjula, B. N.; Acharya, S. A. Non-conservative surface decoration of hemoglobin: influence of neutralization of positive charges at PEGylation sites on molecular and functional properties of PEGylated hemoglobin. *Biochim. Biophys. Acta* **2008**, *1784*, 1395–1401.
- (39)Bova, M. P.; Ding, L. L.; Horwitz, J.; Fung, B. K. Subunit exchange of  $\alpha$ A-crystallin. *J. Biol. Chem.* **1997**, *272*, 29511-29517.
- (40)Cai, S. J.; Inouye, M. Spontaneous subunit exchange and biochemical evidence for *trans*-autophosphorylation in a dimer of *Escherichia coli* histidine kinase (EnvZ). *J. Mol. Biol.* **2003**, *329*, 495-503.
- (41)Sobott, F.; Benesch, J. L.; Vierling, E.; Robinson, C. V. Subunit exchange of multimeric protein complexes. Real-time monitoring of subunit exchange between small heat shock proteins by using electrospray mass spectrometry. *J. Biol. Chem.* **2002**, *277*, 38921-38929.

- (42) Darke, P. L.; Jordan, S. P.; Hall, D. L.; Zugay, J. A.; Shafer, J. A.; Kuo, L. C. Dissociation and association of the HIV-1 protease dimer subunits: equilibria and rates. *Biochemistry* **1994**, *33*, 98-105.
- (43) Portöro, I.; Kocsis, L.; Hermán, P.; Caccia, D.; Perrella, M.; Ronda, L.; Bruno, S.; Bettati, S.; Micalella, C.; Mozzarelli, A.; Varga, A.; Vas, M.; Lowe, K. C.; Eke, A. Towards a novel haemoglobin-based oxygen carrier: Euro-PEG-Hb, physico-chemical properties, vasoactivity and renal filtration. *Biochim. Biophys. Acta* **2008**, *1784*, 1402–1409.
- (44) Misra, H.; Bainbridge, J.; Berryman, J.; Abuchowski, A.; Galvez, K. M.; Uribe, L. F.; Hernandez, A. L.; Sosa, N. R. A Phase Ib open label, randomized, safety study of SANGUINATE™ in patients with sickle cell anemia. *Rev. Bras. Hematol. Hemoter.* **2017**, *39*, 20–27.
- (45) Li, D.; Manjula, B. N.; Acharya, A. S. Extension arm facilitated PEGylation of hemoglobin: correlation of the properties with the extent of PEGylation. *Protein J.* **2006**, *25*, 263–274.
- (46) Highsmith, F. A.; Driscoll, C. M.; Chung, B. C.; Chavez, M. D.; Macdonald, V. W.; Manning, J. M.; Lippert, L. E.; Berger, R. L.; Hess, J. R. An improved process for the production of sterile modified haemoglobin solutions. *Biologicals* **1997**, *25*, 257–268.
- (47) Hu, D.; Kluger R. Functional cross-linked hemoglobin bis-tetramers: geometry and cooperativity. *Biochemistry* **2008**, *47*, 12551–12561.



- (48) Sehgal, L. R.; Rosen, A. L.; Gould, S. A.; Sehgal, H. L.; Moss, G. S. Preparation and *in vitro* characteristics of polymerized pyridoxylated hemoglobin. *Transfusion* **1983**, *23*, 158–162.
- (49) Boykins, R. A.; Buehler, P. W.; Jia, Y.; Venable, R.; Alayash, A. I. *O*-raffinose crosslinked hemoglobin lacks site-specific chemistry in the central cavity: structural and functional consequences of  $\beta$ 93Cys modification. *Proteins* **2005**, *59*, 840–855.
- (50) Meng, F.; Kassa, T.; Jana, S.; Wood, F.; Zhang, X.; Jia, Y.; D'Agnillo, F.; Alayash, A. I. Comprehensive biochemical and biophysical characterization of hemoglobin-based oxygen carrier therapeutics: All HBOCs are not created equally. *Bioconjug. Chem.* **2018**, *29*, 1560-1575.

## Table of contents graphic (TOC)



$K_{eq}$  value is obtained based on the ratios of "clipped" components.