SYNTHESIS, CHARACTERIZATION, AND MODELING OF
NOVEL BOVINE HEMOGLOBIN-BASED OXYGEN CARRIERS

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Abstract

by

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Second generation hemoglobin-based O₂ carriers (HBOCs) are being developed with high O₂ affinity (low \( P_{50} \)) in order to suppress vasoconstriction elicited by over-oxygenating tissues, a problem associated with low O₂ affinity first generation HBOCs. Two novel reactive dialdehydes were synthesized by ring-opening 2-chloroethyl-β-D-fructopyranoside (2-CEFP) and 1-octyl-β-D-glucopyranoside (1-OGP) at the 1,2-diol position, respectively, to yield novel Hb polymerizing reagents. High-affinity polymerized HBOCs were synthesized by reacting R-state bovine hemoglobin (bHb) with ring-opened 2-CEFP and 1-OGP at cross-linker to bHb molar ratios ranging from 10:1 to 30:1. The resulting polymerized bovine HBOCs (bHBOCs) displayed \( P_{50} \)s ranging from 7-18 mmHg, cooperativities ranging from 0.8-1.4, and methemoglobin (metHb) levels ranging from 3-10%. The cross-linking reaction also stabilized the third stepwise Adair
coefficient for 1-OGP20, 1-OGP30, and 2-CEFP30. Additionally, the number-averaged molecular weight of each polymerized bHBOC was larger compared to bHb.

bHb was purified from bovine red blood cells (bRBCs) via anion exchange chromatography preceded by dialysis. Q Sepharose XL, a strong anion exchange resin, was found to have twice the binding capacity for bHb compared to three other anion exchange resins that were studied in this work. MetHb levels remained below 2% with bHb concentrations between 0.7 and 1.7 mM.

The effect that increasing the cross-linker to bHb molar ratio to 100:1 had on metHb levels and size distributions was further studied in detail. Purified bHb was found to elute from a size exclusion chromatography column as a dimer. bHBOCs synthesized with ring-opened 1-OGP and 2-CEFP each showed an increase in bHb species having both decreased and increased \( M_w \) distributions compared to bHb as the ring-opened 1-OGP to bHb molar ratio increased. However, for bHBOCs synthesized with 2-CEFP, the \( M_w \) distributions at cross-linker to bHb molar ratios between 50:1 and 70:1 remained constant. Furthermore, bHBOCs synthesized with 2-CEFP created \( M_w \) species centered at 63.6 and 96 kDa, correlating to intact tetrameric bHb and tetrameric bHb bound to a bHb dimer, respectively.

Autoregulatory theory has been presented as a possible explanation for increased vasoconstriction observed in the transfusion of low-affinity HBOCs, where it is hypothesized that low-affinity HBOCs over-deliver O\(_2\) to tissues surrounding capillaries thereby eliciting vasoconstriction. An \textit{a priori} model has been developed in which the performance of mixtures of acellular HBOCs (having \( P_{50s} \) between 5 and 55 mmHg) and hRBCs has been simulated using a Krogh tissue cylinder model. At normoxic inlet pO\(_2\),
there was no correlation between O$_2$ flux out of the capillary and the O$_2$ affinity of the HBOC. However, a correlation was found between the average pO$_2$ tension in the capillary and the O$_2$ affinity of the HBOC.
In memory of Robert F. Eaton.

I never made Dimino Acids.

Hemoglobin-based oxygen carriers

Will have to do.
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CHAPTER 1:
INTRODUCTION

1.1 Motivation

Each year approximately six million people receive 14 million units of donated blood through blood transfusions in the United States [1]. Considering that the American Red Cross estimated that the price of a unit (1 unit = 1 pint) of transfused blood cost $170 in 2001 [2], the blood transfusion industry is valued at $2.4 billion in the U.S. alone. This cost per unit of blood is constantly increasing as more sophisticated blood screening protocols are put into place to combat the always present threat of infectious agents entering the blood supply.

The safety of allogenic blood is at an all-time high due to careful screening of potential donors, new and more effective blood tests, and better handling practices and tracking systems [3]. However, there are still risks associated with allogenic blood transfusions including mild allergic reactions (1 in 30 patients), delayed hemolytic transfusion reactions (1 in 1,000), transfusion-related acute respiratory disease syndrome (1 in 5,000), fatal acute hemolytic transfusion reactions (1 in 250,000 – 1,000,000), hepatitis B (1 in 30,000 – 250,000) or C (1 in 30,000 – 100,000), and HIV (1 in 500,000) [3]. Hepatitis B, hepatitis C, and HIV are known to have a time period ranging from a few weeks to months before blood screening assays become positive for the virus [1]. Further increases in the safety of the blood supply during the assay time period can be
accomplished through the application of a nucleic acid test to each unit of donor blood; however, this was shown not to be cost-effective by Marshall et al., as it would cost $1.5 million per quality adjusted life year to reduce 37,128, and eight cases, respectively, of hepatitis B, hepatitis C, and HIV each year caused by blood transfusions in the U.S. The increase in safety of the U.S. blood supply comes at a high cost as an increased number of potential donors are turned away during the rigorous screening process [3].

While over 60% of the U.S. population is eligible to donate blood, only 5% do so each year. As of February 2007, 45% of blood centers operated by America’s Blood Centers, which serves 180 million patients at 4,200 healthcare centers each year, had a blood supply that could only last two days or less [4]. The Red Cross would like to maintain the blood supply of all blood types at 10 days or more in the unforeseeable event of natural disasters or terrorist attacks [5]. The current blood shortage is compounded by the fact that over 50% of allogenic blood is used by patients over 65 years of age and that the baby boomer generation is approaching this age category, resulting in a projected blood shortage of 4 million units in the U.S. alone by 2030 [6].

There are several more drawbacks to the use of donor human red blood cells (hRBCs) in blood transfusions, including a maximum shelf-life of 42 days, cross-matching blood types, and new diseases such as the West Nile virus and SARS threatening the blood supply [4, 5]. Due to these drawbacks, there is a need to develop a universal O₂ carrier without these limitations. So far developmental studies of O₂ carriers for application in transfusion medicine and tissue engineering, have focused on perfluorocarbons (PFCs), modified acellular hemoglobins, encapsulated hemoglobins, and hydrogel-based hemoglobins.
PFCs are synthetic molecules that need to be emulsified before they can be injected into the bloodstream to increase the dissolved O\(_2\) content. These emulsions are very unstable, and sometimes need to be stored at -40°C [3, 7, 8]. PFCs also require superphysiological amounts of O\(_2\) in order to increase the amount of O\(_2\) within the plasma to a similar level compared to hRBCs. As a result of the limitations involved in the development of PFCs, the studies described within this dissertation focus on hemoglobin-based O\(_2\) carriers (HBOCs).

1.2 Hemoglobin

Red blood cells (RBCs) are the most prevalent type of cell in the bloodstream, comprising over 95% of the cells in the plasma [9]. RBCs aid in the transportation of O\(_2\) and CO\(_2\) throughout the body [9]. Hemoglobin (Hb) is a tetrameric protein found in RBCs, consisting of two \(\alpha\beta\) dimers. Human Hb (hHb) is nearly spherical with a molecular diameter of 5.5 nm and a molecular weight (\(M_w\)) of 68 kDa [10], while bovine Hb (bHb) has a \(M_w\) of 64 kDa. Mammalian Hb transports the bulk of O\(_2\) and CO\(_2\) throughout the body. Each protein subunit of Hb is composed of a globin chain bound to a prosthetic heme group (Figure 1.1). At the center of the heme group is an iron atom, with a valence of 2\(^+\), that binds to and releases gaseous ligands, including O\(_2\) and CO\(_2\) [11]. Hb exists in two conformations, the tensed state (T-state) and the relaxed state (R-state), which are also known as deoxyHb and oxyHb, respectively [12]. When Hb changes from the T-state to the R-state, the heme pocket is more accessible to O\(_2\). The T-state is stabilized by salt bridges between subunits of Hb and the allosteric effector, which are broken upon conformational change to the R-state [3]. Each type of Hb has a
unique allosteric effector, which reduces the affinity of Hb for O\textsubscript{2}. The allosteric effector for hHb, 2,3-diphosphoglycerate (2,3-DPG), is lost upon lysis of human RBCs, causing the \( P_{50} \) to drop from 26-28 mmHg to 13-14 mmHg, while the allosteric effector of bHb, Cl\textsuperscript{−}, is naturally abundant within the bloodstream [3, 13-15].

When the heme group is oxidized to the \( \text{Fe}^{3+} \) or \( \text{Fe}^{4+} \) states, it forms methemoglobin (metHb) and loses its ability to bind to O\textsubscript{2} [16]. The heme group more readily dissociates from the globin chains in the metHb state. This can lead to endothelial cell death as free heme groups have been shown to be toxic to these cell types [17]. Additionally, Linberg et al. showed that acellular HBOCs containing metHb levels above 10\% are toxic post-infusion [18]. Within RBCs, metHb is reduced to Hb through a system of electron carriers and enzymes [19]. Outside of the RBC, this system is no longer present, and Hb auto-oxidizes to form metHb. MetHb can be reduced using a variety of reducing agents and enzymatic systems \textit{in vitro} [20-24]. Ascorbic acid, a mild reducing agent, has been shown to reduce acellular HBOCs, and different types of Hb at different rates \textit{in vitro} [25]. Additionally, it has been shown that alcohol-based solvents reduce the rate of auto-oxidation of Hb [26, 27].

![Figure 1.1: Structure of the heme group found in each globin chain of the Hb tetramer.](image-url)
1.2.1 Oxygen binding curves

The O\textsubscript{2} binding/dissociation curve for Hb has a sigmoidal shape due to cooperative O\textsubscript{2} binding. This curve can be modeled via the Hill or Adair equations. In the Hill equation, the O\textsubscript{2} dissociation curve is described by an equilibrium reaction (Equation 1.1), where \( n \) is the number of O\textsubscript{2} molecules that bind with Hb to form oxyhemoglobin (Hb(O\textsubscript{2})\textsubscript{n}).

\[
\text{Hb(O}_2\text{)}\textsubscript{n} \rightleftharpoons \frac{k_{-1}}{k_1} \text{Hb} + n\text{O}_2
\]

From this equilibrium reaction, the Hill equation [1.2] can be derived, where \( Y \) is the fraction of Hb that is saturated with O\textsubscript{2}, \( pO_2 \) is the partial pressure of O\textsubscript{2}, and \( P_{50} \) is the pO\textsubscript{2} at which Hb is half-saturated with O\textsubscript{2}.

\[
Y = \frac{(pO_2)^n}{P_{50}^n + (pO_2)^n}
\]

In this form, \( n \) is also known as the Hill number or cooperativity coefficient. A slope of 1.0 represents noncooperative binding and is found in heme proteins having a single subunit, such as myoglobin. Conversely, a slope of 4.0 represents the case where the oxygenation of one subunit leads to an infinite increase in O\textsubscript{2} affinity of all other subunits. Typical cooperativities of mammalian Hbs range between 2.8 and 3.0 [28].

The Adair equation is based on a reaction scheme 1.3 involving unbound Hb and four states of Hb bound to O\textsubscript{2} molecules. The overall Adair equation can then be derived from this reaction scheme to yield Equation 1.4, where \( A_{1-4} \) are the overall Adair constants.
1.3 Hemoglobin-Based Oxygen Carriers

Modified acellular Hbs, encapsulated Hbs, and hydrogel-based Hbs are three types of HBOCs, which are also referred to as artificial blood substitutes. These O₂ carriers can be based on human, animal, or recombinant Hb. One advantage of using an animal Hb, such as bHb, as the building block of an O₂ carrier is the reduced reliance on volunteer blood donations for the Hb source. bHb (composed of 574 amino acids) has approximately the same amount of amino acids as hHb (composed of 578 amino acids) [29-32]. HBOCs are classified as a biologic product in the U.S., and therefore must undergo a three phase clinical trial process to evaluate toxicity and effectiveness [33].

1.3.1 Modified acellular hemoglobins

In the 1930’s, Amberson determined that Hb was still functional outside of RBCs [34]. This discovery led to the initial research on acellular Hbs for use as artificial blood substitutes. These first clinical studies utilizing unmodified acellular Hb as an O₂ carrier failed. This is partly due to the fact that tetrameric Hb (α₂β₂) is in equilibrium with αβ
dimers [35, 36]. These dimers diffuse through the capillary pores more readily than tetrameric Hb, and are quickly cleared by the kidney, eventually leading to kidney failure, since free heme is toxic to the kidney tubules [36, 37]. Another reason for the early failures of unmodified Hbs was that the Hb product was not devoid of RBC debris that are now known to be toxic [35]. Therefore, it was concluded that for a HBOC to be effective, it must be devoid of cell debris (stroma-free) and be modified such that it no longer readily cleared from the kidneys.

Since Amberson’s initial work, a variety of acellular HBOCs have undergone commercial development. They have been modified via cross-linking, oligomerization, polymerization, and conjugation [38]. HBOCs have a variety of applications in the medical field including trauma, surgery, acute normovolemic hemodilution, cardiac surgery, hemorrhagic shock, stroke, nitric oxide-induced shock, radiosensitizers for tumors, erythropoiesis, sickle cell crisis, cell culture, and veterinary use [38].

Chemical modifications can be performed on acellular Hb to prevent the nephrotoxicity observed when transfusing unmodified acellular Hb. Tetrameric Hb has been intramolecularly cross-linked with a specific cross-linking agent to prevent dimer dissociation [39]. Furthermore, Hb can be intermolecularly cross-linked with other Hb tetramers to form oligomeric and polymeric Hb [40]. Finally, it can be conjugated with a high molecular weight species to increase its apparent molecular radius and mask the HBOC from the complement system [38, 39, 41].

One of the first HBOCs to reach clinical trials was HemAssist (Baxter Healthcare; Deerfield, IL). HemAssist consisted of hHb that formed a stable tetrameric Hb using bis-3,5-dibromosalicyl fumarate as the cross-linking agent [39]. It reached Phase III clinical
trials for the treatment of acute blood loss and Phase II clinical trials for the treatment of hemorrhagic shock before it was discontinued due to an increase in clinical mortality rates [38, 39].

Biopure (Cambridge, MA) has synthesized Hemopure, a glutaraldehyde-polymerized hHb to treat hemodilution and sickle cell disease [40]. Glutaraldehyde reacts nonspecifically with Hb both intramolecularly and intermolecularly [42, 43]. Hemopure [33, 44] is a low-affinity O\textsubscript{2} carrier ($P_{50} = 38$ mmHg) being developed for use in elective surgery. It has completed Phase III trials in the U.S., however it is still in the process of answering the FDA’s questions about its biological license application (BLA) [38, 39, 41]. Recently, the FDA did not approve further clinical studies for Hemopure due to an increase in the number of cases of pneumonia, stroke, heart failure, cardiac arrest, and ventricular fibrillation [45]. Additionally, Biopure is being investigated by the Security and Exchange commission for not disclosing the results of the clinical trials to its shareholders.

PolyHeme (Northfield Laboratories; Evanston, IL) [33, 44, 46] is a low-affinity ($P_{50} = 26 – 32$ mmHg) polymerized HBOC that is currently in Phase III clinical trials for use in treating patients suffering from trauma. Northfield Laboratories was involved in controversial trauma clinical trials where unconscious patients were transfused with either PolyHeme or saline solution at the scene of the accident without patient consent. Once the patient was transferred to the hospital, patients that were treated with PolyHeme continued to receive PolyHeme transfusions while patients treated with saline solution received traditional hRBC transfusions. It was controversial due to the fact that patients given PolyHeme were not given the chance to switch to a traditional RBC transfusion
once conscious at the hospital. Furthermore, Northfield Laboratories reported that patients infused with PolyHeme experienced 13.2% mortality rates versus 9.6% mortality rates for patients infused with conventional saline and hRBC treatment [47]. Nonetheless, Northfield Laboratories is still seeking FDA approval for PolyHeme.

Julie Eike has previously worked with modified acellular Hbs within the Palmer research group. Acellular HBOCs were formed via the reaction of glutaraldehyde with bHb, and the reaction was quenched via glycine or sodium borohydride (NaBH₄) [48]. The HBOCs that were quenched with glycine were reacted at a glutaraldehyde to bHb molar ratios between 0:1 and 10:1, while the HBOCs that were quenched with glycine were reacted at glutaraldehyde to bHb molar ratios between 0:1 and 60:1 [48]. The final product of the glycine-quenched HBOCs had size distributions consisting of 35% with a molecular weight ($M_w$) between 32 and 64 kDa and 65% with a $M_w$ between 64 and 128 kDa at a glutaraldehyde to bHb molar ratio of 0:1. For the HBOCs synthesized at a glutaraldehyde to bHb molar ratio of 10:1, the size distributions of the final product consisted of 47% having a $M_w$ between 64 and 128 kDa, 22% with a $M_w$ between 128 and 192 kDa, and 31% with a $M_w$ greater than 192 kDa. Of the NaBH₄-quenched HBOCs, those that were synthesized at glutaraldehyde to bHb molar ratios of 30:1 and 40:1 showed the most promise. At higher glutaraldehyde to bHb molar ratios, the $M_w$ distributions were over 1,000 kDa [48]. The NaBH₄-quenched HBOC reacted at glutaraldehyde to bHb molar ratio of 30:1 had a $P_{50}$ between 13 and 16 mmHg, a $n$ between 1.2 and 1.4, initial metHb levels of 28%, and a size distribution of the final product consisting of 44% with a $M_w$ between 64 and 128 kDa, 17% with a $M_w$ between 128 and 192 kDa, and 39% with a $M_w$ greater than 192 kDa. The metHb level was later
reduced to 2% via the addition of ascorbic acid, a mild reducing agent [49]. The NaBH₄-
quenched HBOC reacted at glutaraldehyde to bHb molar ratio of 40:1 had a $P_{50}$ of 13
mmHg, a $n$ between 1.2 and 1.4, metHb levels of 28%, and a size distribution of the final
product consisting of 1% with a $M_w$ between 320 and 640 kDa, 82% with a $M_w$ between
640 and 960 kDa, and 17% with a $M_w$ greater than 960 kDa [48]. The metHb level was
later reduced to 6% via the addition of ascorbic acid [49].

Eike et al. also investigated the reaction conditions of glutaraldehyde cross-linked
Hbs and found that the reaction time and the NaBH₄ concentration used to quench the
reaction had no effect on the $P_{50}$, $n$, and metHb levels. However, increasing the reaction
time did cause an increase in the degree of cross-linking [49]. Increasing the Cl⁻
concentration caused a decrease in O₂ affinity at low glutaraldehyde to bHb molar ratios;
however, as the size of the HBOCs increased at higher glutaraldehyde to bHb molar
ratios, the allosteric effect of Cl⁻ was less visible [50]. This is most probably due to a
decrease in ability to switch between Hb tetramer conformations.

Eike et al. also investigated the reaction of a variety of ring-opened o-saccharides
with bHb to form HBOCs [51]. Of these cross-linking agents, ring-opened 15 kDa
Dextran, ring-opened 71 kDa Dextran, and ring-opened methylglucopyranoside were
most effective in synthesizing cross-linked HBOCs. Ring-opened 15 kDa Dextran
produced a purely cross-linked tetrameric bHBOC with a $P_{50}$ of 6.1, $n$ of 1.2, and metHb
levels of 24.8% [51]. Ring-opened 71 kDa Dextran produced a purely cross-linked
tetrameric bHBOC with a $P_{50}$ of 8.1, $n$ of 2.0, and metHb levels of 10.8% [51]. Ring-
opened methylglucopyranoside produced a bHBOC that consisted of 39.7% cross-linked
tetrameric bHb and 38.5% higher order species with a $P_{50}$ of 6.1, $n$ of 1.1, and metHb levels of 11.7% [51].

Apex Bioscience (Durham, NC) has synthesized a pyridoxylated human Hb conjugated to polyoxyethylene to treat septic shock, and it is currently in Phase III clinical trials [38, 39, 41]. Enzon (Bridgewater, NJ) has synthesized PEG-Hb, bHb conjugated to poly(ethylene glycol) (PEG) to treat cancer of the stomach which is currently in Phase II clinical trials [38, 39, 41].

The most common side effect of acellular HBOCs in clinical trials has been the development of mild transient hypertension elicited by vasoconstriction [33, 35]. There are two prominent theories as to the exact cause of this effect. In the first theory, HBOCs are believed to scavenge NO from the endothelium, thereby causing smooth muscle cells to contract, which would lead to vasoconstriction [52, 53]. However, Winslow’s group could not find a correlation between the NO scavenging rates of HBOCs and vasoconstriction in a classical stopped flow apparatus [7]. Additionally, Winslow’s group has observed that vasoconstriction occurred more prevalently in HBOCs with low O$_2$ affinities [54-56]. RBCs provide a natural diffusion barrier, in the form of the cell membrane, for O$_2$ to permeate, however acellular HBOCs do have this barrier. This causes O$_2$ to be preferentially delivered to the proximal arterioles, which leads to an autoregulatory mediated constriction of the arterioles and the capillary beds [57]. This autoregulatory response elicits vasoconstriction and systemic hypertension, and increases the mean arterial pressure [7, 56, 58].

From these observations, the autoregulatory hypothesis was formed, which recommended that second generation blood substitutes possess high O$_2$ affinities,
viscosity similar to blood (~ 4 cP), increased oncotic pressure compared to human blood (> 15 mmHg), low concentration of Hb, and long plasma retention [7]. One of the key points of the autoregulatory hypothesis is that acellular HBOCs with low O₂ affinities deliver too much O₂ to surrounding tissues, thereby initiating vasoconstriction [7].

1.3.2 Encapsulated hemoglobins

Encapsulated Hbs are being developed to mimic the overall structure of RBCs. However, due to their increased structural complexity, they have lagged behind in the clinical trials [39]. Neo Red Cells consist of a liposome encapsulated Hb (LEH) and are being developed by Terumo (Kanagawa, Japan) [59-63]. In addition to encapsulating Hb, they also encapsulate an enzymatic reduction system, similar to that found in RBCs in vivo, to maintain low metHb levels [64]. Neo Red Cells offer Hb concentrations of 6 g/dL, \(P_{50}\) ranging from 45-50 mmHg, \(n\) ranging from 1.7-1.9, and half-lives between 24 and 30 hours when infused into rabbits [64]. Considering that it has been found that LEH should have O₂ binding parameters similar to that of RBCs in order to avoid vasoconstriction [65, 66], there is some concern about Neo Red Cells’ low affinity for O₂. These HBOCs have only undergone animal studies, although human clinical studies are expected shortly.

Within the Palmer research group, studies have been conducted on unmodified LEH (ULEH) [67, 68], PEG-conjugated LEH (PEG-LEH) [69], liposome-encapsulated actin-Hb (LEAcHb) [70], and polymersome encapsulated Hb (PEH) [71] by Dian Arifin and Shuliang Li. A brief overview of these studies is subsequently presented.

ULEH was synthesized and observed to have a \(P_{50}\) of 26 mmHg, \(n\) of 2.5, and metHb levels less than 10% [67, 68]. Additionally, it was found to have higher Hb
encapsulation efficiencies and lower metHb levels when extruded in phosphate buffer (PB) as opposed to phosphate buffered saline (PBS). However, ULEH that was extruded with PBS had a more uniform size distribution compared to ULEH extruded with PB. In a similar study, ULEH was observed to have a $P_{50}$ of 28 mmHg, $n$ of 2.5, and spherical shape [70]. The equilibrium O$_2$ binding properties in both studies are in good agreement with each other and similar to that of RBCs. Unmodified liposomes are fragile colloids, and have a tendency to lyze when exposed to shear stress. Chung et al. found that 10-20% of encapsulated Hb is released from unmodified liposome [72]. In order to strengthen the liposome structure, liposomes were PEGylated and encapsulated with an actin matrix.

The PEGylation of a liposome improves its intravascular circulation, biocompatibility, and colloidal state [64, 73-76]. PEG is a bioinert polymer which is approved for clinical use by the FDA. PEG-LEH were synthesized by Arifin et al. and observed to have $P_{50}$ between 17 and 38 mmHg, $n$ between 2.1 and 2.6, and metHb levels less than 5% [69]. Additionally, PEG-LEH was found to be more monodisperse when extruded in PB compared to PBS. However, PEG-LEH showed reduced encapsulation efficiencies compared to ULEH. Finally, while the encapsulation of Hb in unmodified liposomes causes unmodified liposomes to be more susceptible to osmotic pressure gradients, the encapsulation of Hb in PEGylated liposomes did not cause the PEGylated liposomes to show this effect. One drawback of PEG-LEH is that increasing the $M_w$ of PEG results in an increase in the fraction of PEG-lipid molecules that form into a separate micelle phase [77, 78]. A further drawback of liposomes is that their mechanical strength is limited by their thin membrane thickness (3-4 nm) [79, 80].
Liposomes were also encapsulated with an actin matrix within their aqueous core in order to increase their mechanical strength [70]. Hb was then encapsulated within the actin matrix to form LEAcHb which was observed to have a $P_{50}$ between 27 and 29 mmHg and a $n$ between 2.1 and 2.3. Additionally, LEAcHb was found to have a thin disk shape, which has been shown to result in increased half-lives compared to spherical particles.

Hb has also been encapsulated within polymersomes. Polymersomes are vesicles that consist of amphiphilic diblock copolymers [80, 81]. A key advantage of polymersomes compared to PEGylated liposomes is that their entire surface is PEGylated [82-84]. As a result of the inherent PEGylation, polymersomes suppress the activation of the complement system [85]. Additionally, the membrane thickness of polymersomes are generally greater than 4 nm, which results in them being an order of magnitude greater in toughness and ten times less permeable to water compared to liposomes [83, 86]. This helps to reduce lysis caused by osmosis. Arifin et al. synthesized PEH with polyethylene oxide as the hydrophilic block of the diblock copolymer and polybutadiene as the hydrophobic block of the diblock copolymer [71]. PEHs were observed to have $P_{50}$s ranging from 22 to 30 mmHg and $n$s ranging from 2.1 to 2.5.

Encapsulated Hbs have only been studied in select animal studies and there are still many obstacles to overcome before they reach human clinical trials. One of the main obstacles has to do with their limited stability when exposed to shear stress. This could cause unmodified acellular Hb to be released within the plasma where it could readily diffuse through the capillary pores and induce renal failure. As a precaution for this, modified Hbs that have been shown to be effective and safe $O_2$ carriers should be
encapsulated rather than unmodified Hb. Another possible solution is to entrap Hb within a hydrogel matrix to limit its ability to pass through the capillary pores.

1.3.3 Hydrogel-based hemoglobins

Jaqunda Patton synthesized nanoscale hydrogel particles through the photopolymerization of poly(N-isopropylacrylamide) and poly(acrylamide) within liposomal reactors [87-89]. These particles formed via the solubilization of the lipid bilayer of lipogel particles encapsulating bHb. Nanoscale hydrogel particles (NHPs) consist of a three-dimensional network of hydrophilic polymers that swell and absorb aqueous solutions [90]. NHPs are more stable than liposomes, have a greater bHb loading capacity, and their swelling and shrinking properties are highly controllable compared to liposomes [91, 92]. When the NHPs are encapsulated within a liposome, they are referred to as lipogels. Both of these drug delivery systems were encapsulated with Hb to create novel O$_2$ carriers, which prevent acellular Hb from dissociating into dimers. The NHPs encapsulating bHb were observed to have a $P_{50}$ of 16.4 mmHg, $n$ of 2, and metHb levels less than 10% when reduced with N-acetyl cysteine [89]. The lipogels created with poly(N-isopropylacrylamide) possessed $P_{50}$s between 10 and 14 mmHg, $n$ between 2 and 2.1, and metHb levels between 10 and 26%, while the lipogels created with poly(acrylamide) possessed $P_{50}$s between 10 and 12 mmHg, $n$ between 2 and 2.1, and metHb levels between 9 and 15%. The high metHb levels of these particles is of some concern as Linberg et al. showed that metHb levels should not exceed 10% upon infusion into the plasma [18].
1.4 Bovine Hemoglobin Purification

It is essential to develop an efficient and streamlined purification method of bHb to ensure that the starting material for the synthesis of bovine bHBOCs is of high purity and high yield. A brief overview of some of the purification strategies is provided below.

There have been many studies that have focused on the purification of Hb from RBCs. Initial studies focused on the use of centrifugation to first wash the RBCs and remove plasma proteins followed by the addition of a hypotonic buffer to extract Hb from the RBCs [53]. This was sometimes followed by the addition of toluene or another organic solvent to remove cell debris; however, it was found that toluene could remain within the Hb pocket [93]. To avoid the use of organic extracts, some studies have focused on the use of microfiltration, ultrafiltration, and hemolysis [94] as an alternative means of purifying Hbs. However, excess cell debris tends to block the pores in these methods and results in physically compromised membranes. Another technique that has been utilized in bHb purification has been ion exchange chromatography. While there has been much success with conventional ion exchange resins, new resins are continually being developed and should be evaluated to see if they can more effectively purify hemoglobin.

DEAE-Sephadex has been used as an ion exchange resin to purify Hb [95]. A 50 mM Tris gradient was formed (pH = 8.5 – 6.5) to elute the Hb from the column. Newer versions of this resin, such as DEAE-Sepharose have been synthesized and should also be evaluated. Christensen et al. first washed RBCs with isotonic saline solution and then removed the stroma via mixed-bed ion-exchange resin in a Bio-Rex RG501-X8 column [96]. Shorr et al. utilized QMA-Spherosil to purify Hb via ion exchange chromatography
in a flow through method at 3 – 8 °C [97]. In this method, QMA-Spherosil does not capture bHb; instead, DNA and endotoxins bind to the resin while allowing the bHb product to freely elute [97]. This is a concern in that other proteins and lipids could also pass freely into the final product. MetHb levels were reported to be less than 3%. Hb purification has also been performed with Q Sepharose Big Beads by Lu et al. [98]. However, this method was performed at a pH of 6.8 [98], which is of concern since metHb forms rapidly at a pH less than 7 [53]. Additionally, the Hb was not absorbed by the resin which increases the amount of lipid in the final product.

1.5 Oxygen Transport Models

One approach to evaluate the validity of the autoregulatory hypothesis for HBOCs is with an oxygen transport model of HBOCs and hRBCs flowing through a capillary [99-101]. The Palmer research group has previously modeled O₂ transport in hepatic hollow fiber systems [101, 102] and in the hamster skeletal muscle [103]. The first a priori model developed to study O₂ transport in a capillary with more than one type of O₂ carrier was developed recently by Vadapalli et al. [104]. This study was limited to low tissue O₂ consumption rates and did not consider different levels of hRBC loss.

1.6 Research Overview and Outline of Dissertation

1.6.1 Synthesis of high-affinity hemoglobin-based oxygen carriers

Acellular bHBOCs need to be developed with improved tissue pO₂, maintenance of the mean arterial pO₂, and ability to target O₂ delivery to low pO₂ tissues. The focus
of Chapter 2 is the synthesis of acellular bHBOCs with the following design criteria:
high O\textsubscript{2} affinity, methemoglobin (metHb) level < 10%, number-averaged molecular
weight greater than that of bHb [7, 18, 56, 105], and reduced n [106]. Chapter 2
describes the synthesis and characterization of polymerized bHBOCs derived from novel
cross-linking agents for potential application in treating patients suffering from stroke or
hemorrhagic shock.

High-affinity O\textsubscript{2} carriers have been found to target O\textsubscript{2} delivery to tissues with low
pO\textsubscript{2}s [7]. In the case of patients suffering from hemorrhagic shock or stroke, in \textit{vivo}
studies have shown that tissues surrounding the immediate affected areas exhibited low
pO\textsubscript{2}s on the order of 15 mmHg [58]. Hence as a treatment strategy for hemorrhagic
shock or stroke, the first priority during resuscitation should focus on the delivery of O\textsubscript{2}
to these hypoxic tissues in order to avoid the loss of cellular differentiated function and
possible cell death. High-affinity O\textsubscript{2} carriers retain the ability to bind/release O\textsubscript{2} at lower
pO\textsubscript{2}s compared to RBCs, and release more O\textsubscript{2} at pO\textsubscript{2}s less than 15 mmHg compared to
RBCs and low-affinity O\textsubscript{2} carriers. Less O\textsubscript{2} is delivered by high-affinity O\textsubscript{2} carriers at
high pO\textsubscript{2}s, and as a result they can target O\textsubscript{2} delivery to the affected low pO\textsubscript{2} tissue
regions. In contrast, low-affinity O\textsubscript{2} carriers, such as PolyHeme ($P_{50} = 26-32$ mmHg),
Hemopure ($P_{50} = 38$ mmHg), and hRBCs ($P_{50} = 28.6$ mmHg), release more O\textsubscript{2} from their
heme groups at higher pO\textsubscript{2}s [38, 107]. As a result, they cannot target O\textsubscript{2} delivery to low
pO\textsubscript{2} tissues. These studies suggest the need to develop acellular HBOCs with high O\textsubscript{2}
affinities to limit over-oxygenation of tissues, especially in light of the fact that the
modified acellular HBOCs currently in clinical trials have low O\textsubscript{2} affinities.
For high-affinity HBOCs with reduced cooperativities, Kavdia et al. showed that higher mixed venous pO$_2$s are obtained in the capillary [106]. First generation O$_2$ carriers, such as PolyHeme and Hemopure, targeted cooperativities on the order of hRBCs (~2.3). Kavdia’s research also supports the notion that low-affinity HBOCs with increasing cooperativities exhibit higher mixed venous pO$_2$s.

Additionally, acellular bHBOCs should be synthesized with metHb levels less than 10%. MetHb, the oxidized form of bHb, is incapable of binding to O$_2$ and exhibits a high rate of heme release. In an effort to determine the toxicity threshold of metHb levels in vivo, Linberg et al. observed the effects of increasing the metHb levels of transfused acellular Hbs in rats. They found that acellular Hbs with metHb levels less than 10% did not elicit adverse physiological responses post-infusion [18]. In synthesizing acellular bHBOCs, the upper metHb level should be no more than 10%. To limit the formation of metHb, the polymerization reaction occurred under reduced pO$_2$ conditions.

Another design criterion concerns the size of the polymerized acellular bHBOCs. One of the first acellular HBOCs to be studied as an O$_2$ carrier was unmodified acellular Hb. However, the tetramer was prone to dissociate into $\alpha\beta$ dimers, which can readily diffuse through the renal tubules and induce renal failure as a result of heme toxicity. Considering that bHb has a molecular diameter of 5.5 nm (MW = 64 kDa) [108] and that the diameter of the capillary pores is 7 nm (the molecular weight cutoff for a spherical object associated with this diameter is 108 kDa) [109], acellular bHBOCs should be engineered with molecular weights greater than 108 kDa, corresponding to an oligomer consisting of two bHb tetramers. To achieve this goal, bHb was cross-linked both intramolecularly and intermolecularly with ring-opened 1-o-octyl-\(\beta\)-D-glucopyranoside
(1-OGP) and ring-opened 2-chloroethyl-β-D-fructopyranoside (2-CEFP), two novel \( \text{bHb} \) polymerizing agents that were shown to polymerize \( \text{R-state} \) \( \text{bHb} \) after the ring-opening reaction to yield high \( \text{O}_2 \) affinity \( \text{bHBOCs} \).

\( \text{bHb} \) was chosen as the starting material for the \( \text{O}_2 \) carriers, since it is essential to limit the reliance on the use of \( \text{Hb} \) derived from donated human RBCs. The use of \( \text{bHb} \) provides a reliable \( \text{Hb} \) source that does not fluctuate based on donor supplies.

1.6.2 Purification of bovine hemoglobin

Chapter 3 describes the purification of \( \text{bHb} \) via anion exchange chromatography. Four anion exchange resins (Q Sepharose Fast Flow, Q Sepharose XL, ANX Sepharose 4 Fast Flow, and DEAE Sepharose Fast Flow), that have not been previously investigated for \( \text{Hb} \) purification, were purchased from GE Healthcare (Piscataway, NJ) to compare their binding affinity for \( \text{bHb} \). Q sepharose XL was found to have the highest binding affinity for \( \text{bHb} \).

Chapter 3 further describes the purification of \( \text{bHb} \) that entails the extraction of \( \text{bHb} \) from \( \text{bRBCs} \) with a hypotonic PB solution, the removal of cell debris via filtration and dialysis, and the capture of \( \text{bHb} \) with Q Sepharose XL resin at a pH of 7.9 and at room temperature.

1.6.3 Synthesis of HBOCs at increased cross-linker concentrations

In Chapter 4, the \( \text{bHBOCs} \) were synthesized with ring-opened 1-OGP and ring-opened 2-CEFP at cross-linker to \( \text{bHb} \) molar ratios up to 100:1. The met\( \text{Hb} \) levels of the resulting polymerized \( \text{bHBOCs} \) were evaluated. Additionally, the size distributions of
the polymerized bHBOCs were determined via size exclusion chromatography (SEC) and compared to the results obtained by SDS-PAGE and light scattering in Chapter 2.

1.6.4 Model of oxygen transport in a capillary

Chapter 5 describes a numerical model developed to evaluate O$_2$ transport within a capillary having O$_2$ carriers with different affinities for O$_2$. Additionally, possible triggers for the autoregulatory effect were investigated within Chapter 5. The effect of capillary inlet pO$_2$ was investigated, since blood loss is normally correlated with a decrease in capillary inlet pO$_2$. Additionally, the maximal skeletal muscle O$_2$ consumption rate ($V_{max}$) was parameterized, since it can vary based on muscle activity, muscle location, and the fitness of an individual [110]. O$_2$ transport in capillaries experiencing different levels of hRBC loss was modeled by replacing 25%, 50%, and 75% of the hRBC concentration, correlating to a hRBC hematocrit ($Hct$) of 0.113, 0.224, and 0.335, respectively, with either a low-affinity or high-affinity O$_2$ carrier. The total Hb concentration in the form of hRBCs and HBOCs was kept constant.

This model is unique in that flowing hRBCs and HBOCs were studied over a wide range of inlet pO$_2$s, $V_{max}$ and degrees of hRBC loss. Additionally, this model takes into account the equilibrium binding/release of O$_2$ to/from hRBCs/HBOCs via the Adair equation, and models the O$_2$ consumption in muscle tissue with Michaelis-Menten kinetics. The Adair equation more accurately predicts the behavior of experimentally measured O$_2$-hRBC/HBOC dissociation curves compared to the commonly used Hill equation at high and low pO$_2$s [111].
1.6.5 Future studies

Chapter 6 describes the conclusions and future studies that should be pursued with these novel high-affinity bHBOCs. Additionally, my thoughts on the future of O\textsubscript{2} carriers are presented.
CHAPTER 2:
HIGH OXYGEN AFFINITY HEMOGLOBIN-BASED OXYGEN CARRIERS
SYNTHESIZED VIA POLYMERIZATION OF HEMOGLOBIN WITH RING-
OPENED 2-CHLOROETHYL-β-D-FRUCTOPYRANOSIDE AND 1-O-OCTYL-β-D-
GLUCOPYRANOSIDE

2.1 Introduction

Second generation HBOCs are being developed with high O₂ affinities ($P_{50}$s less than hRBCs) in order to suppress vasoconstriction elicited by over-oxygenation of the tissues, a problem associated first generation HBOCs which had lower affinities for O₂ than hRBCs. In this study, two novel reactive dialdehydes were synthesized by ring-opening 2-chloroethyl-β-D-fructopyranoside (2-CEFP) and 1-o-octyl-β-D-glucopyranoside (1-OGP) at the 1,2-diol position to yield novel Hb polymerizing reagents. High-affinity polymerized HBOCs were synthesized by reacting R-state bHb with either ring-opened 2-CEFP or ring-opened 1-OGP at cross-linker to bHb molar ratios ranging from 10:1 to 30:1. The resulting polymerized bovine HBOCs (bHBOCs) displayed $P_{50}$s ranging from 7-18 mmHg, $n$s ranging from 0.8-1.4, and metHb levels ranging from 3-10%. The cross-linking reaction also stabilized the third stepwise Adair coefficient ($a_3$) for bHBOCs reacted with ring-opened 1-OGP at cross-linker to bHb molar ratios of 20:1 and 30:1 and for bHBOCs reacted with ring-opened 2-CEFP at molar ratios of 30:1. Additionally, the number-averaged molecular weight ($M_n$), as determined...
by multi-angle static light scattering, of each polymerized bHBOC was larger compared to bHb. Molecular weight distributions leaning towards larger molecular weight bHBOCs were obtained by increasing the cross-linker to bHb molar ratio. Taken together, the results of this study have identified novel Hb polymerization reagents that are easy to synthesize and are capable of yielding bHBOCs with higher O₂ affinities and \( M_n \) compared to bHb.

2.2 Materials and Methods

2.2.1 Purification of bovine hemoglobin

bRBCs in 3.8% sodium citrate solution (Animal Technologies, Inc; Tyler, TX) were washed three times in isotonic saline solution to remove lysed bRBCs, acellular Hb, and any remaining plasma proteins. Intact bRBCs were then lysed with three equivalents of hypotonic 15 mOsM phosphate buffer [53]. Cell debris was subsequently removed by passing the lysed bRBC solution through a hollow fiber cartridge with a pore size of 0.05 \( \mu \)m. The resultant dilute bHb solution was then concentrated by passing the solution through a hollow fiber cartridge with a pore size of 50 kDa [55]. After extraction, metHb levels in Hb stock solutions were assayed to be between 0.5-2%.

2.2.2 Oxidation of monopyranosides

Fifty millimolar solutions of 1-OGP and 2-CEFP were prepared in 18.1 MΩ deionized water. The monopyranoside solution was oxidized with 2.2 equivalents of sodium m-periodate for one hour at room temperature in order to ring-open the 1,2-diols
and yield two dialdehydes, ring-opened 1-OGP (Figure 2.1A) and ring-opened 2-CEFP (Figure 2.1B) [112, 113]. Sodium bisulfite was then added to redissolve the precipitated iodine. The pH was then brought up to 8.0 to stabilize the solution via the addition of 1 M NaOH.

2.2.3 Synthesis of bovine hemoglobin-based oxygen carriers

bHb was polymerized with either ring-opened 1-OGP or ring-opened 2-CEFP, respectively. Both of the freshly synthesized aldehyde groups on ring-opened 1-OGP and 2-CEFP react with amino groups on bHb, where they cross-link bHb both intramolecularly and intermolecularly, and yield polymerized bHb species. The aldehyde groups react with amino groups to yield the corresponding Schiff base [51]. Ring-opened 1-OGP was reacted in parallel at concentrations of 20 and 30 mM with 1 mM of bHb (as
determined by the cyanomethemoglobin (cyanometHb) method described in Section 2.2.4) in a Quest 210 Parallel Synthesizer (Argonaut Technologies; Redwood City, CA) to yield the polymerized bHb species 1-OGP20 (20:1 ring-opened 1-OGP:bHb molar ratio) and 1-OGP30 (30:1 ring-opened 1-OGP:bHb molar ratio), respectively. A control, 1-OGP0, consisting of bHb in 50 mM Tris buffer (pH 7.2) was run in parallel for comparison. Similarly, ring-opened 2-CEFP was reacted in parallel at concentrations of 10, 20, and 30 mM with 1 mM of bHb to yield the polymerized bHb species 2-CEFP10 (10:1 ring-opened 2-CEFP:bHb molar ratio), 2-CEFP20 (20:1 ring-opened 2-CEFP:bHb molar ratio), and 2-CEFP30 (30:1 ring-opened 2-CEFP:bHb molar ratio), respectively. A control, 2-CEFP0, consisting of bHb in 50 mM Tris buffer (pH 7.2) was also run for comparison. The polymerization reaction was conducted in a low pO₂ environment by bubbling N₂ gas through each reaction vessel for a duration of two hours to limit the rate at which bHb was oxidized to form metHb. Since the environment was not anaerobic, the bHb remained in the oxyHb conformation (R-state) which has a high-affinity for O₂ [12]. This ensured that the polymerized bHBOCs would exhibit high O₂ affinities while limiting the oxygenation of bHb to form metHb. The extent of polymerization was later verified via light scattering (as described in Section 2.2.6) and gel electrophoresis (as described in Section 2.2.7). A two-fold excess of NaBH₄, with respect to the cross-linker, was then added to each reaction vessel for 30 minutes to quench the polymerization reaction by reducing unstable imine bonds formed during polymerization into stable amine linkages [3, 49]. After quenching the polymerization reaction, bHBOC dispersions were dialyzed in 50 mM Tris buffer (pH 7.2, 200 mg/L N-acetyl cysteine, and
20 mM NaCl) to remove excess NaBH₄ and unreacted cross-linker. N-acetyl cysteine was added to act as a reducing agent.

2.2.4 Concentrations of bovine hemoglobin and methemoglobin

The concentration of bHb was determined using the cyanometHb method [114]. CyanometHb has an absorption peak at 540 nm that correlates with an extinction coefficient \( \varepsilon_{540} \) of 11 mM\(^{-1}\)cm\(^{-1} \) [115, 116]. All of the Hb is first converted to metHb via the addition of excess potassium ferricyanide, an oxidizing agent. The metHb is subsequently reacted with excess potassium cyanide to form a solution containing only cyanometHb. The total concentration of Hb \( C_{Hb} \) can be determined with knowledge of the aforementioned extinction coefficient of cyanometHb and Beer’s Law by Equation 2.1, where \( Abs_{540} \) is the measured absorbance at 540 nm, \( l \) is the length of the cuvette, and \( Dilution \) is the dilution factor used in the measurement.

\[
C_{Hb} = \frac{Abs_{540}}{l \cdot \varepsilon_{540}} \cdot Dilution
\]  

[2.1]

The extinction coefficient of metHb \( \varepsilon_{630} \) at 630 nm is 3.7 mM\(^{-1}\)cm\(^{-1} \) [117]. To determine the concentration of metHb \( C_{metHb} \) in the solution, the absorbance of the solution \( Abs_{630A} \) was measured at 630 nm. This solution contained a mixture of bHb and metHb. The metHb was subsequently converted to cyanometHb, which does not absorb at 630 nm, via the addition of excess potassium cyanide. After allowing the reaction to take place for two minutes, the absorbance of the solution \( Abs_{630B} \) was again measured at 630 nm. This absorbance measurement was subtracted from \( Abs_{630A} \) to isolate the absorbance due solely to metHb, and \( C_{metHb} \) was calculated via Equation 2.2.
\[ C_{\text{metHb}} = \frac{Abs_{630,A} - Abs_{630,B}}{\varepsilon_{630} \cdot l} \cdot \text{Dilution} \]  

Each absorbance was measured in triplicate for each bHBOC. The metHb level was calculated as the percent of bHb in the metHb state by Equation 2.3.

\[ \text{metHb level} = \frac{C_{\text{metHb}}}{C_{\text{Hb}}} \times 100\% \]  

2.2.5 Equilibrium oxygen binding parameters of HBOCs

Equilibrium O\(_2\) dissociation curves of bHBOCs were measured using a Hemox Analyzer (TCS Scientific; New Hope, PA) \([118]\). Briefly, a 0.125 mM solution of bHBOC was prepared in Hemox buffer (TCS Scientific), which slowed the auto-oxidation rate of bHb and maintained a constant Cl\(^-\) concentration and pH. The sample was oxygenated to a pO\(_2\) \(\sim\) 150 mmHg using a compressed air stream. Then while the solution was deoxygenated via the addition of N\(_2\) gas, the absorbance of oxyHb in the HBOC solution was recorded as a function of pO\(_2\) via dual wavelength spectroscopy. The absorbance data was then fit to a four parameter \((a_1, a_2, a_3, a_4)\) Adair model, where the absorbance at a pO\(_2\) of 150 mmHg was assumed to represent the absorbance of a completely saturated O\(_2\) carrier \((A_\infty)\) and the absorbance at a pO\(_2\) of 1.9 mmHg was assumed to be the absorbance at an extrapolated pO\(_2\) of 0 mmHg \((A_0)\). (Equation 2.4). In this model \(a_{1-4}\) represent stepwise Adair constants. The stepwise Adair constants of the four parameter Adair model were used as initial guesses for a six parameter Adair model, where \(A_0\) and \(A_\infty\) are no longer fixed parameters. The six parameter stepwise Adair parameters were corrected for statistical binding factors to determine the stepwise
equilibrium constants, \( (K_{1-4}) \) according to Equation 2.5 [111].

\[
Y = \frac{Abs - A_0}{A_\infty - A_0} = \frac{a_1pO_2 + 2a_1a_2pO_2^2 + 3a_1a_2a_3pO_2^3 + 4a_1a_2a_3a_4pO_2^4}{4(1 + a_1pO_2 + a_1a_2pO_2^2 + a_1a_2a_3pO_2^3 + a_1a_2a_3a_4pO_2^4)} 
\]

[2.4]

\[
K_i = a_i \left[ \frac{i}{4 - (i-1)} \right] \quad [2.5]
\]

The \( O_2 \) dissociation curves were also fit to the two parameter \( (P_{50}, n) \) Hill Equation (Equation 2.6), setting \( A_0 \) and \( A_\infty \) to the values regressed from the six parameter Adair model [119].

\[
Y = \frac{Abs - A_0}{A_\infty - A_0} = \frac{P_{50}^n}{pO_2^n + P_{50}^n} 
\]

[2.6]

2.2.6 Molecular weight distribution of HBOCs

In brief, an asymmetric flow field-flow fractionator (AFFFF) coupled in series with a multi-angle static light scattering (MASLS) photometer and a differential interferometric refractometer (DIR) were used to determine the molecular weight distribution of polymerized bHBOCs [51, 71]. Polymerized bHbOCs were separated in the AFFFF channel under non-dissociating conditions (Tris buffer; pH 7.2) at 25°C. These monodisperse fractions are then sent through the flow cell of the MASLS photometer, where the Rayleigh ratio, light scattered intensity \( (R(\theta, c)) \), is measured at 16 different angles \( (\theta) \), [48]. The molecular weight of each monodisperse fraction is determined using light scattering theory developed by Zimm (Equation 2.7) [120, 121].

\[
\frac{K^* c}{R(\theta, c)} = \frac{1}{MP(\theta)} + 2A_2c 
\]

[2.7]
Here, $K^*$ represents the optical constant $4\pi^2(dn/dc)^2n_0^2/N_\lambda\lambda_0^4$, $c$ is the solute concentration, $A_2$ is the second virial coefficient, $n_0$ is the index of refraction of the solvent ($n_0 = 1.3316$ for Tris buffer), $dn/dc$ is the differential refractive index of bHb in Tris buffer with respect to the change in solute concentration ($dn/dc = 0.185$ ml/g), $\lambda_0$ is the vacuum wavelength of the laser ($\lambda_0 = 690$ nm), $N_A$ is Avogadro’s number; $M$ is the molecular weight of an individual fraction, and $P(\theta)$ is the form factor for the angular dependence of scattered light and is represented by Equation 2.8 [68].

$$P(\theta) \approx 1 - \frac{16\pi^2 n_0^2}{3\lambda_0^2} \langle r_s^2 \rangle \sin^2\left(\frac{\theta}{2}\right) + O\left(\sin^4\left(\frac{\theta}{2}\right)\right) - ...$$ [2.8]

As the limit of $\theta$ goes to zero, $P(\theta)$ approaches unity. Therefore, the molecular weight of each monodisperse fraction can be determined by Equation 2.9.

$$M = \frac{R_{\theta \to 0}}{K^* c - 2A_2 c R_{\theta \to 0}}$$ [2.9]

The monodisperse fractions are then sent through the flow cell of the DIR, which measures the change in the refractive index of the monodisperse Hb fraction in Tris buffer compared to pure Tris buffer ($\Delta n$). Using the $dn/dc$ for bHb, we can then calculate the change in the concentration of solute ($\Delta c$) according to Equation 2.10.

$$\Delta c = \frac{\Delta n}{dn/dc}$$ [2.10]

Since the only solute present in the solution is the monodisperse Hb fraction, $\Delta c$ must equal $c_i$. The weight of each slice can then be readily calculated by multiplying the concentration of each slice by the volume of the slice. The measured bHBOC molecular weight distributions were compared to their respective control groups, 1-OGP0 and 2-CEFP0, that were subjected to identical reaction conditions as the synthesized bHBOCs.
2.2.7 Native PAGE and SDS-PAGE

The degree of polymerization was determined via gel electrophoresis in a Mini-
PROTEAN 3 Cell (Bio-Rad; Hercules, CA). bHBOC samples were run on a
discontinuous native PAGE [122]. The purified bHBOCs were suspended in a native gel
sample buffer (Bio-Rad; Hercules, CA) and heated in a water bath at 95°C for 5 minutes.
The samples (0.075 mg per lane) were run at a constant voltage of 75 mV through a 4%
acrylamide stacking gel, and at 150 mV through a 10% acrylamide resolving gel. The
gels were then stained with EZBlue (Sigma-Aldrich, St. Louis, MO) for one hour and
subsequently destained with deionized water. The gels were then imaged on a Kodak
EDAS 290 (Kodak, Rochester, NY) with Kodak 1D v. 3.6 software to determine relative
band intensities.

The samples were also run on a gel consisting of a 6% acrylamide stacking gel
and a 16% acrylamide resolving gel using the SDS-PAGE (Laemmli) buffer system
[123]. The purified bHBOCs were suspended in Laemmli buffer system (Bio-Rad;
Hercules, CA), and denatured in a water bath set at 95°C to ensure that unreacted
tetrameric bHb denatured into its monomeric form. A prestained broad range $M_w$ marker
from Bio-Rad was used to evaluate the degree of cross-linking and consisted of proteins
having molecular weights between 7.1 kDa and 209 kDa. The samples (0.075 mg per
lane) were run at a constant voltage of 75 mV through the stacking gel, and at 150 mV
through the resolving gel. The gels were then stained with EZBlue (Sigma) for one hour
before being destained with deionized water. The gels were subsequently imaged on a
Kodak EDAS 290 (Rochester, NY) with Kodak 1D v. 3.6 software to determine relative
band intensities.
2.3 Results and Discussion

2.3.1 Methemoglobin levels in HBOC dispersions

Polymerized bHBOCs synthesized with ring-opened 1-OGP exhibited metHb levels ranging from 5 to 10% (Figure 2.2A). For polymerized bHBOCs synthesized with ring-opened 2-CEFP, metHb levels ranged between 2 and 10% (Figure 2.2B). Measurements were performed in triplicate for each bHBOC. Recall that 1-OGP0 and 2-CEFP0 are simply bHb that was exposed to reaction conditions identical to bHBOCs in the presence of no cross-linkers. The stock solution represents metHb levels of the purified bHb before the reaction took place. It is essential to measure starting metHb levels of all Hb solutions, since metHb levels do not remain constant. MethHb levels of the bHBOCs and their control were compared to the stock bHb solution by Student’s t-test to determine if the two groups were significantly different from each other. The metHb level of 1-OGP0 was shown to be significantly different ($P < 0.1$) than the stock solution. Since the reaction occurred aerobically at room temperature, it is not unexpected that the metHb levels of the bHb increase under these reaction conditions. Additionally, the metHb levels of 1-OGP20 were shown to be significantly different when compared to a stock solution of bHb ($P < 0.05$). For bHBOCs reacted with ring-opened 2-CEFP, 2-CEFP30 was shown to be significantly different compared to the stock bHb solution ($P < 0.05$).

Infusion of HBOCs with high metHb levels (> 10%) into animals has been demonstrated to increase mortality [124]. In contrast, in vivo studies have shown that infusion of acellular Hbs with metHb levels less than 10% did not elicit adverse physiological responses when infused into rats [18]. Ring-opened 1-OGP and 2-CEFP
Figure 2.2. Methemoglobin levels in HBOC dispersions synthesized with ring-opened 1-OGP (A) and ring-opened 2-CEFP (B). All reactions were compared to a bHb solution before reaction (stock solution) and the same bHb solution after reaction (with no cross-linker) and after dialysis (2-OGP0 and 2-CEFP0). Measurements were performed in triplicate with the error bars representing the standard deviation. ** refers to data with a P value < 0.1, while * refers to data with a P value < 0.05 when compared to the stock bHb solution according to Student’s t-test.

are both mild oxidizing agents; therefore, as the concentration of cross-linker increases, the metHb level of the bHBOC product also increases. To limit the formation of metHb, the cross-linking/polymerization reaction is conducted under reduced pO₂ conditions by bubbling N₂ gas through the reaction vessels. Fortunately, metHb can be reduced to Hb with ascorbic acid, a reducing agent, or other types of reducing agents [20-22, 24, 124]. Preliminary experiments in our group have demonstrated the ability to reduce the metHb levels of acellular bHb by over 90% (from metHb levels of 45.9% to 3.02%) with ascorbic acid (4:1 ascorbic acid to bHb molar ratio) and methylene blue (10⁻³:1 methylene blue to bHb molar ratio). In vitro, methylene blue acts as an electron donor to increase the rate at which ascorbic acid reduces metHb. In vivo, methylene blue is converted to leucomethylene blue in the presence of NADPH which results in the
nonenzymatic reduction of metHb [125]. It is important to note that both ascorbic acid and methylene blue are currently used to treat methemoglobinemia, a rare condition in which a person loses the ability to reduce Hb [125, 126].

These experiments demonstrate the ability of both ring-opened 1-OGP and ring-opened 2-CEFP to yield polymerized bHBOCs with metHb levels at or below 10%. Also, to further reduce metHb levels post reaction, ascorbic acid and methylene blue can be reacted with the final product.

2.3.2 Equilibrium oxygen binding parameters

Novel high-affinity bHBOCs were synthesized by reacting ring-opened 1-OGP and 2-CEFP with R-state bHb. This was confirmed by measuring the O$_2$ dissociation curves of the bHBOCs in a Hemox Analyzer (TCS Scientific, New Hope, PA), which measured the absorbance of the sample as the pO$_2$ was reduced from ~150 to 1.9 mmHg, post-reaction. Additionally, the O$_2$ binding parameters of bRBCs were measured four times to verify the reproducibility of the O$_2$ binding curves. The experimental data were non-linearly fit to the Hill equation, and the $P_{50}$ and $n$ are shown in Figure 2.3. The $P_{50}$ is displayed on the left axis in red, while the $n$ is displayed on the right axis in blue.

The $P_{50}$ describes the pO$_2$ at which half of the heme groups are saturated with O$_2$. High-affinity acellular HBOCs were targeted, since they have been found to better maintain the tissue and mean arterial pO$_2$s and target improved O$_2$ delivery to low tissue pO$_2$s compared to low-affinity acellular HBOCs. To obtain high-affinity HBOCs, the polymerization reaction was performed in the presence of O$_2$ when bHb is in the R-state. Recall that 1-OGP0 and 2-CEFP0, the controls, consisted of unreacted bHb, and as such the O$_2$ binding data of bHb is reported here.
Figure 2.3. Hill equation parameters ($P_{50}$ and $n$) for bHBOCs synthesized in this study and how they compare to bHb and bRBCs. Recall that bHb is the control for both reactions (1-OGP0 and 2-CEFP0, respectively). The $P$ values that the regressed parameters differ from when compared to bHb are as follows: * < 0.05; + < 0.01; and O < 0.005%.

The reaction of ring-opened 1-OGP with bHb produced bHBOCs having high affinities for $O_2$. As the concentration of the ring-opened 1-OGP increased from 0 mM (1-OGP0) to 30 mM, the $P_{50}$ decreased from 22 mmHg to 7 mmHg. Synthesis of these bHBOCs was performed in duplicate to verify reproducibility. The $P_{50}$s of 1-OGP20 and 1-OGP30 were each significantly different from that of bHb ($P < 0.05$) according to Student’s t-test.

Similar results were observed for bHBOCs synthesized via polymerization with ring-opened 2-CEFP. As the ring-opened 2-CEFP concentration increased from 0 mM to
30 mM, the $P_{50}$ decreased from 22 mmHg to 8 mmHg. For these bHBOCs, 2-CEFP10 was reacted in duplicate, and the $P_{50}$ was found to be significantly different from the $P_{50}$ of bHb ($P < 0.15$). These results show that increasing the cross-linker to bHb molar ratio increases the O$_2$ affinity of the bHBOCs synthesized with ring-opened 1-OGP and 2-CEFP, and suggest that further increases in O$_2$ affinity could be achieved by further increasing the cross-linker to bHb molar ratio.

The cooperativity coefficient ($n$) is the second parameter in the Hill equation. For each of the bHBOCs synthesized with ring-opened 1-OGP and 2-CEFP, the cooperativity coefficient showed a significant decrease compared to bRBCs ($n = 2.73$). The cooperativities of 1-OGP20 ($P < 0.05$), 1-OGP30 ($P < 0.01$), and 2-CEFP10 ($P < 0.05$) were significantly different from bHb’s cooperativity according to Student’s t-test. This bodes well for the high-affinity bHBOCs synthesized in this study considering that Kavdia et al. showed that high-affinity HBOCs with reduced cooperativities possessed better mixed venous pO$_2$s [106].

The O$_2$ dissociation curve of bRBCs was measured four times to verify the reproducibility of the Hill data parameters by the Hemox Analyzer (TCS Scientific; New Hope, PA). The $P_{50}$ of bRBCs was determined to be 28.8 mmHg ± 2.88. This $P_{50}$ ($P < 0.05$) and $n$ ($P < 0.005$) were found to be significantly different by Student’s t-test compared to those of bHb.

The raw absorbance data was also fit to the six parameter Adair model, which better models the O$_2$ dissociation curve at high and low pO$_2$s [111]. Stepwise Adair parameters, $a_{1-4}$, were regressed from the six parameter Adair model (Equation 2.4) for each of the bHBOCs (Table 2-I). Additionally, the stepwise Adair constants were
TABLE 2-I

STEPWISE ADAIR AND CORRECTED EQUILIBRIUM O2 BINDING CONSTANTS

<table>
<thead>
<tr>
<th></th>
<th>a1 (mmHg(^{-1}))</th>
<th>a2 (mmHg(^{-1}))</th>
<th>a3 (mmHg(^{-1}))</th>
<th>a4 (mmHg(^{-1}))</th>
<th>K1 (mmHg(^{-1}))</th>
<th>K2 (mmHg(^{-1}))</th>
<th>K3 (mmHg(^{-1}))</th>
<th>K4 (mmHg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-OGP20</td>
<td>0.468 ± 0.3</td>
<td>0.223 ± 0.2</td>
<td>2.67E-2 ± 4E-3</td>
<td>4.12E-2 ± 2E-3</td>
<td>0.117</td>
<td>0.149</td>
<td>0.0401</td>
<td>0.165</td>
</tr>
<tr>
<td>1-OGP30</td>
<td>0.725 ± 0.2</td>
<td>0.295 ± 0.3</td>
<td>6.45E-2 ± 2E-2</td>
<td>3.01E-2 ± 7E-4</td>
<td>0.181</td>
<td>0.197</td>
<td>0.0968</td>
<td>0.120</td>
</tr>
<tr>
<td>2-CEFP10</td>
<td>0.216 ± 0.06</td>
<td>0.0979 ± 0.1</td>
<td>9.00E-9 ± 1E-9</td>
<td>1.50E5 ± 7E4</td>
<td>0.0540</td>
<td>0.0653</td>
<td>1.35E-08</td>
<td>600000</td>
</tr>
<tr>
<td>2-CEFP20</td>
<td>0.00855</td>
<td>2.02</td>
<td>4.50E-9</td>
<td>1.74E5</td>
<td>0.00214</td>
<td>1.347</td>
<td>6.75E-09</td>
<td>696000</td>
</tr>
<tr>
<td>2-CEFP30</td>
<td>0.935</td>
<td>0.248</td>
<td>5.19E-2</td>
<td>3.06E-2</td>
<td>0.234</td>
<td>0.165</td>
<td>0.0779</td>
<td>0.122</td>
</tr>
<tr>
<td>bRBC</td>
<td>0.0184 ± 0.007</td>
<td>0.0378 ± 0.004</td>
<td>6.25E-8 ± 1E-8</td>
<td>4.75E4 ± 5E3</td>
<td>0.00460</td>
<td>0.0252</td>
<td>9.38E-08</td>
<td>190000</td>
</tr>
<tr>
<td>bHb</td>
<td>0.0913</td>
<td>0.0115</td>
<td>9.19E-8</td>
<td>8.48E4</td>
<td>0.0228</td>
<td>0.00767</td>
<td>1.38E-07</td>
<td>339000</td>
</tr>
</tbody>
</table>
corrected for statistical binding factors to determine the stepwise equilibrium constants, $K_{1-4}$ (Equation 2.5). The stepwise Adair constants for unmodified bHb compared well to literature values reported by Johnson et al. [127] for $a_1$, $a_2$, and $a_4$ but differ significantly for $a_3$. This is most likely due to the limitations with spectroscopic measurements [127]. The third stepwise equilibrium constant ($K_3$) is close to zero making the values resolved for $K_3$ and $K_4$ in doubt. At high cross-linker to bHb molar ratios, the value of $K_3$ increases so that it is not close to zero as evidenced by the $K_3$’s of 1-OGP20, 1-OGP30, and 2-CEFP30. Similar results were found by Vandegriff [111] when studying ααHb, a human Hb cross-linked between the α chains. The increases in the $K_3$ values suggest that 1-OGP20, 1-OGP30, and 2-CEFP30 have been stabilized against the formation of αβ dimers compared to uncrosslinked bHb.

2.3.3 Molecular weight distributions

In an attempt to create novel bHBOCs with a $M_n$ greater than 108 kDa, ring-opened 1-OGP and ring-opened 2-CEFP were reacted with bHb at cross-linker to bHb molar ratios between 10:1 and 30:1. Ring-opening 1-OGP and 2-CEFP yielded newly synthesized dialdehydes. It is important to quickly react dialdehydes with bHb, since dialdehydes can self-oligomerize and form a wide range of reactive dialdehyde species. Glutaraldehyde, the dialdehyde used in creating PolyHeme (Northfield Laboratories, Evanston, IL) and Hemopure (Biopure, Cambridge, MA), is known to react at random sites on Hb’s surface. It can react with the amino groups of lysines and arginines; the sulphydryl groups of cysteines; the imidazole rings of histidines; and the phenolic rings of tyrosines [3]. Ring-opened 1-OGP and ring-opened 2-CEFP are hypothesized to react in much the same way, due to the fact that they have two reactive aldehyde groups.
In reacting bHb with dialdehydes, the goal is to create a polymerized bHBOC with a $M_n$ greater than the pore size of the capillary pores (~108 kDa). This size is slightly greater than that of the bHb tetramer (64 kDa), and as such, the $M_n$ of the bHBOCs should be greater than that of a bHb tetramer. The molecular weight distribution was measured in 50 mM Tris buffer, in which tetrameric bHb does not fully dissociate into $\alpha\beta$ dimers. The rationale for using non-dissociating conditions is a result of the fact that high salt conditions can corrode the AFFFF-MASLS-DIR plumbing.

As seen in Figure 2.4, each of the polymerized bHBOCs exhibited higher $M_n$ compared to the control when run under identical reaction conditions. Additionally, the polymerized bHb species that were synthesized at cross-linker to bHb molar ratios of at least 20:1 were shown to have a $M_n$ greater than 108 kDa. Polymerized bHBOCs that were synthesized with ring-opened 2-CEFP showed a higher $M_n$ compared to those synthesized with ring-opened 1-OGP. This could be a result of the longer side chain of ring-opened 1-OGP when compared to that of ring-opened 2-CEFP (8 carbon chains for ring-opened 1-OGP versus 2 carbon chains for ring-opened 2-CEFP) (Figure 2.1). The longer side chain on ring-opened 1-OGP provides more steric hindrance and as a result could lead to a reduction in the number of cross-linking reactions that could possibly occur between the dialdehydes and bHb.

For both 1-OGP20 and 1-OGP30, the $M_n$ was approximately two times greater than that of bHb as determined by AFFFF-MASLS-DIR (Figure 2.4A). It is interesting to note that the $M_n$ is approximately the same for both 1-OGP20 and 1-OGP30 at approximately 135 kDa. One possible explanation of this effect could be a result of the long nonpolar side chain on ring-opened 1-OGP (Figure 2.1). Due to the nonpolar nature
of this side chain, ring-opened 1-OGP could prefer to react with the internal amino acids of bHb where nonpolar residues occur at a higher density. This would lead to a decreased $M_n$ for the bHBOCs synthesized with ring-opened 1-OGP compared to those bHBOCs synthesized with ring-opened 2-CEFP, which does not have a nonpolar side chain. However, knowledge of the amino acid sites that have reacted with the cross-linking agents is essential to draw any definitive conclusions.

For 2-CEFP10, 2-CEFP20, and 2-CEFP30, the $M_n$ was greater compared to bHb (Figure 2.4B). Additionally, as the ring-opened 2-CEFP to bHb molar ratio increases from 10:1 to 30:1, the $M_n$ also increases. As the ring-opened 2-CEFP to bHb molar ratio increases, the intramolecular binding sites that are in close proximity are more likely to be saturated and as a result more intermolecular reactions take place to yield high $M_n$ bHBOCs.
The average $M_n$ of bHb was found to be $79.8 \pm 2.8$ kDa, compared to the accepted value of 64 kDa (Figure 2.4). This is due to limitations in the AFFFF-MASLS-DIR system in measuring molecular weights. Consequently, the $M_n$ of polymerized bHBOCs was compared to the results of the $M_n$ of bHb measured by the AFFFF-MASLS-DIR system the same day. Additionally, polyacrylamide gel electrophoresis (PAGE) studies were performed to verify the conclusions of the light scattering studies.

2.3.4 Native PAGE and SDS-PAGE

bHBOC samples were first analyzed by native PAGE to confirm that they had undergone polymerization (Figure 2.5). Since the sample markers broaden as cross-linking increases, we can conclude that bHb is undergoing reaction with ring-opened 1-OGP and ring-opened 2-CEFP. Native PAGE is of limited use when evaluating modified Hbs since the surface charge on Hb can become altered during the polymerization reaction [128]. As the cross-linker:bHb molar ratio was increased for both the bHBOCs synthesized with ring-opened 1-OGP and ring-opened 2-CEFP, a greater fraction of polymerized bHb was formed. To confirm that the reaction was taking place between monomeric units in the bHb tetramer, bHBOC samples were also run on SDS-PAGE.

For bHBOCs synthesized with ring-opened 1-OGP an increase in the ring-opened 1-OGP to bHb molar ratio resulted in an increase in the relative intensity of bHb dimers (MW ~ 32 and 35 kDa respectively) from 0.13 for 1-OGP0 to 0.29 for 1-OGP30, while the relative intensity of bHb monomers (MW ~ 13 kDa) decreased (Figure 2.6). Additionally, there do not appear to be many higher order species (labeled bHb tetramer +) formed, which supports the results in Section 2.3.3 found by light scattering. Finally,
the cross-linking reaction is shown to be successful as molecular weight species greater than monomeric bHb exist under denaturing conditions.

For bHBOCs synthesized with ring-opened 2-CEFP, an increase in the ring-opened 2-CEFP to bHb molar ratio resulted in an increase in the relative intensities of bHb dimers and higher order species ($M_w > 50$ kDa). 2-CEFP0 had relative intensities of 0.09 and zero for bHb dimers higher order species (labeled bHb tetramer +), respectively, while 2-CEFP30 had relative intensities of 0.32 and 0.05 for bHb dimers and higher order species, respectively (Figure 2.6). Here, it is important to note that increasing the ring-opened 2-CEFP to bHb molar ratio further increases the molecular weight of polymerized bHBOCs, which again lends support to the results acquired by MASLS.

![Native gels of 1-OGP bHBOCs and 2-CEFP bHBOCs.](image)

Figure 2.5. Native gels of (A) 1-OGP bHBOCs and (B) 2-CEFP bHBOCs. The samples were run from top to bottom.
Additionally, the higher order molecular weight species begin to appear towards the top of the gel for 2-CEFP20 and 2-CEFP30. While these bands are faint, they provide evidence of the synthesis of increasing molecular weight species. Higher order molecular weight species are created as intramolecular cross-linking sites become saturated. As these sites become less available at higher cross-linker concentrations, intermolecular bonds occur more frequently to create higher order molecular weight species.

Further purification of both species by gel filtration would be required in order to separate unreacted bHb from polymerized bHBOCs. Additionally, it is believed that the degree of polymerization could be increased for both the synthesized bHBOCs with ring-opened 1-OGP and ring-opened 2-CEFP by increasing the cross-linker to bHb molar ratio above 30:1. However, this could further increase metHb levels and lower the $P_{50}$ of the final product based on the trends observed when increasing the cross-linker to bHb molar ratio from 0:1 to 30:1.

Figure 2.6. SDS-PAGE gels for 2-CEFP bHBOCs and 1-OGP bHBOCs.
2.4 Concluding Remarks

Novel bHBOCs having high affinities for O$_2$ were synthesized with the potential to treat patients suffering from hemorrhagic shock and stroke. bHBOCs exhibited a decrease in cooperativity, which should lead to a higher mixed venous pO$_2$ with infusion of high O$_2$ affinity HBOCs. bHBOCs were synthesized by polymerizing bHb with either ring-opened 1-OGP or 2-CEFP. In doing so, two novel cross-linking agents have been identified for the synthesis of high-affinity bHBOCs. The presence of cross-linking has been confirmed via static light scattering, native PAGE, and SDS-PAGE. Furthermore, the third stepwise Adair constant was stabilized at high cross-linker to bHb molar ratios, suggesting that tetrameric bHb has been stabilized. The molecular weight distribution of bHBOCs can be altered by increasing the cross-linker to bHb molar ratio. Further purification of bHBOCs by gel filtration may be required to better evaluate the molecular weight distribution of polymerized bHBOCs and to separate unreacted bHb from polymerized bHb species.
3.1 Introduction

Previous methods of purifying bHb within the Palmer research group have included using toluene to extract bHb [48-51] and passing bHb through a hollow fiber system [87-89, 129]. Unfortunately, toluene has been found to remain within the hydrophobic core of Hb after purification. Additionally, purification with the hollow fiber system was found to be awkward, and there was some concern that cell debris remained in the final product. A new method for the purification of bHb from bRBCs was developed involving anion exchange chromatography preceded by dialysis. This method is a fast and effective way to obtain bHb from bRBCs using Q Sepharose XL, a strong anion exchange resin. This resin had double the binding capacity for bHb compared to three other anion exchange resins that were studied in this work. MetHb levels remained below 2% with bHb concentrations between 0.7 and 1.7 mM. The high purity of bHb was confirmed via SDS-PAGE and size exclusion chromatography.
3.2 Materials and Methods

3.2.1 Selection of resin

A HiTrap IEX Selection Kit was purchased from GE Healthcare (Piscataway, NJ) to evaluate the binding affinity of bHb to four columns of anion exchange resins: Q Sepharose Fast Flow, Q Sepharose XL, ANX Sepharose 4 Fast Flow, and DEAE Sepharose Fast Flow. Each 1 mL column was overloaded with bHb, before being equilibrated with start buffer (20 mM triethanolamine, pH = 7.9). The bHb was subsequently removed from the column via the addition of 1 M sodium chloride. The total mass of the bHb that was bound to each column was determined from the concentration of Hb and the total volume of fractions containing bHb. The concentration of bHb was determined via the cyanometHb technique which is fully described in Section 2.2.4.

3.2.2 Scale up

Q Sepharose XL had the highest binding capacity for bHb, and was chosen as the anion exchange resin for scale-up studies. A XK50 column (I.D. = 5 cm) and 300 mL of Q Sepharose XL media stored in 20% ethanol were purchased from GE Healthcare in order to scale up the process and obtain the required yield of bHb per purification. The column was packed at a flow rate of 16.3 mL/min, and the final bed height was measured to be 9 cm for a total column volume of 177 mL. The column was loaded with bHb at 30% of its binding capacity and operated at flow rates no greater than 75% of the packing flow rate.
3.2.3 Initial purification

Sterile bRBCs in citrate buffer were purchased from Quad 5 (Ryegate, MT). bRBCs were washed three times with isotonic saline (0.9% w/v) solution to remove acellular Hb and any remaining plasma proteins in a centrifuge for 15 minutes at a speed of 4500 rpm at 4ºC. bHb was subsequently extracted with three equivalents of 15 mOsM PB (pH = 7.2) for one hour in an ice-water bath. The extract was then passed through glass wool three times and qualitative filter paper to remove excess cell debris. The filtrate was placed into a dialysis bag with a molecular weight cutoff of 10 kDa and dialyzed overnight at 4ºC with start buffer (20 mM triethanolamine, pH = 7.90) at a dialysis buffer to filtrate concentration of 35,000 to 1. This process removed small particles from the filtrate, and placed the bHb solution into the proper start buffer so that it would be captured by the FPLC resin.

3.2.4 FPLC purification

The FPLC system was equilibrated with 10 column volumes (CVs) of Start Buffer (20 mM triethanolamine, pH = 7.9) at flow rates of 6, 9, and 12 mL/min. One hundred milliliters of 1 mM bHb solution in Start Buffer was loaded onto the column where it was captured by the Q Sepharose XL resin. A five CV linear gradient, consisting of equal volumes of Start Buffer and Running Buffer (20 mM triethanolamine + 0.5 M NaCl, pH = 7.9) connected by a U-tube, was developed and run through the column at room temperature. The column was subsequently washed with 5 CVs of 1 M NaCl, and then stored with 5 CVs of 20% ethanol. All buffers were filtered through a 0.22 µm filter. Fractions were collected with a fraction collector, and the absorbance and conductivity of each fraction were measured. The absorbance of each fraction was measured at a
wavelength of 280 nm with a Synergy HT 96-well plate reader (Biotek; Winooski, VT) on Costar UV transparent flat bottom plates (Corning; Corning, NY). The fractions that constituted the peak of the bHb product were pooled together as the final purified product. The conductivity of each fraction was measured with a Cole-Palmer (Chicago, IL) conductivity meter.

3.2.5 Concentrations of bovine hemoglobin and methemoglobin

The concentration of bHb was determined using the cyanometHb method [114] described in detail in Section 2.2.4. These measurements were performed in triplicate.

3.2.6 SDS-PAGE

The purity of the bHb was determined via gel electrophoresis in a Mini-PROTEAN 3 Cell (Bio-Rad; Hercules, CA). Samples were collected post-lysis, post-filter, post-dialysis, and from the two fractions closest to the peak of the bHb chromatogram at flow rates of 6, 9, and 12 mL/min. Additionally, for the 12 mL/min purification, two fractions that were not captured by the Q Sepharose XL resin and eluted prior to the bHb peak were run on the gel. The samples were run in a SDS-PAGE (Laemmli) buffer system [123] with a 6% acrylamide stacking gel, and a 16% acrylamide resolving gel. The purified bHBOCs were suspended in Laemmli buffer system (Bio-Rad; Hercules, CA), and denatured in a water bath set at 95°C. bHb appears as a monomeric band due to the denaturant. A prestained broad range molecular weight marker from Bio-Rad was used to evaluate the molecular weight of the fractions and consisted of proteins having molecular weights between 7.1 kDa and 209 kDa. The samples (75 µg per lane) were run at a constant voltage of 75 mV through the stacking
gel, and at 150 mV through the resolving gel. The gels were then stained with EZBlue (Sigma-Aldrich; St. Louis, MO) for one hour before being destained with deionized water and imaged on a Kodak EDAS 290 (Rochester, NY) to determine relative band intensities.

3.2.7 Size exclusion chromatography

The purity of the bHb was also tested via SEC. The final product was dialyzed into 10 L of 0.1 M phosphate buffer (pH = 6.8) with three buffer changes. The purified bHb sample (800 µg) was injected into a Waters’ HPLC (Milford, MA) system that was connected to a Phenomenex (Torrance, CA) SEC column (Biosep-SEC-S 3000; 60 cm × 7.8 mm). The column was run at a flow rate of 1 mL/min, and the chromatogram was evaluated for purity. The column was calibrated with seven proteins having molecular weights between 12 and 670 kDa to determine the size of the bHb species by plotting the log of the molecular weight versus $K_d$ (Figure 3.1). The line of best fit and $R^2$ values are displayed on the graph. Proteins that are labeled red were injected into the SEC column by Phenomenex, while the proteins that are labeled blue were injected into the SEC column in this study. The $K_d$ is defined by Equation 3.1, where $Vol_{unk}$ is the elution volume of the species, $Vol_o$ is the void volume, and $Vol_{inc}$ is the included elution volume. The $Vol_o$ was determined with a high molecular marker to be 10.978 mL, while the $Vol_{inc}$ was determined to be 22.91 mL via the injection of uridine (244.2 Da) into the column.

$$K_d = \frac{Vol_{unk} - Vol_o}{Vol_{inc} - Vol_o}$$ [3.1]
3.2.8 Column cleaning

After each run, the resin was cleaned with 1 M NaCl and then stored in 20% ethanol to prevent bacterial growth. Additionally, thorough cleanings of the resin were performed with 10 mM EDTA in a 0.1% Triton X solution to remove free heme groups that bound to the column. This step can be avoided with careful preparation in the washing and filtering stages of the bHb purification procedure.
3.3 Results and Discussion

3.3.1 Selection of resin

Figure 3.2 shows the binding capacities of each of the resins for bHb. The binding capacity is reported in milligrams of bound bHb per milliliter of resin. Q Sepharose XL had more than twice the binding capacity for bHb compared to Q Sepharose Fast Flow, ANX Sepharose 4 Fast Flow, and DEAE Sepharose Fast Flow. The binding capacities reported here are artificially high, since the columns were overloaded with bHb before equilibrating with Start Buffer and eluting the bHb from the column with 1 M NaCl. Additionally, the Q Sepharose XL purification was able to yield metHb levels less than 2% while operating at a pH of 7.9 and at room temperature.

![Figure 3.2. The binding capacity of the four anion exchange resins at room temperature and a pH of 7.9.](image)
3.3.2 FPLC purification

Figure 3.3 shows the FPLC elution profiles at each of the flow rates within the study, where the absorbance is in red and the conductivity is in blue. Figure 3.3A also shows the different stages of the FPLC purification, starting with the equilibration of the column with Start Buffer to the reestablishment of the baseline with 1 M NaCl. Lipids and metHb have previously been found to elute earlier in the purification process [96], and this peak can be seen best in Figure 3.3A which utilized bRBCs that were four weeks old compared to Figure 3.3C which utilized bRBCs that were two weeks old. Considering that metHb levels increase due to auto-oxidation, this increase in metHb levels is to be expected with an increasing age of the bRBCs. Significant cell debris remained in the product even after filtering the lysate through glass wool and dialyzing this product overnight at 4°C. This was evident by an increase in cloudiness in the initial fractions of the purification fractions post-injection, and suggests that ion exchange chromatography should capture the bHb product to ensure that all cell debris is removed from the final product. The final bHb product was collected from the fractions that were closest to the bHb peak. Typical concentrations of the final bHb product ranged between 0.7 and 1.7 mM with metHb levels between 0.5 and 2% as determined by the cyanometHb technique. It is important to emphasize that the FPLC purification was performed at room temperature and was still able to achieve these low metHb levels.

Figure 3.4 shows the SDS-PAGE results for each step of the bHb purification procedure described above, at flow rates of 6, 9, and 12 mL/min. Bovine hemoglobin represents 90% of the protein content within bRBCs with the remaining protein content consisting of albumin, carbonic anhydrase, and superoxide dismutase [98]. In order to
assess the level of impurities, each lane was overloaded with 75 µg of bHb, with the exception of the last two lanes in the 12 mL/min gel which were loaded directly from two fractions that were not captured by the resin prior to the bHb peak in Figure 3.3C. The bHb travels through the gel as a monomer (α and β globin chains) since it has been denatured by the SDS and is present as a thick band at the bottom of the gel. The post-lysate product is mostly bHb for each flow rate studied; however, a decrease in impurities can be seen throughout the purification process, with the only impurity that remains in the final product occurring at around 32 kDa. This impurity was thought to be either bHb dimers that do not fully denature or remnant superoxide dismutase. There is no
significant difference in the purity of the bHb product at any of the three flow rates within this study.

On the 12 mL/min gel, two lanes were loaded with FPLC fractions containing impurities not captured by the resin. Within these lanes, the bHb concentration is smaller compared to the other lanes as evidenced by the smaller area of the bHb band. These lanes show a significant amount of impurities that were removed from the final bHb product during the FPLC purification with Q Sepharose XL resin. The high purity of the final bHb product was confirmed via SEC on a HPLC system (Figure 3.5), where only one peak was observed. The bHb product was determined to elute from the SEC column as αβ dimers, since its $K_d$ is between the $K_d$ of superoxide dismutase (31.2 kDa) and
ovalbumin (44 kDa) in Figure 3.1. Additionally, superoxide dismutase was observed to have a retention time of 19.056 minutes within the SEC column. However, no peak was observed at this retention time for the final bHb product, suggesting that the impurities seen in the SDS-PAGE gel at 32 kDa (Figure 3.4) were $\alpha\beta$ dimers rather than superoxide dismutase.

3.4 Concluding Remarks

The purification of bHb is an essential first step in the synthesis of HBOCs for use as artificial blood substitutes and in tissue engineering applications. This study determined that Q Sepharose XL had a superior binding capacity for bHb compared to the other resins within this study. It was determined that it is essential to have the ion exchange resin capture the bHb in order to ensure the removal of all cell debris.
Furthermore, anion exchange chromatography provides an avenue to separate metHb from oxyHb and retain low metHb levels. Indeed, metHb levels were kept below 2% at room temperature, which reduces energy costs compared to purifications that operate in a cold room.
CHAPTER 4:

THE EFFECTS OF INCREASING THE RING-OPENED MONOPYRANOSIDE TO BOVINE HEMOGLOBIN MOLAR RATIO HAS ON THE METHEMOGLOBIN LEVELS AND MOLECULAR WEIGHT DISTRIBUTIONS OF BOVINE HEMOGLOBIN-BASED OXYGEN CARRIERS

4.1 Introduction

In Chapter 2, the synthesis of novel bHBOCs via the cross-linking of bHb with ring-opened 1-OGP and 2-CEFP at cross-linker to bHb molar ratios from 0:1 to 30:1 was discussed. The size distributions of these bHBOCs were characterized via light scattering, native PAGE, and SDS-PAGE. The effect of further increasing the cross-linker to bHb molar ratios to 100:1 has on the metHb levels and size distributions are described in this chapter. Increasing the cross-linker to bHb molar ratio caused the metHb levels of bHBOCs synthesized with ring-opened 1-OGP to decrease. However, this effect was not seen for bHBOCs synthesized with ring-opened 2-CEFP, where the bHBOCs were observed to have higher metHb levels between 44% and 54%.

The size distributions of bHBOCs were determined via SEC and compared to the results of the SDS-PAGE and light scattering studies described in Chapter 2. Purified bHb was found to elute from the SEC column as a dimer in Chapter 4. bHBOCs synthesized with ring-opened 1-OGP and ring-opened 2-CEFP showed an increase in bHb species having both decreased and increased $M_w$ distributions compared to bHb as
the ring-opened 1-OGP to bHb molar ratio increased. However, for bHBOCs synthesized with 2-CEFP, the $M_w$ distributions at cross-linker to bHb molar ratios between 50:1 and 70:1 remained constant. Furthermore, bHBOCs synthesized with 2-CEFP created $M_w$ species centered at 63.6 and 96 kDa, correlating to intact tetrameric bHb and tetrameric bHb bound to a bHb dimer, respectively. The results for the size distributions calculated by SEC were compared to the size distributions calculated by SDS-PAGE and light scattering (Chapter 2).

4.2 Materials and Methods

4.2.1 Purification of bovine hemoglobin

bHb was purified according to the Materials and Methods that was described in detail in Chapter 3. In brief, sterile bRBCs (Quad 5) were washed three times in isotonic saline to remove any remaining plasma proteins. bHb was then extracted from the bRBCs via the addition of hypotonic 15 mOsM PB (pH = 7.2), filtered through glass wool and a paper filter, and dialyzed in Start Buffer (20 mM triethanolamine, pH = 7.9). The bHb was then purified via anion exchange chromatography on a XK50 column packed with Q Sepharose XL resin with a 5 CV linear gradient of Start Buffer and Running Buffer (20 mM triethanolamine, 0.5 M NaCl, pH = 7.9). The fractions that eluted at the peak of the chromatogram were collected for the final bHb product. The concentration and metHb levels of the final bHb product were determined via the cyanomethemoglobin technique (See Section 2.2.4).
4.2.2 Oxidation of monopyranosides

1-OGP and 2-CEFP were oxidatively ring-opened via the addition of sodium periodate. A full description of this reaction can be found in Section 2.2.2.

4.2.3 Synthesis of bovine hemoglobin-based oxygen carriers

Purified bHb was reacted with ring-opened 1-OGP and 2-CEFP in 50 mM Tris buffer (pH = 7.2) for two hours at cross-linker to bHb molar ratios from 20:1 to 100:1 via parallel synthesis according to the methods described in Section 2.2.3 to create bHBOCs. bHBOCs synthesized with ring-opened 1-octyl-β-D-glucopyranoside were evaluated via size exclusion chromatography (SEC) at cross-linker to bHb molar ratios of 0:1 (1-OGP0), 20:1 (1-OGP20), 30:1 (1-OGP30), 40:1 (1-OGP40), 50:1 (1-OGP50), 60:1 (1-OGP60), 70:1 (1-OGP70), 80:1 (1-OGP80), 90:1 (1-OGP90), and 100:1 (1-OGP100). bHBOCs synthesized with ring-opened 2-chloroethyl-β-D-fructopyranoside were evaluated via SEC at cross-linker to bHb molar ratios of 0:1 (2-CEFP0), 20:1 (2-CEFP20), 40:1 (2-CEFP40), 50:1 (2-CEFP50), 60:1 (2-CEFP60), 70:1 (2-CEFP70), 80:1 (2-CEFP80), 90:1 (2-CEFP90), and 100:1 (2-CEFP100).

4.2.4 Concentrations of bovine hemoglobin and methemoglobin

The concentration of bHb and metHb within the bHBOCs were assessed via the cyanomethemoglobin technique that was previously described in Section 2.2.4. The metHb levels were subsequently calculated. Each measurement was performed in triplicate.
4.2.5 Size exclusion chromatography

The size distributions of bHBOCs were also measured via SEC. All of the bHBOCs synthesized with ring-opened 1-octyl-β-D-glucopyranoside were injected into the SEC column and evaluated. The following bHBOCs synthesized with ring-opened 2-chloroethyl-β-D-fructopyranoside were evaluated via SEC: 2-CEFP0, 2-CEFP20, 2-CEFP40, 2-CEFP50, 2-CEFP60, and 2-CEFP70. 2-CEFP80, 2-CEFP90, and 2-CEFP100 were not evaluated due to the fact that the SEC column displayed an increase in back pressure after the injection of 2-CEFP70. The bHBOCs (800 µg) were suspended in 0.1 M PB (pH = 6.8) and injected into a Waters’ HPLC system (Milford, MA) that was connected to a Phenomenex (Torrance, CA) SEC column (Biosep-SEC-S 3000; 60 cm × 7.8 mm). The column was run at a flow rate of 1 mL/min with 0.1 M PB (pH = 6.8), and the chromatogram was evaluated to determine the molecular weight distributions. The column was calibrated with seven proteins having molecular weights between 12 and 670 kDa by plotting the log of the molecular weight versus the $K_d$, and the line of best fit and $R^2$ values are displayed in Figure 3.1. bHb was determined to elute as a dimer from the protein standards. The $K_d$ is defined by Equation 5.1, where $Vol_{unk}$ is the elution volume of the species, $Vol_o$ is the void volume, and $Vol_{inc}$ is the included elution volume. The $Vol_o$ was determined with high molecular weight standards to be 10.978 mL, while the $Vol_{inc}$ was determined to be 22.91 mL via the injection of uridine (244.2 Da) into the column. Chromatograms comparing the elution volume to the absorbance at 280 nm were processed from the HPLC system and then subsequently processed into graphs comparing the $K_d$ to the absorbance at 280 nm. The $K_d$ at each elution volume was
calculated and then correlated to a molecular weight distribution by the line of best fit described in Figure 3.1.

\[ K_d = \frac{Vol_{unk} - Vol_{o}}{Vol_{inc} - Vol_{o}} \]  \[4.1\]

4.3 Results and Discussion

4.3.1 Purification of bovine hemoglobin

bHb was purified the day before the synthesis of bHBOCs via cross-linking. Figure 4.1A shows the chromatogram of bHb purification purified via FPLC that was to be reacted with ring-opened 1-OGP. Fractions 183-191 (in blue) were pooled together as the purified bHb product and were found to have a final bHb concentration of 1.34 mM with metHb levels of 0.44%. This final product was used as the stock bHb solution for bHBOCs synthesized with ring-opened 1-OGP. Figure 4.1B shows the chromatogram of the FPLC purification of bHb that was to be reacted with ring-opened 2-CEFP. Fractions 172-177 were pooled together as the purified bHb product and were found to have a final bHb concentration of 0.77 mM with metHb levels of 1.06%. This final product was used as the stock bHb solution for bHBOCs synthesized with ring-opened 2-CEFP.

4.3.2 Methemoglobin levels

Figure 4.2 shows the metHb levels of bHBOCs synthesized with ring-opened 1-OGP. The control, 1-OGP0, consisted of bHb undergoing identical reaction conditions as
the bHBOCs synthesized with ring-opened 1-OGP. The metHb levels of 1-OGP0 increased from 0.44% to 4.07% after undergoing identical reaction conditions as the bHBOCs polymerized with ring-opened 1-OGP and overnight dialysis into PB (pH = 6.8). 1-OGP20 was observed to have a metHb level of 58.33%. Increasing the ring-opened 1-OGP to bHb molar ratio surprisingly caused the metHb levels to decrease as 1-OGP100 was observed to have metHb levels of 4.86%. This could be a result of the increasing concentrations of NaBH₄ (40 mM was used to quench 1-OGP20, while 200 mM was used to quench 1-OGP100) that was added to quench the reaction between ring-opened 1-OGP and bHb.

Figure 4.3 shows the metHb levels of bHBOCs synthesized with ring-opened 2-CEFP. The control, 2-CEFP0, consisted of bHb undergoing identical reaction conditions as bHBOCs synthesized with ring-opened 2-CEFP. The metHb levels of 2-CEFP0 increased from 1.06% to 20.69% after undergoing identical reaction conditions as the bHBOCs polymerized with ring-opened 2-CEFP and overnight dialysis into PB (pH =
Figure 4.2. MetHb levels of bHBOCs reacted with ring-opened 1-OGP.

Figure 4.3. MetHb levels of bHBOCs reacted with ring-opened 2-CEFP.
6.8). This high increase in metHb levels is most likely due to the fact that for this reaction, the bHBOCs were dialyzed over the course of 48 hours compared to the typical 16 hour dialysis duration. MetHb levels for the bHBOCs synthesized with ring-opened 2-CEFP remained between 54% and 64%. Increasing the amount of NaBH$_4$ did not have any effect on the metHb levels for these bHBOCs. This could be due to the initial increase in metHb levels of 2-CEFP0, the control. These metHb levels are also high compared to those found in Chapter 2 due to the fact that the bHBOCs were synthesized without the presence of N-acetyl-cysteine, a reducing agent. Decreases in the metHb levels can also be obtained through the addition of ascorbic acid.

4.3.3 Size exclusion chromatography

Figure 4.4 shows the molecular weight distributions of the bHBOCs synthesized with ring-opened 1-octyl-β-D-glucopyranoside compared to a control, 1-OGP0, which consists of bHb that has undergone identical reaction conditions as the bHBOCs. The elution time was transformed into a molecular weight ($M_w$) as described in Section 4.2.5. 1-OGP0 elutes from the SEC column at a $M_w$ centered at 33.8 kDa, which is in good agreement to the accepted $M_w$ of a bHb dimer (~32 kDa). The absorbance intensity centered at the bHb dimer declined in 1-OGP20, 1-OGP30, 1-OGP40, 1-OGP50, 1-OGP60, and 1-OGP70 as the cross-linker to bHb molar ratio increased. Additionally, a bHb species centered at a $M_w$ of 24.2 kDa elutes from the SEC column when the aforementioned bHBOCs were injected into the column, suggesting that at low cross-linker to bHb molar ratios some of the bHb becomes denatured and breaks into smaller molecules. As the cross-linker to bHb molar ratio increases, there is a smaller amount of the low $M_w$ bHb species. Furthermore, an increase in higher order $M_w$ species is seen
when increasing the cross-linker to bHb molar ratio from 20:1 to 70:1. Finally, a high $M_w$ aggregate elutes from the column with a $M_w$ distribution centered at 2500 kDa as the cross-linker to bHb molar ratio increases from 20:1 to 70:1. This provides more evidence that at low cross-linker to bHb molar ratios, ring-opened 1-octyl-β-D-glucopyranoside denatures bHb causing the final product to have high $M_w$ aggregates. 1-OGP80, 1-OGP90, and 1-OGP100 display a broad spectrum of $M_w$s, centered at 33.8 kDa, having smaller amounts of low $M_w$ bHb species and increasing amounts of high $M_w$ bHb species. Additionally, there appears to be less high $M_w$ aggregates in these bHBOCs. This suggests that at high cross-linker to bHb molar ratios, the bHb product becomes stabilized and forms increasing $M_w$ bHb species. Finally, the broad peak seen in 1-OGP80, 1-OGP90, and 1-OGP100 shows the formation of bHb species having $M_w$s as high as 200

![Figure 4.4. SEC column profiles of bHBOCs synthesized with ring-opened 1-OGP.](image)
Figure 4.5. SEC column profiles of bHBOCs synthesized with ring-opened 2-CEFP.

This could suggest that ring-opened 1-o-octyl-β-D-glucopyranoside reacts nonspecifically to bHb and yields a wide $M_w$ distribution of bHb species.

Figure 4.5 shows the molecular weight distributions of the bHBOCs synthesized with ring-opened 2-chloroethyl-β-D-fructopyranoside compared to a control, 2-CEFP0, which consists of bHb that has undergone identical reaction conditions as the bHBOCs. 2-CEFP0 elutes from the SEC column at a $M_w$ distribution centered at 33.4 kDa, which is in good agreement with both the literature value for bHb dimers (~32 kDa) and the $M_w$ distribution of 1-OGP0 (33.8 kDa). The absorbance intensity that centered at 33.4 kDa decreased as the cross-linker to bHb molar ratio was increased, suggesting an increase in the amount of bHb that has undergone reaction. While there is evidence of an increase in the degree of low $M_w$ bHb distributions centered at 21.5 kDa, there are also two individual high $M_w$ bHb species that are being formed with $M_w$ distributions centered at 63.6 and 96 kDa, respectively. These $M_w$s are significant, since they are evenly spaced at
approximately 32 kDa, the accepted literature value for a bHb dimer. With this knowledge, we can conclude that the $M_w$ distribution centered at 63.6 kDa represents an intact bHb tetramer, while the $M_w$ distribution centered at 96 kDa represents an intact tetramer bound to a dimer. No significant difference in the degree of polymerization was observed as the cross-linker to bHb molar ratio increased from 40:1 to 70:1. This could be a result of saturation of the binding sites on the bHb molecule by cross-linker molecules.

The results of the SEC study supported the SDS-PAGE study that was discussed in Section 2.3.4. The SDS-PAGE gel (Figure 2.6) shows that 1-OGP20 and 1-OGP30 consist of mostly unmodified bHb monomer with the remainder consisting of cross-linked bHb dimer. This is confirmed by the SEC study in Figure 4.4, where the great majority of the $M_w$ distributions for 1-OGP20 and 1-OGP30 are dimers with a small amount of higher order species. The SDS-PAGE gel (Figure 2.6) also shows that more of the bHb monomer is converted into higher order species when the cross-linker is ring-opened 2-chloroethyl-β-D-fructopyranoside compared to 1-octyl-β-D-glucopyranoside at a cross-linker to bHb molar ratio of 20:1. This is confirmed in the SEC study, where 2-CEFP20 has a greater fraction of bHb eluting at a $M_w$ distribution greater than a dimer compared to 1-OGP20.

Taken together, the results of the SDS-PAGE and SEC studies do not lend support to the accuracy of the previously discussed light scattering study (Section 2.2.6). First, the light scattering study does not show that a majority of the reacted bHb is not modified by the cross-linking agents. Additionally, the $M_w$ distributions in the light scattering study seem artificially high when compared to those obtained in the PAGE and SEC
studies. While the light scattering data does show similar $M_w$ profiles in terms of shape as the SEC data, it appears that the light scattering data does not have the capabilities to resolve the multiple peaks seen in the chromatograms of bHBOCs reacted with 2-chloroethyl-β-D-fructopyranoside. This could be a limitation of the AFFF separation method which results in monodisperse fractions not being separated prior to entering the MASLS apparatus.

4.4 Concluding Remarks

From the SEC studies, it can be concluded that ring-opened 2-CEFP is a more promising cross-linking agent compared to ring-opened 1-OGP. At cross-linker to bHb molar ratios of 50:1, ring-opened 2-CEFP yields more higher order $M_w$ species than its counterpart. Additionally, the polymerized bHBOCs that were created with ring-opened 2-CEFP had $M_w$ distributions that were centered at 63.6 kDa and 96 kDa, while the polymerized bHBOCs created with ring-opened 1-OGP had one broad $M_w$ distribution centered at 32 kDa. The high metHb levels observed in bHBOCs synthesized with ring-opened 2-CEFP can be reduced via the addition of a mild reducing agent, such as ascorbic acid. Further purification would be required to separate the bHb dimers from the polymerized bHBOCs to make an effective $O_2$ carrier.
CHAPTER 5:
HEMOGLOBIN-BASED OXYGEN CARRIER OXYGEN AFFINITY AND CAPILLARY INLET PARTIAL PO\(_2\) ARE IMPORTANT FACTORS THAT INFLUENCE OXYGEN TRANSPORT IN A CAPILLARY

5.1 Introduction

Hemopure (Biopure; Cambridge, MA) and PolyHeme (Northfield Laboratories; Evanston, IL) are two acellular HBOCs currently in phase III clinical trials for use as RBC substitutes in transfusion applications. The most common adverse side effect that these HBOCs exhibit is increased vasoconstriction. Autoregulatory theory has been presented as a possible explanation for this physiological effect, where it is hypothesized that low-affinity HBOCs over-deliver O\(_2\) to tissues surrounding capillaries thereby eliciting vasoconstriction. An *a priori* model has been developed to evaluate possible triggers of the autoregulatory theory. At normoxic inlet pO\(_2\)s, there was no correlation between O\(_2\) flux out of the capillary and the O\(_2\) affinity of the HBOC. However, a correlation was found between the average pO\(_2\) tension in the capillary and the O\(_2\) affinity of the HBOC. Hence, it is hypothesized that an increased pO\(_2\) tension in the capillary acts as the trigger for the autoregulatory response rather than increased O\(_2\) flux into the skeletal muscle tissue.

Mixtures of acellular HBOCs, having both lower and higher affinities for O\(_2\) compared to hRBCs, and hRBCs were modeled in a Krogh tissue cylinder model...
(KTCM) that comprised a capillary surrounded by a capillary membrane and skeletal muscle tissue with specified tissue $O_2$ consumption rates in the tissue space having Michaelis-Menten kinetics. The HBOCs studied possessed $P_{50}$s spanning 5 - 55 mmHg. The equilibrium binding/release of oxygen to/from the HBOCs was modeled using the Adair equation. In this chapter, the total Hb (hRBCs and HBOCs) concentration was kept constant. Changing the inlet $pO_2$ greatly affected which HBOC, having a unique $O_2$ affinity, best delivered $O_2$ to the surrounding tissue. These findings suggest that the $O_2$ affinity of the HBOC should be carefully selected based on the capillary inlet $pO_2$ tension for a specific transfusion application.

5.2 Materials and Methods

5.2.1 Synthesis of bovine hemoglobin-based oxygen carriers

bHb was polymerized with ring-opened 1-octyl-$\beta$-D-glucopyranoside (1-OGP) and ring-opened 2-chloroethyl-$\beta$-D-fructopyranoside (2-CEFP), respectively according to the methods described in Section 2.2.3. In addition to the HBOCs synthesized in Chapter 2, ring-opened 1-OGP was reacted with acellular bHb at a ring-opened 1-OGP to bHb molar ratio of 40:1 to yield 1-OGP40.

5.2.2 Equilibrium oxygen binding properties of bHBOCs

The overall Adair constants used to model the $O_2$ binding curves of polymerized bovine Hb (PolyBvHb) [56], $\alpha\alpha$ cross-linked Hb ($\alpha\alpha$Hb) [130], and hRBCs [107] were obtained from the literature and presented in Table 5-I, while the high $O_2$ affinity
TABLE 5-I

P$_{50}$ AND OVERALL ADAIR PARAMETERS USED IN KTCM

<table>
<thead>
<tr>
<th>O$_2$ Carrier</th>
<th>P$_{50}$ (mmHg)</th>
<th>A$_1$ (mmHg$^{-1}$)</th>
<th>A$_2$ (mmHg$^{-2}$)</th>
<th>A$_3$ (mmHg$^{-3}$)</th>
<th>A$_4$ (mmHg$^{-4}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyBvHb [56]</td>
<td>54.2</td>
<td>4.365×10$^{-2}$</td>
<td>1.299×10$^{-3}$</td>
<td>1.637×10$^{-5}$</td>
<td>1.023×10$^{-7}$</td>
</tr>
<tr>
<td>ααHb [130]</td>
<td>33.8</td>
<td>2.220×10$^{-2}$</td>
<td>9.510×10$^{-4}$</td>
<td>1.340×10$^{-11}$</td>
<td>1.050×10$^{-6}$</td>
</tr>
<tr>
<td>hRBC [107]</td>
<td>28.6</td>
<td>1.530×10$^{-2}$</td>
<td>1.100×10$^{-3}$</td>
<td>1.240×10$^{-7}$</td>
<td>1.810×10$^{-6}$</td>
</tr>
<tr>
<td>2-CEFP20 [129]</td>
<td>15.2</td>
<td>9.000×10$^{-3}$</td>
<td>1.800×10$^{-2}$</td>
<td>9.000×10$^{-11}$</td>
<td>2.000×10$^{-5}$</td>
</tr>
<tr>
<td>1-OGP20 [129]</td>
<td>11.3</td>
<td>4.675×10$^{-1}$</td>
<td>1.043×10$^{-1}$</td>
<td>2.800×10$^{-3}$</td>
<td>1.000×10$^{-4}$</td>
</tr>
<tr>
<td>2-CEFP30 [129]</td>
<td>7.98</td>
<td>9.000×10$^{-1}$</td>
<td>2.250×10$^{-1}$</td>
<td>1.170×10$^{-2}$</td>
<td>4.000×10$^{-4}$</td>
</tr>
<tr>
<td>1-OGP40</td>
<td>5.15</td>
<td>3.900×10$^{-1}$</td>
<td>2.808×10$^{-1}$</td>
<td>2.110×10$^{-2}$</td>
<td>8.000×10$^{-4}$</td>
</tr>
</tbody>
</table>

bHBOCs (1-OGP20, 2-CEFP20, and 2-CEFP30 [129]) synthesized in the Palmer research group measured using a Hemox Analyzer (TCS Scientific, PA) [118] according to the methods described in Section 2.2.5. The equilibrium O$_2$ binding data of 1-OGP40 was determined for this study in a similar manner. Stepwise Adair constants (Equation 5.1) can be readily transformed into overall Adair constants (Equation 5.2). These results are discussed in [129] and the overall Adair constants that were used to model these O$_2$ carriers can be found in Table 5-I. The P$_{50}$ was determined by calculating the pO$_2$ at which the O$_2$ carrier was half-saturated with O$_2$.

\[
Y = \frac{a_1pO_2 + 2a_2pO_2^2 + 3a_3pO_2^3 + 4a_4pO_2^4}{4(a_1pO_2 + a_2pO_2^2 + a_3pO_2^3 + a_4pO_2^4)} \quad [5.1]
\]

\[
Y = \frac{A_1pO_2 + 2A_2pO_2^2 + 3A_3pO_2^3 + 4A_4pO_2^4}{4(A_1pO_2 + A_2pO_2^2 + A_3pO_2^3 + A_4pO_2^4)} \quad [5.2]
\]
5.2.3 Modeling O\textsubscript{2} transport in a capillary by a mixture of hRBCs and HBOCs

O\textsubscript{2} delivery to skeletal muscle tissue facilitated by a mixture of hRBCs and HBOCs was modeled with a KTCM for a single capillary. The model is composed of three separate subdomains: the lumen (Subdomain I), the capillary membrane (Subdomain II) and the tissue space (Subdomain III). The dimensions of the KTCM used in this study are listed in Table 5-II, where \( L\textsubscript{c} \) is the capillary length; \( r\textsubscript{c} \) is the capillary radius; \( r\textsubscript{m} \) is the radius of the capillary wall; and \( r\textsubscript{t} \) is the Krogh tissue cylinder radius. Subdomains I (Equation 5.3), II (Equation 5.4), and III (Equation 5.5) have unique partial differential equations (PDEs) in cylindrical coordinates describing oxygen transport in these regions.

\[
\nu \frac{\partial pO_2}{\partial r} = \left(1 + m\text{RBC}D\text{RBC}^C + m\text{HBOC}D\text{HBOC}^C \right) \frac{1}{\left(1 + m\text{RBC} + m\text{HBOC} \right)} \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial pO_2}{\partial r} \right) + \frac{\partial^2 pO_2}{\partial z^2} \right]
\]

\[ [5.3] \]

\[
0 = D\text{O}_2^m \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial pO_2}{\partial r} \right) + \frac{\partial^2 pO_2}{\partial z^2} \right]
\]

\[ [5.4] \]

\[
0 = \left(1 + p\text{Mb}D\text{Mb}^T \right) \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial pO_2}{\partial r} \right) + \frac{\partial^2 pO_2}{\partial z^2} \right] + R_{\text{cons}} H^T_{O_2}
\]

\[ [5.5] \]

**TABLE 5-II**

**GEOMETRY OF THE KTCM**

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Size (µm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L\textsubscript{c} )</td>
<td>500</td>
<td>[110]</td>
</tr>
<tr>
<td>( r\textsubscript{c} )</td>
<td>2.5</td>
<td>[131]</td>
</tr>
<tr>
<td>( r\textsubscript{m} )</td>
<td>3.0</td>
<td>[109]</td>
</tr>
<tr>
<td>( r\textsubscript{t} )</td>
<td>26</td>
<td>[110]</td>
</tr>
</tbody>
</table>
Figure 5.1 shows a schematic of the KTCM along with the boundary conditions used in the simulations. The model allows specification of the capillary inlet $pO_2$ ($pO_{2,in}$) and calculates the outlet $pO_2$ in the capillary via the finite element method. At the capillary centerline, the symmetry boundary condition is applied. Additionally, it accounts for the fact that there is no convective flux around the entire membrane and tissue space. The continuity equation is used at the capillary and capillary membrane interface, as well as at the capillary membrane and tissue space interface.

In the lumen, $O_2$ exists in three states: dissolved $O_2$, bound to hRBCs, and bound to HBOCs. $O_2$ carriers are not allowed to diffuse through the capillary membrane in this model, resulting in $O_2$ only existing as dissolved $O_2$ in the capillary membrane. Finally, in the tissue space, $O_2$ can exist as dissolved $O_2$ or bound to myoglobin (Mb), an $O_2$ carrier found in skeletal muscle that aids in $O_2$ transport.

5.2.4 Henry’s law constants

The solubility of dissolved $O_2$ in the presence of Hb ($\alpha_{O_2-Hb}$) is the inverse of the Henry’s law constant for dissolved $O_2$ in the presence of Hb ($H_{O_2-Hb}$). The solubility of dissolved $O_2$ in the lumen was determined using the relationship described by Bouwer et al. in Equation 5.6 [132].

$$\alpha_{O_2-Hb} = \alpha_{O_2-H_2O} \left(1 + 0.00312 \frac{dL}{g} C_{sat,Hb} - 0.3 \frac{1}{M} [salt]\right)$$ [5.6]

The Henry’s law constant for $O_2$ in the tissue space ($H_{O_2}^T$) was measured by Mahler et al. in skeletal muscle [133].
Figure 5.1. A schematic (not drawn to scale) of the KTCM used to model oxygen transport from the capillary to the surrounding tissues by a mixture of hRBCs and HBOCs.
5.2.5 Diffusion coefficients

The diffusion coefficient of dissolved O\textsubscript{2} in the capillary (\(D_{O_2}^C\)) was calculated from the correlation described by Bouwer et al. (Equation 5.7) \[132\]. Here \(D_{0,O_2}\) is the diffusion coefficient of O\textsubscript{2} in the presence of no Hb; \(C_{sat,Hb}\) is the Hb concentration in g/dL; and \(C_{1,O_2}\) and \(C_{2,O_2}\) are constants that were obtained by a least squared fit of the diffusion data for O\textsubscript{2} by Bouwer et al. \[132\]. These constants can be found in Table 5-III.

\[
D_{i}^C = D_{0,i} \left(1 - \frac{C_{sat,Hb}}{C_{1,i}}\right) \left(\frac{10^{-C_{sat,Hb}}}{C_{2,i}}\right)
\]

[5.7]

The diffusion coefficient of dissolved O\textsubscript{2} in the capillary membrane region (\(D_{O_2}^M\)) was estimated from a correlation \[109\] based on the solute radius (\(a\)), capillary pore radius (\(r_p\)), and diffusivity of O\textsubscript{2} in water (Equation 5.8).

\[
D_{O_2}^M = \frac{D_{O_2}^W}{r_p} \left(1 - \frac{a}{r_p}\right) \left[1 - 2\left(\frac{a}{r_p}\right) + 2.09 \left(\frac{a}{r_p}\right) - 0.95 \left(\frac{a}{r_p}\right)^5\right]
\]

[5.8]

The diffusion coefficient of dissolved O\textsubscript{2} in the tissue space (\(D_{O_2}^T\)) was obtained from the measured literature value for mammalian striated muscle measured by Bentley et al. (Table 5-III) \[134\].

The diffusivity coefficients of Hb contained within hRBCs (\(D_{RBC}^C\)) and HBOCs (\(D_{HBOC}^C\)) in the capillary were assumed to be equal to that of free Hb. They were subsequently calculated using the correlation described by Bouwer et al. (Equation 5.7) \[132\], where \(D_{0,Hb}\) is the diffusion coefficient of Hb in the presence of no Hb, \(C_{sat,Hb}\) is the Hb concentration in g/dL, and \(C_{1,Hb}\) and \(C_{2,Hb}\) are constants that were obtained by a
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&lt;V&gt;$; [cm/s]</td>
<td>0.225</td>
<td>[110]</td>
</tr>
<tr>
<td>$D_{0,O_2}$; [cm$^2$/s]</td>
<td>$2.07\times10^{-5}$</td>
<td>[132, 135]</td>
</tr>
<tr>
<td>$C_{1,O_2}$; [g/dL]</td>
<td>100</td>
<td>[132, 135]</td>
</tr>
<tr>
<td>$C_{2,O_2}$; [g/dL]</td>
<td>119</td>
<td>[132, 135]</td>
</tr>
<tr>
<td>$D_{0,Hb}$; [cm$^2$/s]</td>
<td>$1.03\times10^{-6}$</td>
<td>[132, 135]</td>
</tr>
<tr>
<td>$C_{1,Hb}$; [g/dL]</td>
<td>46</td>
<td>[132, 135]</td>
</tr>
<tr>
<td>$C_{2,Hb}$; [g/dL]</td>
<td>128</td>
<td>[132, 135]</td>
</tr>
<tr>
<td>$C_{sat,Hb}$; [μM] heme</td>
<td>8800</td>
<td>[10]</td>
</tr>
<tr>
<td>$α_{O_2-H_2O}$; [M/mmHg]</td>
<td>$1.412\times10^{-6}$</td>
<td>[136]</td>
</tr>
<tr>
<td>$D_{O_2}^T$; [cm$^2$/s]</td>
<td>$2.92\times10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>$r_p$; [nm]</td>
<td>3.5</td>
<td>[109]</td>
</tr>
<tr>
<td>$D_{O_2}^T$; [cm$^2$/s]</td>
<td>$2.44\times10^{-5}$</td>
<td>[134]</td>
</tr>
<tr>
<td>$D_{Mb}^T$; [cm$^2$/s]</td>
<td>$1.73\times10^{-7}$</td>
<td>[110]</td>
</tr>
<tr>
<td>$C_{sat,Mb}$; [μM]</td>
<td>383</td>
<td>[110]</td>
</tr>
<tr>
<td>$H^T_{O_2}$; [mmHg/μM]</td>
<td>0.77</td>
<td>[133]</td>
</tr>
<tr>
<td>$P_{50, Mb}$; [mmHg]</td>
<td>3.2</td>
<td>[110]</td>
</tr>
<tr>
<td>$K_m$; [mmHg]</td>
<td>0.44</td>
<td>[137, 138]</td>
</tr>
<tr>
<td>$V_{max}$; [μM/s]</td>
<td>5, 26, 45, 75, 100, 125, 150</td>
<td></td>
</tr>
<tr>
<td>$Hct$</td>
<td>0.113, 0.224, 0.335, 0.448</td>
<td></td>
</tr>
<tr>
<td>$pO_2_{in}$; [mmHg]</td>
<td>5, 10, 15, 20, 25, 30, 45, 65, 95, 115</td>
<td></td>
</tr>
</tbody>
</table>
least squared fit of the diffusion data for Hb. These constants can likewise be found in Table 5-III. It is assumed that the HBOCs and hRBCs are only present in the lumen. This is a reasonable assumption since hRBCs are much larger than the capillary pores (~7 nm), and HBOCs should be designed to be larger than the capillary pores.

Mb is an O\textsubscript{2} carrier that is found in skeletal muscle and aids in the transport of O\textsubscript{2} within the tissue space. For this model, Mb is physically limited to the tissue space. The concentration of Mb in the tissue space (\(C_{sat,Mb}\)) and the diffusion coefficient of Mb in the tissue space (\(D_{Mb}^T\)) were determined by McGuire and Secomb [110] and are listed in Table 5-III.

5.2.6 Velocity profile

The velocity profile in the capillary is described by Poiseuille flow [131] (Equation 5.9), where \(<V>\) represents the average velocity of the blood (Table 5-III) and \(r_c\) represents the capillary radius (Table 5-II).

\[
V = 2 <V> \left[ 1 - \left( \frac{r}{r_c} \right)^2 \right] \quad [5.9]
\]

Within the capillary membrane and the tissue space, it is assumed that O\textsubscript{2} transport is due solely to diffusion.

5.2.7 Reaction kinetics

Within the lumen and the capillary membrane, it is assumed that there is no O\textsubscript{2} consumption. However, within the tissue space, O\textsubscript{2} is consumed by the skeletal muscle according to Michaelis-Menten kinetics (Equation 5.10). The term \(R_{cons}\) represents the
rate of O\text sub{2} consumption by skeletal muscle tissue, where K\text sub{m} is the Michaelis-Menten constant for O\text sub{2} being consumed by skeletal muscle tissue and V\text sub{max} is the maximum O\text sub{2} consumption rate of skeletal muscle tissue. The maximum O\text sub{2} consumption rate was varied between 5 and 150 µM/s, which is listed in Table 5-III.

\[
R_{cons} = \frac{V_{max} pO_2}{K_m + pO_2} \tag{5.10}
\]

5.2.8 Differential mass balances

A differential mass balance on dissolved O\text sub{2} was performed in the lumen (Equation 5.11), where C is the concentration of dissolved O\text sub{2} in the lumen, R_{O_2-HBOC} is the rate at which O\text sub{2} binds to the HBOCs in the lumen, and R_{O_2-RBC} is the rate at which O\text sub{2} binds to hRBCs in the lumen.

\[
V \frac{\partial C}{\partial r} = D_{O_2}^C \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) + \frac{\partial^2 C}{\partial z^2} \right] + R_{O_2-RBC} + R_{O_2-HBOC} \tag{5.11}
\]

Similarly, differential mass balances on Hb contained within hRBCs and Hb contained within HBOCs were performed in the lumen to yield Equation 5.12 and Equation 5.13, respectively, where C\text sub{RBC} is the concentration of heme groups in the hRBCs, R_{RBC-O_2} is the rate at which O\text sub{2} is released by the hRBCs, C\text sub{HBOC} is the concentration of heme groups in the HBOCs, and R_{HBOC-O_2} is the rate at which O\text sub{2} is released by the HBOCs.

\[
V \frac{\partial C_{RBC}}{\partial r} = D_{RBC}^C \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_{RBC}}{\partial r} \right) + \frac{\partial^2 C_{RBC}}{\partial z^2} \right] + R_{RBC-O_2} \tag{5.12}
\]

\[
V \frac{\partial C_{HBOC}}{\partial r} = D_{HBOC}^C \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_{HBOC}}{\partial r} \right) + \frac{\partial^2 C_{HBOC}}{\partial z^2} \right] + R_{HBOC-O_2} \tag{5.13}
\]
It was assumed that hRBCs and HBOCs bound and released O\textsubscript{2} at equilibrium. In making this assumption, the differential mass balances of hRBCs and HBOCs were able to be converted into a differential mass balance on dissolved O\textsubscript{2}. The change in the concentration of O\textsubscript{2} and dissolved O\textsubscript{2} \((m_i)\) for the O\textsubscript{2} carrier is represented by Equation 5.14, where \(x_i\) is the mole fraction of heme groups, \(C_{\text{sat},Hb}\) is the total concentration of heme groups in the lumen, and \(\frac{\partial Y_i}{\partial pO_2}\) is the change in the \(i\)th O\textsubscript{2} carrier’s equilibrium curve (Equation 5.2) with respect to the partial pressure of O\textsubscript{2} \((pO_2)\). The \(pO_2\) is related to the dissolved O\textsubscript{2} concentration by Henry’s law.

\[
m_i = x_i \frac{\partial C_i}{\partial C} = x_i H_{O_2-Hb} C_{\text{sat},Hb} \frac{\partial Y_i}{\partial pO_2}
\]

[5.14]

The term \(m_i\) was multiplied across the differential mass balances of hRBCs and HBOCs to yield Equation 5.15 and Equation 5.16, respectively, so that the differential mass balances on hRBCs and HBOCs were both in terms of dissolved O\textsubscript{2}.

\[
m_{\text{RBC}} V \frac{\partial C}{\partial r} = m_{\text{RBC}} D_{\text{RBC}}^C \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) + \frac{\partial^2 C}{\partial z^2} \right] + R_{\text{RBC-O}_2}
\]

[5.15]

\[
m_{\text{HBOC}} V \frac{\partial C}{\partial r} = m_{\text{HBOC}} D_{\text{HBOC}}^C \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) + \frac{\partial^2 C}{\partial z^2} \right] + R_{\text{HBOC-O}_2}
\]

[5.16]

Equation 5.12, Equation 5.15, and Equation 5.16 were summed together to obtain the overall differential mass balance in the lumen (Equation 5.3). Since it was assumed that the O\textsubscript{2} binding/release reactions occurred at equilibrium, \(R_{\text{RBC-O}_2}\) and \(R_{\text{O}_2-\text{RBC}}\) must have the same magnitude but opposite signs and sum to zero. This is also true for \(R_{\text{HBOC-O}_2}\) and \(R_{\text{O}_2-\text{HBOC}}\).

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A differential mass balance on dissolved O\(_2\) was also performed in the capillary membrane (Equation 5.4). Here, it is assumed that there is no convective flow in the capillary membrane, the transport of dissolved O\(_2\) is solely due to diffusion, and the subdomain is free of O\(_2\) carriers. Since dissolved O\(_2\) is the only source of O\(_2\) in this region, the differential mass balance on dissolved O\(_2\) is also the overall differential mass balance on O\(_2\) within the capillary membrane.

A differential mass balance on dissolved O\(_2\) was performed in the tissue space (Equation 5.17), where \(R_{O_2-Mb}\) is the rate at which O\(_2\) binds to Mb in the tissue space and \(R_{cons}^T\) is the O\(_2\) consumption rate in the skeletal muscle tissue as represented by Michaelis-Menten kinetics (Equation 5.10).

\[
0 = D_{O_2}^T \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) + \frac{\partial^2 C}{\partial z^2} \right] + R_{O_2-Mb} + R_{cons}^T \tag{5.17}
\]

It was assumed that there is no flow in the tissue space. Mb is an O\(_2\) carrying protein found within skeletal muscle. A differential mass balance on oxygenated Mb (MbO\(_2\)) was performed in the tissue space (Equation 5.18), where \(C_{MbO_2}\) is the concentration of MbO\(_2\) in the tissue space and \(R_{Mb-O_2}\) is the rate at which O\(_2\) is released by Mb in the tissue space.

\[
0 = D_{Mb}^T \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_{MbO_2}}{\partial r} \right) + \frac{\partial^2 C_{MbO_2}}{\partial z^2} \right] + R_{Mb-O_2} \tag{5.18}
\]

The change in the concentration of Mb with respect to the change in concentration of dissolved O\(_2\) (\(p_{Mb}\)) is represented by Equation 5.19, where \(C_{Sat, Mb}\) is the total
concentration of Mb and $\frac{\partial Y_{Mb}}{\partial pO_2}$ is the change in the Mb equilibrium curve (Equation 5.20) with respect to the pO₂.

$$p_{Mb} = \frac{\partial C_{MbO_2}}{\partial C} = H_{O_2}^T C_{sat, Mb} \frac{\partial Y_{Mb}}{\partial pO_2}$$  \[5.19\]

$$Y_{Mb} = \frac{pO_2}{P_{50, Mb} + pO_2}$$  \[5.20\]

This $p_{Mb}$ term was multiplied across the differential mass balance of Mb to yield Equation 5.21. Both differential mass balances were in terms of dissolved O₂, and they were summed together to yield the overall mass balance in the tissue space (Equation 5.5). Since it was assumed that the binding of Mb to O₂ occurred at equilibrium, $R_{Mb-O_2}$ and $R_{O_2-Mb}$ must have equal magnitudes but opposite signs and sum to zero.

$$0 = p_{Mb}D_{Mb}^T \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) + \frac{\partial^2 C}{\partial z^2} \right] + R_{Mb-O_2}$$  \[5.21\]

5.2.9 Parameters studied

The PDEs for the three subdomains were solved simultaneously on Comsol Multiphysics 3.1 using the finite element method for two low-affinity HBOCs (PolyBvHb and ααHb), hRBCs, and four high-affinity HBOCs (2-CEFP20, 1-OGP20, 2-CEFP30, and 1-OGP40). A summary of the finite elements used in this study are listed in Table 5-IV. The hematocrit (Hct), $pO_{2, inst}$, and $V_{max}$ were varied in the O₂ transport simulations according to Table 5-III for each HBOC. After simulations were conducted, surface and volume integrals were evaluated to determine the surface averaged outlet pO₂ ($<pO_{2, out}>$), surface averaged pO₂ at the capillary membrane ($<pO_{2, wall}>$), and volume
averaged $pO_2$ ($<pO_2>$) for each subdomain. The total average $O_2$ saturation ($<Y_{Hb}>$) of HBOCs and hRBCs in the capillary was calculated for each simulation from the volume averaged $pO_2$ in the capillary, the average $O_2$ saturation of HBOCs ($<Y_{HBOC}>$), and the average $O_2$ saturation of hRBCs ($<Y_{hRBC}>$).

The flux of $O_2$ ($J_{O_2}$) into the tissue space originating from the capillary space was evaluated by Equation 5.22, where $Q$ represents the blood flow rate, $[Hb]_{ARBC}$ represents the Hb concentration of the hRBCs, and $[Hb]_{HBOC}$ represents the Hb concentration of the HBOCs. Each Hb concentration must be multiplied by a factor of four, since a single Hb molecule is composed of four heme groups that can each bind to one $O_2$ molecule.

$$J_{O_2} = Q \cdot \left[ \alpha_{Hb-O_2} pO_{2,in} + 4HctY_{in}^{RBC}[Hb]_{RBC} + 4(1 - Hct)Y_{in}^{HBOC}[Hb]_{Cap} \right] - \left[ \alpha_{Hb-O_2} pO_{2,out} + 4HctY_{out}^{RBC}[Hb]_{RBC} + 4(1 - Hct)Y_{out}^{HBOC}[Hb]_{Cap} \right]$$

[5.22]

The $pO_2,in$ was specified in all simulations, while the $pO_2,out$ was calculated by performing a surface integral on the $pO_2$ at the capillary exit. The normalized $O_2$ delivery rate was calculated by dividing the flux of $O_2$ into the tissue space by the $O_2$ demand of the tissue space as defined by Equation 5.23.
\[ \text{O}_2 \text{ Demand} = \text{Vol}_{\text{iss}} \text{ V}_{\text{max}} \]  \hspace{1cm} [5.23]

The overall mass transfer coefficient of O\(_2\) \((k_o)\) was calculated by Equation 5.24, where the \(<pO_2>_{\text{cap}}\) is the volume averaged pO\(_2\) in the capillary and \(pO_{2,\text{wall}}\) is the surface averaged pO\(_2\) at the capillary wall.

\[ k_o = \frac{J_{O_2}}{\langle pO_2 \rangle_{\text{cap}} - pO_{2,\text{wall}}} \]  \hspace{1cm} [5.24]

5.3 Results & Discussion

5.3.1 Effect of hematocrit

The total concentration of Hb (contained in hRBCs and HBOCs) within the lumen was kept constant at 2200 \(\mu\text{M/s}\) for all oxygen transport simulations. Figure 5.2 focuses on how the replacement of hRBCs with PolyBvHb, the O\(_2\) carrier with the lowest affinity for O\(_2\) within this study, and 1-OGP40, the O\(_2\) carrier with the highest affinity for O\(_2\) in this study, changes the volume averaged pO\(_2\) in the capillary \(<pO_2>_{\text{cap}}\) for different degrees of blood loss and \(V_{\text{max}}\) at an inlet pO\(_2\) of 95 mmHg. In the simulations where PolyBvHb replaces the loss of hRBCs, increasing the amount of PolyBvHb in the capillary increases the average pO\(_2\) of the capillary with respect to a hRBC Hct of 0.448 (no blood loss). Simulations involving the replacement of hRBCs with 1-OGP40 show the opposite effect, where decreasing the degree of blood loss from 75% to 0% causes the average pO\(_2\) in the capillary to approach the average pO\(_2\) with respect to simulations where there is no loss of blood.
Figure 5.2. Comparison of the average capillary pO$_2$ between PolyBvHb, a low-affinity HBOC, and 1-OGP40, a high-affinity HBOC, at an inlet pO$_2$ of 95 mmHg and a constant total concentration of Hb of 2200 µM.
5.3.2 Oxygen saturation of hemoglobin-based oxygen carriers

Here, simulations involving equal volume mixtures of hRBCs (Hct = 0.224) and HBOCs with a total Hb concentration of 2200 μM are highlighted. These simulations were compared to those that involved no blood loss (a hRBC Hct of 0.448). Simulations involving the replacement of hRBCs with high-affinity HBOCs retained the most O2 at inlet pO2s less than or equal to 45 mmHg for all of the O2 consumption rates in this study. Figure 5.3 shows this effect at $V_{max}$ values of 26, 45, and 150 µM/s, where the hRBC Hct was kept constant at 0.224. Increasing the $V_{max}$ causes less O2 to be bound to the HBOCs and hRBCs. For each of the $V_{max}$ studied, simulations replacing hRBCs with low-affinity HBOCs retained less bound O2 compared to simulations that either did not replace hRBCs or replaced hRBCs with high-affinity O2 carriers. At all O2 consumption rates, the total Hb-bound O2 remained higher for the simulations that replaced hRBCs with high-affinity O2 carriers compared to simulations with no blood loss or that replaced hRBCs with low-affinity O2 carriers. At low inlet pO2s, hRBCs and low-affinity HBOCs have already lost much of their ability to bind to O2. However, at higher inlet pO2s of 65 and 95 mmHg, there is little difference in the total amount of O2 bound to Hb amongst the HBOCs, with the exception of PolyBvHb. PolyBvHb is unique amongst the other O2 carriers, such that at atmospheric pressure (pO2 ~ 150 mmHg) it has the lowest amount of Hb-bound O2 and does not approach complete saturation at atmospheric pressure.

Targeting O2 delivery to tissues possessing low pO2s is one possible application of high-affinity HBOCs, since these O2 carriers retain more O2 at low pO2s compared to hRBCs. Cancer cells are known to have lower inlet pO2s compared to normoxic cells [99]. Conventional treatment of cancer cells calls for the presence of more O2. Low-
Figure 5.3. Average total saturation of hRBCs and HBOCs in the capillary for each of the HBOCs studied as a function of the capillary inlet pO$_2$ at a $V_{\text{max}}$ of (A) 26 µM/s , (B) 45 µM/s, and (C) 150 µM/s.
affinity O₂ carriers and hRBCs would continue to deliver O₂ to unaffected regions (experiencing normoxic oxygenation), while high-affinity O₂ carriers would be able to target O₂ delivery to the affected regions having low pO₂s, since they release more Hb-bound O₂ at lower inlet pO₂s compared to hRBCs. Additionally, HBOCs are much smaller in size (diameter ~7-28 nm) compared to hRBCs (diameter ~ 8 um), and can more readily enter partially blocked blood vessels.

5.3.3 pO₂ profiles

The volume averaged pO₂ in each region was calculated for each HBOC (at a specific pO₂,in, Vₘₐₓ, and Hct). Varying the Hct of hRBCs while keeping the total Hb concentration constant did not affect the shape of the pO₂ profiles. As such, the discussion is limited to hRBCs that were replaced by an equal volume of HBOCs (Hct = 0.224). The volume averaged pO₂ profile, <pO₂>, was calculated in the three model regions, namely: capillary, membrane, and tissue space.

The inlet pO₂ was kept constant while varying the degree of O₂ consumption and the O₂ binding parameters of different HBOCs. For low capillary inlet pO₂s (pO₂,in ≤ 15 mmHg), the volume averaged pO₂ profiles of the capillary, membrane, and tissue space were highest in simulations examining high-affinity O₂ carriers. This effect can be seen within the capillary in Figure 5.4A. At a capillary inlet pO₂ of 15 mmHg, the volume averaged pO₂ profiles in each subdomain was highest in simulations where 2-CEFP20 (P₅₀ = 15.2 mmHg) replaced lost hRBCs. 2-CEFP20 retains more O₂ at low pO₂s compared to low-affinity O₂ carriers (PolyBvHb and αα-Hb) and hRBCs. Additionally, at this inlet pO₂, 2-CEFP20 exhibits the greatest change in its O₂ binding curve
Figure 5.4. Average pO$_2$ in the capillary as a function of the $V_{max}$ at a Hct of 0.224 for inlet pO$_2$s of (A) 15 mmHg and (B) 30 mmHg.
represented by \( \frac{\partial Y_{HBOC}}{\partial pO_2} \) (Figure 5.5). Moreover, Figure 5.4B shows that simulations involving no loss of hRBCs maintained the highest volume averaged pO\(_2\) profiles in the capillary at inlet pO\(_2\)s between 30 and 45 mmHg as a consequence of exhibiting the greatest change in \( \frac{\partial Y_{HBOC}}{\partial pO_2} \) amongst the O\(_2\) carriers in this study, which can be seen in Figure 5.5. At inlet pO\(_2\)s above 65 mmHg, simulations involving low-affinity O\(_2\) carriers exhibited the greatest change in \( \frac{\partial Y_{HBOC}}{\partial pO_2} \), which supports the fact that simulations containing \( \alpha \alpha \)Hb and PolyBvHb exhibit the highest pO\(_2\) profiles throughout the simulations. Figure 5.6 shows the pO\(_2\) profiles for each of the simulations replacing hRBCs with a HBOC at an inlet pO\(_2\) of 95 mmHg and \( V_{\text{max}} \) of 26, 45, and 150 \( \mu \)M/s. Simulations that replaced hRBCs with low-affinity O\(_2\) carriers exhibited higher pO\(_2\) profiles than simulations that replaced hRBCs with high-affinity O\(_2\) carriers. Simulations replacing hRBCs with low-affinity O\(_2\) carriers also show higher pO\(_2\) profiles compared to simulations having not replaced hRBCs. This can be readily observed by looking at the location of the four contour lines (pO\(_2\) = 10, 30, 50, and 70 mmHg) at each \( V_{\text{max}} \). At a \( V_{\text{max}} \) of 26 \( \mu \)M/s, all of the pO\(_2\) profiles show only one contour line (pO\(_2\) = 70 mmHg) indicating that these simulations maintain a pO\(_2\) greater than 50 mmHg throughout the KTCM. Simulations with low-affinity O\(_2\) carriers as a replacement for hRBCs show a greater amount of each region retaining a pO\(_2\) of at least 70 mmHg compared to simulations where no hRBCs have been replaced, and simulations that have replaced hRBCs with high-affinity O\(_2\) carriers. At a \( V_{\text{max}} \) of 45 \( \mu \)M/s all of the pO\(_2\) profiles show
Figure 5.5. The change in the O₂-HBOC equilibrium curve as a function of the pO₂ for each of the HBOCs in this study.
Figure 5.6. \( \text{pO}_2 \) profiles of simulations that were conducted with \( \text{O}_2 \) carriers replacing 50% of hRBCs at a constant inlet \( \text{pO}_2 \) of 95 mmHg at \( V_{\text{max}} \) of 26, 45, and 150 µM/s, respectively.
two contour lines (pO$_2$ = 50, 70 mmHg) indicating that these simulations maintain a pO$_2$ greater than 30 mmHg throughout the KTCM. Again, simulations with low-affinity O$_2$ carriers replacing hRBCs show increased pO$_2$s compared to simulations showing no loss of hRBCs. At a $V_{\text{max}}$ of 150 µM/s, all of the pO$_2$ profiles show all four contour lines indicating that at this high of a $V_{\text{max}}$, an anoxic region is unavoidable for a KTCM with these dimensions. Simulations with $\alpha\alpha$Hb replacing hRBCs showed increased pO$_2$ profiles compared to simulations showing no loss of hRBCs. Interestingly, PolyBvHb did not show an increase in the pO$_2$ profiles. This is most likely due to the fact that at atmospheric pressure, much less O$_2$ is bound to PolyBvHb than the other O$_2$ carriers within this study. Increased pO$_2$ profiles could be the trigger for the autoregulatory hypothesis. However, it is essential to also look at the O$_2$ flux out of the capillary as a possible trigger for this effect.

5.3.4 Oxygen flux out of the capillary

When the flux of O$_2$ entering the tissue space from the capillary meets the demand for O$_2$ in the tissue space, the normalized O$_2$ delivery rate is greater than one. The $V_{\text{max}}$ was varied between 5 and 150 µM/s and the ratio of the O$_2$ flux from the capillary to the O$_2$ demand in the tissue space was calculated. When the $V_{\text{max}}$ in the tissue space was 5 µM/s, all of the O$_2$ carriers deliver sufficient amounts of O$_2$ to meet the demand for O$_2$ by the skeletal muscle tissue. As the $V_{\text{max}}$ was increased from 5 to 150 µM/s, the O$_2$ carriers no longer meet the skeletal muscle’s O$_2$ demand at low inlet pO$_2$s. Figure 5.7 shows this effect in the case of no blood loss, where increasing $V_{\text{max}}$ resulted in simulations with higher inlet pO$_2$s not meeting the O$_2$ demands of the skeletal muscle tissue. In fact,
increasing $V_{max}$ resulted in the decreased ability of the O$_2$ carriers to supply sufficient O$_2$ to muscle tissue at increasing inlet pO$_2$s.

- High-affinity HBOCs

At low capillary inlet pO$_2$s ($pO_{2,in} < 20$ mmHg), simulations where high-affinity HBOCs replaced hRBCs delivered more O$_2$ to the tissue space compared to simulations that replaced hRBCs with low-affinity HBOCs or simulations where no hRBCs were replaced. Figure 5.8A shows that at a $V_{max}$ of 5 $\mu$M/s, all O$_2$ carriers delivered sufficient O$_2$ to meet the skeletal muscle’s O$_2$ demand at all inlet pO$_2$s as the normalized O$_2$ delivery rate is greater than one. As the $V_{max}$ increased to 26 and 45 $\mu$M/s, simulations where high-affinity O$_2$ carriers replaced hRBCS supplied skeletal muscle tissue with up
to twice the amount of O\(_2\) compared to simulations where no blood was replaced or low-affinity O\(_2\) carriers replaced hRBCs (Figure 5.8B). However, at higher inlet pO\(_2\)s, all O\(_2\) carriers delivered sufficient O\(_2\) to meet the demands of the skeletal muscle tissue. This in effect means that the trigger for the autoregulatory hypothesis is more likely to be increased pO\(_2\) profiles rather than an increase in O\(_2\) flux into the tissue space, since at normoxic pO\(_2\)s all of the O\(_2\) carriers within this study deliver similar amounts of O\(_2\) to the tissue space.

Simulations where 2-CEFP20 (\(P_{50} = 15.2\) mmHg) replaced hRBCs delivered the most O\(_2\) to skeletal muscle tissue at inlet pO\(_2\)s between 10 and 20 mmHg. This is probably due to the fact that at pO\(_2\)s between 4.4 and 15.2 mmHg, 2-CEFP20 releases a greater fraction of bound O\(_2\) from bHb at the highly sloped region of the O\(_2\)-HBOC equilibrium binding curve compared to the other HBOCs in this study (Figure 5.5). At a capillary inlet pO\(_2\) of 5 mmHg, simulations where 1-OGP40 replaced hRBCs delivered the most O\(_2\) to skeletal muscle tissue. This is supported by Figure 5.5 where at pO\(_2\)s between 1.4 and 4.4 mmHg, 1-OGP40 releases the greatest fraction of its bound O\(_2\) compared to the other HBOCs in this study. At pO\(_2\)s between 0 and 1.4 mmHg, 2-CEFP30 releases the greatest portion of its bound O\(_2\) within this limited pO\(_2\) window. However, the capillary inlet pO\(_2\) was not sufficiently lowered for the simulations in which hRBCs were replaced with 2-CEFP30 to show the greatest O\(_2\) flux into the tissue space.
Figure 5.8. Normalized O$_2$ flux out of the capillary for each HBOC studied at a Hct of 0.224 at $V_{max}$ of (A) 5 µM/s, (B) 45 µM/s, and (C) 150 µM/s, respectively.
• hRBCs

Simulations in which hRBCs were not replaced with HBOCs exhibit a smaller O\textsubscript{2} flux compared to simulations where hRBCs have been replaced with high-affinity HBOCs at inlet pO\textsubscript{2}s below 20 mmHg (Figure 5.8). At inlet pO\textsubscript{2}s below 20 mmHg, hRBCs (\(P_{50} = 28.6\) mmHg) have lost over half of its bound O\textsubscript{2}, which results in hRBCs having less O\textsubscript{2} to deliver to skeletal muscle tissue compared to high-affinity O\textsubscript{2} carriers. For inlet pO\textsubscript{2}s between 20 and 45 mmHg, simulations involving no blood loss delivered the most O\textsubscript{2} to skeletal muscle tissue compared to simulations where hRBCs were replaced with HBOCs. This is supported by Figure 5.5, since hRBCs release the most Hb-bound O\textsubscript{2} between pO\textsubscript{2}s of 15 and 35 mmHg of the O\textsubscript{2} carriers studied in this work.

• ααHb

For capillary inlet pO\textsubscript{2}s at or below 25 mmHg, simulations that replaced hRBCs with a high-affinity O\textsubscript{2} carrier showed an increase in O\textsubscript{2} delivery to skeletal muscle compared to simulations that replaced hRBCs with ααHb. For capillary inlet pO\textsubscript{2}s between 25 and 45 mmHg, simulations that replaced hRBCs with ααHb showed an increase in O\textsubscript{2} delivery to skeletal muscle tissue compared to simulations that replaced hRBCs with a high-affinity O\textsubscript{2} carrier.

• PolyBvHb

Lastly, for most of the range of capillary inlet pO\textsubscript{2}s studied, simulations with high-affinity O\textsubscript{2} carriers showed increased O\textsubscript{2} delivery to the tissue space compared to those with PolyBvHb. PolyBvHb has the lowest affinity for O\textsubscript{2} (\(P_{50} = 54.2\) mmHg) of all
the O\textsubscript{2} carriers in this study. At capillary inlet pO\textsubscript{2}s between 20 and 45 mmHg, simulations in which no blood was replaced or \(\alpha\)Hb replaced hRBCs showed the greatest amount of O\textsubscript{2} delivery to the tissue space. This is most likely due to the fact that a greater fraction of Hb-bound O\textsubscript{2} is released from the heme groups compared to PolyBvHb in this pO\textsubscript{2} window based on the O\textsubscript{2}-HBOC equilibrium curves of hRBCs and \(\alpha\)Hb. Based on the change in slope of the O\textsubscript{2}-PolyBvHb equilibrium curve, one would expect simulations replacing hRBCs with PolyBvHb to deliver more O\textsubscript{2} to muscle tissue at pO\textsubscript{2}s greater than 74 mmHg; however, hRBCs, \(\alpha\)Hb, and PolyBvHb each deliver sufficient O\textsubscript{2} to meet the demands set by the muscle tissue at capillary inlet pO\textsubscript{2}s greater than 45 mmHg at a \(V_{\text{max}}\) of 150 \(\mu\)M/s, the largest \(V_{\text{max}}\) in this study. Consequently, there was no scenario in this study where PolyBvHb exhibited the highest O\textsubscript{2} flux into the tissue space.

5.3.5 Possible applications of HBOCs with different oxygen affinities

HBOCs can have different therapeutic applications based on their O\textsubscript{2} affinity. For example, cancerous tissue have been observed to possess capillary inlet pO\textsubscript{2}s as low as 10 mmHg [99]. At this capillary inlet pO\textsubscript{2}, 2-CEFP20 most effectively delivers O\textsubscript{2} and would be the O\textsubscript{2} carrier of choice for use in oxygenating the cancerous growth, thus making it more susceptible to conventional treatment plans, such as radiation or chemotherapy. However, it is possible that some cancerous tissue may be fed by capillaries with capillary inlet pO\textsubscript{2}s as low as 5 mmHg. In this case, 1-OGP40 would be the best choice as an O\textsubscript{2} delivery agent to these cells, while at even lower inlet pO\textsubscript{2}s 2-CEFP30 could be the preferred O\textsubscript{2} carrier. Additionally, there is growing evidence that much of the O\textsubscript{2} released from the microcirculation system occurs in the arterioles (blood vessels...
with diameters ranging from 60 to 120 μm) [139]. Shibata et al. found that smaller arterioles (D < 60 μm) had average pO₂ tensions of 46.6 mmHg, while the larger arterioles (D = 80-120 μm) had average pO₂s of 74.6 mmHg [140]. So it is safe to assume that the entering capillary pO₂ will be between 46.6 mmHg and 95 mmHg (the exiting pO₂ of the plasma from the lungs). In the case of hemorrhagic shock, the inlet pO₂s of the capillaries could subsequently drop below these pO₂ levels due to significant loss of hRBCs. For these oxygenation scenarios, 2-CEFP20 releases the most O₂ in this pO₂ window, and was shown to best deliver O₂ to skeletal muscle tissue in the preceding simulations. Finally, O₂ consumption rates have been found to be an order of magnitude greater in 2-D cultures of hepatocytes [141] compared to in vivo conditions. If tissue cultures involving 2-D plates of skeletal muscle tissue also possess O₂ consumption rates an order of magnitude greater than the in vivo rates, then the \( V_{max} \) would be greater than 150 µM/s. In this case low-affinity O₂ carriers would best deliver O₂ to the surrounding tissue cells and should be chosen as the O₂ carrier of choice.

5.3.6 Overall mass transfer coefficient of oxygen

The overall O₂ mass transfer coefficient \( (k_o) \) was calculated as a function of the average saturation of hRBCs, \( <Y_{hRBC}> \), which is related to the average pO₂ in the capillary. The overall mass transfer coefficient is a ratio of the flux into the tissue space to the difference in the pO₂ tension between the volume averaged pO₂ in the capillary and the surface averaged pO₂ at the capillary wall. The difference in the pO₂ tension is what results in the influx of O₂ into the tissue space. The \( k_o \) of O₂ was evaluated over a wide range of \( V_{max}s \), Hcts, and O₂ affinities and plotted it against \( <Y_{hRBC}> \).
Figure 5.9. Overall mass transfer coefficient for hRBCs Hct = 0.448 ) at each Vmax studied.

The effect of varying $V_{\text{max}}$ on the $k_o$ of O$_2$ is shown in Figure 5.9 for simulations where no hRBCs were replaced. The x-axis consists of the $<Y_{\text{hRBC}}>$, which is proportional to the average pO$_2$ in the capillary. This normalizes the data so that it goes from zero to one. Changing $V_{\text{max}}$ did not affect the $k_o$ of O$_2$ as the overall shape of the $k_o$ remains constant at increasing $V_{\text{max}}$. As such, in studying the effect that O$_2$ affinity and Hct had on the $k_o$ of O$_2$ within the capillary, $V_{\text{max}}$ was kept constant at 5 µM/s.

Figure 5.10 shows the $k_o$s of O$_2$ for simulations where the hRBC Hct was 0.113 and the HBOCs had a corresponding Hct of 0.335. 1-OGP40 has the highest O$_2$ affinity of the O$_2$ carriers studied, and consequently it has the lowest $k_o$ of O$_2$ at high hRBC O$_2$ saturations. The high O$_2$ affinity causes the difference in the pO$_2$ tension between the volume averaged pO$_2$ in the capillary and the surface averaged pO$_2$ at the capillary wall
Figure 5.10. Overall O\textsubscript{2} mass transfer coefficient as a function of the Hb saturation in the hRBCs for each of the HBOCs studied at a \textit{Hct} of 0.113.

to increase, while the O\textsubscript{2} flux into the capillary remains relatively constant at high $<Y\text{\textsubscript{hRBC}}>$s. In general, it was found that decreasing the O\textsubscript{2} affinity of the HBOCs increased the $k_o$ of O\textsubscript{2} at high $<Y\text{\textsubscript{hRBC}}>$s. There were two exceptions to this rule. First, 1-OGP20 has a smaller affinity for O\textsubscript{2} than 2-CEFP30; however, the $k_o$ of O\textsubscript{2} for simulations where hRBCs were replaced by 1-OGP20 was lower than the $k_o$ of O\textsubscript{2} for simulations where hRBCs were replaced by 2-CEFP30 at $<Y\text{\textsubscript{hRBC}}>$ near one. At atmospheric pO\textsubscript{2}s (pO\textsubscript{2} ~ 150 mmHg), 2-CEFP30 maintains a higher $<Y\text{\textsubscript{HBOC}}>$ than 1-OGP20 resulting in more O\textsubscript{2} bound to 2-CEFP30. This results in simulations where hRBCs have been replaced by 2-CEFP30 having a larger $k_o$ of O\textsubscript{2} compared to simulations where hRBCs have been replaced by 1-OGP20 at $<Y\text{\textsubscript{hRBC}}>$ near one.
Secondly, PolyBvHb did not have the largest $k_o$ of O$_2$ despite having the smallest O$_2$ affinity. This is most probably due to the fact that PolyBvHb is much less saturated at atmospheric pO$_2$s compared to the other O$_2$ carriers in this study and does not level off at atmospheric pressure. Therefore, it does not need to release as much O$_2$ to reach its $P_{50}$ resulting in a decreased change in the slope of the O$_2$-HBOC equilibrium curve.

At low $<Y_{hRBC}>$s, the opposite effect is observed, where O$_2$ carriers with the highest affinity for O$_2$ have the highest $k_o$s of O$_2$. Here, high-affinity HBOCs have the highest change in their O$_2$-HBOC equilibrium curve resulting in decreased pO$_2$ gradients within the capillary. The one notable exception is that PolyBvHb does not have the lowest $k_o$ of O$_2$ at low $<Y_{hRBC}>$s. Of the O$_2$ carriers studied, only three were shown to be cooperative: ααHb, hRBC, and 2-CEFP20. This is evident by their sigmoidal shape at low pO$_2$s that can be seen in Figure 5.11. Since PolyBvHb is non cooperative, it has a greater change in its O$_2$-HBOC equilibrium curve at low $<Y_{hRBC}>$s compared to ααHb and hRBC. This results in smaller pO$_2$ gradients throughout the capillary and increased $k_o$s of O$_2$.

Since high-affinity HBOCs have higher $k_o$s of O$_2$ at low $<Y_{hRBC}>$s compared to hRBCs, they can target delivery of O$_2$ to tissues with low pO$_2$ tensions when hRBCs have begun to lose much of their effectiveness. An added benefit of using hRBCs in combination with high-affinity HBOCs is that at higher $<Y_{hRBC}>$s, hRBCs have higher $k_o$s of O$_2$ and can help in maintaining normoxic pO$_2$ tensions in the capillaries and surrounding tissues.
5.4 Concluding Remarks

The capillary model permits evaluation of more than one O\textsubscript{2} carrier in a modified KTCM using the finite element method. This allows for a more realistic study of O\textsubscript{2} transport in a capillary, since in practice an O\textsubscript{2} carrier would have to be mixed with hRBCs during a blood transfusion. The effects that varying the Hct, \( V_{\text{max}} \), capillary inlet pO\textsubscript{2}, and the O\textsubscript{2} affinity of the O\textsubscript{2} carrier had on the pO\textsubscript{2} distribution, O\textsubscript{2}, \( J_{O_2} \), and \( k_o \) of O\textsubscript{2} were studied in detail.

Low-affinity O\textsubscript{2} carriers exhibited vasoconstriction in clinical trials, and this has been theorized to be an autoregulatory response due to an increase in \( J_{O_2} \). However, no
evidence was found of an increase in the O\textsubscript{2} flux from the capillary for low-affinity HBOCs compared to hRBCs at normoxic pO\textsubscript{2}s. The simulations suggest that replacing hRBCs with low-affinity HBOCs causes an increase in pO\textsubscript{2} profiles throughout the KTCM compared to simulations with no blood loss. Based on this fact, the increase in pO\textsubscript{2} profiles could be the trigger for the autoregulatory response. This increase in pO\textsubscript{2} profiles is most likely due to the increased slope of the O\textsubscript{2}-HBOC equilibrium curve of the low-affinity O\textsubscript{2} carriers compared to hRBCs and high-affinity O\textsubscript{2} carriers at normoxic pO\textsubscript{2}s. At normoxic pO\textsubscript{2}s, all seven HBOCs in this study delivered sufficient O\textsubscript{2} to meet the demand of the skeletal muscle tissue specified in the model.

By studying the slope of the O\textsubscript{2}-HBOC equilibrium binding curve, different O\textsubscript{2} carriers were found to release the largest fraction of O\textsubscript{2} in a specific pO\textsubscript{2} window. To avoid an increase in pO\textsubscript{2} in the capillaries and the possible autoregulatory response that could be associated with it, HBOCs should be selected to have $P_{50}$s less than that of hRBCs. Additionally, tissues at a particular capillary inlet pO\textsubscript{2} can be targeted for O\textsubscript{2} delivery by choosing an O\textsubscript{2} carrier with the greatest change in the O\textsubscript{2}-HBOC at or just below the specified inlet pO\textsubscript{2} in capillaries that are experiencing blood loss.
6.1 Conclusions

First generation acellular HBOCs for use in transfusion applications were designed to have low affinities for \( \text{O}_2 \). Hemopure (Biopure) and PolyHeme (Northfield Laboratories) are two low-affinity HBOCs that have reached Phase III clinical trials. However, both of these companies have run into problems at this stage of the clinical phase process. Hemopure is no longer approved for future clinical studies by the FDA due to an increase in the number of cases of pneumonia, stroke, heart failure, cardiac arrest, and ventricular fibrillation [45]. In the latest clinical trials for PolyHeme, Northfield Laboratories reported that trauma patients infused with PolyHeme experienced 13.2% mortality rates versus 9.6% mortality rates for trauma patients infused with conventional saline and hRBC treatment [47].

In addition to the complications seen by low-affinity acellular HBOCs in Phase III clinical trials, Winslow’s group has shown that low-affinity acellular HBOCs cause vasoconstriction and systemic hypertension post-infusion. Interestingly, high-affinity acellular HBOCs did not cause this effect. Second generation acellular HBOCs should be developed with high affinities for \( \text{O}_2 \). We have identified two cross-linking agents that yield high-affinity HBOCs when cross-linked with stroma-free Hb. Additionally, through an \textit{a priori} model, we have identified that increased pO\textsubscript{2} tensions within the
vascular network rather than increased O$_2$ delivery to the tissue space could be the trigger for the autoregulatory theory. Further purification and *in vitro* complement activation studies should be performed before testing these novel high-affinity HBOCs in animal studies.

6.2 Future Studies

6.2.1 Isolation and pasteurization of polymerized HBOCs

The polymerized bHBOCs within these studies should be isolated from the unmodified bHb via gel filtration. Monodisperse fractions should be collected and their O$_2$ binding properties, metHb levels, and size distributions should be reevaluated. Also, bHb should be reacted with the cross-linking agents for longer time periods than the two hours within this study to decrease the amount of unmodified bHb present. Additionally, long term stability studies should be undertaken to determine to what degree the metHb levels and size distributions of the polymerized bHBOCs change over time.

After the bHBOCs have been separated from unmodified bHb, they should be pasteurized. Sakai et al. showed that bHb was able to be pasteurized for greater than 10 hours at 60°C [142]. Additionally, bHb was found to be more thermally stable compared to hHb which bodes well for pasteurization of bHBOCs. Once the final bHBOC product has been isolated from unmodified bHb and pasteurized, it can then be applied in tissue culture applications and evaluated for complement activation prior to evaluation in animal studies.
6.2.2 Tissue culture

HBOCs also have applications within tissue cell cultures as a means to supplement O$_2$ within a bioreactor. Within the Palmer research group, Jesse Sullivan [101, 102] and Jason Gordon [143, 144] have studied the delivery of O$_2$ to hepatic cells within a hollow fiber bioreactor in detail. Within these studies, hepatic cells are cultured within the extracellular space of a hollow fiber bioreactor. O$_2$ delivery is enhanced through the supplementation of RBCs or HBOCs within the lumen. Sullivan found that O$_2$ delivery is most effective at inlet pO$_2$s near the $P_{50}$ of the supplemented O$_2$ carrier [102]. For hepatic cells, an elevated pO$_2$ (> 95 mmHg) has been found to best promote in vivo like conditions for hepatic cell growth. As such, low-affinity O$_2$ carriers (high $P_{50}$) should be developed to have increased $P_{50}$s. Interestingly, it has been found that embryonic stem cells from the peripheral and central nervous system proliferate and differentiate best at low pO$_2$ levels [145, 146]. The high-affinity bHBOCs within this study could be candidates to supplement O$_2$ delivery in stem cells cultured within a hollow fiber bioreactor.

6.2.3 Complement activation

The complement system is comprised of over 30 plasmatic components, which result from the cleavage of complement proteins [147]. Upon activation, this system undergoes a series of reactions that leads to the killing of susceptible bacteria and protozoa, the lysis of erythrocytes, the promotion of immune-adherence reactions, and the onset of phagocytosis [148]. O$_2$ carriers to be transfused into the bloodstream should be designed to limit complement activation. The extent of complement activation can be determined in vitro by measuring the amount of complement required to lyze 50% of
sheep RBCs ($CH_{50}$) in the presence of HBOCs. Determining the amount of complement activation in vitro is quicker, more economical, and lessens the amount of animal suffering compared to in vivo complement activation protocols.

An in vitro assay to test complement activation has been previously developed that relies on the ability of sheep RBCs to be sensitized by rabbit antibodies directed against sheep RBCs [147]. In this assay, human serum was added to the sensitized sheep RBCs to determine the amount of lysis that occurs after incubation for 45 minutes at 37°C, body temperature. The determination of the amount of RBCs lyzed was achieved through cell counts, or by measuring the optical density of the solution in a UV/Vis spectrophotometer at 541 nm [147]. To determine the degree that HBOCs activate the complement system, HBOCs will be added to human serum before allowing it to react with the sensitized sheep RBCs. After incubation for 45 minutes, the $CH_{50}$ of the solutions containing HBOCs would be measured and compared to the control $CH_{50}$. If there is a left shift in this value, the complement system has been activated, which would suggest immunogenic response in a living organism. Only those HBOCs that produce little to no left shift of the $CH_{50}$ will be used for animal studies. A possible solution in the event that the HBOCs cause a significant amount of complement activation is to conjugate the HBOCs with PEG [55, 149, 150] after polymerization with cross-linking agents.

6.2.4 Animal studies

Once candidate high-affinity bHBOCs are isolated and shown to limit the activation of the complement system, the pharmacokinetic properties should be studied in Sprague-Dawley rats [149, 151]. The biodistribution of the O$_2$ carriers will be measured
by sacrificing the rats at various time-points, and determining the distribution of O\textsubscript{2} carriers in the major rat organs [149]. Rats will also be subjected to varying degrees of hemorrhagic shock and resuscitated with an equal volume of O\textsubscript{2} carrier [152]. Additionally, the mean blood pressure and heart rate of rats will be monitored during these experiments to ensure that vasoconstriction does not develop following transfusion. The \textit{a priori} model that was discussed in Chapter 5 should be modified to model O\textsubscript{2} transport within Sprague-Dawley rats. This would allow for comparison between the \textit{a priori} pO\textsubscript{2} profiles and experimental pO\textsubscript{2} profiles.

To determine some of the pharmacokinetic parameters, ten percent of a rat’s plasma volume will be bled from the rat’s vein tail and replaced with an equal volume of HBOCs. Over the course of six days post-injection, 100 \textmu L samples of blood will be bled from the rat’s tail vein at scheduled time intervals under the supervision of an animal safety expert. The amount of HBOC present in the blood can be determined in one of three ways. Before injection, the HBOCs can be labeled with a fluorescent marker such as fluorescein isothiocyanate, and evaluated with flow cytometry. The HBOCs can also be radioactively labeled before injection with an isotope such as iodine monochloride [153]. The amount of HBOCs in the plasma can also be determined using UV/Vis spectrophotometry [151]. In this method, the HBOCs do not need to be labeled before injection. Three optical absorbances will be measured at 570, 576, and 590 nm to calculate a corrected absorbance, correlating to the amount of acellular Hb in the plasma. The retention time of the HBOCs in the blood stream can then be calculated.

The blood pressures and heart rates will be monitored to ensure that the HBOCs do not cause systemic hypertension. Both of these parameters have been continually
measured in previous studies via the placement of a catheter in the femoral artery [154]. Within this catheter, a pressure transducer is able to continually measure both the heart rate and arterial blood pressure. The pO$_2$ profiles can also be measured by measuring the phosphorescence emission lifetime in order to calculate pO$_2$s in vivo [155]. Within this method, rats are anesthetized while a window of their skeletal muscle is exposed. A membrane impermeable to O$_2$ is then placed over this region so that it can be visually monitored. An O$_2$ probe, which is composed of palladium meso-tetra [4-carboxyphenyl]porphine, can then be injected into multiple sites within the vascular network to determine the pO$_2$s in real time. This technique has also been used to determine that the critical pO$_2$ within skeletal muscle of rats is between 2.4 and 2.9 mmHg [156]. The pO$_2$ profiles of the vascular network can then be compared to an a priori model similar to the one discussed in Chapter 5. After six days, the rats will be sacrificed and their organs collected to determine HBOC accumulation in the tissues. The polymerized HBOCs will be considered effective O$_2$ carriers if they are able to exhibit long half-lives, have little accumulation in the major rat organs, show no significant increase in the mean arterial blood pressure, and do not have any pO$_2$ measurements smaller than the critical pO$_2$.

6.3 Future of Oxygen Carriers

First generation acellular HBOCs have stalled in progress in clinical trials. The time window for the development of an acellular HBOC for traditional transfusion applications is shrinking as stem cell research is advancing. Indeed, Olivier et al. have recently published a procedure for the large-scale production of RBCs from embryonic stem cells [157]. They were able to produce 5 million mature RBCs from 50,000
embryonic stem cells. Nonetheless acellular HBOCs still have many applications available to them.

Acellular HBOCs can be applied to tissue culture applications to better oxygenate the cell cultures. Tissue cultures have been found to prefer different pO₂ levels. Since acellular HBOCs have been found to best deliver O₂ at their P₅₀, they can be designed to have O₂ affinities that would best deliver O₂ to a specific cell culture application. High-affinity HBOCs are designed to release O₂ at low pO₂s. Considering that tissues surrounding cancer cells have been found to have low inlet pO₂s (~ 15 mmHg) and that traditional cancer treatments require the presence of O₂, high-affinity HBOCs might be able to aid in this treatment. While Northfield Laboratories’ most recent clinical trial of PolyHeme did show increased mortality rates for patients transfused with solely PolyHeme (13.2%) compared to traditional saline and RBC transfusion protocols (9.6%), PolyHeme could still have applications as an O₂ carrier on the battlefield. Unlike RBCs, PolyHeme can be transported to the front lines where it can be readily transfused into soldiers. Another advantage of an O₂ carrier, such as PolyHeme, over RBCs is the fact that troops would not have to give up their position to receive a transfusion application. In this setting, an increase in mortality rates of wounded soldiers may be an acceptable cost of war compared to the cost of giving up the position of a squadron.

In closing, it is my opinion that we are still years away from seeing artificial blood being used for transfusion applications. By this time, stem cell research may produce the answers to the blood shortages seen in the U.S. However, there are many other possible applications for O₂ carriers that should be explored.


