Biophysical Properties and Oxygenation Potential of High-Molecular-Weight Glutaraldehyde-Polymerized Human Hemoglobins Maintained in the Tense and Relaxed Quaternary States

Ning Zhang, M.S.,¹ Yiping Jia, Ph.D.,² Guo Chen, Ph.D.,¹ Pedro Cabrales, Ph.D.,³ and Andre F. Palmer, Ph.D.¹

Recent clinical evaluation of commercial glutaraldehyde-polymerized hemoglobins (PolyHbs) as transfusion solutions has demonstrated several adverse side effects. Chief among these is the hypertensive effect. Fortunately, previous studies have shown that the hypertensive effect can be attenuated by removing free hemoglobin (Hb) and low-molecular-weight (low-MW) PolyHbs from the PolyHb mixture. In this work, polymerized human Hb (PolyhHb) solutions were synthesized in two distinct quaternary states with high MW and subjected to extensive diafiltration to remove free Hb and low-MW PolyhHb components (<500 kDa). The resultant PolyhHb solutions possessed high MW, distinct quaternary state, distinct reactivities with O₂ and CO, similar NO deoxygenating rate constants, distinct autoxidation rate constants, high viscosity, and low colloid osmotic pressure. To preliminarily assess the ability of PolyhHb solutions to oxygenate surrounding tissues fed by a blood vessel, we evaluated the ability of PolyHbs to transport O₂ to cultured hepatocytes in a mathematical model of a hollow fiber bioreactor. The structure of individual hollow fibers in the bioreactor is similar to that of a blood vessel and provides an easy way to assess the oxygenation potential of PolyHbs without the need for expensive and time-consuming animal studies. It was observed that PolyHbs with low O₂ affinities were more effective in oxygenating cultured hepatocytes inside the bioreactor than high O₂ affinity PolyHbs. Taken together, our results show that it is possible to synthesize high-MW PolyHbs with no free Hb and low-MW PolyhHb components that are capable of transporting O₂ to cultured cells/tissues.

Introduction

HEMOGLOBIN-BASED OXYGEN CARRIERS (HBOCs) are being developed as red blood cell substitutes. However, vasoconstriction and hypertension still remain significant side effects.¹⁻⁴ Although the exact mechanism of vasoconstriction upon HBOC transfusion is not known, there are two major hypotheses in the literature, namely, nitric oxide (NO) scavenging (by far the most popular one of the two) and oxygen oversupply.⁵⁻⁷ To limit/prevent vasoconstriction by either mechanism, the size (i.e., molecular weight, MW) of the HBOC should be increased and free hemoglobin (Hb) and low-MW components should be removed from solution to reduce the interaction of the HBOC with the endothelium.^{6,8,9} Therefore, strategies targeted toward increasing the size of HBOCs should be able to reduce these unwanted side effects. Polymerization of Hb represents such an approach.

Two commercial polymerized Hb (PolyHb)-based HBOCs were developed for clinical use, Hemopure[®] (OPK Biotech, Cambridge, MA) and PolyHeme[®] (Northfield Laboratories Inc., Evanston, IL). Hemopure[®] had an average MW of 250 kDa^{10,11} and was composed of <5% of free Hb,¹² whereas PolyHeme[®] had an average MW of 150 kDa¹³ and was composed of <1% of free Hb.¹⁴ Unfortunately, both PolyHbs elicited substantial hypertension *in vivo* upon transfusion.^{4,11,15}

Fortunately, a path exists to improve the safety of PolyHbs. Sakai *et al.* was the first to demonstrate that the extent of vasoconstriction and hypertension was inversely proportional to the size of the HBOC.¹⁶ Unfortunately, this study was performed on a wide variety of HBOCs synthesized with different chemistries ranging from acellular Hbs to cellular Hbs. Hence, it was not clear if this principle would be applicable to HBOCs synthesized with the same chemistry. However recently, Cabrales *et al.* demonstrated the same

¹William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio.

²Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), Bethesda, Maryland.

³Department of Bioengineering, University of California, San Diego, La Jolla, California.

heuristic on a small library of tense-state PolyHbs (>500 kDa) of varying size synthesized with the same polymerization chemistry, thereby verifying Sakai's observations.⁹ The results of Cabrales *et al.*'s study identified two tense-state PolyHbs (50:1 and 40:1) with MWs of 1.36–23.71 MDa,⁹ whereas another study identified a zero-link cross-linked bovine Hb (ZL-HbBv) with an average MW of 42 MDa¹⁷; both were reported to elicit no vasoconstriction *in vivo*. Therefore, polymerization of high-MW Hb seems like an effective and simple strategy to limit/prevent vasoconstriction *in vivo*.

This current work will expand on the study by Cabrales *et al.*,⁹ which focused on bovine Hb, and focus solely on the synthesis and *in vitro* biophysical characterization of polymerized human Hb (PolyhHb) solutions in two distinct quaternary states, with both high MW and no free Hb or low-MW PolyhHbs in solution. Glutaraldehyde will be used to nonspecifically cross-link/polymerize hHb to yield high-MW PolyhHb solutions that are frozen in either the tense (T) or relaxed (R) quaternary states. The PolyhHb solutions will be subjected to extensive diafiltration on a 500 kDa filter to remove free Hb and low-MW PolyhHbs. The biophysical properties of the PolyhHbs will then be extensively characterized and used in a mathematical model that describes O₂ transport in a hollow fiber (HF) bioreactor. The HF bioreactor is composed of individual HFs that mimics the structure of a blood vessel. Therefore, the mathematical model will be used to preliminarily assess the ability of PolyhHbs' to oxygenate cultured cells in the device and by extension tissues fed by a blood vessel. The results of these simulations will inform the proper selection of PolyhHbs for applications ranging from tissue engineering to transfusion medicine.

Experimental Procedures

Materials

Glutaraldehyde (70%), synapinic acid, NaCl (sodium chloride), NaOH (sodium hydroxide), Na₂S₂O₄ (sodium dithionite), NaCNBH₃ (sodium cyanoborohydride), NaBH₄ (sodium borohydride), KCl (potassium chloride), CaCl₂ (calcium chloride), sodium lactate, N-acetyl-L-cysteine (NALC), Na₂HPO₄ (sodium phosphate dibasic), and NaH₂PO₄ (sodium phosphate monobasic) were purchased from Sigma-Aldrich (St. Louis, MO). KCN (potassium cyanide), KFe(CN)₆ (potassium ferricyanide), and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). HF cartridges were obtained from Spectrum Labs (Rancho Dominguez, CA).

Hb purification

hHb was purified by tangential flow filtration as described previously in the literature.^{18–20}

PolyhHb synthesis

T-state PolyhHb was synthesized as previously described in the literature.^{9,21–23} hHb was diluted with 20 mM phosphate buffer (PB; pH 8.0) to yield 1.5 L of 0.3 mM hHb that was stored in a 2 L glass bottle submerged in an ice bath. The contents of the glass bottle were then subjected to vacuum for 1 min, followed by argon purging for 1 min. This gas

exchange step was repeated a total of 10 times. The partially deoxygenated hHb solution was then purged with argon for 40 min. After another two short gas exchange steps and long argon purging step, Na₂S₂O₄ was used to remove any residual O₂ in the hHb solution. A stock solution of Na₂S₂O₄ was prepared by dissolving 300 mg Na₂S₂O₄ in 200 mL of cold PB, which was kept on ice in the dark. The Na₂S₂O₄ stock solution was injected into the hHb solution under constant stirring, while the pO₂ of the hHb solution was monitored after each 30 mL infusion of Na₂S₂O₄ using a RapidLab 248 Blood Gas Analyzer (Siemens USA, Malvern, PA). Once the pO₂ was out of range (<0 mm Hg), the infusion of Na₂S₂O₄ was terminated. Next, deoxygenated glutaraldehyde (70%) was added to the deoxygenated hHb solution to polymerize hHb at the following glutaraldehyde to hHb tetramer molar ratios: 50:1 and 40:1. The polymerization reaction was continued for 2 h in the dark at 37°C, and then quenched with 22.5 mL of 2 M NaBH₄ in 20 mM PB to yield T-state PolyhHb.

R-state PolyhHb was synthesized as previously described in the literature.^{21–23} About 1.5 L of 0.3 mM hHb in 20 mM PB was purged with pure O₂ for 2 h in an ice bath, and its pO₂ was continuously monitored with a RapidLab 248 Blood Gas Analyzer. After the pO₂ was out of range (>749 mm Hg), glutaraldehyde was added at the following glutaraldehyde to hHb molar ratios—30:1 and 20:1. At the end of the 2 h polymerization period in the dark at 37°C, 3.5 mL of 8 M NaCNBH₃ in 20 mM PB was added to the reaction mixture to reduce the resultant Schiff bases and methemoglobin (metHb; i.e., oxidized form of Hb). The reaction mixture was then stirred on ice for another 30 min, and then 13.5 mL of 2 M NaBH₄ was added to quench the reaction to yield R-state PolyhHb.

Diafiltration of PolyhHb

Both T-state PolyhHb and R-state PolyhHb were first passed through a column packed with glass wool to remove any large particles from solution. The clarified PolyhHb solution was then subjected to 4 cycles of diafiltration on a 500 kDa MW cutoff HF cartridge (Spectrum Labs) with a modified lactated Ringer's buffer (NaCl 115 mM, KCl 4 mM, CaCl₂ 1.4 mM, NaOH 13 mM, sodium lactate 27 mM, and NALC 2 g/L). The concentrated PolyhHb solution was then stored at –80°C.

Protein concentration of PolyhHb

The total protein concentration of hHb/PolyhHb was measured by the Bradford method²⁴ using the Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL).

MetHb level of PolyhHb

The metHb level of hHb/PolyhHb was measured by the cyanomethemoglobin method.²⁵

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PolyhHb

Twenty-five micrograms of hHb or PolyhHb was mixed with an equal volume of denaturation sample buffer (Bio-Rad, Hercules, CA), and boiled for 5 min before being loaded on the sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) gel. Electrophoresis was conducted in a Mini Format 1-D Electrophoresis System (Bio-Rad) using a 12% SDS-PAGE gel. After electrophoresis, the gel was fixed and stained in Staining Buffer (Bio-Rad), and destained before image analysis. Raw photos were processed by Quantity One (Bio-Rad) software.

Native-PAGE of PolyhHb

Twenty micrograms of hHb or PolyhHb was mixed with a 1/4 volume of native-PAGE Sample Buffer (Invitrogen, Carlsbad, CA), and loaded on a Novex[®] 4%–12% Tris-Glycine gel (Invitrogen). After electrophoresis, the gel was fixed and stained in Staining Buffer (Bio-Rad), and destained before image analysis. Photos were taken and processed by Quantity One (Bio-Rad) software.

Absolute MW distribution of PolyhHb

The absolute MW distribution of hHb and PolyhHb solutions was characterized by size exclusion chromatography coupled with multi-angle static light scattering as described previously in the literature.^{9,26}

Matrix-assisted laser desorption/ionization of PolyhHb

Protein samples were diluted with deionized water to a concentration of 0.2 mg/mL, and mixed in 1:1:1 (v/v/v) ratio with 0.1 M HCl and saturated sinapinic acid in 50% (v/v) acetonitrile solution on a matrix-assisted laser desorption/ionization (MALDI) plate (Bruker Inc., Billerica, MA). Results were obtained on a Bruker Microflex machine (Bruker Inc.).

O₂ affinity and cooperativity of PolyhHb

The equilibrium O₂ binding properties of hHb/PolyhHb were measured using a Hemox-Analyzer (TCS Scientific Corporation, New Hope, PA) at 37°C. Samples were prepared by thoroughly mixing 50–100 µL of hHb/PolyhHb with 5 mL of Hemox buffer (pH 7.4), 20 µL of Additive-A, 10 µL of Additive-B, and 10 µL of anti-foaming agent (all purchased from TCS Scientific Corporation). The samples were allowed to saturate to a pO₂ (i.e., partial pressure of O₂) of 145 ± 2 mm Hg using compressed air. After giving the sample enough time to equilibrate, the gas stream was switched to pure N₂ to deoxygenate the sample. The absorbance of oxygenated and deoxygenated Hb in solution was recorded as a function of pO₂ via dual wavelength spectroscopy. O₂-hHb/PolyhHb equilibrium curves were fit to a four-parameter (A_0 , A_∞ , P_{50} , and n) Hill model (Equation 1). Where Y is the fractional saturation of hHb/PolyhHb (Y has no units and ranges from 0 to 1, a value of 0 corresponds to fully deoxygenated hHb/PolyhHb, while a value of 1 corresponds to fully deoxygenated hHb/PolyhHb), while A_0 and A_∞ represent the absorbance at 0 mm Hg and full saturation, respectively. P_{50} represents the pO₂ at which the heme binding sites in the Hb sample are 50% saturated with O₂, while n is the Hill coefficient, a measurement of cooperativity in O₂ binding.

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

The goodness of fit was verified by comparing the experimental data ($Y = \frac{Abs - A_0}{A_\infty - A_0}$ versus pO₂) with the curve fitted data ($Y = \frac{pO_2^n}{pO_2^n + P_{50}^n}$ versus pO₂).

Circular dichroism spectroscopy of PolyhHb

The circular dichroism (CD) spectra of hHb/PolyhHb was measured on an AVIV CD spectrometer (Lakewood, NJ). hHb/PolyhHb was first diluted to 1.7 mg/mL in 20 mM PB (pH 8.0), and scanned from 500 to 250 nm in a 0.1 cm path length quartz cell. Samples were also diluted to 0.085 mg/mL in 20 mM PB (pH 8.0), and scanned from 250 to 200 nm in a 0.1 cm path length quartz cell. Data were collected and analyzed by AVIV data collection software. Each curve was an average of 3 scans.

Fast kinetic analysis of PolyhHb and ligand reactions

Fast kinetic measurements of gaseous ligand reactions with hHb/PolyhHb were carried out in an Applied Photophysics SF-17 micro-volume stopped-flow instrument as previously described.²⁷ O₂ dissociation kinetics were measured by rapidly mixing 30 µM of O₂-hHb/PolyhHb (heme concentration) with 1.5 mg/mL of sodium dithionite (British Drug House, Poole, England), and the deoxygenation process was monitored by absorbance changes at 437.5 nm in 0.05 M Tris buffer, pH 7.4, at 25°C. The averaged kinetic traces were fit to exponential equations using Marquardt-Levenberg fitting routines in the Applied Photophysics program. The kinetics of CO association with deoxygenated hHb/PolyhHb were measured in the stopped-flow instrument following absorbance changes at 437.5 nm in 0.05 M Tris buffer, pH 7.4, at 25°C in the presence of 1.5 mg/mL of Na₂S₂O₄. The CO saturated stock solution (about 1 mM) was prepared by flowing prewashed CO gas through degassed buffer solution.

The fast kinetics of NO oxidation with O₂-hHb/PolyhHb solutions were measured in the stopped-flow instrument as previously described.²⁸ NO gas prewashed in deoxygenated 1 M NaOH and buffer solutions was bubbled through deoxygenated 0.05 M Tris buffer, pH 7.4, to make an NO-saturated stock solution (~2 mM) in a gas-tight serum bottle. Specific amounts of the NO stock solution were then transferred with a Hamilton syringe into a gastight syringe containing deoxygenated buffer solution to make appropriate NO concentrations for the reaction with O₂-hHb/PolyhHb solutions. O₂-hHb/PolyhHb solutions (1 µM heme) were mixed with NO solutions (0–50 µM), and the absorbance changes of the reaction were followed at 420 nm. Multiple traces were taken and averaged for each reaction. The averaged trace was fit to exponential equations to obtain the reaction rate constant.

Spontaneous oxidation of PolyhHb solutions

All hHb/PolyhHb solutions were reduced to O₂-hHb/PolyhHb immediately before autoxidation experiments. Experiments were carried out with 20–25 µM hHb/PolyhHb (heme concentration) in sealed cuvettes with room air-equilibrated 50 mM Chelex-treated PB at 37°C. Absorbance changes in the range of 450–700 nm due to spontaneous

oxidation of O₂-hHb/PolyhHb were recorded up to 24 h 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). A multi-component analysis was performed to calculate the oxy and ferric species based on their individual extinction coefficients.²⁹ Autoxidation rates were obtained from plots of the loss of O₂-hHb/PolyhHb versus time using nonlinear least squares curve fitting with a double exponential equation in Sigma-Plot (SPSS, Chicago, IL).

Viscosity and colloid osmotic pressure of PolyhHb

hHb/PolyhHb 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/second. The colloid osmotic pressure (COP) of hHb/PolyhHb was measured using a Wescor 4420 Colloid Osmometer (Wescor, Logan, UT).

Statistical analysis

All data were analyzed by t-test, and a *p*-value of 0.05 or less was considered significant.

Simulation of PolyhHb facilitated O₂ transport in a HF bioreactor

The main function of these materials is to store and transport O₂. Therefore, the purpose of the mathematical model is to preliminarily determine the degree to which these polymerized human Hb (PolyhHb) molecules are capable of transporting O₂ to surrounding cells cultured in the HF bioreactor and by extension tissues fed by a blood vessel without performing expensive and time-consuming *in vitro* (bioreactor) or *in vivo* (animal) studies. In reality, the HF

bioreactor is used to facilitate cell culture; however, it can also be used to simulate the structure of a blood vessel surrounded by tissue. The main advantage of the O₂ transport model is that the model parameters have been experimentally measured for all PolyhHbs.

The PolyhHb selection criteria for transfusion versus bioreactor applications will be different and will mainly depend on a variety of factors such as inlet pO₂, flow rate of solution, viscosity of solution, concentration of PolyhHb, size of PolyhHb, O₂ affinity of PolyhHb, O₂ dissociation rate constant of PolyhHb, and cell/tissue O₂ consumption rate. A convenient feature of this model is that all of these parameters can be varied in the simulation and its effect on oxygenation of the surrounding cells or tissues can be examined, to select PolyhHbs for either of these two applications.

Therefore, the aim of these simulations is to explore the oxygenation potential of PolyhHbs in oxygenating a tissue-engineered construct (i.e., a bioartificial liver assist device) and by extension tissue fed by a cylindrical blood vessel. A mathematical model previously developed by Chen and Palmer was used to assess the ability of PolyhHbs in oxygenating mammalian cells (C3A hepatocytes) cultured in a HF bioreactor.³⁰ These results should yield some preliminary insight into how PolyhHbs would oxygenate tissues when transfused *in vivo*.

The O₂ transport model is based on the geometry of a single HF in a HF bioreactor, which mimics the *in vivo* capillary/sinusoid structure (Fig. 1). In this geometry, cell culture medium supplemented with HBOC enters the HF bioreactor at the entrance of the lumen to provide nutrients to cultured cells, which reside in the extra capillary space (ECS), and leave the HF bioreactor through the exit port of the lumen carrying away waste products. The cell culture medium recirculates throughout the entire HF bioreactor system. The HF membrane has a MW cut-off of 35 kDa and therefore confines HBOCs (MW > 64 kDa) within the

FIG. 1. Geometry of HF bioreactor system. (A) Schematic of the entire HF bioreactor system. (B) Closer view of the HF bioreactor. (C) Diagram of a single HF used in O₂ transport simulations. HF, hollow fiber. Color images available online at www.liebertonline.com/tea.

luminal space of the HF without contacting the cells residing in the ECS.

The velocity profile in each of the three subdomains (lumen, membrane, and ECS) can be calculated from a set of momentum transport partial differential equations, shown in Equation 2.

Navier-Stokes equation (lumen):

$$(\vec{v}' \cdot \nabla) \vec{v}' = - \left[\frac{\mu}{l_0 v_0 \rho} \right] \nabla P' + \left[\frac{\mu}{l_0 v_0 \rho} \right] \nabla^2 \vec{v}' \quad (2-A)$$

Brinkman's Equation (membrane and ECS):

$$\left[\frac{l_0^2}{\kappa} \right] \vec{v}' = \nabla(-P') + \nabla^2 \vec{v}' \quad (2-B)$$

Where $\vec{v}' = \frac{\vec{v}}{v_0}$, $P' = \frac{P}{\mu v_0 / l_0}$. $\vec{v}'$ is the dimensionless velocity

vector and P' is the dimensionless pressure. l_0 , v_0 , ρ , and μ , represent the reference length, reference velocity, fluid density, and viscosity, respectively.

Equation 3 shows the mass conservation equations, which describe the transport of dissolved O_2 , total HBOC, and O_2 -HBOC in dimensionless form. C' can either represent the dimensionless O_2 partial pressure (p_{O_2}), total HBOC concentration, or O_2 -HBOC concentration. C_0 can either represent the reference O_2 partial pressure, total HBOC concentration, or O_2 -HBOC concentration. D can either represent the diffusivity of O_2 or HBOC. R represents the rate of formation of O_2/O_2 -HBOC. The HBOC diffusivity is estimated from the PolyhHb MW using Equation 4.

$$\nabla \cdot (-\nabla C') = \left[\frac{l_0^2}{D} \cdot \frac{R}{C_0} \right] - \left[\frac{v_0 l_0}{D} \right] \vec{v}' \cdot \nabla C' \quad (3)$$

$$D = 1.013 \times 10^{-4} (MW)^{-0.46} (cm^2/s) \quad (4)$$

The reaction between O_2 and HBOC is described by Equation 5, where m is the number of O_2 binding sites (i.e., heme groups) on a single HBOC molecule. The number of O_2 binding sites on PolyhHb can be calculated by dividing the weight average MW of PolyhHb by the MW of tetrameric Hb multiplied by a factor of 4, which represents the number of heme groups per Hb tetramer. Given the thermodynamic relationship describing the equilibrium between HBOC and O_2 (Equation 6, where a_1 - a_4 are the Adair constants), the rate of formation of O_2 (R_{O_2} [mm Hg/s]) or O_2 -HBOC ($R_{oxyHBOC}$ [mol/m³/s]) in the lumen is shown in Equation 7. S is defined as the HBOC saturation; that is, the molar fraction of HBOC that is saturated with O_2 and S_{eq} is the HBOC saturation at equilibrium. $[O_2]$ is the concentration of dissolved O_2 , $[HBOC]$ is the concentration of total HBOC and α is the solubility of O_2 in the aqueous medium. The O_2 consumption rate in the ECS is described by Michaelis-Menten (M-M) kinetics.

$$S_{eq} = \frac{a_1 \cdot pO_2 + 2a_2 \cdot pO_2^2 + 3a_3 \cdot pO_2^3 + 4a_4 \cdot pO_2^4}{4(1 + a_1 \cdot pO_2 + a_2 \cdot pO_2^2 + a_3 \cdot pO_2^3 + a_4 \cdot pO_2^4)} \quad (6)$$

$$R_{oxyHBOC} = -\frac{\alpha}{m} \cdot R_{O_2} = k^- \cdot [HBOC] \left(\frac{S_{eq}}{1 - S_{eq}} (1 - S) - S \right) \quad (7)$$

Further details about the mathematical model and parameters/constants used in the simulations can be found in the literature.³⁰ The experimentally measured biophysical properties of the hHb/PolyhHb solutions were used in these simulations (i.e., MW, P_{50} , n , and k^-). The coupled set of partial differential equations was solved by the finite element method in Comsol Multiphysics (COMSOL, Inc., Burlington, MA) yielding numerical solutions.

Results

Polymerization of hHb

The major chemical reaction in the polymerization of hHb with glutaraldehyde involves Michael addition between α , β -unsaturated oligomeric aldehydes and primary amine groups on lysine residues that are present on the surface of hHb (Fig. 2A). Some Schiff bases form during the polymerization step, but they are all reduced via the addition of NaBH₄ or NaCNBH₃ at the end of the polymerization reaction. Therefore, polymerization of hHb is based on stable C-N bonds that will not hydrolyze in solution.

Figure 2B shows the SDS-PAGE of hHb and all PolyhHb solutions under denaturing conditions. After sample denaturation, hHb yields two bands in the gel. The lower band corresponds to individual α/β subunits, whereas the upper band corresponds to α - α/β - α/β dimers that do not dissociate into individual subunits. In contrast, all PolyhHb solutions under denaturing conditions display strong bands >250 kDa and weak bands close to 15, 30, 45, and 60 kDa in MW corresponding to individual α/β subunits, α - α/β - α/β dimers, α/β trimers, and α/β tetramers. In fact, PolyhHb's with higher cross-link density locked in the same quaternary state exhibit less individual α/β subunits, dimers, trimers, and tetramers compared to lower cross-link density PolyhHb.

Figure 2C shows the absolute MW distribution of hHb and PolyhHb solutions under nondenaturing conditions. Tetrameric hHb displays a weight averaged MW of ~62 kDa, whereas all PolyhHbs display weight averaged MWs >62 kDa. The weight averaged MW of hHb and all PolyhHbs is displayed in Table 1. Light scattering results clearly show that both T- and R-state PolyhHbs possess no individual α/β subunits, dimers, trimers, and tetrameric hHb in solution.

Figure 2D shows the native PAGE analysis of hHb and PolyhHb solutions under nondenaturing conditions. The position of the hHb tetramer in the gel is labeled by an arrow. None of the PolyhHb solutions display bands at or lower than the position of the hHb tetramer. This demonstrates that PolyhHb is free of Hb tetramers, trimers, dimers, or individual α/β subunits in solution monomers.

Figure 2E shows the MALDI mass spectral analysis of hHb and PolyhHb solutions. It is evident that hHb is composed of 2 peaks corresponding to individual α and β subunits. There is no peak corresponding to dimers as indicated in the SDS-PAGE analysis of hHb (Fig. 2B). Also, there are no peaks corresponding to α/β subunits, dimers, trimers, or tetramers for all PolyhHb solutions, in stark contrast to the SDS-PAGE results (Fig. 2B).

FIG. 2. Schematic of reaction between hHb and glutaraldehyde, and methods used to assess the extent of polymerization. **(A)** Reaction scheme showing the chemical reaction between primary amine groups on hHb and reactive moieties on glutaraldehyde. **(B)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of hHb and PolyhHb solutions under denaturing conditions. **(C)** Absolute MW distribution of hHb and PolyhHb solutions under nondenaturing conditions. **(D)** Native gel of hHb and PolyhHb solutions under nondenaturing conditions. **(E)** Matrix-assisted laser desorption/ionization mass spectra of hHb and PolyhHb. hHb, human hemoglobin; PolyhHb, polymerized human hemoglobin; MW, molecular weight. Color images available online at www.liebertonline.com/tea.

TABLE 1. BIOPHYSICAL PROPERTIES OF NATIVE HUMAN HEMOGLOBIN AND POLYMERIZED HUMAN HEMOGLOBIN SOLUTIONS

Solution	Weight averaged molecular weight	P_{50} (mm Hg)	Hill coefficient	Methemoglobin level (%)
hHb	62.29 ± 1.63 kDa	13.27 ± 0.55	2.59 ± 0.12	0.88 ± 0.33
40:1 T-state PolyhHb	3.68 ± 1.33 MDa	37.19 ± 1.49	0.81 ± 0.05	4.32 ± 0.41
50:1 T-state PolyhHb	18.44 ± 7.23 MDa	48.84 ± 4.22	0.87 ± 0.16	4.99 ± 1.26
20:1 R-state PolyhHb	1.10 ± 0.33 MDa	1.45 ± 0.14	0.70 ± 0.06	2.97 ± 0.97
30:1 R-state PolyhHb	5.54 ± 2.00 MDa	0.76 ± 0.50	0.91 ± 0.51	5.81 ± 3.51

hHb, human hemoglobin; PolyhHb, polymerized human hemoglobin.

To estimate the stability of PolyhHb during long-term storage, the MW distribution of 40:1 T-state PolyhHb was analyzed immediately after synthesis, after 8 months of storage at -80°C , and after 8 months of storage at -80°C coupled with incubation at 37°C for 5 days (Supplementary Figure S1; Supplementary Data are available online at www.liebertonline.com/ten). Eight months of storage at -80°C had no influence on the MW distribution of the 40:1 T-state PolyhHb, whereas incubation of the 40:1 T-state PolyhHb at 37°C for 5 days significantly increased the MW of the PolyhHb. Analysis of the MW of the 40:1 T-state PolyhHb under all conditions yielded no free Hb tetramers, trimers, dimers, or α/β subunits in solution. For this study the PolyhHb was in the oxygenated form and not the deoxygenated form. Therefore, it is more subjective to oxidative reactions for prolonged exposures to high temperatures unlike the deoxygenated version of the same compound.

O₂ binding affinity and cooperativity of PolyhHb

Figure 3A shows the O₂-hHb/PolyhHb equilibrium curves. T-state PolyhHb solutions are right-shifted compared to the control of hHb, whereas R-state PolyhHb solutions are

left-shifted compared to the control of hHb. Figure 3B shows the regressed O₂ affinity (P_{50}) of T- and R-state PolyhHbs. Compared to the control of hHb (13.27 ± 0.55 mm Hg), T-state PolyhHbs display much higher P_{50} s. In contrast, R-state PolyhHbs has extremely low P_{50} s compared to both hHb and T-state PolyhHbs. Both T- and R-state PolyhHbs display cooperativity coefficients <1 , whereas unmodified hHb has a cooperativity coefficient of 2.59 ± 0.12 (Fig. 3C). The O₂ affinity and cooperativity coefficient of hHb and all PolyhHbs are listed in Table 1.

MetHb level of PolyhHb

Figure 4 displays the metHb levels of hHb, T-, and R-state PolyhHb solutions. Both T- and R-state PolyhHbs display metHb levels $<10\%$, but higher than the metHb level of hHb ($0.88\% \pm 0.33\%$). Table 1 summarizes the biophysical properties of hHb and PolyhHb solutions.

CD spectroscopy of PolyhHb

The influence of glutaraldehyde polymerization on hHb structure is determined by CD spectroscopy in the

FIG. 3. O₂ binding affinity and cooperativity of PolyhHb solutions. **(A)** O₂ equilibrium curves of native hHb and PolyhHb solutions, where Y is the fraction of heme sites saturated with O₂. **(B)** Regressed O₂ affinity (P_{50}) of native hHb and PolyhHb solutions. **(C)** Regressed cooperativity coefficient (n) of native hHb and PolyhHb solutions. Error bars represent the standard deviation from triplicate reactions. Color images available online at www.liebertonline.com/tea.

FIG. 4. Methemoglobin levels of native hHb and PolyhHb solutions. Error bars represent the standard deviation from triplicate reactions.

far-ultraviolet and near-ultraviolet regions. The influence of hHb polymerization on the secondary structure of hHb is determined by far-ultraviolet CD spectroscopy in the wavelength range 200 to 250 nm (Fig. 5A). In the far-ultraviolet region, all hHb/PolyhHb spectra share a common peak at ~ 220 nm with similar spectral intensity. In contrast, the influence of hHb polymerization on the heme environment is determined by near-ultraviolet CD spectroscopy in the wavelength range 250 to 500 nm (Fig. 5B). In the near-ultraviolet region, all hHb/PolyhHb spectra share common peaks at ~ 260 and 420 nm, with slight differences in intensity among all hHb and PolyhHb solutions.

Stopped-flow kinetics of PolyhHb and ligand reactions

Figure 6 shows a representative time course of CO binding to deoxygenated Hb and the CO concentration dependence of the apparent reaction rate constants for deoxygenated native hHb and PolyhHb solutions. Unlike the slightly lower CO binding rate constants of T-state PolyhHb solutions compared to that of hHb, R-state PolyhHb solutions exhibits much higher CO reactivities characterized by the large increase (>20 -fold) in bimolecular rate constants. Table 2 summarizes the CO binding ($k'_{\text{on,CO}}$), O_2 dissociation (k_{off}), and NO binding/oxidation ($k'_{\text{ox,NO}}$) rate constants as the mean of a minimum of three separate measurements. The O_2 dissociation rate constants of T- and R-state PolyhHb solutions differ from that of native hHb by either a $\sim 30\%$ increase or reduction, respectively, whereas the NO reactivities are very similar among all hHb/PolyhHb solutions in our study.

FIG. 5. CD spectra of hHb and PolyhHb solutions. (A) CD in the far-ultraviolet region ranging from 200 to 250 nm. (B) CD in the near-ultraviolet region ranging from 250 to 500 nm. Each curve represents the average of three measurements. CD, circular dichroism. Color images available online at www.liebertonline.com/tea.

FIG. 6. Representative time course of CO binding to deoxygenated hHb. The inset shows the CO concentration dependence of the apparent reaction rate constants for native hHb and PolyhHb solutions.

TABLE 2. KINETIC PARAMETERS OF GASEOUS LIGAND REACTIONS WITH HUMAN HEMOGLOBIN AND POLYMERIZED HUMAN HEMOGLOBIN SOLUTIONS

Solution	k_{off} (s^{-1})	$k'_{on,CO}$ ($\mu M^{-1}s^{-1}$)	$K'_{ox,NO}$ ($\mu M^{-1}s^{-1}$)
hHb	40.4	0.214	18.5
40:1 T-state PolyhHb	49.7	0.181	19
50:1 T-state PolyhHb	51.7	0.184	20.1
20:1 R-state PolyhHb	31.4	4.76	21.8
30:1 R-state PolyhHb	25.5	4.88	20.2

Autoxidation kinetics of PolyhHb

The autoxidation rate constants of oxygenated hHb and PolyhHb solutions are summarized in Table 3. The high O_2 affinity PolyhHb solutions exhibit similar spontaneous oxidation rate constants compared to that of unmodified hHb. Conversely, the low O_2 affinity PolyhHb solutions show much elevated spontaneous oxidation rates under the same experimental conditions. Additionally, PolyhHb solutions show much less of a response compared to hHb in the presence of the antioxidant enzymes superoxide dismutase (SOD) and catalase.

Viscosity and COP of PolyhHb

Table 4 shows the viscosity and COP of hHb as well as T- and R-state PolyhHb solutions. The viscosity of both PolyhHbs in a distinct quaternary state increases with increasing cross-link density, whereas the COP is virtually unaffected by cross-link density. However, T-state PolyhHbs exhibit a much lower COP versus R-state PolyhHbs.

Simulation results

Figure 7A shows the normalized O_2 consumption rate of C3A hepatocytes as a function of the inlet pO_2 ($pO_{2,in}$). The value of the normalized O_2 flux starts at 15–20 at low $pO_{2,in}$ s (~ 5 mm Hg) and then decreases as the $pO_{2,in}$ increases for all HBOCs. At higher $pO_{2,in}$ s (~ 150 mm Hg), O_2 transport is still at least 2–3 times greater versus the case with no HBOC supplementation.

The pO_2 profiles within the HF bioreactor (including lumen, membrane, and ECS) are shown in Figure 7B for all HBOCs at varying concentrations. Each unit represents the cross-sectional view of a single HF (Fig. 1C). The top horizontal boundary represents the HF centerline, whereas the left boundary represents the inlet of the lumen and the right boundary represents the lumen exit. The maximum heme

concentration ([Heme]) in the HF bioreactor is normalized by the average heme concentration in human blood (8800 μM). Without any HBOC supplementation in the HF bioreactor, most of the ECS is hypoxic, with pO_2 levels below 20 mm Hg. As the HBOC concentration increases, O_2 transport in the HF improves and the hypoxic region in the ECS gradually reduces in size. Among all the HBOCs simulated, R-state PolyhHbs show the least capacity to oxygenate the ECS versus hHb and T-state PolyhHbs. However, T-state PolyhHbs are better at oxygenating the ECS versus native hHb and R-state PolyhHb.

Similar oxygenation results are observed for the ECS zonation breakdown plots (Fig. 7C). As mentioned previously, in the absence of HBOCs, the hypoxic region (<25 mm Hg) dominates the majority ($\sim 95\%$) of the ECS volume, and only a small percentage of hepatocytes ($\sim 5\%$) are subjected to *in vivo* pO_2 levels (25–70 mm Hg). The hypoxic region gets smaller in size when HBOCs are present in the cell culture media. However, under the simulation conditions, only T-state PolyhHbs successfully improve O_2 transport and recapitulated the *in vivo* pO_2 environment in the ECS with virtually no part of the ECS experiencing hypoxic oxygenation ($<5\%$). In contrast, a significant volume of the ECS is hypoxic with supplementation of hHb ($\sim 55\%$) and R-state PolyhHbs (65%–80%).

Discussion

Light scattering, native-PAGE, and MALDI analysis of both T- and R-state PolyhHb solutions show that they exhibit large weight averaged MWs, without the presence of hHb tetramers, trimers, $\alpha\beta$ dimers, and individual α/β subunits in solution in approximately physiological conditions. On the other hand, SDS-PAGE shows the presence of a small percentage of individual α/β subunits and $\alpha\beta/\alpha\alpha/\beta\beta$ dimers, trimers, and tetramers in the PolyhHb solutions under denaturing conditions. This apparent contradiction is caused by the different environmental conditions to which the sample is exposed to during these measurements.

In SDS-PAGE, hHb/PolyhHb solutions are subjected to harsh denaturing conditions, which should dissociate α/β monomers and dimers that are not cross-linked to the PolyhHb superstructure. However, these harsh sample processing conditions actually chemically cross-link α/β subunits into higher order structures, that is, dimers, trimers, and tetramers. Evidence of this phenomenon is found in the SDS-PAGE of hHb, where only one band corresponding to α/β subunits should be present in the gel. Instead, the SDS-PAGE of hHb has an additional band corresponding to dimers. There are no chemical cross-links between the individual subunits of unmodified hHb. Therefore, SDS-PAGE of

TABLE 3. AUTOXIDATION RATE CONSTANTS OF HUMAN HEMOGLOBIN AND POLYMERIZED HUMAN HEMOGLOBIN SOLUTIONS IN THE PRESENCE AND ABSENCE OF SUPEROXIDE DISMUTASE AND CATALASE

Solution	k_{autox} (min^{-1})	$k_{autox} + SOD/catalase$ (min^{-1})
hHb	$5.54 \times 10^{-4} \pm 3.44 \times 10^{-5}$	$3.02 \times 10^{-4} \pm 1.64 \times 10^{-5}$
40:1 T-state PolyhHb	$1.45 \times 10^{-3} \pm 4.45 \times 10^{-5}$	$1.35 \times 10^{-3} \pm 7.33 \times 10^{-5}$
50:1 T-state PolyhHb	$1.34 \times 10^{-3} \pm 8.58 \times 10^{-5}$	$1.16 \times 10^{-3} \pm 2.03 \times 10^{-5}$
20:1 R-state PolyhHb	$4.98 \times 10^{-4} \pm 1.35 \times 10^{-5}$	$4.47 \times 10^{-4} \pm 1.92 \times 10^{-5}$
30:1 R-state PolyhHb	$6.86 \times 10^{-4} \pm 1.02 \times 10^{-4}$	$6.0 \times 10^{-4} \pm 9.64 \times 10^{-6}$

SOD, superoxide dismutase.

TABLE 4. VISCOSITY AND COLLOID OSMOTIC PRESSURE OF HUMAN HEMOGLOBIN AND POLYMERIZED HUMAN HEMOGLOBIN SOLUTIONS

Solution	Concentration (g/dl)	Viscosity (cp)	COP (mm Hg)
hHb	10	1.6	38
40:1 T-state PolyhHb	9.9	8.7	4
50:1 T-state PolyhHb	10	9.6	4
20:1 R-state PolyhHb	9.1	3.2	37
30:1 R-state PolyhHb	9.6	6.6	24

COP, colloid osmotic pressure.

hHb should only yield 1 band corresponding to α/β subunits. The fact that there are 2 bands indicates that the sample preparation condition for SDS-PAGE induces chemical cross-links between subunits. This calls into question the actual presence of cross-linked dimers, trimers, and tetramers in PolyhHb solutions as determined by SDS-PAGE. These cross-linked species are not present in solution when PolyhHbs are analyzed by light scattering, native-PAGE, and MALDI mass spectral analysis.

Therefore, it is safe to conclude that the final PolyhHb products are free of α/β subunits, dimers, trimers, and tetramers in solution. However, the PolyhHb products are composed of some uncross-linked α/β subunits that are not chemically integrated into the PolyhHb superstructure. At a fixed quaternary state, SDS-PAGE, native-PAGE, light scattering, and MALDI all show an increase in the MW of PolyhHb with increased cross-link density. This result is expected since the number of potential chemical cross-links increases with increasing cross-link density.

Compared to other HBOCs reported in the literature, T- and R-state PolyhHb solutions possess smaller MWs (1.10–18.44 MDa) than 50:1 T- and 40:1 R-state PolybHb (16.59–26.33 MDa)^{21,22} and ZL-HbBv (42 MDa),¹⁷ but larger MWs than O-raffinose cross-linked hHb (O-R-polyHbA₀, 64–600 kDa)³¹ and Oxyglobin® (87.2–502.3 kDa).³² The two commercially manufactured PolyHb products, Hemopure® and PolyHeme®, possess average MWs of 250 and 150 kDa, respectively.^{11,13} Hence, PolyhHb products are up to 70 times larger than Hemopure®, and 120 times larger than PolyHeme®. In addition, Hemopure® and PolyHeme® both have <5% unreacted hHb tetramers and $\alpha\beta$ dimers in solution. Free hHb tetramers, $\alpha\beta$ dimers, and low-MW PolyHbs are believed to elicit vasoconstriction and systemic hypertension *in vivo*. All PolyhHbs are free of hHb, $\alpha\beta$ dimers, and low-MW PolyHbs and should generate limited/no vasoconstriction compared to current commercial PolyHbs.

T- and R-state PolyhHbs exhibit vastly different O₂ affinities. T-state PolyhHbs possess P₅₀s > 35 mm Hg, which are considerably higher than that of unmodified hHb (13.27 ± 0.55 mm Hg), hHb inside human red blood cells (26–28 mm Hg), PolyHeme® (29 mm Hg),¹² and ZL-HbBv (6.4 mm Hg),¹⁷ but comparable to Hemopure® (38 mm Hg),¹² O-R-polyHbA₀ (50.9 mm Hg),³³ and Oxyglobin® (35.1 mm Hg).³² Since hHb is totally deoxygenated before, during and after polymerization (but not during storage), the resultant T-state PolyhHbs are conformationally frozen in the T-state after polymerization. Hence, T-state PolyhHbs exhibit higher P₅₀s compared to hHb. In contrast, R-state PolyhHbs exhibit

P₅₀s < 2 mm Hg, indicating their extremely high O₂ affinity. This is because hHb is fully saturated with pure O₂ before, during and after polymerization. Therefore, the resultant R-state PolyhHbs are conformationally frozen in the R-state after polymerization. Hence, R-state PolyhHbs exhibit lower P₅₀s compared to hHb. This pattern is consistent with PolybHb (the P₅₀ is 41 mm Hg for 50:1 T-state PolybHb and 0.66 mm Hg for 40:1 R-state PolybHb).^{21,22}

Both PolyhHbs show no cooperativity. Polymerization of hHb freezes the structure of hHb in a well-defined quaternary state. Since, the individual subunits are cross-linked to each other, quaternary structure changes, which would occur during normal O₂ binding/offloading are hindered by the presence of chemical cross-links. This results in the loss of cooperative O₂ binding to the hHb tetramer for all PolyhHbs. The two commercially produced PolyHbs, Hemopure® ($n = 1.4$) and PolyHeme® ($n = 1.7$),¹² and other reported HBOCs, PolybHb ($n < 1$),²¹ ZL-HbBv ($n = 1.2$),¹⁷ O-R-polyHbA₀ ($n = 1$),³³ and Oxyglobin® ($n = 1.4$),³² also show reduced cooperativity compared to native hHb ($n = 2.59 \pm 0.12$) or bHb ($n = 2.5$).²¹ These results indicate that the extent of polymerization of PolyhHb is significantly greater than that of commercial PolyHbs.

The metHb level of T- and R-state PolyhHbs is higher than that of hHb. However, R-state PolyhHbs have a higher metHb level than T-state PolyhHbs. This observation is consistent with the fact that R-state PolyhHbs are maintained in an oxygenated environment, which enhances autooxidation of the heme prosthetic group. On the other hand, current HBOCs in phase III trials possess similar metHb levels. For example, Hemopure® has a metHb level <10%, and PolyHeme® has a metHb level <8%.¹² Therefore, T- and R-state PolyhHbs exhibit metHb levels that are acceptable for clinical evaluation. Nonetheless, the metHb level of PolyhHb solutions could be further reduced after polymerization by incubating these solutions with additional NaCNBH₃, a mild reducing agent.

The CD spectra of hHb and PolyhHb solutions show no significant differences either in the far-ultraviolet region or in near-ultraviolet region. This indicates that the influence of polymerization on the secondary structure and heme environment of hHb is negligible.

Fast kinetic analysis of gaseous ligand binding with PolyhHb solutions shows that the O₂ dissociation rate constant of PolyhHbs (49.7–51.7 s⁻¹ for T-state PolyhHbs and 31.4–25.5 s⁻¹ for R-state PolyhHbs) are comparable to PolybHb (53 s⁻¹ for 50:1 T-state PolybHb, and 22 s⁻¹ for 40:1 R-state PolybHb)²¹ and ZL-HbBv (27.4 s⁻¹),¹⁷ but smaller than the rate constants for O-R-polyHbA₀ (130 s⁻¹)³³ and Oxyglobin® (61.8 s⁻¹).³² The CO association rate constants of T-state PolyhHbs (0.181–0.184 $\mu\text{M}^{-1}\text{s}^{-1}$) are comparable to 50:1 T-state PolybHb (0.18 $\mu\text{M}^{-1}\text{s}^{-1}$),²¹ ZL-HbBv (0.28 $\mu\text{M}^{-1}\text{s}^{-1}$),¹⁷ and Oxyglobin® (0.19 $\mu\text{M}^{-1}\text{s}^{-1}$).³² In contrast, the CO association rate constant of R-state PolyhHbs (4.76–4.88 s⁻¹) are comparable to 40:1 R-state PolybHb (4.84 s⁻¹)²¹ and O-R-polyHbA₀ (1.2 s⁻¹).³³ Both T-state PolyhHbs possess O₂ dissociation rate constants that are larger than that of hHb (40.4 s⁻¹) and CO binding rates smaller than that of hHb (0.214 s⁻¹). On the contrary, both R-state PolyhHbs possess O₂ dissociation rate constants that are smaller than that of hHb, and CO association rate constants that are much larger than hHb. These results are consistent with the O₂ affinities of PolyhHbs, which are in turn reflected by their P₅₀ values

FIG. 7. Simulation of PolyhHb facilitated O_2 transport in a hepatic HF bioreactor. **(A)** Normalized O_2 consumption rate of HBOC supplemented HF bioreactors, normalized by a control HF bioreactor (no HBOC supplementation). **(B)** pO_2 profiles within a single HF (lumen, membrane, and ECS) for all HBOCs at varying heme concentrations. Each rectangle represents the region depicted in Figure 1C. **(C)** ECS Zonation within the HF bioreactor for all HBOCs at [Heme] = 100%. HBOCs, hemoglobin-based oxygen carriers; ECS, extra capillary space. Color images available online at www.liebertonline.com/tea.

obtained under equilibrium conditions. NO binding/oxidation of oxygenated PolyhHb solutions occurs very rapidly on the order of $10^7 \text{ M}^{-1}\text{s}^{-1}$, and remains largely unchanged after glutaraldehyde polymerization. However, the kinetic param-

eters for O_2 dissociation and CO binding of PolyhHb solutions indicate the reduced or increased O_2 affinities after T-state or R-state PolyhHb modification, respectively. More specifically, the large reduction in the P_{50} of R-state PolyhHb solutions

is mostly reflected in their increased CO binding rate constants, whereas the increase in the P_{50} values of T-state PolyhHb solutions are in agreement with their O_2 dissociation rate constants. These physicochemically characteristic changes are determined by the nature of the glutaraldehyde cross-linking reactions under oxygenated or deoxygenated conditions.

Hb autoxidation is an intrinsic characteristic of the heme group, and is usually affected by chemical modification of the Hb molecule. The autoxidation rate constant of low O_2 affinity T-state PolyhHbs ($0.00145\text{--}0.00134\text{ min}^{-1}$) is higher than that of R-state PolyhHbs ($0.00050\text{--}0.00069\text{ min}^{-1}$). The elevated autoxidation rates, especially for low O_2 affinity PolyhHb solutions, are consistent with that of previously reported cross-linked and PolyHbs. For example, the autoxidation rate constant of ZL-HbBv is three times greater than that of bHb, whereas the rate of Oxyglobin[®] autoxidation is 1.3 times greater than that of bHb. Low O_2 affinity 50:1 T-state PolybHb has an increased rate of autoxidation compared to high O_2 affinity 40:1 R-state PolybHb and native bHb.²¹ This undesirable change is one of the biggest challenges facing HBOC development. The increased Hb oxidation not only lowers its capacity to carry O_2 , but also introduces enhanced toxicity that could elicit cell and tissue damage. The weakened protection by SOD and catalase against autoxidation of PolyhHb solutions underlines the complexity of the issues faced by this chemical modification.

The viscosity of both types of PolyhHb solutions increases with increasing cross-linking density and is higher than that of whole blood (~ 3 cp), Hemopure[®] (1.3 cp at 13 g/dL), and PolyHeme[®] (2.1 cp at 10 g/dL),¹² but similar to that of PolybHb (11.4 cp for 50:1 T-state PolybHb and 7.8 cp for 40:1 R-state PolybHb at a concentration of 10 g/dL).²¹ High-viscosity HBOCs are preferred for transfusion, since they can elicit the generation of endothelial-derived relaxing factors, through flow-mediated endothelial mechanotransduction.³⁴ This can potentially neutralize the vasoconstrictive effect caused by NO scavenging or O_2 oversupply upon PolyHb infusion, if it still exists. The COP of both PolyhHb solutions is less than the COP of whole blood (27 mm Hg).¹² However, R-state PolyhHbs exhibit COPs comparable to Hemopure[®] (25 mm Hg) and PolyHeme[®] (23 mm Hg).¹² T-state PolyhHbs exhibit COPs comparable to PolybHb (1 mm Hg for 50:1 T-state PolybHb and 7 mm Hg for 40:1 R-state PolybHb).²¹ Low COP fluids can facilitate the outward flow of intravascular fluid across blood vessels into the tissue space, thereby reducing the blood volume. However, this problem could be remediated by supplementing human serum albumin to the low COP PolyhHb solution, thereby increasing the PolyhHb solution COP.³⁵

Among all the HBOCs we studied via simulation, the O_2 equilibrium curves of R-state PolyhHbs are left-shifted corresponding to low P_{50} values, whereas T-state PolyhHbs are right-shifted with correspondingly high P_{50} values, compared to native hHb. It is apparent from Figure 7A that all PolyhHb solutions have slightly less capacity for improving O_2 transport at lower inlet pO_2 s (<40 mm Hg) compared to native hHb. This is probably due to the low cooperativities and large diffusion coefficients of these molecules that are conferred upon polymerization. However, there is not much difference between all PolyhHb solutions and native hHb in the normalized O_2 consumption rate at higher inlet pO_2 s,

especially for T-state PolyhHbs, which possess higher P_{50} s. This becomes more evident in the pO_2 profile and ECS zonation plots (Fig. 7B and C). Generally, T-state PolyhHbs (high P_{50}) enhance O_2 transport to the ECS in the HF bioreactor. Under the simulated conditions (inlet $pO_2 = 80$ mm Hg), T-state PolyhHbs can release a considerable amount of O_2 to the HF ECS especially as the local pO_2 drops below the P_{50} of the HBOC. However, for R-state PolyhHbs, which have very high O_2 affinities, the pO_2 needs to be below 1 mm Hg to make these HBOCs offload their store of O_2 . At a pO_2 below 1 mm Hg, hepatocytes will suffer from extensive hypoxia. In this case, R-state PolyhHbs perform like myoglobin which primarily stores O_2 and only releases it under extreme hypoxia in the tissue. Therefore, R-state PolyhHbs are not suitable for tissue engineering applications where it is important to recapitulate *in vivo* O_2 levels and gradients.

Conclusion

We have demonstrated that high-MW PolyhHbs can be synthesized in a defined quaternary state without the presence of free hHb and low-MW PolyhHb species in solution. Our results show that the PolyhHb MW and O_2 affinity can be easily regulated by controlling the glutaraldehyde cross-link density and hHb quaternary state. O_2 transport simulations indicate that T-state PolyhHbs oxygenated the ECS of a HF bioreactor to a greater extent versus R-state PolyhHbs. Taken together, these results support the use of PolyhHbs as efficacious O_2 carrying solutions with possible applications in transfusion medicine and tissue engineering.

Acknowledgments

This work was supported by National Institutes of Health Grants R01HL078840 and R01DK070862 to A.F.P. The authors would like to thank David R. Harris for purifying the hHb for these studies.

Disclaimer

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy.

Disclosure Statement

No competing financial interests exist.

References

- Freilich, D., Pearce, L.B., Pitman, A., Greenburg, G., Berzins, M., Bebris, L., Ahlers, S., and McCarron, R. HBOC-201 vasoactivity in a phase III clinical trial in orthopedic surgery subjects—extrapolation of potential risk for acute trauma trials. *J Trauma* **66**, 365, 2009.
- Savitsky, J.P., Doczi, J., Black, J., and Arnold, J.D. A clinical safety trial of stroma-free hemoglobin. *Clin Pharmacol Ther* **23**, 73, 1978.
- Lamy, M.L., Daily, E.K., Brichant, J.F., Larbuisson, R.P., Demeyere, R.H., Vandermeersch, E.A., Lehot, J.J., Parsloe, M.R., Berridge, J.C., Sinclair, C.J., Baron, J.F., and Przybelski, R.J. Randomized trial of diaspirin cross-linked hemoglobin solution as an alternative to blood transfusion after cardiac

- surgery. The DCLHb Cardiac Surgery Trial Collaborative Group. *Anesthesiology* **92**, 646, 2000.
4. Moore, E.E., Moore, F.A., Fabian, T.C., Bernard, A.C., Fulda, G.J., Hoyt, D.B., Duane, T.M., Weireter, L.J., Jr., Gomez, G.A., Cipolle, M.D., Rodman, G.H., Jr., Malangoni, M.A., Hides, G.A., Omert, L.A., and Gould, S.A. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. *J Am Coll Surg* **208**, 1, 2009.
 5. McCarthy, M.R., Vandegriff, K.D., and Winslow, R.M. The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers. *Biophys Chem* **92**, 103, 2001.
 6. Rice, J., Philbin, N., Light, R., Arnaud, F., Steinbach, T., McGwin, G., Collier, S., Malkevich, N., Moon-Massatt, P., Rentko, V., Pearce, L.B., Ahlers, S., McCarron, R., Handrigan, M., and Freilich, D. The effects of decreasing low-molecular weight hemoglobin components of hemoglobin-based oxygen carriers in swine with hemorrhagic shock. *J Trauma* **64**, 1240, 2008.
 7. Rohlfis, R.J., Bruner, E., Chiu, A., Gonzales, A., Gonzales, M.L., Magde, D., Magde, M.D., Jr., Vandegriff, K.D., and Winslow, R.M. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. *J Biol Chem* **273**, 12128, 1998.
 8. Sampei, K., Ulatowski, J.A., Asano, Y., Kwansa, H., Bucci, E., and Koehler, R.C. Role of nitric oxide scavenging in vascular response to cell-free hemoglobin transfusion. *Am J Physiol Heart Circ Physiol* **289**, H1191, 2005.
 9. Cabrales, P., Sun, G., Zhou, Y., Harris, D.R., Tsai, A.G., Intaglietta, M., and Palmer, A.F. Effects of the molecular mass of tense-state polymerized bovine hemoglobin on blood pressure and vasoconstriction. *J Appl Physiol* **107**, 1548, 2009.
 10. Levy, J.H., Goodnough, L.T., Greilich, P.E., Parr, G.V., Stewart, R.W., Gratz, I., Wahr, J., Williams, J., Comunale, M.E., Doblar, D., Silvay, G., Cohen, M., Jahr, J.S., and Vlahakes, G.J. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. *J Thorac Cardiovasc Surg* **124**, 35, 2002.
 11. Marret, E., Bonnin, P., Mazoyer, E., Riou, B., Jacobs, T., Coriat, P., and Samama, C.M. The effects of a polymerized bovine-derived hemoglobin solution in a rabbit model of arterial thrombosis and bleeding. *Anesth Analg* **98**, 604, 2004.
 12. Napolitano, L.M. Hemoglobin-based oxygen carriers: first, second or third generation? Human or bovine? Where are we now? *Crit Care Clin* **25**, 279, 2009.
 13. Gould, S.A., and Moss, G.S. Clinical development of human polymerized hemoglobin as a blood substitute. *World J Surg* **20**, 1200, 1996.
 14. Gould, S.A., Moore, E.E., Hoyt, D.B., Burch, J.M., Haenel, J.B., Garcia, J., DeWoskin, R., and Moss, G.S. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. *J Am Coll Surg* **187**, 113, 1998.
 15. Sprung, J., Kindscher, J.D., Wahr, J.A., Levy, J.H., Monk, T.G., Moritz, M.W., and O'Hara, P.J. The use of bovine hemoglobin glutamer-250 (Hemopure (R)) in surgical patients: results of a multicenter, randomized, single-blinded trial. *Anesth Analg* **94**, 799, 2002.
 16. Sakai, H., Hara, H., Yuasa, M., Tsai, A.G., Takeoka, S., Tsuchida, E., and Intaglietta, M. Molecular dimensions of Hb-based O(2) carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* **279**, H908, 2000.
 17. Jia, Y., and Alayash, A.I. Effects of cross-linking and zero-link polymerization on oxygen transport and redox chemistry of bovine hemoglobin. *Biochim Biophys Acta* **1794**, 1234, 2009.
 18. Elmer, J., Buehler, P.W., Jia, Y., Wood, F., Harris, D.R., Alayash, A.I., and Palmer, A.F. Functional comparison of hemoglobin purified by different methods and their biophysical implications. *Biotechnol Bioeng* **106**, 76, 2010.
 19. Elmer, J., Harris, D.R., Sun, G., and Palmer, A.F. Purification of hemoglobin by tangential flow filtration with diafiltration. *Biotechnol Prog* **25**, 1402, 2009.
 20. Palmer, A.F., Sun, G., and Harris, D.R. Tangential flow filtration of hemoglobin. *Biotechnol Prog* **25**, 189, 2009.
 21. Buehler, P.W., Zhou, Y., Cabrales, P., Jia, Y., Guoyong, S., Harris, D.R., Tsai, A.G., Intaglietta, M., and Palmer, A.F. Synthesis, biophysical properties and pharmacokinetics of ultrahigh molecular weight tense and relaxed state polymerized bovine hemoglobins. *Biomaterials* **31**, 3723, 2010.
 22. Cabrales, P., Zhou, Y., Harris, D.R., and Palmer, A.F. Tissue oxygenation after exchange transfusion with ultrahigh-molecular-weight tense- and relaxed-state polymerized bovine hemoglobins. *Am J Physiol Heart Circ Physiol* **298**, H1062, 2010.
 23. Palmer, A.F., Sun, G., and Harris, D.R. The quaternary structure of tetrameric hemoglobin regulates the oxygen affinity of polymerized hemoglobin. *Biotechnol Prog* **25**, 1803, 2009.
 24. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248, 1976.
 25. Derviz, G.V. A method of determining methemoglobin in the blood on the basis of its cyanide (cyanomethemoglobin). *Lab Delo* **9**, 527, 1966.
 26. Zhang, Y., Bhatt, V.S., Sun, G., Wang, P.G., and Palmer, A.F. Site-selective glycosylation of hemoglobin on Cys beta93. *Bioconjug Chem* **19**, 2221, 2008.
 27. Antonini, E., and Brunori, M. Kinetics of Reactions of Hemoglobin and Myoglobin with Ligands. Amsterdam: North-Holland Pub. Co., 1971.
 28. Alayash, A.I., Summers, A.G., Wood, F., and Jia, Y. Effects of glutaraldehyde polymerization on oxygen transport and redox properties of bovine hemoglobin. *Arch Biochem Biophys* **391**, 225, 2001.
 29. Winterbourn, C.C. CRC Handbook of Methods for Oxygen Radical Research. Boca Raton, Fla.: CRC Press, 1985.
 30. Chen, G., and Palmer, A.F. Hemoglobin-based oxygen carrier and convection enhanced oxygen transport in a hollow fiber bioreactor. *Biotechnol Bioeng* **102**, 1603, 2009.
 31. Boykins, R.A., Buehler, P.W., Jia, Y., Venable, R., and Alayash, A.I. O- α -D-glucopyranosyl hemoglobin lacks site-specific chemistry in the central cavity: structural and functional consequences of beta93Cys modification. *Proteins* **59**, 840, 2005.
 32. Buehler, P.W., Boykins, R.A., Jia, Y., Norris, S., Freedberg, D.I., and Alayash, A.I. Structural and functional characterization of glutaraldehyde-polymerized bovine hemoglobin and its isolated fractions. *Anal Chem* **77**, 3466, 2005.

33. Jia, Y., Ramasamy, S., Wood, F., Alayash, A.I., and Rifkind, J.M. Cross-linking with O-raffinose lowers oxygen affinity and stabilizes haemoglobin in a non-cooperative T-state conformation. *Biochem J* **384**, 367, 2004.
34. Palmer, R.M., Ferrige, A.G., and Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524, 1987.
35. Cabrales, P., Tsai, A.G., and Intaglietta, M. Isovolemic exchange transfusion with increasing concentrations of low oxygen affinity hemoglobin solution limits oxygen delivery due to vasoconstriction. *Am J Physiol Heart Circ Physiol* **295**, H2212, 2008.

Address correspondence to:

Andre F. Palmer, Ph.D.

William G. Lowrie

Department of Chemical and Biomolecular Engineering

The Ohio State University

Columbus, OH 43210

E-mail: palmer.351@osu.edu

Received: June 14, 2010

Accepted: October 27, 2010

Online Publication Date: January 5, 2011