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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 O_2 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.

1. 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 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-bourn 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.

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

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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 αβ 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 cross-link/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 P_{50} of 38 mm Hg and MW ranging from 130 to 500 kDa [13,36–38]. In contrast, PolyHeme® (Northfield Laboratories Inc., Evanston, IL) consists of a pyridoxylated polymerized human hemoglobin with a P_{50} of 28–30 mm Hg and a MW ranging from 128 to 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 (P_{50}) 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.

2. Methods

2.1. Materials

Glutaraldehyde (70%), NaCl, KCl, NaOH, $Na_2S_2O_4$, NaCl (USP), KCl (USP), $CaCl_2$–$2H_2O$ (USP), NaOH (NF), sodium lactate (USP), *N*-acetyl-L-cysteine (USP), $NaCNBH_3$ and $NaBH_4$ 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 h to degrade any endotoxin present, followed by thorough rinsing with HPLC grade water.

2.2. 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 h. 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