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Synthesis, Biophysical Properties and Pharmacokinetics of Ultrahigh Molecular Weight Tense and Relaxed State Polymerized Bovine Hemoglobins

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Abstract

Hemoglobin-based oxygen carriers (HBOC) are currently being developed as red blood cell (RBC) substitutes for use in transfusion medicine. Despite significant commercial development, late stage clinical results of polymerized hemoglobin (PolyHb) solutions hamper development. We synthesized two types of PolyHbs with ultrahigh molecular weights: tense (T) state PolyHb ($M_W = 16.59$ MDa and $P_{50} = 41$ mm Hg) and relaxed (R) state PolyHb ($M_W = 26.33$ MDa and $P_{50} = 0.66$ mm Hg). By maintaining Hb in either the T- or R-state during the polymerization reaction, we were able to synthesize ultrahigh molecular weight PolyHbs in distinct quaternary states with no tetrameric Hb, high viscosity, low colloid osmotic pressure and the ability to maintain O₂ dissociation, CO association and NO dioxygenation reactions. The PolyHbs elicited some *in vitro* RBC aggregation that was less than 6% dextran (500 kDa) but more than 5% human serum albumin. *In vitro*, T-state PolybHb autoxidized faster than R-state PolybHb as expected from previously reported studies, conversely, when administered to guinea pigs as a 20% exchange transfusion, R-state PolybHb oxidized faster and to a greater extent than T-state PolybHb, suggesting a more complex oxidative processes *in vivo*. Our findings also demonstrate that T-state PolybHb exhibited a longer circulating half-life, slower clearance and longer systemic exposure time compared to R-state PolybHb.

Introduction

In the United States, allogeneic red blood cell (RBC) transfusion has long been considered an important treatment option for patients suffering from blood loss [1]. However, the recent emergence of infectious agents such as the H1N1 influenza virus and others has put the blood supply at risk [2]. Currently, the American Red Cross tests donated blood for hepatitis B and C viruses, human immunodeficiency virus (HIV), human T-cell lymphotropic virus, syphilis, West Nile virus and the agent of Chagas disease [3-6]. As a result, the safety of the U.S. blood

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supply in terms of transfusion-transmitted diseases is quite good. However as new infectious agents emerge the costs of a unit of blood increases; since additional screening tests may have to be conducted before blood can be distributed to health care providers. Of more concern is the fact that donated blood may contain yet to be identified infectious agents [3]. Moreover, transfusion-related adverse events, both short- and long-term, are among the costliest contributors to health care expenditures [7]. In addition, there are new concerns regarding the safety of blood transfusions following extended durations of storage [8,9].

The safety of the blood supply in developing countries is even more problematic, since serious concerns still exist about the risks associated with blood transfusion including: potential contamination by blood-borne pathogens; fatal immunological reactions; acute lung injury and even mistransfusion [10]. To further compound the problem, the availability of human blood is even more limited in emergency situations such as wars or natural disasters [11]. Therefore, it has been a long-term goal of scientists and engineers to develop an efficacious and safe universal RBC substitute for use in transfusion medicine.

In the RBC, hemoglobin (Hb) is the protein responsible for storage and transport of oxygen and other gaseous ligands [12]. Hb is also the precursor material for synthesis/formulation of Hb-based oxygen carriers (HBOCs) that can be used as RBC substitutes [13-15]. It is not surprising that the first HBOC to be developed as a RBC substitute consisted of stroma-free Hb [16,17]. However, transfusion of Hb leads to two major side-effects [18-22].

In the circulation, tetrameric Hb ($\alpha_2\beta_2$) easily dissociates into two pairs of $\alpha\beta$ dimers [19,20], which are extremely prone to oxidation [22] and enhanced renal excretion [19,23,24]. The process of oxidation of Hb to methemoglobin (metHb) promotes unfolding of the globin chains and releases cytotoxic heme in the circulation leading to kidney tubule damage and eventual renal failure [19,20]. MetHb can also generate harmful reactive oxygen species (ROS) [18]. These ROS can initiate a series of oxidative cascades that can damage cell membranes, oxidize nucleic acids and proteins [21].

The presence of extracellular Hb in the circulatory system can also elicit vasoconstriction and systemic hypertension. This is thought to occur via two mechanisms [25-28]. The first hypothesis suggests that Hb can extravasate through the blood vessels and scavenge nitric oxide (NO), which acts as a vasodilator to the surrounding smooth muscle cells [25,26]. Another hypothesis promotes an "autoregulatory" response in which extracellular Hb facilitates oxygen transport in the lumen of the blood vessel and overoxygenates surrounding tissues, thereby eliciting vasoconstriction in order to reduce blood flow [27,28]. Regardless of the exact mechanism for the development of vasoconstriction and systemic hypertension, stroma-free Hb must be modified in order to eliminate or reduce the above adverse effects. Polymerization of Hb with difunctional cross-linking reagents can potentially resolve the concerns listed above, since polymerized Hbs (PolyHbs) will be larger in size compared to tetrameric Hb. The increased size of PolyHbs should be able to prevent the undesired extravasation/interaction of Hb through/with the blood vessel wall and prolong the HBOC's half-life [29].

Issues surrounding the safety of modified Hb used as RBC substitutes/Hb therapeutics remain an important focus of attention [30,31]. However, of equal and related importance is the design of modified Hbs with optimal pharmacokinetic behavior for their intended indications. For example, preparation of modified Hbs as short-term oxygen bridging agents in trauma and related disease requires rapid optimization of tissue oxygenation and a relatively short systemic exposure time. Conversely, preparation of modified Hbs for use as true RBC replacements in genetic/acquired anemia and when blood is unavailable requires long-term oxygen delivery with extended systemic exposure times.

Glutaraldehyde has been widely employed to non-specifically crosslink/polymerize Hb [32-35]. Recently, there have been two glutaraldehyde PolyHbs which have undergone phase III clinical trials. Hemopure® (HBOC-201) (Biopure Corp., Cambridge, MA) consists of polymerized bovine hemoglobin with a P₅₀ of 38 mm Hg and MW ranging from 130-500 kDa [13,36-38]. In contrast, PolyHeme® (Northfield Laboratories Inc., Evanston, IL) consists of a pyridoxylated polymerized human hemoglobin with a P₅₀ of 28-30 mm Hg and a MW ranging from 128-400 kDa [14,39,40].

Despite commercial development of glutaraldehyde-polymerized Hbs, hypertension and other important safety concerns remain critical impedances to further clinical use of HBOC-201 and PolyHeme® in the U.S. [41,42]. These safety issues may be attributed to vasoactivity and/or oxidative events caused by PolyHb solutions. Hb interactions with the vascular endothelium or sub-endothelium can occur with certain modified Hbs either by extravasation through or interaction with the vascular endothelium. Interactions may include NO scavenging, increased facilitated diffusion of oxygen to surrounding tissues and oxidative side reactions at the endothelial layer or within sub-endothelial compartments. Recently, it was pointed out that an acceptable HBOC should have a diameter of at least 7 nm in order to prevent extravasation [43] and reduce the facilitated diffusion of oxygen to surrounding tissues [27]. With this in mind, the next generation of PolyHbs should be synthesized with larger molecular weights compared to HBOC-201 and PolyHeme®.

To our knowledge, high molecular weight (MW) glutaraldehyde-polymerized Hbs frozen in a well-defined quaternary state have never been synthesized. In previous studies, the effect of glutaraldehyde concentration on the degree of Hb polymerization was quantitatively investigated [44,45]. It was shown that the degree of polymerization increased proportionally to the molar ratio of glutaraldehyde to Hb (G:Hb) [44]. However, in these studies Hb was not polymerized exclusively in a well-defined quaternary state [44,45].

In a recent study, we polymerized Hb exclusively in either the tense (T) or relaxed (R) quaternary states at different cross-link densities [46]. However, in this study no attempt was made to separate unpolymerized Hb from Hb polymers. Despite this fact, we demonstrated control over the PolyHb's oxygen affinity (P₅₀) and absolute MW. In another study, we separated T-state PolyHb mixtures that had been polymerized at different cross-link densities into two fractions: one above 500 kDa in MW and another below 500 kDa in MW [47]. We observed that the PolyHb fraction above 500 kDa in MW with the highest cross-link density (50:1) yielded no vasoconstriction and the lowest increase in the mean arterial pressure compared to other PolyHb fractions examined in this study [47]. The results from this previous study support Sakai et al.'s observations, in which it was shown that vasoconstriction and hypertension were inversely proportional to the size of the HBOC [29].

Therefore, synthesis/formulation of HBOCs with large molecular sizes may enhance Hb compartmentalization within the vascular space, extend exposure times and limit vasoconstriction/hypertension. This new design approach satisfies the two potential mechanisms for the development of vasoconstriction and hypertension upon administration of HBOCs and may optimize circulation times for extended duration therapeutic applications.

In this study, we synthesized ultrahigh MW PolyHbs in both the T- or R-state and characterized the biophysical, rheological, pharmacokinetic properties and *in vitro/in vivo* oxidative tendencies of these Hb preparations.

Methods

Materials

Glutaraldehyde (70%), NaCl, KCl, NaOH, Na₂S₂O₄, NaCl (USP), KCl (USP), CaCl₂·2H₂O (USP), NaOH (NF), sodium lactate (USP), N-acetyl-L-cysteine (USP), NaCNBH₃ and NaBH₄ were purchased from Sigma-Aldrich (Atlanta, GA). Sephadex G-25 resin was purchased from GE Healthcare (Piscataway, NJ). KCN, KFe(CN)₆, and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

In preparation for experiments, all glassware and plasticware were immersed in 1 mol/L NaOH solution for more than 6 hours to degrade any endotoxin present, followed by thorough rinsing with HPLC grade water.

Hb Purification

Fresh bovine blood stored in 3.8% sodium citrate solution at a final concentration of 90:10 v/v (bovine blood:sodium citrate solution) was purchased from Quad Five (Ryegate, MO). Bovine Hb (bHb) was purified from lysed bovine RBCs (bRBCs) via tangential flow filtration (TFF) [48,49]. bRBCs were initially washed 3 times with 3 volumes of isotonic saline solution (0.9%) at 4°C. bRBCs were subsequently lysed on ice with 2 volumes of hypotonic, 3.75 mM phosphate buffer (PB) at pH 7.4 for 1 hour. The RBC lysate was then filtered through a glass column packed with glass wool to remove the majority of cell debris. Clarified bRBC lysate was then passed through 50 nm and 500 kDa hollow fiber cartridges (Spectrum Labs, Rancho Dominguez, CA) to remove additional cell debris and impurity proteins. Purified bHb was collected and concentrated on a 100 kDa hollow fiber cartridge (Spectrum Labs) to yield the raw material for synthesis of polymerized bHb (PolybHb).

Polymerization of bHb

T-state PolybHb was synthesized according to a previously described method [46,47]. To generate fully deoxygenated or T-state bHb, 30 grams of purified bHb was diluted with PB (20 mM, pH 8.0) to yield 1200 mL of bHb solution. The bHb solution was placed inside an airtight bottle and connected to a vacuum manifold. The entire system was kept below 4°C in an ice water bath. The bHb solution was then subjected to several cycles of vacuum and argon (Ar) purging to remove the majority of O₂ from solution. After 4 hours of vacuum and Ar cycling, Na₂S₂O₄ solution (1.5 mg/mL) was titrated into the bHb solution with a syringe pump (Razel Scientific, St. Albans, VT), while the pO₂ of the solution was simultaneously measured using a RapidLab 248 (Siemens, Malvern, PA) blood gas analyzer until the pO₂ of the bHb solution attained a value of 0 mm Hg. At this point, an additional 30 mL of 1.5 mg/mL Na₂S₂O₄ solution was added to the T-state bHb solution to maintain the pO₂ at 0 mm Hg during and after the polymerization reaction. A 30 mL syringe was used to titrate glutaraldehyde preequilibrated with Ar into the sealed glass bottle under continuous stirring. A 50:1 molar ratio of glutaraldehyde to bHb was used for the T-state polymerization reaction.

Relaxed (R) state bHb was prepared in a similar manner to T-state bHb using the same vacuum manifold system. 1500 mL of 0.3 mmol/L bHb solution was saturated with pure O₂ for 2 hours in an ice-water bath and the pO₂ was monitored using a RapidLab 248 blood gas analyzer. When the pO₂ measured was well over the 749 mm Hg measurement range of the RapidLab 248 blood gas analyzer, a 30 mL syringe was used to titrate glutaraldehyde in the sealed glass bottle under continuous stirring. A 40:1 molar ratio of glutaraldehyde to bHb was used for the R-state polymerization reaction.

The resulting T- or R-state bHb solutions were then allowed to react with glutaraldehyde in the dark at 37°C for 2 hours, and were stirred and equilibrated with either pure Ar (50:1 T-state

bHb) or O₂ (40:1 R-state bHb). At the end of the 2 hour reaction period, for the 50:1 T-state bHb solution, 20 mL of 2 M NaBH₄ in PB buffer (20 mM, pH 8.0) was injected into the glass bottle to quench the polymerization reaction. For the 40:1 R-state bHb solution, 5 mL of 8 M NaCNBH₃ in PB buffer (20 mM, pH 8.0) was injected into the glass bottle to reduce the Schiff base and reduce the metHb level of the PolybHb solution. The PolybHb solution was continuously stirred for 30 min in an ice-water bath. Subsequently, 20 mL of 2M NaBH₄ in PB buffer (20 mM, pH 8.0) was injected into the glass bottle to quench the polymerization reaction. The pO₂ of the bHb solution before polymerization, after polymerization, and after quenching with NaBH₄ was measured using a RapidLab 248 blood gas analyzer. All reactions were repeated in triplicate.

Clarification and Separation of PolybHb Solutions

Initially, each PolybHb solution was clarified by filtering it through a glass column packed with glass wool that had been autoclaved at 250°C for 30 min [50] in order to degrade any endotoxin present in the glass wool. The clarified PolybHb solution was then separated into two distinct molecular weight (MW) fractions with a 500 kDa hollow fiber cartridge (Spectrum Labs). The retentate mostly contained PolybHb molecules that were larger than 500 kDa.

Buffer Exchange of PolybHb Solutions

After clarification and separation, the PolybHb was suspended in PB buffer along with reduced glutaraldehyde and excess NaBH₄ (and excess NaCNBH₃ for R-state PolybHb). The PolybHb solution underwent buffer exchange to remove cytotoxic glutaraldehyde, NaCNBH₃ and NaBH₄ [51] with a modified lactated Ringer's solution (NaCl (USP) 115 mmol/L, KCl (USP) 4 mmol/L, CaCl₂-2H₂O (USP) 1.4 mmol/L, NaOH (NF) 13 mmol/L, sodium lactate (USP) 27 mmol/L and N-acetyl-L-cysteine (USP) 2 g/L). The buffer exchange was conducted using an ÄKTA Explorer 100 system controlled by Unicorn 5.1 software (GE Healthcare). An XK 50/30 (300 mm in length, 50 mm I.D.) column (GE Healthcare) was packed with 500 mL of Sephadex G-25 medium resin at room temperature. After equilibrating the column with modified lactated Ringer's solution at a flow rate of 8 mL/min, the PolybHb solution was injected into the XK 50/30 column via a superloop (50 mL, GE Healthcare) at a flow rate of 5 mL/min. 100 mL of sample was injected each time and then eluted with modified lactated Ringer's solution. The protein concentration was detected at a wavelength of 280 nm, while the salt concentration was monitored with a conductivity detector. During the buffer exchange process, the UV signal increased as PolybHb eluted from the column, while the conductivity decreased when reduced glutaraldehyde and NaBH₄ (and NaCNBH₃ for R-state PolybHb) eluted from the column. The buffer exchanged PolybHb solution was collected as the UV signal increased, but before the conductivity signal decreased. The PolybHb fraction was then concentrated with a 100 kDa hollow fiber cartridge (Spectrum Labs).

MetHb Level and Protein Concentration of PolybHb Solutions

The metHb level of bHb/PolybHb solutions was measured via the cyanomethemoglobin method [52]. Total protein concentration was measured using the Bradford method [53] using the Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL).

SDS-PAGE of PolybHb Solutions

The MW distribution of bHb/PolybHb solutions was initially assessed via gel electrophoresis using a Mini-PROTEAN 3 Cell (Bio-Rad; Hercules, CA). All samples were mixed with an equal volume of sample buffer (Bio-Rad) containing 5% v/v β-mercaptoethanol, and then boiled for 5 min. A 4% stacking gel with a 12% resolving gel was assembled on a minivertical gel apparatus and each lane was loaded with 25 µg of protein. The gel was run at 120 V for approximately 1 hour. After electrophoresis, the gel was stained with Coomassie blue R250

(stain buffer, Bio-Rad) for one hour and then destained with a buffer consisting of 10% acetic acid and 20% methanol. The gel was scanned on a Gel Doc XR (Bio-Rad) imaging system for further analysis.

Size Exclusion Chromatography Coupled with Multi-Angle Static Light Scattering (SEC-MASLS) Analysis of PolybHb Solutions

The absolute MW distribution of bHb/PolybHb solutions was measured using a SEC column (Ultrahydrogel linear column, 10 μ m, 7.8 $\times$ 300 mm, Waters, Milford, MA) driven by a 1200 HPLC pump (Agilent, Santa Clara, CA), controlled by Eclipse 2 software (Wyatt Technology, Santa Barbara, CA) connected in series to a DAWN Heleos (Wyatt Technology) light scattering photometer and an OptiLab Rex (Wyatt Technology) differential refractive index detector. The mobile phase consisted of 20 mM PB (pH 8.0), 100 ppm NaN₃, and 0.2 M NaCl (Fisher Scientific) in HPLC grade water that was filtered through a 0.2 μ m membrane filter. PolybHb solutions were diluted to 1 mg/mL with the mobile phase, and 60 μ L of sample was injected into the column via a 1200 Autosampler (Agilent). All data were collected and analyzed using Astra 5.3 (Wyatt Technology) software.

O₂-PolybHb Equilibria

The O₂ affinity and cooperativity coefficient of bHb/PolybHb solutions were regressed from O₂-PolybHb equilibrium curves measured on a Hemox Analyzer (TCS Instruments, Southampton, PA) at 37°C.

Samples were prepared by thoroughly mixing 100 μ L of sample with 5 mL of Hemox buffer (pH 7.4, TCS Instruments), 20 μ L of Additive-A, 10 μ L of Additive-B and 10 μ L of anti-foaming agent. The bHb/PolybHb sample was allowed to equilibrate to a pO₂ of 145 $\pm$ 2 mm Hg using compressed air. After equilibrating the sample for 45 minutes, the gas stream was switched to pure N₂ to deoxygenate the bHb/PolybHb sample. The absorbance of oxy- and deoxy-Hb in solution was recorded as a function of pO₂ via dual wavelength spectroscopy. O₂-PolybHb equilibrium curves were fit to a four-parameter (A_0 , A_∞ , P_{50} , n) Hill model (Equation 1). In this model, A_0 and A_∞ represent the absorbance at 0 mm Hg and full saturation, respectively. The cooperativity coefficient is represented by n , and the pO₂ at which the bHb/PolybHb is half-saturated with O₂ is represented by the O₂ affinity or P_{50} .

$$Y = \frac{Abs - A_0}{A_\infty - A_0} = \frac{pO_2^n}{pO_2^n + P_{50}^n} \quad (1)$$

Stopped Flow Kinetic Analysis of PolybHb Solutions

The rapid kinetics of gaseous ligand reactions with bHb/PolybHb were measured in an Applied Photophysics SF-17 micro-volume stopped-flow apparatus as previously described [54]. Hb solutions (30 μ M) were rapidly mixed with equal volumes of 1.5 mg/mL sodium dithionite (British Drug House, Poole, England), and oxygen dissociation was monitored by the absorbance changes at 437.5 nm in 0.05 M bis-Tris buffer, pH 7.4. Multiple kinetic traces were averaged for each reaction, and fit to exponential equations using Marquardt-Levenberg fitting routines in the Applied Photophysics software. The kinetics of CO binding with deoxygenated Hbs were measured at 437.5 nm in 50 mM bis-Tris buffer at pH 7.4 at 25°C in the presence of sodium dithionite. The CO stock solution (about 1 mM) was prepared by saturating the degassed buffer solution with the flow of pre-washed CO gas.

The kinetics of NO oxidation with oxy-bHb/PolybHb solutions were carried out in the stopped-flow instrument as previously described [32]. NO stock solutions (~2 mM) were prepared by

saturation deoxygenated 0.05 M Tris buffer, pH 7.4, in a gas-tight serum bottle with NO gas that was pre-washed with deoxygenated 1M NaOH and buffer solutions. The NO stock solution was then transferred with a Hamilton syringe to a gastight syringe containing deoxygenated buffer solution to make appropriate concentrations of NO solutions. Hb solutions (1 μM) were mixed with NO solutions ($\leq 50 \mu\text{M}$), and the absorbance changes of the reaction were followed at 420 nm. Multiple traces were averaged for each reaction, and fit to exponential equations to obtain reaction rate constants.

PolybHb Viscosity and Colloid Osmotic Pressure

PolybHb viscosity was measured in a cone and plate viscometer DV-II plus with a cone spindle CPE-40 (Brookfield Engineering Laboratories, Middleboro, MA), at a shear rate of 160/sec. The colloid osmotic pressure (COP) of PolybHb was measured using a Wescor 4420 Colloid Osmometer (Wescor, Logan, UT) [55].

RBC Aggregation in PolybHb Solutions

The extent of RBC aggregation in PolybHb solutions under stasis was measured using a transparent cone-plate shearing instrument that uses the light transmission method [56]. This instrument consists of a transparent horizontal plate and rotating cone between which the blood sample is placed, with a light source and photocell arranged vertically (i.e., perpendicular to the plane of the cone and plate) to measure light transmission through the sample. The degree of RBC aggregation was assessed from triplicate measurements on a 0.35 mL sample of heparinized Syrian hamster blood mixed with the test solution at a volume ratio of 1:1, with the photometric rheoscope (Myrenne Aggregometer, Myrenne, Roetgen, Germany). The Myrenne "M" aggregation parameter is determined as follows: The sample is first exposed to a brief period of high shear (600 s^{-1}) to disrupt any preexisting RBC aggregates. The rotation is then stopped, and the light transmittance through the blood sample is recorded for 10 s; the average change in light transmission over this period is taken as the M value (units are arbitrary). If no aggregation occurs, then the light transmission remains constant, and $M = 0$. Aggregation of the RBCs reduces scattering and allows more of the light to reach the photocell, giving a positive M value, the magnitude of which increases with the degree of aggregation. The use of this technique as well as comparisons of this index of aggregation (M) with other methods and with different animal species has been described previously [57,58]. M indexes in 5% human serum albumin (no aggregation) and 6% dextran 500 kDa (aggregation) were used as control solutions to compare with PolybHb solutions.

In Vitro Autoxidation of PolybHb Solutions

All PolybHb samples were converted to ferrous (Fe^{2+}) or oxy-PolybHb immediately prior to autoxidation experiments. Experiments were carried out with 20-25 μM heme in sealed cuvettes with room air equilibrated with 50 mM Chelex-treated potassium phosphate buffer at 37 °C. Absorbance changes in the range of 450-700 nm due to spontaneous oxidation of Hb were recorded in a temperature-controlled photodiode array spectrophotometer (Hewlett Packard 8453, Palo Alto, CA). Similar oxidation assays were also performed in the presence of superoxide dismutase (4.6 U/mL) and catalase (414 U/mL). Autoxidation reactions were followed to near completion ($\sim 24 \text{ h}$), at which time 22 μM potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) was added to completely oxidize the remaining ferrous PolybHb. A multicomponent analysis was performed to calculate the ferrous and ferric species based on their individual extinction coefficients. Autoxidation rates were obtained from plots of the loss of ferrous to ferric PolybHb versus time using nonlinear least-squares curve fitting (single-exponential, two parameter decay) techniques in Sigma-Plot (SPSS, Chicago IL).

Animals and Surgical Preparation

Male Hartley guinea pigs were purchased from Charles Rivers Laboratories (Wilmington, MA) and acclimated for 1 week upon arrival to the FDA/Center for Biologics Evaluation and Research (CBER) animal care facility. All animals were fed normal diets throughout the acclimation period and weighed 350-450 g at the time of study. Animal protocols were approved by the FDA/CBER Institutional Animal Care and Use Committee with all experimental procedures performed in adherence to the National Institutes of Health guidelines on the use of experimental animals.

On days of surgery, guinea pigs were anesthetized via the i.p. route with a cocktail of ketamine HCl (100 mg/kg) and xylazine HCl (5 mg/kg) (Phoenix Scientific Inc., St. Joseph, MO). Under aseptic conditions a midline incision was made around the neck region allowing for blunt dissection and exposure of the right common carotid artery and the left external jugular vein. Saline filled catheters containing 50 IU of heparin per mL prepared from sterile PE50 tubing (Clay Adams of Becton-Dickenson, Sparks, MD) were placed in each vessel and tunneled under the skin to the back of the neck. Immediately following surgeries animals were administered a subcutaneous dose of ketoprofen (10 mg/kg) (Fort Dodge Pharmaceuticals, Fort Dodge, IA, USA) and allowed 24 hours of recovery prior to experimentation. Animals were randomized to receive a 20% blood volume exchange (ET) transfusion with either 50:1 T state or 40:1 R state PolybHb (n=4 guinea pigs / group). Fully conscious and freely moving guinea pigs underwent a 20% ET replacing blood with PolybHb. Arterial and venous catheters were extended, tethered and connected to separate syringe pumps (Model 11 Harvard Apparatus, Holliston, MA) set on withdrawal (1 mL/min) and infuse (1 mL/min), respectively. 20% ET volume in the guinea pig was estimated using the equation described by Ancill RJ [59] ($20\% \text{ ET (mL)} = (0.07 \text{ (mL/g)} \times \text{body weight (g)}) / 2$). Plasma from blood in the heparinized withdrawal syringe for each transfused animal was obtained to determine the total PolybHb removed during the exchange transfusion period (approximately 12 minutes). Each PolybHb solution was transfused as a 100 mg/mL protein solution. Blood samples (0.2 mL) were obtained from the arterial catheter prior to infusion (baseline) and at the end of ET (time 0), and at 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 hours. Plasma was used for evaluation of: (1) total PolybHb; (2) ferrous PolybHb and (3) ferric PolybHb using a photodiode array spectrophotometer (Model 8453 Hewlett Packard, Palo Alto, CA) [60]. The circulating T- and R-state PolybHb polymer distribution was evaluated as a function of time using a previously described method [61]. In brief, plasma samples (50 μ L) were evaluated by size exclusion chromatography (SEC). Samples were run on a BioSep-SEC-S3000 (600 mm $\times$ 7.5 mm) SEC column (Phenomenex, Torrance, CA) attached to a Waters Delta 600 pump and Waters 2499 dual-wavelength detector, controlled by a Waters 600 controller using EmpowerTM2 software (Waters Corp., Milford, MA).

Pharmacokinetic Analysis of PolybHb Solutions

The dose (mg) of PolybHb received by each animal at the end of ET was determined by subtracting the total amount of PolybHb in the plasma from whole blood collected in the ET syringe from the total amount of infused PolybHb according to the following equation:

$$\text{dose}_{\text{received}} = ([\text{PolybHb}]_{\text{infused}} \times V_{\text{infused}}) - ([\text{PolybHb}]_{\text{totalET}} \times V_{\text{ET}})$$

Where dose $[\text{PolybHb}]_{\text{infused}}$ is the concentration of PolybHb (mg/mL) infused, V_{infused} is the PolybHb infusion volume (mL), $[\text{PolybHb}]_{\text{totalET}}$ is the concentration of PolybHb (mg/mL) from plasma sampled out of the withdrawal syringe and V_{ET} is the volume (mL) collected in

the withdrawal syringe. Pharmacokinetic (PK) parameters were determined for total PolybHb, ferrous PolybHb (oxy/deoxy) and ferric PolybHb. Noncompartmental methods employed by WinNonlin version 4.1 (Pharsight Corp., Mountain View, CA, USA) were used to calculate PK parameter estimates. The area under the plasma concentration time curve ($AUC_{0-\infty}$) was estimated using the linear trapezoidal rule to the last measurable concentration ($AUC_{0-C_{last}}$). Extrapolation to infinity ($AUC_{C_{last}-\infty}$) was accomplished by dividing C_{last} by the negative value of the terminal slope (k) of the log-linear plasma concentration-time curve. Thus $AUC_{0-\infty}$ is equal to the sum of $AUC_{0-C_{last}}$ and $AUC_{C_{last}-\infty}$. Additional parameters were calculated as follows: the plasma clearance (CL) was calculated as the dose divided by $AUC_{0-\infty}$, the mean residence time (MRT) was calculated as k^{-1} , the apparent volume of distribution (V_{dss}) was calculated as the product of CL and MRT and half-life ($t_{1/2}$) was calculated as $\ln(2)$ divided by k .

Results

bHb was polymerized using glutaraldehyde (G) as the cross-linking reagent at a G:Hb molar ratio of 50:1 for T-state PolybHb and 40:1 for R-state PolybHb. After polymerization, each PolybHb mixture was fractionated with a 500 kDa hollow fiber cartridge (Spectrum Labs) to remove tetrameric Hb and Hb oligomers with MW less than 500 kDa. PolybHb fractions with MW above 500 kDa were used in this study.

pO₂ of PolybHb Solutions

To ensure that PolybHb was in the T- or R-state, the pO₂ at various stages of the bHb polymerization process was measured and shown in Figure 1. For T-state PolybHb, the pO₂ of the bHb solution was reduced to 0 mm Hg by argon purging and subsequent titration of Na₂S₂O₄ before polymerization and remained at 0 mm Hg after the polymerization reaction and subsequent quenching with reducing agents. These results show that under the protection of an inert atmosphere, bHb was polymerized in an oxygen-free environment and maintained in the deoxygenated state (T-state) during the polymerization process. For R-state PolybHb, the pO₂ before and after polymerization was kept above the measurement range of the oxygen detector. This shows that bHb was saturated with oxygen and bHb was maintained in the R-state. After quenching the R-state bHb polymerization reaction with NaCNBH₃ and NaBH₄, the pO₂ of the R-state PolybHb solution dropped to 0 mm Hg. This pO₂ drop is due to the displacement of O₂ from solution by the H₂ gas generated by the NaBH₄.

SDS-PAGE and MW Distribution of PolybHb Solutions

Figure 2 shows the SDS-PAGE of native bHb and high MW PolybHbs. Both T- and R-state PolybHb show a strong band above 250 kDa and very weak bands around 15 and 30 kDa suggesting that T- and R-state PolybHb mostly consists of inter- and intramolecular cross-links. Therefore, these results show the presence of very little α and β monomers (these correspond to the two bands around 15 kDa, the lower band represents α subunits while the upper band represents β subunits) as well as $\alpha_2/\beta_2/\alpha\beta$ dimers in solution (these correspond to the band at 30 kDa), confirming that the TFF process was very effective in removing small MW Hb species less than 500 kDa in MW. The majority of both R- and T-state PolybHb solutions was above 250 kDa in MW with the R-state PolybHb being slightly larger (Figure 2). Light scattering results confirmed that R-state PolybHb has a larger MW distribution compared to T-state PolybHb (Figure 3). Despite this difference, both PolybHb solutions possess large MWs ranging from 16.59 to 26.33 MDa (260~400 bHb tetramers). The light scattering results also indicate that there is no free Hb in the PolybHb solution, since there is no peak corresponding to bHb tetramers. This shows that all bHb tetramers were polymerized in the reaction. However, the SDS-PAGE results indicate that an extremely small fraction of the PolybHb solutions possess uncross-linked α and β monomers as well as $\alpha_2/\beta_2/\alpha\beta$ dimers.

While all the tetrameric Hb is polymerized, there are some α and β monomers as well as $\alpha\beta/\alpha_2/\beta_2$ dimers that are not cross-linked within the PolybHb superstructure.

P₅₀ and n of PolybHb Solutions

Figure 4 shows the equilibrium oxygen dissociation curves of native bHb, T- and R-state PolybHb. Compared to the O₂-bHb equilibrium curve, the O₂-PolybHb equilibrium curve of T-state PolybHb is shifted to the right, while the O₂-PolybHb equilibrium curve of R-state PolybHb is shifted to the left. The regressed P₅₀ and cooperativity coefficient (*n*) of bHb and fractionated PolybHb solutions are shown in Figure 5. The P₅₀ of R-state PolybHb is approximately 0.66 mm Hg, which is much lower than that of T-state PolybHb which is approximately 41 mm Hg. The cooperativity coefficients of both T- and R-state PolybHb solutions are less than 1.

MetHb Level of PolybHb Solutions

The metHb level of native bHb, T- and R-state PolybHb is shown in Figure 6. The metHb level of native bHb is very low (<1%), since it was purified from fresh bRBCs and stored at -80°C. T- and R-state PolybHb had similar metHb levels, which were both below 4%.

Stopped Flow Kinetic Analysis of PolybHb Solutions

Representative time courses for CO binding to deoxygenated bHb/PolybHb was obtained on the stopped-flow instrument and plotted in Figure 7 for comparison. While CO binding to deoxygenated T-state PolybHb is slightly slower compared to native bHb, the binding of CO to deoxygenated R-state PolybHb occurs much more rapidly. This is supported by the CO concentration dependence of the apparent CO association reaction rates shown in the insert of Figure 7. Table 1 summarizes the kinetic parameters for oxygen dissociation from oxy-bHb/PolybHb, and the second order rate constants of CO association and NO dioxygenation with deoxygenated and oxygenated bHb/PolybHb, respectively. Both T- and R-state PolybHb oxygen dissociation rate constants deviated from that of native bHb by either a 50% increase or 40% reduction, respectively. The main difference in gaseous ligand binding properties was observed in CO association to deoxygenated R-state PolybHb, which resulted in an ~ 20-fold increase of the binding rate constant with respect to bHb. CO binding with T-state PolybHb is only slightly lower than that of native bHb. Conversely, the NO reaction remains largely unchanged among native bHb, T- and R-state PolybHb.

Viscosity and COP of PolybHb Solutions

The viscosity and COP of PolybHb solutions is shown in Table 2. The viscosity of both PolybHb solutions increased with increasing PolybHb concentration. In contrast, the COP of PolybHb solutions was fairly insensitive to PolybHb concentration. However, the T-state PolybHb solutions displayed lower COP versus R-state PolybHb solutions.

RBC Aggregation of PolybHb Solutions

The M index of aggregation of heparinized hamster blood mixed with PolybHb solutions is shown in Figure 8. The aggregation of RBCs induced by PolybHb solutions was significantly less than the aggregation induced by 6% dextran 500 kDa (a high viscosity plasma expander, with viscosity of 6.3 cP and COP of 38 mm Hg), but higher than the aggregation induced by 5% human serum albumin solution. The blood smears show that mixtures of blood and 10% PolybHb generate minor rouleaux or rouleaux-rouleaux complexes compared to 6% dextran 500 kDa. The observed aggregation was very mild compared to dextran. The general trends for both PolybHb samples were very similar, both PolybHbs promoted static RBC aggregation compared to 5% albumin, however, significantly less than 6% dextran 500 kDa, both of them clinically used plasma expanders.

Pharmacokinetic Analysis of PolybHb Solutions

Plasma heme concentrations versus time for T- and R-state PolybHbs following a 20% exchange transfusion are shown in Figure 9. The pharmacokinetic parameter estimates for total PolybHb, ferrous PolybHb and ferric PolybHb following a 20% T- and R-state PolybHb exchange transfusion are listed in Table 3. Doses received by animals ($n=4/\text{group}$) determined at the completion of exchange transfusion were similar at 506.4 ± 0.42 mg (T-state PolybHb) and 508.5 ± 0.77 mg (R-state PolybHb). Transfusions resulted in total plasma PolybHb maximum plasma concentrations ($C_{\max}$) of 14.4 ± 0.44 mg/mL and 13.1 ± 0.50 mg/mL for T- and R-state preparations, respectively. The volume of distribution (V_{dss} (mL)) was determined to be low (42.98 ± 5.5 (T-state PolybHb) and 44.3 ± 0.67 (R-state PolybHb)) suggesting a limited distribution from the central compartment for both preparations. Data plotted in Figure 9 and inset photos of representative plasma sets from T- and R-state PolybHb dosed guinea pigs indicate visual differences in rates of circulatory clearance between the two preparations. Pharmacokinetic parameters calculated from plotted data demonstrate a significantly increased rate of total circulatory clearance (Cl (mL $\cdot$ h $^{-1}$)) of approximately 2 fold and decreased exposure expressed as area under the concentration versus time curve ($AUC_{0-t_{\text{last}}}$ and $AUC_{0-\infty}$ (mg $\cdot$ h $\cdot$ mL $^{-1}$)) of approximately 2 fold following administration of R-state PolybHb compared to T-state PolybHb. The circulating half-life ($t_{1/2}$) of T-state PolybHb was increased by approximately 1.5 fold compared to R-state PolybHb.

In vivo generation of ferric (Fe^{3+}) from ferrous (Fe^{2+}) R- and T-state PolybHb solutions demonstrated unique differences. The $C_{\max}$ of ferric (Fe^{3+}) R-state PolybHb was 4.57 ± 0.31 mg/mL occurring at a $T_{\max}$ of 4.0 ± 2.0 hours, while the $C_{\max}$ of ferric (Fe^{3+}) T-state PolybHb was 3.27 ± 0.32 at a $T_{\max}$ of 8.0 ± 2.3 hours. Areas under the plasma concentration versus time curves were significantly greater for T-state PolybHb. This finding is a function of the overall greater circulation time of the T-state PolybHb, since the area under the ferric (Fe^{3+}) T- and R-state PolybHb concentration versus time curves were determined to be 40% of their respective total PolybHb exposures.

Plasma PolybHb Polymer Dissociation

The plasma polymer distribution of T- and R- state PolybHb over time is shown in Figure 10. Plasma samples from representative T- and R-state PolybHb transfused guinea pigs were analyzed on an analytical BioSep-S3000 size exclusion chromatography column and compared to elution profiles of PolybHb and bHb prior to infusion. Both T- and R-state PolybHb demonstrate similar polymer elution patterns over the time following the end of transfusion indicating limited hydrolysis of stabilized glutaraldehyde bonds in the T- and R-state PolybHb within the systemic circulation.

Oxidation of T- and R-State PolybHb

The differences in autoxidation rates obtained at 37°C and 24 hours were derived from a single-exponential, two parameter decay and are shown in Table 3 as the slope of the second parameter. The percent of ferric (Fe^{3+}) formation at 2, 4 and 24 hours from *in vitro* and *in vivo* studies of oxidation are shown in Figure 11. The high oxygen affinity R-state PolybHb solution demonstrates a significantly reduced rate of *in vitro* autoxidation and significantly less ferric Hb formation (R-state 2 hr: $8.23 \pm 0.80\%$, 4 hr: $13.6 \pm 1.03\%$) compared to bHb (2 hr: $13.7 \pm 0.21\%$, 4 hr: $21.8 \pm 0.44\%$) and the low oxygen affinity T-state PolybHb (2 hr: $32.8 \pm 0.62\%$, 4 hr: $46.2 \pm 0.78\%$) in the initial hours and at the 24 hour time point of autoxidation (bHb: $64.7 \pm 1.36\%$, T-state: $79.2 \pm 7.59\%$, R-state: $35.4 \pm 2.0\%$). A clear disconnect between *in vitro* autoxidation and *in vivo* oxidation is seen in Figure 11 A (*in vitro* autoxidation) and B (*in vivo* oxidation), where *in vivo* R-state PolybHb oxidizes more rapidly to its ferric (Fe^{3+}) form compared to T-state PolybHb. In a dynamic *in vivo* situation involving both oxidative and clearance processes R-state PolybHb accumulated to $50.4 \pm 1.4\%$ at a $T_{\max}$ of 4 hours.

This was significantly greater than ferric (Fe^{3+}) T-state PolybHb accumulation of 37.8 ± 2.8 % at a T_{max} occurring 8 hours post transfusion.

Discussion

Biophysical Properties of T- and R-state PolybHb Solutions

The goal of this study was to synthesize high MW T- and R-state PolyHbs with no tetrameric Hb and large molecular sizes ($>500\text{kDa}$). bHb was polymerized in distinct quaternary states by carefully controlling the pO_2 of the solution during the polymerization process. In addition, we characterized certain biophysical as well pharmacokinetic properties of the PolyHbs.

For T-state PolybHb, the bHb solution was thoroughly deoxygenated before polymerization and polymerized under an inert argon atmosphere. The PolybHb obtained in this manner was maintained in the homogeneous T-state via intra- and inter-molecular glutaraldehyde cross-links. To produce R-state PolybHb, bHb was first transformed into the R-state by completely oxygenating the bHb solution with O_2 and subsequently polymerizing the bHb under an O_2 saturated environment to ensure that the bHb was polymerized in the homogeneous R-state. Therefore, after fractionating the PolybHb mixture with a 500 kDa TFF cartridge, both T- and R-state PolybHb solutions contained no tetrameric Hb. To our knowledge, this is the first time that homogeneous T- and R-state PolybHb solutions were synthesized. The two commercially manufactured PolyHbs, HBOC-201 and PolyHeme®, make no mention of the pO_2 during the Hb polymerization process [13,39,62]. Therefore, these commercial products must be considered heterogeneous with respect to the composition of the PolyHb quaternary state. Therefore, our homogeneous T- and R- state PolybHbs can provide a better model for clinical evaluation.

The MW distribution of R-state PolybHb is slightly higher than that of T-state PolybHb even though the polymerization reaction was conducted at a lower G:Hb molar ratio. There are two reasons why this makes sense. First, it has been reported that the reactivity of glutaraldehyde to oxy-Hb is much greater than that to deoxy-Hb [33,34]. Thus, R-state polymerization can generate bigger aggregates of bHb compared to T-state polymerization. Our results are consistent with that in the literature with respect to this phenomenon. The second reason is due to the presence of $\text{Na}_2\text{S}_2\text{O}_4$ in the bHb solution. $\text{Na}_2\text{S}_2\text{O}_4$ was used in the T-state polymerization process to scavenge oxygen from the bHb solution, and therefore maintain the pO_2 at 0 mm Hg. $\text{Na}_2\text{S}_2\text{O}_4$ can react with free aldehyde groups[63], thereby quenching some of the glutaraldehyde and reducing the actual G:Hb molar ratio to a level lower than the reported level of 50:1, hence, reducing the MW of T-state PolybHb compared to R-state PolybHb.

Despite the difference in the MW distribution of our T- and R-state PolybHbs, both of them possess large MWs ranging from 16.59 to 26.33 MDa and no tetrameric Hb in solution (Figure 3). Tetrameric Hb was removed from the PolybHb solution by filtering it through a 500 kDa TFF cartridge. The high MW of the PolybHb solutions and the absence of tetrameric Hb are important for several reasons. First, the tetrameric component of these PolyHb solutions is able to extravasate through the pores in blood vessels or interact more closely with endothelial cells covering the vascular lumen and can scavenge NO from the surrounding endothelial cells or the sub-endothelial compartments. This will cause the smooth muscle cells to constrict leading to vasoconstriction in the microcirculation and eventual systemic hypertension [64]. These side-effects can be aggravated in a dose-dependent manner. The T- and R-state PolybHbs synthesized in this study possess no tetrameric Hb and perhaps more importantly exceed 500 kDa in MW. Secondly, high MW PolyHbs may exhibit longer circulation times compared to smaller MW PolyHbs. It has been shown that the half-life of PolyHbs is proportional to the MW of the PolyHb [62,65] and reached about 12-15 h for the 192 kDa fraction of Hemolink (Hemosol Corp., Mississauga, Canada) [65] to 20 h for the 576 kDa fraction of HBOC-201

[13]. Both of our PolybHbs possess MWs 50-fold greater than the 576 kDa fraction of HBOC-201. Thus, it is reasonable to predict that our R-state and T-state PolybHbs should display longer circulation times when dosed at equal concentration and volume.

After curve fitting the O₂-PolybHb equilibrium data to the Hill equation, the P₅₀ of both fractionated samples varied greatly (Figure 5). The P₅₀ of the high MW T-state PolybHb was around 41 mm Hg similar to the reported value of 38 mm Hg for HBOC-201 [13,37], suggesting that the T-state PolybHb may have similar oxygen transporting ability to HBOC-201. The P₅₀ of R-state PolybHb is 0.66 mm Hg which demonstrates that cross-linking Hb in the R-state can greatly increase the oxygen affinity of the product in a polymerization-dependent manner [33,34]. Restitution of the oxygen carrying capacity of low P₅₀ materials compared to normal blood does not affect the total amount of oxygen transported, but affects the amount of oxygen released at each segment of the circulation. Therefore, for the purpose of transfusion medicine, a moderate increase in oxygen carrying capacity with decreased oxygen affinity HBOCs should provide more effective oxygen delivery. Furthermore, a mixture of HBOCs with different P₅₀s may provide an even more efficient mechanism for restoring optimal oxygen delivery with a minimal amount of material.

The cooperativity of the two PolybHb solutions is <1 (Figure 5) compared to the reported value for unmodified bHb (2.5) [46]. This is due to the intra- and inter-molecular glutaraldehyde cross-links, which freezes the quaternary structure of Hb and reduces its structural flexibility. Therefore, the quaternary structure changes which otherwise would occur during normal oxygen binding/offloading are hindered by the cross-links. This results in a significant loss of cooperative O₂ binding to the Hb tetramer.

The metHb level of both PolybHbs were lower than 4% (Figure 6), which fulfill the standard 10% metHb requirement that is frequently cited in the literature [66]. Several steps were taken to retard autoxidation. First, the initial purified Hb had a metHb level lower than 1%. Second, the processes for deoxygenating and oxygenating bHb as well as fractionation of the PolybHb mixture by TFF were all conducted in an ice bath in order to slow down oxidation of the heme. Third, PolybHb solutions were buffer exchanged against modified lactated Ringer's solution containing N-acetyl-L-cysteine, an antioxidant [67], thereby limiting heme oxidation.

Stopped-flow kinetic analysis revealed some interesting changes in the gaseous ligand binding properties which resulted from the polymerization of Hb with the cross-linking reagent glutaraldehyde. Although NO oxidation of oxygenated Hb is usually not sensitive to Hb conformational changes or chemical modifications, the kinetic parameters describing oxygen dissociation and CO binding are good indicators of Hb oxygen affinity. Not surprisingly, the differences found in oxygen dissociation (k_{off}) and CO binding ($k'_{\text{on, CO}}$) of T- and R-state PolybHb with native bHb are consistent with their equilibrium oxygen binding properties (i.e. oxygen affinity (P₅₀)). Moreover, the large drop in the P₅₀ of R-state PolybHb compared to bHb is mostly reflected by its 20 fold increase in the CO binding rate constant, suggesting a much more open conformation assumed by the R-state PolybHb that leads to higher heme pocket accessibility. A previous study examined the fast kinetics of the glutaraldehyde polymerized bHb veterinary product Oxyglobin® (Biopure Corp., Cambridge, MA) and its 4 component fractions (F1(multi-tetramer)-F4 (single tetramer)) ranging in molecular size from tetrameric (~64 kDa) to multi-tetrameric (~500 kDa) [68]. The starting material (bHb) in this study exhibited a similar k_{off} ($33.5 \pm 0.1 \text{ s}^{-1}$) and $k'_{\text{on, CO}}$ ($0.22 \pm 0.02 \mu\text{M}^{-1}\text{s}^{-1}$) to the bHb used in the present study (see Table 2). k_{off} values for Oxyglobin® ($60.0 \pm 3.2 \text{ s}^{-1}$) and fractions (F1, $63.3 \pm 1.2 \text{ s}^{-1}$, F2, $57.2 \pm 1.0 \text{ s}^{-1}$, F3, $63.7 \pm 1.4 \text{ s}^{-1}$, and F4, $62.8 \pm 2.5 \text{ s}^{-1}$) were nearly identical to the T-state PolybHb described in the current study. Similarly, $k'_{\text{on, CO}}$ values for Oxyglobin® ($0.15 \pm 0.01 \mu\text{M}^{-1} \text{ s}^{-1}$) and fractions (F1, $0.19 \pm 0.01 \mu\text{M}^{-1} \text{ s}^{-1}$, F2, 0.18 ± 0.01

$\mu\text{M}^{-1} \text{s}^{-1}$, F3, $0.20 \pm 0.02 \mu\text{M}^{-1} \text{s}^{-1}$, and F4, $0.19 \pm 0.02 \mu\text{M}^{-1} \text{s}^{-1}$) were nearly similar to the T-state PolybHb described in the current study (see Table 2) [68].

At PolybHb concentrations of 5 and 10 g/dL, the viscosity of both PolybHb solutions is higher than that of blood (~ 3 cp). In this study, the viscosity of both PolybHb solutions increases as the PolybHb concentration increases. This should come as no surprise, since molecular interactions between PolybHb molecules will be enhanced as the concentration increases in solution. Originally blood substitutes were designed with the assumption that lower blood viscosity is always beneficial. However, blood viscosity directly influences blood vessel diameter due to the shear stress interaction with the endothelium [69]. It is known that a decrease in blood viscosity induces vasoconstriction. Hence, blood viscosity is an important determinant of vasoactivity, as shown in our experiments [70]. PolybHbs can interact mechanically in terms of shear stress, presumably leading to a difference in mechanotransduction with the endothelium. Transfusion of these high viscosity solutions may be advantageous, since these solutions could stimulate NO generation via mechanotransduction of the endothelium [71]. The release of NO would relax the tone of blood vessels and alleviate the vasoconstrictive effect. Additionally, as the PolybHb solutions are proposed to be used during anemic conditions, where blood viscosity is already low, the increase in plasma viscosity induced by the PolybHb will not increase peripheral vascular resistance.

Both polymers showed very similar effects on RBC aggregation. Although the degree of RBC aggregation was substantially less when compared to a high viscosity plasma expander (6% dextran 500 kDa). The ability of the larger bHb polymers to promote RBC aggregation was microscopically evident, and the aggregates tended to form rounded and compressed clumps rather than elongated rouleaux.

Interestingly, the COP of both PolybHbs solutions is low (< 10 mm Hg) (Table 1). This is due to two reasons. First, there is no free Hb present in the PolybHb solution [62]. Second, filtering the PolybHb solutions through the 500 kDa TFF column substantially removed Hb species smaller than 500 kDa. The COP of the PolybHb solutions is lower than that of normal blood (27 mm Hg), and can be adjusted to physiological levels by supplementing PolybHb solutions with human serum albumin solution. When transfused, this should enable simultaneous oxygen transport and blood volume expansion. Moreover, oxygen carrying capacity is in principle a direct function of the concentration of functional Hb molecules. The present PolybHb formulations have the highest oxygen carrying capacity due to their extremely low COP. Therefore, these solutions will not elicit autotransfusion and dilute the HBOC concentration in the blood, since their COP is lower than that of blood. As for other commercial HBOCs, augmenting their concentration is not an option in order to increase their oxygen carrying capacity, since they have increased COP. The high COP will promote the flow of interstitial fluid into the circulation, diluting the HBOC, thus lowering the circulating concentration of Hb and increasing the blood volume, a self-limiting process. In contrast, the PolybHbs described in this work constitute a set of HBOCs that could potentially overcome these problems.

Pharmacokinetics of T- and R-state PolybHb Solutions

Pharmacokinetic evaluation of both T- and R-state PolybHbs revealed an extended circulation time for T- versus R-state PolybHb. All pharmacokinetic estimates of clearance and exposure, excluding C_{max} which was intentionally matched, indicated that R-state PolybHb was removed from the circulation at a significantly greater rate than T-state PolybHb. This finding is somewhat contrary to the expected result that increased molecular size (R-state ~ 26 MDa versus T-state ~ 17 MDa) contributes to a greater circulation time. Interestingly, while behaving as expected from the perspective of *in vitro* autoxidation, R-state (high oxygen affinity) oxidized *in vivo* to a greater extent than T-state (low oxygen affinity) PolybHb. The

observation of increased oxidation of R-state PolybHb within an earlier time frame following transfusion may have contributed to the greater overall clearance of this protein in circulation.

Conclusion

In this work, ultrahigh molecular weight tense state PolybHb ($M_W = 16.59$ MDa and $P_{50} = 41$ mm Hg) and relaxed state PolybHb ($M_W = 26.33$ MDa and $P_{50} = 0.66$ mm Hg) were synthesized with no tetrameric Hb and low methHb levels (<4%). Both PolybHbs possessed high viscosities and low COPs. In addition, transfusion of these PolybHbs indicated limited PolybHb dissociation, which is a good preliminary indicator that these PolybHb will not extravasate through blood vessels and scavenge NO. In light of these results, these PolybHbs should not elicit vasoconstriction/hypertension and are a good basis for future HBOC development.

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Figure 1. pO_2 at various stages of the bHb polymerization process for T- and R-state PolybHb solutions. The error bar represents the standard deviation from triplicate reactions

Figure 2.
SDS-PAGE of native bHb, T- and R-state PolybHb solutions

Figure 3.
Absolute molecular weight distribution of native bHb, T- and R-state PolybHb solutions.

Figure 4. Equilibrium O_2 -bHb/PolybHb binding curves of native bHb, T- and R-state PolybHb solutions.

Figure 5.

Oxygen affinity (P_{50}) and cooperativity coefficient (n) of native bHb, T- and R-state PolybHb solutions. The error bar represents the standard deviation from triplicate reactions. * $p < 0.05$ with respect to native bHb.

Figure 6. MethHb level of native bHb, T- and R-state PolybHb solutions. The error bar represents the standard deviation from triplicate reactions. * $p < 0.05$ with respect to native bHb.

Figure 7.

Rapid kinetics of CO binding with native deoxygenated bHb, T- and R-state PolybHb. Stopped-flow time courses of 15 μM deoxygenated bHb/PolybHb (after mixing) reacting with 250 μM CO solutions for native bHb (○) and T-state PolybHb (□) solutions, or 50 μM CO solution for R-state PolybHb (Δ) were monitored at 437.5 nm in 50 mM phosphate buffer at pH 7.4 and room temperature. The solid lines are from the nonlinear least-square curve fitting of the time courses to the exponential equation. The obtained apparent rate constants were plotted versus CO concentration in the insert for each bHb/PolybHb to generate the second order rate constants of the reaction between deoxygenated bHb/PolybHb and CO (Table 1).

Figure 8.

A typical blood smear for aggregation studies obtained from heparinized hamster blood mixed in a 1:1 volume ratio with test solution. 5% albumin does not produce aggregation (above), however 6% dextran 500 kDa increases aggregation (below).

Figure 9.

Pharmacokinetics of T- (panel A) and R- (panel B) state PolybHb: top – photograph of (1) bHb, (2) T- and R-state PolybHb (prior to transfusion), (3) plasma (prior to transfusion), (4) end of transfusion, (5) 0.25 hour, (6) 0.5 hour, (7) 1 hour, (8) 2 hour, (9) 4 hour, (10) 8 hour, (11) 12 hour, (12) 24 hour and (13) 48 hour. Insets show representative visible spectra of plasma from the end of transfusion until 48 hours from 450 to 700 nm. The concentration (heme) versus time (hours) plot shows the plasma concentration $\pm$ sem ($n=4$ /time point) of T- and R-state PolybHb as total heme (black), ferrous heme (red) and ferric heme (blue).

Figure 10.

Circulating T- and R-state PolybHb polymer distribution over time: (A) Size exclusion chromatography of (.....) bHb, (—) T-state PolybHb and plasma samples from the end of transfusion to 48 hours (shown as dark grey fading to white). (B) Size exclusion chromatography of (.....) bHb, (—) R-PolybHb and plasma samples from the end of transfusion to 24 hours (shown as dark grey fading to white).

Figure 11.

(A) *In vitro* autoxidation studies show a significant ($p < 0.05$) attenuation in % R-state PolybHb ferric Hb formation compared to % bHb (*) and % T-state PolybHb (†) ferric Hb formation over 24 hours at 37°C. (B) % ferric Hb in plasma from *in vivo* studies show a significant ($p < 0.05$) increase in % ferric R-state PolybHb compared to T-state PolybHb (†) within an early time frame following transfusion.

Table 1

Rapid kinetic parameters of gaseous ligand reactions with native bHb, T- and R-state PolybHb: oxygen dissociation rate constant (k_{off}), CO binding rate constant ($k'_{\text{on, CO}}$), and the rate of NO dioxygenation ($k'_{\text{ox, NO}}$).

	k_{off} (s^{-1})	$k'_{\text{on, CO}}$ ($\mu\text{M}^{-1}\text{s}^{-1}$)	$k'_{\text{ox, NO}}$ ($\mu\text{M}^{-1}\text{s}^{-1}$)
Native bHb	36.1	0.22	18.3
T-state PolybHb	53.0	0.18	18.9
R-state PolybHb	22.0	4.84	17.5

Table 2

The viscosity and COP of T- and R-state PolybHb.

Sample	Concentration (g/dL)	Viscosity (cP)	COP (mmHg)
T-PolybHb (50:1)	10	11.4	1
R-PolybHb (40:1)	10	7.8	7
T-PolybHb (50:1)	5	5.2	1
R-PolybHb (40:1)	5	3.6	4

Table 3

Pharmacokinetic parameter estimates following T- and R-state PolybHb transfusion.

Estimate	Total PolybHb	PolybHb (Fe ²⁺)	PolybHb (Fe ³⁺)
T-State PolybHb			
<i>Dose</i> (mg)	506.4 ± 0.42	489.1 ± 0.36	1.3 ± 0.10
<i>C_{max}</i> (mg•mL ⁻¹)	14.4 ± 0.44	12.0 ± 0.43	3.27 ± 0.32
<i>T_{max}</i> (h)	0.17	0.17	8.0 ± 2.3
<i>AUC_{0-tlast}</i> (mg•h•mL ⁻¹)	214.9 ± 9.91	124.7 ± 6.52	90.1 ± 5.78 (41.9% total)
<i>AUC_{0-∞}</i> (mg•h•mL ⁻¹)	252.4 ± 34.1	139.4 ± 14.5	113.0 ± 20.9 (44.3% total)
<i>Cl</i> (mL•h ⁻¹)	2.1 ± 0.24	3.6 ± 0.36	
<i>Vd_{ss}</i> (mL)	42.98 ± 5.5	61.3 ± 7.2	
<i>t_{1/2}</i> (h)	11.1 ± 0.22	6.85 ± 0.07	
<i>MRT_{i.v.}</i> (h)	22.15 ± 5.9	17.91 ± 4.0	
<i>In vitro k_{ox}</i> (min ⁻¹)	1.23•10 ⁻³ ± 3.37•10 ⁻⁵		
R-State PolybHb			
<i>Dose</i> (mg)	508.5 ± 0.77	491.1 ± 1.0	1.0 ± 0.03
<i>C_{max}</i> (mg•mL ⁻¹)	13.1 ± 0.50	10.4 ± 0.70	4.57 ± 0.31 [†]
<i>T_{max}</i> (h)	0.17	0.17	4.0 ± 2.0 [†]
<i>AUC_{0-tlast}</i> (mg•h•mL ⁻¹)	127.4 ± 11.2 [†]	75.4 ± 12.1 [†]	51.9 ± 5.71 [†] (40.5% total)
<i>AUC_{0-∞}</i> (mg•h•mL ⁻¹)	158.5 ± 25 [†]	94.8 ± 24.1 [†]	63.7 ± 8.3 [†] (41.5% total)
<i>Cl</i> (mL•h ⁻¹)	3.8 ± 0.40 [†]	7.0 ± 1.2 [†]	
<i>Vd_{ss}</i> (mL)	44.3 ± 0.67	69.4 ± 1.0	
<i>t_{1/2}</i> (h)	7.31 ± 0.53 [†]	5.60 ± 1.3	
<i>MRT_{i.v.}</i> (h)	13.6 ± 1.9 [†]	13.3 ± 3.2	
<i>In vitro k_{ox}</i> (min ⁻¹)	9.0•10 ⁻⁴ ± 3.34•10 ⁻⁵ *		

[†]Significant difference between T- and R-state PolybHb parameter estimates (p<0.05)* = Significant difference between T- and R-state PolybHb *in vitro* oxidation (p<0.05)