Comprehensive Characterization of Tense and Relaxed State Glutaraldehyde Polymerized Bovine Hemoglobin as a Function of Cross-link Density

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April 28, 2020

Abstract

Previously, our lab developed high molecular weight (MW) tense (T) state glutaraldehyde polymerized bovine hemoglobins (PolybHbs) that exhibited reduced vasoactivity in several small animal models. In this work, we prepared PolybHb in the T- and relaxed (R) quaternary state with ultrahigh MW (> 500 kDa) with varying cross-link densities and investigated the effect of MW on key biophysical properties (i.e., O2 affinity, cooperativity coefficient, hydrodynamic diameter, polydispersity, polymer composition, viscosity, gaseous ligand-binding kinetics, autoxidation, and haptoglobin-binding kinetics). To further optimize current PolybHb synthesis and purification protocols, we performed a comprehensive meta-data analysis to evaluate correlations between procedural parameters (i.e. cross-linker:bovine Hb (bHb) molar ratio, gas/liquid exchange time, temperature during dithionite addition, and number of diafiltration cycles) and the biophysical properties of both T-state and R-state PolybHbs. Our results showed that, the duration of the fast-step autoxidation phase of R-state PolybHb increased with decreasing glutaraldehyde:bHb molar ratio. Additionally, T-state PolybHb exhibited significantly higher biomolecular rate constants for binding to haptoglobin and unimoleular O2 offloading rate constants compared to R-state PolybHb. The methemoglobin (metHb) level in the final product was insensitive to the molar ratio of glutaraldehyde to bHb for all PolybHb. During tangential flow filtration processing of the final product, 14 diafiltration cycles was found to yield the lowest metHb level.

1 Introduction

Hemoglobin (Hb)-based oxygen (O_2) carriers (HBOCs) are a class of O_2 therapeutics that are currently in development (A. F. Palmer & Intaglietta, 2014). Among the myriad of methods used to produce HBOCs, glutaraldehyde-based protein crosslinking is the most frequently employed due to its low cost. Glutaraldehyde has been used to synthesize commercial polymerized bovine Hb (PolybHb) based HBOCs (Oxyglobin® (Cabrales, Tsai, & Intaglietta, 2008) and Hemopure® (Rice et al., 2008); Biopure Corporation, Cambridge, MA) and polymerized human Hb (PolyhHb) based HBOCs (PolyHeme® (Day, 2003; Sehgal, Gould, Rosen, Sehgal, & Moss, 1984); Northfield Laboratories, Evanston, IL). O-raffinose, another common cross-linking agent, has also been used to synthesize PolyhHb (Hemolink (Cheng et al., 2004; Leytin, Mazer, Mody, Garvey, & Freedman, 2003); Hemosol Ltd, Toronto, Canada). Due to the observation of deleterious side-effects in phase III clinical trials, which included vasoconstriction, systemic hypertension and oxidative tissue injury, none of these commercial products are FDA approved for clinical applications in the United States (Moradi, Jahanian-Najafabadi, & Roudkenar, 2016).

Vasoactivity via nitric oxide (NO) scavenging and oxidative tissue injury via tissue deposition of iron was determined to result from extravasation of low molecular weight (MW) polymerized Hb (PolyHb) and tetrameric Hb ($\alpha_2\beta_2$) from the blood vessel lumen into the tissue space (Marret et al., 2004). For example, the aforementioned commercial PolyHbs with an average MW of 150-250 kDa and 1-5% unmodified Hb induced hypertension, stroke, myocardial infarction, renal toxicity, and even death in clinical trials (Gould & Moss, 1996; Levy et al., 2002; Marret et al., 2004; Napolitano, 2009b). The harmful side-effects of these small molecular diameter commercial PolyHbs in clinical trials underscore the need to eliminate these low MW fractions in future generations of PolyHbs. (Meng et al., 2018a). Moving forward, revived interest in PolyHb-based HBOCs as oxygen therapeutics must incorporate the lessons learned from these failed trials. Zhang*et al.* integrated these lessons into their method of synthesis and purification of PolyHb by polymerizing hHb at higher glutaraldehyde:hHb molar ratios and removing low MW PolyHb species with a high cutoff MW filter (Zhang, Jia, Chen, Cabrales, & Palmer, 2011). The PolyhHb synthesized in that study had an average MW of 1.1 - 18 Mda. Similar to Zhang et al.'s work, Zhou et al. synthesized PolybHb at various glutaradehyde:bHb molar ratios which had an averaged MW of 0.1 – 6.3 MDa (Zhou et al., 2011). The small library of glutaraldehyde polymerized bHb (PolybHb) evaluated by Baek *et al.* demonstrated a direct correlation between the cross-link density (i.e. glutaraldehyde:Hb molar ratio) and PolybHb MW (Baek et al., 2012). Administration of these PolybHbs *in vivo* confirmed the vasoactive effects of low MW PolybHbs. Low MW PolybHb also displayed reduced circulatory lifetime, and increased renal tissue deposition (Baek et al., 2012). Therefore, PolybHbs with MW greater than 500 kDa are expected to be less vasoactive and exihibit less tissue toxicity compared to PolybHbs with MW under 500 kDa. Unfortunately, not all PolybHb fractions greater than 500 kDa are equally safe. For PolybHb fractions greater than 500 kDa, there is an inverse relationship between PolybHb MW and vasoactivity at the same concentration (Baek et al., 2012). This inverse ratio also suggests that the polymer distribution has an impact on the toxiciological properties of HBOCs (Baek et al., 2012; Cabrales et al., 2009; Rice et al., 2008).

In this work, we synthesized a library of low O_2-affinity tense quarternary state (T-state) PolybHbs and high O_2-affinity relaxed quarternary state (R-state) PolybHbs of varying sizes with low batch-to-batch variability. These materials had very low levels of small MW PolybHbs and improved batch-to-batch consistency of biophysical properties via clarification with a 0.2 μm hollow fiber (HF) filter and diafiltration with a 500 kDa HF filter (Cabrales, Zhou, Harris, & Palmer, 2010; A. F. Palmer, Sun, & Harris, 2009). To investigate the nano-structure of PolybHb, transmission electron microscopy (TEM) was performed for the first-time on our material. Furthermore, to optimize the PolybHb synthesis protocol, we conducted a meta-data analysis of the procedural data during both polymerization and TFF purification to evaluate the correlation between procedural parameters and PolybHb biophysical properties. For example, increasing the number of diafiltration cycles resulted in higher final product purity, but produced materials with high methemoglobin (metHb) levels since the longer processing time resulted in increased PolybHb oxidation. In this study, we found the optimal number of diafiltrations (i.e. 14 diacycles), that led to the lowest metHb level, while not significantly affecting overall product yield and purity. Such findings could be used as future guidance to minimize batch-to-batch variances when synthesizing PolyHbs.

2 Materials and methods

2.1 Materials.

Sodium citrate anticoagulated whole blood was collected aseptically from adult cattle (Quad Five, Ryegate, MT). Glutaraldehyde (70%), sodium lactate, sodium dithionite ($Na_2S_2O_4$), calcium chloride ($CaCl_2{\cdot}2H_2O$), potassium chloride (KCl), sodium cyanoborohydride ($NaCNBH_3$), sodium hydroxide (NaOH), sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), and sodium phosphate dibasic (Na_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO). HF modules of 50 kDa, 100 kDa, 500 kDa, and 0.2 μm (MW cut offs) were obtained from Spectrum Laboratories (Rancho Dominguez, CA). Acetonitrile (C_2H_3N), α-cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA), potassium cyanide (KCN), potassium

ferricyanide ($K_3Fe(CN)_6$) were procured from Fisher Scientific (Pittsburgh, PA). Coomassie brilliant blue was purchased from Bio-Rad Labs (Irvine, CA). 10-20% Novex^{TM} Tris-glycine gel, electrophoresis supplies and 0.2 μm syringe filters were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2 PolybHb Synthesis.

Bovine red blood cells (RBCs) were separated and washed by centrifugation with 0.9% saline and lysed with phosphate buffer (PB) (3.75 mM, pH 7.4). Tangential flow filtration (TFF) cartridges with MWCOs of 500 kDa and 50 kDa were then used to purify and concentrate bHb as described in the literature (Cabrales et al., 2010; A. F. Palmer et al., 2009).

Initially, 30 g of bHb was diluted in 1.5 L phosphate buffer saline (PBS, 0.1 M, pH 7.4) and placed into an airtight, amber-tinted reactor vessel with continuous stirring. To produce T-state PolybHb, the bHb solution was completely deoxygenated via continuous recirculation through the liquid side of a 3M MiniModule gas/liquid exchange module (Maplewood, MN) while the gas side was fed with pure nitrogen gas (N_2). The partial pressure of O_2 in solution (pO_2) was measured using a RapidLab 248 Blood Gas Analyzer (Siemens USA, Malvern, PA). When the $pO_2$was lower than 20.0 mm Hg, 300 mg of sodium dithionite dissolved in N_2 purged PBS (0.1 M, pH 7.4) was injected into the bHb solution via a needleless valve and allowed to mix for 15-20 minutes. To ensure complete deoxygenation of the bHb solution, four additional 1 mL injections of 50.0 mg sodium dithionite in N_2 purged PBS at pH 7.4 were delivered after the initial bolus injection. The polymerization of bHb in the T-state was not initiated until the pO_2 attained a value of 0.0 mm Hg.

Deoxygenated bHb was maintained throughout the T-state polymerization process by continuous purging of the reactor headspace with N_2. The same reactor vessel configuration was used to synthesize R-state PolybHb via complete oxygenation of bHb. Instead of using pure nitrogen on the gas side of the gas/liquid exchange membrane, pure O_2 was used. Polymerization was not initiated until the pO_2 was above 745 mm Hg. Oxygenation was maintained throughout the R-state polymerization process by continuous purging of the reactor headspace with O_2.

T- and R-state PolybHb were both polymerized at glutaraldehyde:bHb molar ratios of 25:1, 30:1, and 35:1. Glutaraldehyde was added dropwise to the constantly stirring bHb solution at a rate of 2 mL/min to achieve a total volume of 50.0 mL and then allowed to react for 2 hours at 37 °C. To quench the polymerization reaction, a bolus addition of $NaCNBH_3$ was injected into the reactor vessel to reduce the linkage of amine ligand and aldehyde (Schiff bases) and mixed for 30 minutes as the reactor temperature was cooled to ambient temperature (20 °C). $NaCNBH_3$ was prepared at a 7:1 molar ratio to glutaraldehyde in PBS (pH 7.4). The reactor vessel was then placed into a refrigerator and maintained at 4°C overnight. A schematic of the reactor setup, and timeline for reagent addition is shown in**Figure 1** .

2.3 PolybHb Clarification and Purification.

Both T- and R-state PolybHb were sterile filtered via TFF on a 0.2 μm HF module as described previously (Cabrales et al., 2010; Elmer, Harris, & Palmer, 2011). A schematic of this setup is shown in **Figure 2.**The PolybHb was then buffer exchanged into a modified lactated Ringer's solution (115 mM NaCl, 4 mM KCl, 1.4 mM $CaCl_2.2H_2O$, 13 mM NaOH, 12.3 mM N-acetyl-L-cysteine, 27 mM sodium lactate, pH 7.4) via diafiltration. 35:1 and 30:1 T-state PolybHb and 30:1 and 25:1 R-state PolybHb were diafiltered and concentrated on a 500 kDa HF module. 35:1 R-state PolybHb was diafiltered and concentrated on a 0.2 μm HF module. 25:1 T-state PolybHb was diafiltered and concentrated on a 100 kDa HF module to retain most of the product.

2.4 Characterization of PolybHb

2.4.1 Size Quantification

2.4.1.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

bHb and PolybHb samples were diluted to ~1 mg/mL in deionized (DI) water and mixed with tris-glycine buffer in a 1:1 v/v ratio. 100 µL of 1M diothiothreitol (DTT) was added to run the samples under reducing conditions. 20 µL of sample was loaded into a 10-20% Novex tris-glycine gel and incubated for 40 mins. Gels were stained with 1 × Coomassie brilliant blue and destained (3:1:6 v/v/v methanol:acetic acid:DI mixture) overnight. Images were obtained using an image scanner.

2.4.1.2 Dynamic Light Scattering (DLS).

The hydrodynamic diameter of bHb and PolybHb was measured using a BI-200SM Goniometer (Brookhaven Instruments, Holtsville, NY) as described in literature (Zhou, 2011). All bHb, T-state, and R-state PolybHb samples were diluted to ~ 1 mg/mL with deionized water.

2.4.1.3 Size Exclusion HPLC (HPLC-SEC).

The molecular weight (MW) distribution of bHb and PolybHb was estimated via a Thermo Scientific Dionex UltiMate 3000 UHPLC/HPLC system coupled with an Acclaim SEC-1000 column. The samples were filtered through a 0.2 µm syringe filter before injection into the column. Each sample was run at the flow rate of 0.35 mL/min in the mobile phase (PB, 50 mM, pH 7.4). To represent the size of PolybHb molecules, the polymer order (n_0) is defined as:

$$M = 2^{n_o} \quad (1)$$

where n_0 is an integer ranging from 0-5, and M is the total number of individual bHb molecules polymerized to yield one PolybHb molecule. The MW of each polymer order species was estimated by analyzing HPLC-SEC spectra with a deconvolution algorithm (Belcher, Cuddington, Martindale, Pires, & Palmer, 2020; Cuddington, Moses, Belcher, Ramesh, & Palmer, 2020). The quantification of the fraction of each polymer order was performed based on the absorbance at 413 nm.

2.4.1.4 Transmission Electron Microscopy (TEM) Analysis.

The structural morphology of PolybHb was analyzed via transmission electron microscopy (TEM) using a FEI Tecnai G2 Spirit TEM (FEI, Hillsboro, OR). 1 mL of PolybHb sample at ~100 mg/ml was first diluted to 0.2 mg/ml with DI water and vortexed to disaggregate high MW PolybHb polymers. Then the PolybHb solution was filtered through a 0.2 µm syringe filter to remove aggregates. 10 µL of the filtered solution was placed on a CF300-CU TEM grid (EMS, Hatfield, PA), dried overnight and imaged the next day. During imaging, the PolybHb grid was initially placed on a specimen holder and then inserted into the airlock cylinder. The condenser apertures was centered at a magnification of ~10000×, followed by direct alignment. The image of PolybHb was recorded at a voltage of 80 kV and 39000× magnification.

2.4.2 Rheology.

The viscosity of bHb and PolybHb were measured in a DV3T-CP viscometer with a cone spindle CPA-40Z (Brookfield AMETEK, Middleboro, MA) (Belcher et al., 2018). 0.5 mL PolybHb solution (~50 mg/mL) was loaded onto the cone spindle. All PolybHb samples were then measured at a shear rate of 150 s^{-1}.

2.4.3 Autoxidation Experiments.

The initial metHb level (%) of all samples was lower than 5%. Autoxidation kinetics at 37 °C was measured by incubating PolybHb samples in PB (50 mM, pH 7.4) over a 24 h period. To determine the autoxidation rate of bHb and PolybHbs (0.775 mM heme basis, the concentration of HBOCs in the systemic circuit after intravenous transfusion (Jahr, MacKenzie, Pearce, Pitman, & Greenburg, 2008)), the absorbance was monitored from 350-700 nm in parafilm-sealed cuvettes within a temperature-controlled HP 8452A diode

array UV-visible spectrometer (Olis, Bogart, GA). Extinction coefficients of oxyHb and metHb were used to determine the molar concentration of the corresponding species. First order autoxidation rate constants were estimated by performing a biphasic linear regression on the natural log of the normalized concentration of PolybHb/bHb.

2.4.4 Ligand-Binding Kinetics.

Fast kinetic measurements of gaseous ligand reactions with bHb, T-state, and R-state PolybHbs were carried out via an Applied Photophysics SF-17 micro-volume stopped-flow instrument (Applied Photophysics Ltd., Surrey, United Kingdom) (Belcher et al., 2018; Jahr et al., 2008; Rameez & Palmer, 2011). O_2 dissociation kinetics were determined by rapidly mixing 12.5 μM of bHb or PolybHbs (heme-based concentration) with sodium dithionite (1.5 mg/mL). The absorbance changes at 437.5 nm were recorded in 0.1 M PBS (pH 7.4, 25 °C). The O_2 dissociation kinetics traces were avergared and fit to monoexponential equations in the Applied Photophysics program.

For the analysis of haptoglobin (Hp) binding to bHb/PolybHb, a Hp mixture containing Hp2-1 and Hp2-2 was purified from human Cohn Fraction IV. The kinetics of Hp binding to bHb/PolybHbs was recorded as described in the literature (Baek et al., 2012; Meng et al., 2018b). To calculate Hp-Hb binding biomolecular rate constants, the fluorescence emission was measured by exciting at 285 nm and moniotring the emission at 310 nm. The resulting fluorescence intensity was fit to a monoexponential equation to determine the pseudo-first-order Hp-Hb binding rate constant. To determine the bimolecular rate constant, a linear fit was then performed on the pseudo first order rate constant as a function of PolybHb/bHb concentration to regress the slope.

2.4.5 MetHb Level.

The metHb level of cell-free bHb, T- and R-state PolybHb was determined using the cyanomethemoglobin assay (Drabkin, DL ; Austin, 1935).

2.4.6 Circular Dichroism (CD) Spectroscopy.

The CD spectra of bHb, T-state, and R-state PolybHb were measured using an JASCO J-815 CD (JASCO, Easton, MD) spectrometer. All samples were first diluted to 0.5 mg/mL in PB (50 mM, pH 7.4). The CD specta was recorded from 190 nm to 260 nm in a quartz cuvette (1 mm path length) and analyzed by the JASCO software (Zhang et al., 2011).

2.4.7 MALDI-TOF Analysis.

Protein samples were diluted to 0.5 mg/mL protein basis in DI water. A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution was prepared in a 50% v/v mixture of acetonitrile (99.9%) and trifluoroacetic acid (0.1%). 1 μL of the mixture of the matirix and protein solution was deposited on a matrix assisted laser desorption/ionization (MALDI) plate and run in a Microflex MALDI-TOF MS system (Bruker, Billerica, MA).

2.4.8 Statistical Analysis.

Either t-test or one-way ANOVA was used to analyze the variance among all data, and a p-value of <0.05 was considered significant. A least-squares linear regression analysis was used to study the correlation between procedural parameters and PolybHb biophysical properties.

3 Results and Discussion

After synthesis and purification, the biophysical properties of PolybHb were characterized via various techniques. The resulting O_2 affinity, cooperativity coefficient, hydrodynamic diameter, ligand binding/release kinetics, autoxidation kinetics, and average MW are listed in **Table 1** .

3.1 Oxygen Affinity and Offloading.

In this study, the effect of the quaternary state of bHb during polymerization on oxygen affinity (P_{50}) and Hill cooperativity coefficient (n) were explored. **Figure 3A** displays the oxygen equilibrium curves of bHb, T- and R-state PolybHb. Fitting the OECs to the Hill equation yields a P_{50} of 41.3 ± 3.3 mm Hg, 43.4 ± 7.0 mm Hg and 38.9 ± 2.5 mm Hg for T-state PolybHb 25:1, 30:1, and 35:1, respectively. These P_{50} values are comparable to the commercial PolybHb HBOC-201® (˜38 mm Hg) (Biopure, Cambridge, MA) (J.S., M., & J.C., 2008; Pearce, Gawryl, Rentko, Moon-Massat, & Rausch, 2006). The P_{50}s of the T-state PolybHbs prepared in this study are, on average, higher than the commercial human PolyHbs, PolyHeme® (28-30 mm Hg) (Northfield Laboratories, Evanston, IL) and Hemolink® (33.5 mm Hg) (Hemosol Inc., Toronto, Canada) (Lou Carmichael et al., 2000; Rice et al., 2008). The right-shifted O_2 equilibrium curve of T-state PolybHb can be ascribed to deoxygenation of bHb during PolybHb synthesis. Conversely, the left-shifted O_2 equilibrium curve of R-state PolybHb was due to oxygenation of bHb during PolybHb synthesis. Interestingly, the molar ratio of glutaraldehyde to bHb during polymerization did not have a significant effect on the P_{50} and n ($p < 0.05$). In comparison to T-state PolyhHbs and unmodified bHb, R-state PolybHbs exhibit much lower P_{50}s. In **Figure 3C** , we found significant differences ($p < 0.05$) between the P_{50} of bHb and PolybHb in both quaternary states. In **Figure 3D** , the Hill cooperativity coefficient showed no significant difference ($p < 0.05$) between T- and R-state PolybHbs. Both T- and R-state PolybHbs displayed no cooperativity (n˜1.0), which is lower than PolyHeme® (1.7) and HBOC-201® (1.4) (Day, 2003; Lou Carmichael et al., 2000; Napolitano, 2009a). The absence of cooperativity implies that the α and β globin subunits can no longer go through conformational changes which is indicative of the higher cross-link density of PolybHb in this study. This can be explained by the "locking effect" that the protein cross-linker glutaraldehyde has on the quaternary structure of Hb (Buehler et al., 2005).

Figure 3B shows the O_2 offloading kinetics for bHb and PolybHbs at various glutaraldehyde:bHb molar ratios. T-state PolybHbs released O_2 faster than R-state PolybHbs. Both T-state and R-state PolybHbs released O_2 slower than native bHb. **Figure 3E** displays the effect of the glutaraldehyde:bHb molar ratio on $k_{off,\ O_2}$. R-state PolybHb 25:1 exhibited a significantly higher$k_{off,\ O_2}$ than R-state PolybHb 30:1 and 35:1, indicating that the $k_{off,\ O_2}$ of R-state PolybHb can be engineered by varying the glutaraldehyde:bHb molar ratio. The$k_{off,\ O_2}$ of T-state PolybHb was not sensitive to the glutaraldehyde:bHb molar ratio.

Figure 3F displays the effect of quaternary state on$k_{off,\ O_2}$. T-state PolybHb possessed significantly higher $k_{off,\ O_2}$ than R-state PolybHb ($p < 0.05$), suggesting that the $k_{off,\ O_2}$ of PolybHb can be modified by maintaining the quaternary state of bHb before and during polymerization. The $k_{off,\ O_2}$ of bHb was significantly higher than PolybHb ($p < 0.05$), suggesting that polymerization regulates O_2 offloading kinetics. As a result, PolybHbs will not inherit the cooperative O_2offloading feature of native bHb. In comparison to Hemolink® (130 ± 3.5 s^{-1}) and Oxyglobin® (61.8 ± 1.6 s^{-1}) (Chen, Chen, Liou, & Chao, 2015; Jia, Ramasamy, Wood, Alayash, & Rifkind, 2004), both T-state and R-state PolybHbs synthesized at higher cross-link density with negligible fraction of free bHb resulted in a reduced $k_{off,\ O_2}$. R-state PolybHb exhibited increasing $k_{off,\ O_2}$ with decreasing glutaraldehyde:bHb molar ratio.

3.2 Size and Molecular Weight.

Figure 4A displays the size distribution of bHb, T-state PolybHb, and R-state PolybHb at glutaraldehyde:bHb molar ratios of 25:1, 30:1, and 35:1. All PolybHb solutions contained negligible free Hb tetramers (0 to 1.2%, 0^{th} order species, i.e. free Hb), which is considerably less than previous PolybHbs synthesized

from our lab (Zhou et al., 2011). A right-shifted distribution of PolybHb MW was observed with increasing glutaraldehyde:bHb molar ratio, suggesting that the size of PolybHb is tunable. Both 35:1 T-state and 35:1 R-state PolybHb possessed the highest average MW (1194 ± 106 kDa, 1316 ± 81 kDa respectively) compared to the other PolybHbs synthesized in this study. However, the increased size of 35:1 R-state PolybHb resulted in negligible passage through 0.2 μm HF filter modules. Zhang *et al* . synthesized 40:1 and 50:1 T-state PolyhHb which had a weight averaged MW of 3.7 and 18 MDa, respectively (Zhang et al., 2011). Since the PolyhHbs in that study were synthesized at higher glutaraldehyde:Hb molar ratios than the 30:1 ratio in this study, it should have contained a larger fraction of polymers > 0.2 μm in size than the PolybHbs produced in this study. Previously, Liu *et al* . found that nanoparticles with the diameter > 300 nm tended to trigger macrophage uptake in the reticuloendothelial system (Liu, Mori, & Huang, 1992). Thus, the PolybHbs synthesized in this study may be less likely to be quickly cleared versus PolyHbs that were synthesized in previous work (Zhang et al., 2011). In **Figure 4B** , analysis of the polymer order of the majority of T-state and R-state PolybHbs yielded less than 1% free bHb in solution (0^{th} order, 1.31 ± 0.87%). In comparision, the commercial HBOCs, PolyHeme® and Hemopure® contained less than 5% of free Hb (Levy et al., 2002; Marret et al., 2004). The average MW of PolyHeme® and Hemopure® were 250 kDa and 150 kDa, respectively. Unfortunately, both commercial HBOCs induced severe hypertension when transfused *in vivo* . Fortunately, 35:1 T-state and R-state PolybHbs possessed MWs 4-fold higher than PolyHeme®and Hemopure® (Gould et al., 1998; Moore et al., 2009).

All PolybHbs have less than 0.9% of 128 kDa 1^{st} order polymers (0.14 +- 0.32%). T-state PolybHb at a 25:1 cross-link density contained a significantly higher fraction of 3^{rd} and 4^{th} order PolybHb (27.6 +- 8.7%; 51.7 +- 2.0%) than those at a 30:1 cross-link density (8.7 +- 3.7%; 33.1 +- 2.1%) and 35:1 cross-link density (5.0 +- 3.4%; 22.2 +- 2.9%). All PolybHbs possessed similar amounts of 5^{th} order polymers (25.8 +- 6.9%). The fraction of 5^{th} order polymers in both the T- or R-state PolybHb increased with increasing glutaraldehyde:bHb molar ratio. The fraction of lower polymer orders (2^{nd} and 3^{rd}) exhibited the opposite trend, since smaller polymers were crosslinked to form larger clusters with increasing glutaraldehyde:bHb molar ratio. Previously, it was observed that the presence of free Hb was responsible for the renal toxicity, vasoconstriction, and hypertension observed when PolyHb was transfused*in vivo* [26, 37–39]. Thus, both T-state and R- state PolybHb synthesized in this study are unlikely to induce those side-effects, because of the extremely low levels of stroma-free bHb in the final products. It is interesting to note that the 35:1 T-state PolybHb and 30:1 R-state PolybHb have a similar MW distribution even though they were syntheszied at different glutaraldehyde:bHb molar ratios. The shift in MW distribution observed via HPLC-SEC is consistent with the hydrodynamic diameter measured via DLS and is shown in **Figure 4D** . In contrast, **Figure 4B** shows that there was a minor difference between the polymer-order composition of T-state PolybHb 35:1 and R-state PolybHb 30:1. T-state PolybHb 35:1 contained a higher fraction of 2^{nd} and 5^{th} order polymers compared to R-state PolybHb 30:1.

Figure 4C shows the SDS-PAGE of bHb, T-state PolybHb, and R-state PolybHb synthesized in this study. All lanes show a band at 32 kDa, representing $\alpha\beta$ dimers. For bHb (control) in lane 1, a clear band at approximately 16 kDa was observed, which indicates the presence of α (15.043 kDa) and β (15.946 kDa) subunits that dissociated from tetrameric native bHb ($\alpha_2\beta_2$). Lanes 2 and 3 display both T-state and R-state PolybHb at a 25:1 glutaraldehyde:bHb molar ratio, where T-state PolybHb migrated as a broader band compared to R-state PolybHb. This pattern is also observed at other glutaraldehyde:bHb molar ratios (30:1 and 35:1). This finding aligns with the polymer order distribution in **Figure 4B** as in general, T-state PolybHb had a smaller fraction of $4^{th}$and 5^{th} order of polymers compared to R-state PolybHb at the same cross-link density. Within the same quaternary state in lanes 3, 6 and 9 (or 2, 5 and 8), the MW of T-state (or R-state) PolybHb increased with increasing glutaraldehyde:bHb molar ratio.

Figure 4D displays the hydrodynamic diameter of bHb and PolybHb. The diameter of PolybHb grew with increasing glutaraldehyde:bHb molar ratio, which is consistent with **Figures 4A, 4B and 4C** . Both 35:1 T-state and 35:1 R-state PolybHb had the largest effective hydrodynamic diameters (64.1 ± 9.3 and 166.9 ± 4.1 nm, respectively) compared to 25:1 and 30:1 PolybHbs. PolybHb is 5-10 times on average larger in size compared to Oxyglobin® (5.85–10.49 nm), Hemolink® (5.28–11.13 nm), HBOC-201® (5.73–11.10

nm) and PolyHeme® (5.59–10.31 nm) (Day, 2003; R. M. J. Palmer, Ferrige, & Moncada, 1987). In**Table 1** , the polydispersity index (PDI) of PolybHb is shown. All PolybHbs possess a PDI lower than 0.4, which corresponds to moderate polydispersity.

3.3 Viscosity.

The viscosity of T-state and R-state PolybHbs synthesized in this study is listed in **Table 1** . Increasing the glutaraldehyde:bHb molar ratio increased the viscosity of PolybHbs. Both T-state PolybHb 35:1 and R-state PolybHb 30:1 possesed a higher viscosity at 5 g/dL compared to PolyHeme® (˜2.1 cP) and Hemopure® (˜1.3 cP) at approximately a protein concentration of 10 g/dL (Napolitano, 2009b). PolybHbs with high viscosity are desirable for intravenous transfusion, since they induce the release of endothelial-derived relaxing factors (EDRFs) via mechanotransduction, which might further mitigate the vasoconstrictive effects due to PolyHb NO scavenging or O_2 oversupply (R. M. J. Palmer et al., 1987). However, the ultra-high viscosity of R-state PolybHb 35:1 might induce retinal dilation and conjunctival hemorrhage if transfused *in vivo* (Gertz & Kyle, 1995). Thus, R-state PolybHb 35:1 may not serve as an ideal RBC substitute.

3.4 PolybHb-Haptoglobin Binding Kinetics.

Free Hb in plasma tends to dissociate into two pairs of αβ dimers, which are scavenged via the Hb binding protein haptoglobin (Hp). The resulting Hb-Hp complex can then bind to the CD163+ macrophages and monocytes. The CD163 receptor scavenges both Hb and Hb-Hp and essentially cleares Hb from the systemic circulation by receptor endocytosis into macrophages and monocytes (Etzerodt & Moestrup, 2013). To determine the potential for PolybHb to be cleared via CD163 mediated endocytosis, the ligand-binding kinetics of Hp with bHb/PolybHb was monitored by rapidly mixing Hp with bHb/PolybHbs at various concentrations.

The kinetics of Hp-bHb/PolybHb binding is shown in **Figure 5A** . It was evident that both T-state and R-state PolybHbs quenched a lower number of Hb binding sites in Hp compared to bHb. By fitting the kinetic traces in **Figure 5A** to a mono-exponential equation, the pseudo first order binding rate constants at different concentrations of bHb and PolybHbs were plotted in **Figure 5B** . To calculate the 2nd order (bHb/PolybHb)-Hp binding rate constant ($k_{\text{Hp-Hb}}$), we performed a linear fit to the data in **Figure 5B** to determine the slope of the pseudo first order reaction rate constant as a function of PolybHb/bHb concentration. Overall, the $k_{\text{Hp-Hb}}$ of T-state (0.0136 – 0.0228 μM^{-1} s^{-1}) and R-state PolybHbs (0.0069 – 0.0097 μM^{-1} s^{-1}) were significantly reduced compared to unmodified bHb (0.145 μM^{-1} s^{-1}). The$k_{\text{Hp-Hb}}$ of Hp-bHb binding was consistent with a previously reported value of 0.15 $\mu M^{-1}s^{-1}$ (Meng et al., 2018a). The$k_{\text{Hp-Hb}}$ of T-state PolybHb was significantly ($p < 0.05$) greater than that of R-state PolybHb at the same glutaraldehyde:bHb molar ratio. The $k_{\text{Hp-Hb}}$ for 25:1 and 30:1 R-state PolybHb were lower than Oxyglobin® (0.011 μM^{-1} s^{-1}) as reported previously in the literature (Meng et al., 2018a). The $k_{\text{Hp-Hb}}$for 25:1 T-state PolybHb is similar to Oxyglobin® (0.011 μM^{-1} s^{-1}) (Meng et al., 2018a). The$k_{\text{Hp-Hb}}$ of the rest of PolybHbs was similar to Oxyglobin® (0.011 μM^{-1} s^{-1}) (Meng et al., 2018a).

3.5 Autoxidation Rate.

Autoxidation of bHb, T-state PolybHb, and R-state PolybHb is shown in**Figure 5C and 5D** . We found that both T- and R-state PolybHbs displayed two-phase autoxidation kinetics which was not observed for native bHb. The fast-step autoxidation rate constant of all PolybHbs except 25:1 T-state PolybHbs was higher than that of bHb ($k_{ox} = 0.021 \pm 0.003$ h^{-1}). This finding aligns with previous studies which demonstrated that HBOCs with chemical modifications exhibit higher autoxidation rate constants compared to unmodified Hb (Gertz & Kyle, 1995; Meng et al., 2018a). For the slow-step autoxidation kinetics, all PolybHbs yielded a decreased rate of autoxidation compared to native bHb. R-state PolybHbs had autoxidation rates 2-fold lower than that of bHb. These results might be attributed to the different autoxidation rate between α and β subunits (Tsuruga, Matsuoka, Hachimori, Sugawara, & Shikama, 1998). The slow-step autoxidation rate

constant of T-state PolybHbs (<0.0162 h^{-1}) was higher than that of R-state PolybHbs (<0.0069 h^{-1}). The higher autoxidation rate constants of T-state PolybHbs compared to R-state PolybHb are consistent with that of previously reported HBOCs (Zhang et al., 2011). For instance, it was reported that 50:1 T-state PolybHb had an increased k_{ox} in comparison to 40:1 R-state PolybHb (Zhang et al., 2011). For T-state PolybHb, the duration of slow-step phase was found to increase when decreasing the glutaraldehyde:bHb molar ratios.

3.6 CD Spectra Analysis.

Figure 6A displays the CD spectra of both T-state and R-state PolybHb in the far-UV region (190 - 260 nm). No significant differences ($p < 0.05$) between PolybHb and bHb were observed, suggesting that the secondary structure of bHb remained unchanged in both T-state and R-state PolybHbs.

3.7 MALDI-TOF MS Analysis.

The results of MALDI-TOF mass spectral analysis is shown in**Figure 6B, 6C and 6D** . In **Figure 6B** , the spectrum in the 0-3000 Da m/z mass range for bHb exhibited a peak around 616 Da that corresponded to the heme group (Bonaventura, Henkens, Alayash, & Crumbliss, 2007; Etzerodt & Moestrup, 2013). A heme peak around 616 Da was also observed for both T- and R-state PolybHb along with other unidentified low abundance peaks in the 0-3000 Da m/z range (data not shown).

Figure 6C displays the mass spectra of bHb and R-state PolybHb in the in the 10,000-80,000 Da m/z mass range. The mass spectrum of bHb exhibited two distinct peaks at 15,043 and 15,946 m/z that correspond to the α and β subunits of bHb (Elmer et al., 2011). The spectra for all PolybHb showed a flat noisy line with low intensity in the mass range 20-80 kDa signifying the virtual absence of smaller species (αβ dimers or $\alpha_2\beta_2$ tetramers) in the polymerized samples. These results align with the findings from HPLC-SEC and SDS-PAGE analysis which showed that <1% free bHb was present in most of T- and R-state PolybHb preparations. Furthermore, the sample preparation conditions (acetonitrile and TFA) did not induce substantial Hb monomer formation as observed by the low-intensity peaks in the monomer region for PolybHb samples. This further confirmed that the crosslinked bHb was extremely stable and did not dissociate upon ionization in the mass spectormeter. In the inset of **Figure 6C** , R-state PolybHbs exhibited a low-intensity peak at 15,043 m/z, representing uncrosslinked α subunits (<1%). While for T-state PolybHb, two distinct low-intensity peaks were observed at both 15,043 and 15,946 m/z which correspond to the α and β subunits. This difference indicated that R-state PolybHb contains more β-β crosslinked polymers compared to T-state PolybHb.

3.8 Meta-Data Analysis.

In **Figure 7A** , for both T-state and R-state PolybHbs, the gas-liquid exchange contact time did not correlate with the metHb level. Figure 7B shows that increasing the temperature in the reactor during dithionite injection lead to higher metHb levels. In Figure 7C, while the PolybHb yield seemed to decrease linearly with the number of diafiltration cycles, no significant effect of diafiltration cycles on yield was observed ($p < 0.05$). Hence, we can enhance the purity of PolybHb without being concerned about lowering the PolybHb yield. Figure 7D displays the effect between the number of diafiltration cycles and metHb level, where a significant variance ($p < 0.05$) was found between the group with 14 diafiltraion cycles compared to others. 14 diafiltration cycles yielded the lowest metHb for both T-state and R-state PolybHb. Figure 7E and 7F show the correlation between glutaraldehyde:bHb molar ratio and yield of PolybHbs. R-state PolybHb 35:1 and T-state PolybHb 35:1 both exhibit significantly higher yields than the other molar ratios ($p<0.05$). Unfortunately, 35:1 R-state PolybHb was diafiltered on a 0.2 μm HF filter instead of a 500 kDa HF filter compared to the other PolybHbs. Thus, only 30:1 R-state PolybHb and 35:1 T-state PolybHb are desirable PolybHbs with clearly higher yield. In Figure 7G and 7H, neither the quaternary state nor glutaraldehyde:bHb molar ratio influenced the PolybHb metbHb level (3.7 ± 1.5 %).

4 Conclusion

In this study, high MW R-state and T-state PolybHb were synthesized with negligible amounts of free Hb and low MW polymer order species (i.e. 1^{st} order polymer, 128 kDa) in the final product, which is a significant improvement compared to previously failed commercial PolyHbs. Although the 35:1 T-state PolybHb and 30:1 R-state PolybHb had similar MW, the polymer order composition was found to be different. 35:1 T-state PolybHb was found to contain a higher fraction of 2^{nd} and 5^{th} order polymers than 30:1 R-state PolybHb. By using a 0.2 μm HF module for product clarification and sterile filtration, we were able to sterile filter the PolybHb solutions and remove ultrahigh MW polymer order species which might induce macrophage uptake in the reticuloendothelial system. For ligand-binding kinetics, T-state PolybHb exhibited significantly higher Hp-PolybHb binding rate constant and O_2 offloading rate constant compared to R-state PolybHb. MALDI analysis indicated that R-state PolybHbs contain more β-β crosslinked polymers compared to T-state PolybHb.

Furthermore, for synthesis and TFF optimization, our results show that the metHb level in the final product of T-state PolybHb was sensitive to the temperature during sodium dithionite addition. During TFF of the final product, 14 difiltration cycles was identified as being optimal, since it resulted in the lowest PolybHb metHb level. Taken together, these results provide comprehensive guidance for synthesis and purification of PolybHbs as a HBOC.

Acknowledgements

Dr. Crystal Bolden-Rush for helping with synthesizing PolybHb. Dr. Jim Lee (Dept. of Chemical and Biomolecular Engineering, The Ohio State University) for allowing us to use his dynamic light scattering (DLS) spectrometer. Pengfei Jiang for helping us to run the Tecnai G2 30 TWIN Transmission Electron Microscopse (Campus Microscopy & Imaging Facility, The Ohio State University). This work was supported by NIH grants: R56HL123015, R01HL126945, R01EB021926.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Parameter	bHb (n=7)	R 25:1 (n=4)	R 30:1 (n=11)	R 35:1† (n=3)	T 25:1* (n=3)	T 30:1 (n=5)	T 35:1 (n=10)
P_{50} (mm Hg)	25.8 ± 0.9	1.6 ± 0.6	1.2 ± 0.4	1.0 ± 0.4	41.4 ± 3.3	43.4 ± 7.0	38.9 ± 2.5
Cooperativity	2.6 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
MW (kDa)	64 ± 1	557 ± 19	1131± 97	1316 ± 81	484 ± 58	796 ± 13	1194 ± 106
D_{eff} (nm)	5.5 ± 0.7	44.2 ± 2.0	62.4 ± 12.5	166.9 ± 4.1	26.8 ± 6.2	49.9 ± 2.8	64.1 ± 9.3
PDI	0.20 ± 0.10	0.30 ± 0.01	0.24 ± 0.02	0.33 ± 0.05	0.26 ± 0.04	0.21 ± 0.00	0.19 ± 0.02
MetHb level (%)	1.0 ± 0.3	3.4 ± 1.3	3.7 ± 1.1	3.7 ± 1.1	3.2 ± 1.2	3.2 ± 1.2	3.2 ± 1.2
Viscosity (cP)	1.6‡	3.6 ± 0.7	9.9 ± 1.0	> 15.33¥	2.8 ± 0.3	9.2 ± 0.2	7.9 ± 2.0
$k_{off,\ O_2}$ (s^{-1})	44.86 ± 1.93	19.70 ± 0.73	15.12 ± 1.60	13.66 ± 1.86	26.78 ± 6.22	31.05 ± 6.00	35.13 ± 9.90
$k_{Hb\text{-}Hp}$ ($\mu M^{-1} s^{-1}$)	0.1448	0.0069	0.0076	0.0097	0.0136	0.0198	0.0228
$k_{ox,\ fast}$ (h^{-1})	0.021 ± 0.003	0.057 ± 0.039	0.063 ± 0.005	0.033 ± 0.027	0.008 ± 0.002	0.033 ± 0.008	0.090 ± 0.059
$k_{ox,\ slow}$ (h^{-1})	0.021 ± 0.012	0.008 ± 0.002	0.005 ± 0.001	0.004 ± 0.001	0.004 ± 0.002	0.017 ± 0.001	0.007 ± 0.003
Yield (%)	60.9 ± 0.08	26.4 ± 9.6	38.1 ± 11.0	48.3 ± 7.4	29.2 ± 1.1	29.8 ± 4.2	50.0 ± 9.7

Figure Legends

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