Targeted Delivery of Gaseous Ligands (CO and NO) for the Treatment of Ischemia Reperfusion Injury

DISSERTATION

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By

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Hemorrhagic shock is a clinical condition characterized by the sudden loss of intravascular blood volume and reduction in organ/tissue perfusion due to trauma. This in turn induces tissue ischemia, which is characterized by limited oxygen (O$_2$) transport to tissues coupled with an accumulation of tissue excretory products eventually leading to cell death. To treat hemorrhagic shock, clinicians normally stop the bleeding and transfuse the patient with either red blood cells (RBCs) or plasma expanders in order to restore the lost blood volume. Hence, re-establishment of blood flow and restoration of tissue oxygenation is known as reperfusion. This abrupt change in tissue oxygenation, though considered beneficial, can paradoxically induce cell apoptosis, tissue inflammation, infarct formation and has been shown to elicit multiple organ dysfunctions by activating cytotoxic injury processes such as reactive O$_2$ species (ROS) generation, neutrophil-endothelium interactions, and hypercontracture (i.e. intracellular overloading of cardiomyocytes with Ca$^{2+}$ ions). These collective set of side-effects that occur during the ischemic and reperfusion phases is classified as ischemia reperfusion injury (IRI) and is a major obstacle towards successful treatment of hemorrhagic shock. In 2010, IRIs affected 2.4 million patients in the United States (US), and was responsible for ca. 15.3% of total deaths in the US. World Health Organization (WHO) predicts that by 2020, IRIs will be the leading cause of mortality in the world. Therefore, it is vital to develop novel therapeutics to protect the cardiovascular system during acute ischemic and post-ischemic phases that occur during resuscitation from hemorrhagic shock.
Neutrophils and their interactions with adhesion molecules play a major role in exacerbating tissue injury post reperfusion. Neutrophils, activated by elevated ROS levels post reperfusion, infiltrate into the extravascular compartment by interacting directly with integrins and damage the endothelium by secreting cytotoxic molecules such as proteases, ROS, hypochlorous acid, arachidonic metabolites, and cytokines. The ROS and cytokines released lead to cell apoptosis and inflammation. The damaged endothelium may further aggravate tissue injury by the suppression of vasoactive molecules such as nitric oxide (NO), adenosine, and endothelin; which can disrupt homeostasis of the coronary vasculature and lead to reduced tissue perfusion. In light of the anti-apoptotic, anti-inflammatory and vasoactive properties of carbon monoxide (CO) and NO, their presence in the systemic circulation could inhibit the release of cytotoxic molecules during reperfusion.

Previously, inhalation-based delivery of NO to ischemic tissues has been successfully used to treat IRI. However, this approach increases the methemoglobin (metHb) level in the blood and delivers NO systemically throughout the body. Another treatment strategy involves transfusion of CO saturated RBCs to prevent sudden oxygenation of ischemic tissues and reduce IRI. Therefore, RBCs seem like a logical carrier for \textit{in vivo} delivery of NO and CO. However, in order to circumvent potential issues associated with RBC transfusion (i.e. short storage shelf life (42 days), limited supply and possibility of transmission of yet to be identified pathogens), hemoglobin (Hb)-based O\textsubscript{2} carriers (HBOCs) have been used to deliver exogenous CO to ischemic tissues in rat models. Further, attractive properties of HBOCs such as low viscosity and smaller size are expected to improve microcirculation upon reperfusion. The promising attributes of HBOCs motivated us to investigate these RBC substitutes as potential carriers of beneficial gaseous molecules to ischemic tissues.
In this study, we hypothesize that HBOCs can be used to deliver potent gaseous ligands such as CO and NO to ischemic tissues in order to treat IRIs encountered during resuscitation from hemorrhagic shock. Initially, the ligand loaded HBOCs are expected to supply beneficial gaseous molecules to ischemic tissues in order to mitigate IRI. Transfusion of these particles will also help in restoring blood volume and shear stress, thereby promoting increased endothelial NO synthesis. After the beneficial gaseous ligands dissociate from the ligand carriers, the particles are expected to store and transport O\textsubscript{2}. Thus, the Hb-based ligand carriers will perform multiple functions at different stages of the proposed treatment.

This dissertation presents economically feasible, large-scale methods for the production of Hb-based ligand carriers. The methods described herein have been developed in our lab and can be easily scaled-up to produce industrial sized batches. The dissertation also outlines detailed characterization techniques used to establish the efficacy of these ligand carriers. These carriers were characterized in terms of their O\textsubscript{2}-equilibria, size, Hb content, interaction with physiologically important gaseous ligands, and metHb levels. In addition, this manuscript describes the methods involved in loading therapeutic NO and CO gas onto these Hb-based carriers via degassing and gassing techniques developed in our lab.

All in all, the novel therapeutic proposed in this study can be an effective and economical alternative for current IRI treatment strategies.
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CHAPTER 1  Introduction

1.1  Background

1.1.1 Oxygen Homeostasis

Oxygen (O$_2$) plays a pivotal role in cellular respiration in aerobic organisms [1-4]. Cellular respiration can be described as a “catabolic pathway to generate energy as adenosine triphosphate (ATP) from consumed food” [5, 6]. Cellular respiration involves three main pathways namely, glycolysis, the citric acid cycle (Kreb’s cycle), and electron transport [6]. O$_2$ is the eventual electron acceptor during the electron transport cycle; during which the passage of “high energy” electrons to oxygen eventually leads to the production of ATP [5, 6]. This process, known as oxidative phosphorylation, is also mediated by enzymes such as ATP synthase which convert adenosine diphosphate (ADP) and inorganic phosphate to ATP [5]. Oxidative phosphorylation eventuates in the inner membrane (cristae) of the mitochondria [7]. ATP is the “energy currency” of cells [7, 8], and is consumed during various energy intensive cellular functions such as signal transduction [9-14], transport of nutrients and molecules across cellular membranes and barriers [15-17], intracellular synthesis of biomolecules (e.g. DNA and RNA) [18-22], cellular movement (e.g. movement of chromosomes and flagella), and muscle contraction [23-27]. However, the beneficial role of O$_2$ in cellular respiration and subsequent ATP synthesis is only part of the story. Patho-physiological effects of increases in O$_2$ tension are also well documented in the literature [28-41]. It has been
shown that breathing hyperbaric (O$_2$ at pressures higher than 1 atm) O$_2$ has deleterious effects on the central nervous system [38-42] and also induces pulmonary toxicity [28, 29, 31-36]. Additionally, increases in O$_2$ tension have been associated with tissue inflammation [28, 43]. In vivo, O$_2$ is known to trigger reactive oxygen species (ROS) formation. ROS, such as superoxide ion, hydroxyl radical, hydrogen peroxide, and peroxynitrite possess one or more unpaired electrons are extremely unstable [4, 28]. O$_2$ molecules accept free electrons to form reactive superoxide radicals (O$_2^-$). Superoxide ions can generate other harmful ROS such as the hydroxyl radical and hydrogen peroxide after accepting more free electrons in alkaline and acidic conditions respectively (Figure 1.1) [28, 44-46]. Furthermore, superoxide reacts with nitric oxide (NO) to yield highly toxic peroxynitrite (ONOO$^-$) (Figure 1.1) [28, 44-46]. ROS are known to cause severe oxidative damages at both cellular and systemic levels due to their volatile nature [28]. ROS can oxidize cellular lipids, proteins and nucleic acids, and can eventually lead to cell death via apoptotic and necrotic pathways [46, 47]. Further, at high concentrations, O$_2$ free radicals overpower the inherent detox systems of the body causing irreparable oxidative damages [28]. This type of toxicity is referred to as oxidative tissue toxicity.

Therefore, though O$_2$ is quintessential in supporting life in aerobic organisms, the detrimental effects caused by high O$_2$ levels in the tissues cannot be neglected [4]. All in all, these contrasting traits of O$_2$ demand a strict regulation of O$_2$ homeostasis within the body [4]. Extensive research has been conducted to understand the correlations between the molecular, cellular and physiological factors involved in maintaining in vivo O$_2$ balance [4, 30, 43, 48, 49]. It is well known that blood, a “specialized bodily fluid”, is essentially responsible for delivering and transporting O$_2$ to cells and tissues [50-52]. Therefore, a thorough comprehension of the functionalities of blood and its components
is necessary to fully understand the issues associated with intracellular maintenance of O$_2$ homeostasis [4].

Figure 1.1: Schematic showing reactions involved in ROS generation. Superoxide and hydrogen peroxide act as substrates to yield more toxic hydroxyl radical and peroxynitrite. These free radicals oxidize intracellular lipids, proteins and nucleic acids causing extensive oxidative tissue toxicity. Reprinted from Paediatric Respiratory Reviews, 15(2), Louise Thomson & James Paton, Oxygen Toxicity, p. 120-123, Copyright (2014), with permission from Elsevier [28].

1.1.2 Blood

1.1.2.1 Components of Blood

Blood is a non-Newtonian fluid comprising of blood cells and plasma [52, 53]. Plasma is the liquid constituent of blood, and it transports blood cells, nutrients, and water to the tissues [53]. Plasma accounts for about 55% of total blood volume [52]. It is
essentially water (~92%) containing dissolved sugars, proteins, carbon dioxide, hormones, and minerals [52, 53]. There are three major types of blood cells, namely, red blood cells (RBCs), white blood cells (WBCs), and platelets.

WBCs or leukocytes are the blood cells representing the immune system of the body. They are classified into two main groups: granulocytes and mononuclear cells [54]. Granulocytes have granules or lysozymes in their cytoplasm necessary for digesting and degrading phagocytosed foreign bodies such as bacteria and other pathogens [54]. Granulocytes are further classified into neutrophils, eosinophils, and basophils based on their staining characteristics [54, 55]. Neutrophils are the most abundant granulocytes, and they stain with neutral dyes; whereas eosinophils and basophils stain with acidic and basic dyes respectively [54, 55]. Mononuclear cells or agranulocytes are characterized by their un-segmented nuclei and the absence of granules in their cytoplasm [54]. They are classified into two main subclasses, namely, lymphocytes and monocytes [54, 55]. Lymphocytes are further classified into thymus derived or T-lymphocytes and bone marrow derived or B-lymphocytes. T-lymphocytes provide cellular immunity from viruses and fungi and are also responsible for “rejections of foreign tissue grafts” [54]. B-lymphocytes on the other hand are accountable for antibody mediated humoral immunity [54]. Monocytes, also known as macrophages, are primarily responsible for phagocytosis and production of antigens in chronic conditions of inflammation and injury [54]. WBCs account for about 1% of total blood volume and there are approximately $4 \times 10^{10}$ WBCs per liter of blood [52].

Platelets or thrombocytes are the coagulating agents of blood [52]. Platelets release fibrinogen which gets converted to fibrin [52]. Crosslinked fibrin ultimately results in clot or thrombus formation [56]. Blood clotting is an inherent defense mechanism to prevent excessive bleeding of injured tissues and blood vessels [56].
RBCs or erythrocytes are the most abundant cells in blood [50, 52]. RBCs are produced in the bone marrow at a rate of ~2.4 million cells per second [57]. On an average these blood cells circulate in the body for a period of 100 – 120 days before being captured by macrophages and eventually degraded in the liver and in spleen [57]. RBCs are donut-shaped cells predominantly composed of hemoglobin (Hb), an iron-containing protein. Hb accounts for about 97% of RBC’s dry weight [52]. Hb molecules bind O₂ and hence are directly responsible for the O₂ carrying abilities of RBCs. Intra-RBC Hb concentration (mean corpuscular Hb concentration, MCHC) is around 32 – 36 g/dL and approximately 27 – 31 pg Hb (mean corpuscular Hb, MCH) is contained in each RBC [58].

1.1.2.2 Introduction to Hb

The Hb tetramer is constituted of 4 subunits (polypeptide chains): two α chains and two β chains [59-63]. The α and β subunits are made up of 141 and 146 amino acids respectively [60, 61, 64-67]. The Hb tetramer is essentially a dimer of αβ dimers [68]. The α₁β₁ and α₂β₂ dimerizations are stabilized by 17 – 19 hydrogen (H) bonds between 35 or so amino acids which come in contact for each of these dimers [61-63, 69]. Finally, the tetramer results from the stabilization these dimers by salt bridges between positively charged nitrogen atoms and negatively charged oxygen atoms [59-63, 69, 70]. In order to preserve the salt bridges and avoid dissociation of the tetramer, it is critical to suspend Hb in buffers having high salt concentrations (>5mM) upon purification [59-61]. Figure 1.2A shows schematic structure of human hemoglobin bound to 4 O₂ molecules. The α and β subunits are shown as gray and scarlet ribbons respectively. The heme molecules are shown in green; and the O₂ molecules are represented in blue. The structure was created with DeepView v4.1 S/W (Swiss-
PDBViewer, developed by Guex et al.) using data and coordinates obtained from the protein data bank (PDB) file: 1GZX (PDB ID) developed by Research Collaboratory for Structural Bioinformatics (RCSB) [71-73]. The 3D coordinates in the PDB file 1GZX were determined from x-crystallography studies performed by Paoli et al. [74].

Each Hb subunit fold into 8 helical secondary structures referred to as Helix A through Helix H [61-63, 70]. These helices further fold into globular tertiary structures [61-63, 70]. Housed in a hydrophobic pocket formed by Helix E and Helix F, each Hb subunit holds an iron (Fe$^{2+}$) containing protoporphyrin IX molecule known as the heme molecule [59-63, 70]. Therefore, there are 4 heme molecules per Hb molecule and thus each Hb molecule can bind to a maximum of 4 O$_2$ or other gas molecules [51].

Porphyrrins are aromatic macrocycles incorporating 4 pyrrole rings interlinked by methine (= CH – ) bridges [59-63]. Protoporphyrins are porphyrin macrocycles substituted with 4 methyl, 2 vinyl, and 2 propionate side chains [50, 59]. The iron atom (Fe$^{2+}$ state) of the heme molecule sits at the center of the protoporphyrin ring and is covalently bound to the 4 nitrogen (N) atoms of the 4 pyrrole subunits [60-63, 70]. Further, the iron atom is connected via coordination bonds to histidine residues on F helices of the polypeptide chains [60-63, 70]. In α subunits, the iron forms coordination bonds with histidine 87 side chain; while, coordination linkages occur between iron and histidine 92 in β chains [60-63]. Figure 1.2B shows the chemical structure of a heme molecule. The figure was downloaded from the RCSB-PDB website; it is available as a ligand for structure ID: 1GZX [71]. Convenient niche afforded by the hydrophobic pockets (also known as heme pockets) mitigates oxidation of the iron atoms in heme molecules from Fe$^{2+}$ state to Fe$^{3+}$ state; thereby reducing methemoglobin (metHb) formation [59-61]. MethHb or Hb with its iron atoms in oxidized Fe$^{3+}$ state is unable to bind O$_2$ and is, therefore, undesirable [59-61].
Figure 1.2: (A) A schematic of human oxyhemoglobin. The α and β subunits are shown as gray and scarlet ribbons respectively. The heme molecules are shown in green; and the O$_2$ molecules are represented in blue. The structure was created using DeepView v4.1 (Swiss-PDBViewer) S/W (developed by Guex et al.) using data and coordinates obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (PDB) file: 1GZX (PDB ID). The 3D coordinates in the PDB file 1GZX were determined from x-crystallography studies performed by Paoli et al. *Crystal structure of T state haemoglobin with oxygen bound at all four haems.* J. Mol. Biol. 256(4) pp 775-92 (Mar 8, 1996). (B) Chemical structure of heme group reported as ligands in PDB 1GZX.
1.1.2.3 Transport and delivery of physiological gases

As noted earlier, blood is responsible for delivering nutrients and O\textsubscript{2} to cells and tissues. Additionally, cells and tissues also use blood to get rid of metabolic wastes such as carbon dioxide (CO\textsubscript{2}), lactate, nicotinamide adenine dinucleotide (NADH), long chain acyl-coenzyme A, K\textsuperscript{+} and H\textsuperscript{+} ions [75-80]. In this section, we will review the mechanisms involved in the transport and delivery of important gases (O\textsubscript{2} and CO\textsubscript{2}) by blood via Hb encapsulated within the RBCs.

The composition of air (78% N\textsubscript{2}, 21% O\textsubscript{2} and 1% trace gases) is well-established [4]. Inhaled air having 21% O\textsubscript{2} (pO\textsubscript{2} – 160 mm Hg) comes in contact with the blood circulation in the alveoli of the lungs [4]. The gas exchange is diffusion driven and occurs across the membranes of the alveolar sacs. In the alveoli, CO\textsubscript{2} is offloaded from the Hb molecules encapsulated within the RBCs and O\textsubscript{2} is loaded instead [4, 50]. Interestingly, the pO\textsubscript{2} inside the lungs has been observed to be ~100 mm Hg which is comparatively lesser than the pO\textsubscript{2} levels of inhaled air [4, 62, 63]. However, this anomaly can be explained by the significant contributions made to the total pressure by the high concentrations of CO\textsubscript{2} and water vapor in the lungs; thereby causing the pO\textsubscript{2} to drop [4]. The pO\textsubscript{2} of venous blood is ~35 mm Hg [62, 63, 81]. Therefore, in spite of the lower pO\textsubscript{2} levels (as compared to inhaled air) in lungs, about 98% of Hb in venous blood is saturated with O\textsubscript{2} in vivo [50, 59]. Also, it has been shown that Hb molecules deliver approximately 66% of the transported O\textsubscript{2} to deoxygenated tissues where pO\textsubscript{2} is much lower at ~20 mm Hg [50, 59]. It has been further reported that Hb molecules are able to successfully offload bulk of the bound O\textsubscript{2} molecules as a result of the relative differences in pO\textsubscript{2} levels in arterial blood (pO\textsubscript{2} – 95 mm Hg) and deoxygenated tissues [50, 59]. Interestingly, not all of the O\textsubscript{2} picked up by the Hb molecules in the alveoli are used for cell respiration and ATP generation [4]. A part of the Hb bound O\textsubscript{2} is committed to a
different protein called myoglobin (Mb) in muscle tissues for storage and future use [4]. Mb is also a heme based protein and has a structure similar to the β subunit of Hb [62, 63, 69, 82, 83]. However, Mb is a much simpler molecule and consists of only one polypeptide chain and a single heme molecule [62, 63, 69, 82, 83].

As noted earlier 4 O₂ molecules can bind to one Hb molecule. The sigmoidal shape of Hb-O₂ equilibrium plots suggests that O₂ binds to Hb using a positive cooperative approach [50, 59, 62, 63, 84]. In other words, though deoxyHb (tense or T-State) is somewhat disinclined to accept the first O₂ molecule, binding of the first O₂ molecule leads to structural modifications to the overall protein which result in comparatively higher O₂ affinities of the remaining heme moieties [84]. Conversely, release of the first O₂ molecule from the heme of an oxyHb (relaxed or R-state) molecule causes the remaining heme molecules to also lose their O₂ molecules more easily [62, 63, 84]. This behavior of Hb with regards to O₂ binding is known as cooperativity. The tangent to the maximum slope of the Hb-O₂ equilibrium curve is referred to as Hill coefficient (n) and is a measure of cooperativity (sigmoidal shape) of Hb [50, 62, 63]. A higher cooperativity indicates that a larger fraction of the O₂ bound to Hb can be released with ease [62, 63]. Another important aspect while describing O₂ binding to Hb is the partial pressure of O₂ at which 50% Hb is fully saturated [50, 62, 63]. This concentration of O₂ is known as P₅₀ and is a measure of O₂ affinity [50, 62, 63]. O₂ affinity depends on various factors such as protons (also expressed as [H⁺] or pH), CO₂ concentration, Cl⁻ concentration, 2, 3-diphosphoglycerate (2, 3- DPG), and temperature [62, 63]. An increase in temperature or the concentrations of any of these species lowers O₂ affinities but increases cooperativities [62, 63].

It has been observed that a drop in pH results in lowering O₂ affinities [62, 63]. It is well known that metabolic activities of the cell produce lactic acid and carbonic acid
and in turn these acids release H\(^+\) ions or protons. Perutz reported that these protons “right shift the Hb-O\(_2\) equilibrium curve and lower O\(_2\) affinity of Hb” by favouring the transmission of R-state to T-state Hb. Perutz reasoned that “protons increase the number of positively charged N atoms on Hb subunits and thereby strengthen or build new salt bridges to stabilize the T or deoxyHb structure”. Therefore, when oxygenated RBCs carried by arterial blood reach deoxygenated tissues, an excess of acidic intracellular metabolic wastes result in offloading of O\(_2\) from Hb via proton donation.

Bohr reported that Hb accepts a proton for every 2 O\(_2\) molecules released; and conversely, releases a proton upon binding 2 O\(_2\) molecules [85-87]. According to Perutz, “this reciprocal behavior, better known as the Bohr effect, forms the basis of the underlying mechanisms involved in the transport of CO\(_2\) from tissues to the lungs” [62, 63]. CO\(_2\) is released as a product of cellular respiration [59, 61-63, 69]. Solubility of CO\(_2\) in plasma is negligible and therefore CO\(_2\) cannot be transported to the lungs as a dissolved gas [62, 63]. However, CO\(_2\) reacts with water to form soluble bicarbonate ions (HCO\(_3^-\)) and protons according to the following reversible reaction [62, 63]:

\[
CO_2 + H_2O \leftrightarrow HCO_3^- + H^+
\]

In the tissues, oxygenated Hbs inside the RBCs of arterial blood tend to accept protons and adopt a deoxy conformation [62, 63]. Removal of protons (H\(^+\) ions) from the system favors the forward reaction and therefore augments the conversion of CO\(_2\) to HCO\(_3^-\) [62, 63]. The HCO\(_3^-\) ions are much more soluble and can be transported easily by plasma [62, 63]. In the lungs, due to elevated pO\(_2\) levels, O\(_2\) binds to Hb and thereby releases protons as per Bohr’s effect [4, 62, 63]. This favors the backward reaction and forces the conversion of HCO\(_3^-\) to CO\(_2\) [4, 62, 63, 84]. The negligible solubility of CO\(_2\) in plasma
causes CO$_2$ to come out of the solution phase and exist as gas [4, 62, 63]. Gaseous CO$_2$ is then exhaled to the atmosphere [4, 62, 63].

Previous discussions in this chapter underline the importance of O$_2$ as a gaseous molecule which supports life. Inhaled O$_2$ molecules are picked up in the lungs by deoxygenated RBCs suspended in venous blood. RBCs are primarily composed of O$_2$ binding protein – Hb. Blood performs vital functions such as delivery and transport of O$_2$ to tissues and organs, deliver nutrients to cells, and dispose cellular metabolic wastes. Thus, disruptions in blood flow can lead to shortages of O$_2$ and nutrients at the cellular level and can have devastating consequences. Sudden loss of intravascular blood volume and reduction in organ/tissue perfusion pressure due to trauma is a clinical condition known as hemorrhagic shock [88, 89]. This in turn induces tissue ischemia, which is characterized by limited O$_2$ transport to tissues coupled with an accumulation of tissue excretory products, such as CO$_2$, lactate, NADH, long chain acyl-coenzyme A, K$^+$ and H$^+$ ions [75-80]. The following sections describe the menaces of ischemia and issues related with its treatment.

1.1.3 Ischemia

During hemorrhagic shock, the O$_2$ supply to various tissues and organs is severely depleted compared to baseline levels. This state is known as ischemia, and is accompanied by the accumulation of anaerobic cell metabolites such as CO$_2$, lactic acid, glycolytic intermediates, K$^+$ and H$^+$ ions [75-80]. Depletion of O$_2$ and other cellular nutrients can eventually lead to cell death and dysfunction via necrotic and apoptotic pathways [4, 90-92]. The durations of time for which O$_2$ supply to cells are affected due to disruptions in blood flow determine the extent of tissue damage and the austerity of ischemia [4, 93, 94]. The normalization of blood flow and perfusion pressure is referred
to as reperfusion [95]. However, this sudden supply of O$_2$ can invoke adverse reaction cascades in the tissues and can elicit inflammation and apoptosis [96]. These collective set of side-effects that occur during the ischemic and reperfusion phases is classified as ischemia reperfusion injury (IRI), and is a major obstacle towards successful treatment of hemorrhagic shock [46, 95]. The pathophysiology of reperfusion injury and common treatment strategies of IRI will be discussed in a following section. In this section we analyze the various events which might lead to ischemia. Additionally, we will review the physiological and functional changes in ischemic tissues.

1.1.3.1 Common Causes of Ischemia

Ischemia is defined as a reduction in arterial blood flow causing oxygen demand-supply imbalance thereby forcing the cells to resort to anaerobic respiration [77]. Myocardial infarction, a condition resulting from myocardial cell death due to prolonged ischemia, is the most common form of ischemia [97]. Thus, the loss of perfusion pressure in coronary blood vessels is the main cause of ischemic injuries each year. The website of Mayo Clinic (an institution ranked #3 overall in hospitals across the US by U.S. News and World Report [98]) lists some of the main factors responsible for occlusion of arterial blood supply leading to ischemia as follows [99]:

1. **Atherosclerosis** – Deposition of lipid-laden plaques on arterial walls leading to restricted blood flow [100].

2. **Thrombosis/blood clot formation** – Rupture of plaques formed in atherosclerosis can cause blood clots. These clots, known as emboli, can block small arteries and fine capillaries leading to ischemia. Thrombosis can be caused by other abnormalities such as endothelial cell injury or hypercoagulability [100].
3. **Coronary artery spasm** – Sudden contraction of muscles of the arterial walls.

Coronary artery spasms are more common in individuals suffering from high blood pressure, excessive tobacco usage, and high blood cholesterol levels.

4. **Hypotension** – Abnormally low blood pressure resulting from severe illness, infections, and loss of blood.

Arterial blood flow can also decrease due to tachycardia (abnormally rapid beating of heart), hypoglycemia (lower than normal blood glucose levels), and external compression of blood vessels (tumor growth adjacent to blood vessel) [99, 101]. Established risk factors which make an individual vulnerable to ischemia include (but are not limited to) smoking, age, raised plasma concentrations of low-density lipoprotein (LDL) cholesterol, decreased plasma concentrations of high-density lipoprotein (HDL) cholesterol, abnormal blood glucose levels, and high blood pressure [99, 101].

### 1.1.3.2 Pathophysiology of Ischemia

As noted earlier, with loss in oxygen supply the cells switch from aerobic respiration to anaerobic glycolysis for ATP production during ischemia. This coupled with the lack of blood flow leads to accumulation of tissue excretory products, such as carbon dioxide ($\text{CO}_2$), lactate, NADH, long chain acyl-coenzyme A, $\text{K}^+$, and $\text{H}^+$ ions [75-80, 102]. Additionally, transition from aerobic to anaerobic respiration causes rapid exhaustion of cellular energy currencies such as ATP, cytidine-5’-triphosphate (CTP), uridine-5’-triphosphate (UTP), guanosine-5’-triphosphate (GTP), and phosphocreatine [4, 103, 104].

**Depletion of Cellular ATP Levels**

As noted by Mirzakhani and Nozari of Dept. of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School (Boston, MA),
“anaerobic respiration through glycolytic pathways leads to the generation of H\(^+\) and lactate ions; while lack of blood flow results in accumulation of these species in the cytoplasm lowering cytosolic pH levels and causing severe acidosis [4]. Low pH levels interfere with the activities of enzyme 6-phosphofructokinase, further thwarting ATP production” [4]. In their chapter titled “Ischemia”, included in “Chemistry and Biochemistry of Oxygen Therapeutics – From Transfusion to Artificial Blood” (edited by Mozzarelli and Bettati, and published by John Wiley and Sons, Ltd.), Mirzakhani and Nozari indicate that “depletion of cellular ATP levels causes dysfunction of ATP-driven ion pumps such as Na\(^+\)/K\(^+\) and Na\(^+\)/Ca\(^{2+}\) exchange pumps leading to intracellular accumulation of Na\(^+\) and Ca\(^{2+}\) ions” [4]. According to researchers, other manifestations of reduction in cellular ATP levels include “vacuole formation in cytoplasm, impaired protein synthesis due to ribosomal disengagement resulting from endoplasmic reticulum dilation and cell membrane damage, osmotic influx of water leading to edema, and subsequent cell disintegration” [4, 105-107].

**Loss of Calcium Homeostasis**

Calcium ions are indispensible to cells as they are important constituents of endoplasmic reticulum (ER) and mitochondria [4]. Ca\(^{2+}\) ions are widely recognized as intracellular “messengers” as they act as links between various intracellular reactions due to their inherent ability to attach to various ligand binding sites [108]. However, cells regulate cytosolic Ca\(^{2+}\) concentrations at levels significantly lower (3 – 4 folds) than extracellular Ca\(^{2+}\) concentrations by multiple ATP-driven processes such as membrane pumps, Na\(^+\)/Ca\(^{2+}\) exchange pumps, and accumulation inside mitochondria and endoplasmic reticulum [4, 108-110]. ATP shortage during ischemia interrupts these energy-driven mechanisms involved in maintaining intracellular Ca\(^{2+}\) ion concentration
resulting in a loss of Ca\textsuperscript{2+} homeostasis [4]. Increased cytosolic Ca\textsuperscript{2+} ion concentration leads to the activation of various deleterious pathways such as protein kinases, phospholipases, proteases and endonucleases [4, 107, 111, 112]. Phospholipases hydrolyze membrane phospholipids and cause membrane disintegration by releasing important membrane constituent arachidonic acid [4, 113, 114]. Protein kinases release enzymes such as NO synthase and xanthine oxidase which oxidize cellular proteins and cause chromatin fragmentation [4, 115-119]. Further, proteases and endonucleases result in cytoskeleton deconstruction and nucleic acid degradation respectively [4, 120-122]. Excessive accumulation of Ca\textsuperscript{2+} ions within the mitochondria adversely affects ATP synthesis by the opening of mitochondrial permeability-transition pores (PTPs) [4, 123-127]. Under normal circumstances, these pores allow the passage of smaller species such as Ca\textsuperscript{2+} ions across the mitochondrial membrane and are pH operated [126]. Ichas et al. reported that during ischemia the “saturation of internal Ca\textsuperscript{2+} binding sites of PTPs result in a conformational transition of these pores” [4, 126]. With a more open conformation during ischemia, these pores allow influx of larger molecules such as water into the mitochondrial matrix causing the mitochondria to swell up [4, 126]. Additionally, opening of PTPs result in minimizing potential difference across the inner mitochondrial membrane [4, 124]. Taken together, these environmental changes in the mitochondria leads to the stagnation of ATP production, outer mitochondrial membrane damage, and generation of pro-apoptotic factors such as calpain, caspase 9 and cytochrome C [4, 123, 127, 128].

**Tissue Inflammation**

Ischemia is known to cause tissue inflammation by the activation of various pro-inflammatory entities such as cytokines (i.e. interleukin (IL)-1, tumor necrosis factor
(TNF-α), chemokines, growth factors, adhesion molecules (i.e. E-Selectin, vascular cell adhesion molecule (VCAM), and intercellular adhesion molecule (ICAM)-1) and enzymes (i.e. cyclooxygenase-2) [4, 129, 130]. Cell adhesion molecules (CAMs) promote the adhesion of leukocytes on vascular endothelium. Post adhesion, activation of neutrophils can lead to the release of cytotoxic enzymes and microvascular occlusion [4, 131-141]. Activated neutrophils infiltrate into the extravascular compartment by interacting directly with integrins and damage the endothelium by secreting cytotoxic molecules such as proteases, ROS, hypochlorous acid, arachidonic metabolites, and cytokines (i.e. interleukin-8, (IL-8)) [46, 142-146]. The ROS and cytokines released may further lead to cell apoptosis and inflammation, thereby damaging the endothelium [147, 148].

1.1.4 Reperfusion Injury

To treat hemorrhagic shock, clinicians normally stop the bleeding and transfuse the patient with either RBCs or plasma expanders in order to restore the lost blood volume. Hence, re-establishment of blood flow and restoration of tissue oxygenation is known as reperfusion. This abrupt change in tissue oxygenation, though considered beneficial, can paradoxically induce cell apoptosis, tissue inflammation, infarct formation and has been shown to elicit multiple organ dysfunction by activating cytotoxic injury processes such as ROS and reactive nitrogen species (RNS) generation, neutrophil-endothelium interactions, and hypercontracture (i.e. intracellular overloading of cardiomyocytes with Ca\(^{2+}\) ions) [46, 95, 96]. Reperfusion injuries are commonly observed in cases of acute myocardial infarction, organ transplants and resections and during hemorrhagic shock resuscitation.
As noted before, the collective set of side-effects that occur during the ischemic and reperfusion phases is classified as ischemia reperfusion injury (IRI) [4]. Organ damage in the brain, heart, kidney, liver and lungs as a result of IRI is well documented in the literature [95]. It is believed that IRI propagates by damaging intracellular mitochondria. During ischemia, the mitochondria can deviate from its normal functions (i.e. ATP production and Ca\(^{2+}\) homeostasis) in the presence of detrimental signals such as ROS, protein kinases, high Ca\(^{2+}\) ion concentration, calpain and other pro-apoptotic factors such as caspase 9 and cytochrome C. In addition, the intracellular H\(^+\) ion concentration increases due to accumulation of lactic acid produced during anaerobic glycolysis and also by hydrolysis of ATP. The accumulation of H\(^+\) ions overload the Ca\(^{2+}\) ions to be exchanged with the extracellular Na\(^+\) ions during reperfusion, leading to the disruption of Ca\(^{2+}\) homeostasis [123, 128]. The following section discusses the pathophysiology of IRI.

1.1.4.1 Pathophysiology of Ischemia/Reperfusion Injury (IRI)

It is proposed that abnormal regulation of local blood flow is a determining factor in the propagation of IRI [96]. Disrupting the balance of vasoactive signaling molecules can hamper the normal functions of the microvasculature. The presence of ROS in the endothelium can up-regulate production of vasoconstrictive signaling molecules such as endothelin, protein kinases, eicosanoids, and isoprostanes [46, 149]; thereby suppressing the production of vasorelaxation factors such as NO and CO [96, 149-152]. Such alterations in blood vessel function can lead to local loss of autoregulation; thereby promoting vascular cell permeability [153], cytotoxic enzyme release [154], cytokine release, changes in vascular tone and can eventually elicit cell injury or even cell death [149, 155]. It has also been noted that the heterogeneity of blood flow promotes IRI [96,
The human body is equipped to prevent IRI to a certain extent by the expression of cytoprotective enzymes such as heme oxygenase (HO), nitric oxide synthase [157] and via other physiologically important vasorelaxing, anti-apoptotic and anti-inflammatory gaseous molecules such as CO [158-161], NO [162], H₂S [163], and HNO [164-167]. However if the extent of endothelial damage is massive, these inherent mechanisms to prevent aggravation of IRI are rendered ineffective [168]. Most significant manifestations of IRI include cell death by necrotic and apoptotic pathways and tissue inflammation at microcirculatory levels [4, 169].

**ROS Generation and Cell Death by Apoptosis/Necrosis**

A group of proteins, located on the inner membrane of the mitochondria and constituting the electron transport chain (ETC), facilitate the movement of electrons from donors (NADH) to acceptors (O₂) during oxidative phosphorylation [4]. The ETC also ‘pumps’ protons into the intermembrane space from the mitochondrial matrix [4]. Thus, a potential difference is created between the mitochondrial intermembrane space and the matrix of the mitochondria due to increased proton concentration in the intermembrane space and the differences in electric potentials across the inner mitochondrial membrane [4, 109, 170, 171]. Energy released during the transport of electrons by the ETC within the mitochondrial intermembrane space is utilized to channel a flow of protons back in to the matrix through the F-ATPase ion transporters [4, 170, 171]. The F-ATPase enzyme complexes use the proton gradient to generate ATP from ADP and inorganic phosphates [4, 170, 171].

Even under physiological conditions some electrons dodge the ETC to combine with molecular O₂ to yield toxic superoxide (O₂⁻) ions [4, 172, 173]. However, in low concentrations, this deleterious oxidising agent is eventually reduced by intrinsic
reducing agents such as superoxide dismutase (SOD) to form less harmful H$_2$O$_2$ [4, 172, 173]. During ischemia the ETC is severely damaged and the mitochondria do not function normally [4]. However, sudden reperfusion can elevate electron leakages leading to a surge in O$_2^-$ ion production [4, 172]. Increased O$_2^-$ ion concentrations can overpower the inherent reducing agents and can inflict severe oxidative damage to cellular proteins and membrane lipids [4, 172]. The action of enzymes such as NADPH oxidase and xanthine oxidase also generate O$_2^-$ ions [4]. During ischemia, intracellular Ca$^{2+}$ ion overloading boosts the conversion of xanthine dehydrogenase to xanthine oxidase [4, 174, 175]. Reperfusion provides O$_2$ and subsequently the formed xanthine oxidase can catalyze the conversion of hypoxanthine and O$_2$ to xanthine and O$_2^-$ ions [4, 174, 175]. As noted earlier O$_2^-$ ions are reduced by SOD to yield H$_2$O$_2$ [4]. Though comparatively less toxic, H$_2$O$_2$ reacts with heavy metal ions (Fe$^{2+}$ and Cu$^+$) present in various cellular proteins such as cytochromes, hemoglobin, myoglobin to form highly reactive hydroxyl ions (•OH) [4, 176-179]. Moreover, H$_2$O$_2$ reacts with physiological halides to form hypohalous acids; which are known to damage the cytoskeleton and the extracellular matrix [4, 180-183]. Activated neutrophils can potentially trigger the production of H$_2$O$_2$ during ischemia [4, 184]. High intracellular H$_2$O$_2$ concentrations can further inhibit the reduction of O$_2^-$ ions by SOD upon reperfusion by promoting the reverse reaction. ROS causes severe damages to proteins constituting ion transporter channels and membrane phospholipids. These coupled with exhausted ATP levels during ischemia lead to intracellular Ca$^{2+}$ ion overloading. As noted earlier, high concentrations of O$_2^-$ and Ca$^{2+}$ ions can open mitochondrial PTPs resulting in ionic equilibria [4, 123-127]. This causes a drop in proton gradient and further disables ATP production [4]. Opening of mitochondrial PTPs also allow an influx of water into the
mitochondria causing it to swell up and eventually rupture [4, 126]. Significantly, electron transfer protein cytochrome c is released into the cytosol where it activates various apoptotic cascades [4, 185].

Further, $O_2^-$ ions react with nitric oxide (NO) to yield highly toxic peroxynitrite ($\text{ONOO}^-$) free radicals [28, 44-46]. Peroxynitrite free radicals attach to the 3-position phenol ring of tyrosine residues in proteins to form 3-nitrotyrosine [4, 186]. Protein nitration can have various bio-physiological outcomes such as loss of cellular functionalities by inhibition of tyrosine phosphorylation [187, 188], vulnerability of modified proteins to proteolytic degradation [48, 187, 189-191], alterations in protein activities [48, 192], and activation of immune response cascades [193-196]. Nitration of mitochondrial SOD during IRI was shown to exacerbate renal injury in animal models and promote nitration of other mitochondrial proteins [197, 198]. Nitration of mitochondrial proteins can further result in impaired ATP production, rupture of mitochondrial outer-membrane and subsequent cell death via apoptotic pathways [172, 199, 200]. Peroxynitrite interacts with membrane lipids to result in membrane rupture and eventual cell death via necrotic pathways [185, 201-203]. Moreover, this free radical induces DNA mutations following its reaction with deoxyribose [185, 201, 203, 204].

**Tissue Inflammation and No-Reflow**

During the inceptive phases of reperfusion, increased intracellular ROS levels lead to incorporation of activated platelets onto reperfused tissues [205, 206]. The activated platelets express P-selectin cell adhesion molecules which attach to P-selectin glycoprotein ligand-1 located on leukocytes to facilitate adhesion of these hematopoietic cells onto post-ischemic tissues [205, 207]. Furthermore, the tethered leukocytes express L-selectin adhesion molecules which mediate the adherence of more leukocytes
At elevated ROS levels endothelial tissues express P-selectins and E-selectins which mediate direct attachment of leukocytes onto the endothelium [208]. Once attached to reperfused tissues, these activated leukocytes (T-lymphocytes, neutrophils, monocytes) secrete inflammation producing cytokines, ROS, NO and lysozymes to exacerbate initial reperfusion injuries [206]. During reperfusion, activated platelets and inflammatory molecules released by leukocytes adhering to microvascular endothelium can lead to thrombosis (blood clotting) [169, 175, 209-213]. The thrombus (clot) can circulate as an embolus and subsequently block fine capillaries [169, 214]. This leads to localized loss of blood flow and limits normalization of perfusion pressure [169, 215]. This phenomenon, known as “no-reflow”, is a major concern while treating IRI [169, 216, 217].

1.2 Motivation for this research

1.2.1 Current IRI Treatment Strategies

In 2010, IRIs affected 2.4 million patients in the United States (US), and was responsible for ca. 15.3% of total deaths in the US [97, 218]. According to the World Health Organization (WHO), by 2020, IRIs will be the leading cause of mortality in the world [219]. Therefore, there is a critical need to develop therapeutic strategies to protect the cardiovascular system during the acute ischemic and post-ischemic phases that occur during resuscitation from hemorrhagic shock. This section reviews some of the current treatment strategies used by doctors and clinicians to counteract IRI.
1.2.1.1 Ischemic Pre- and Post-Conditioning

The most popular strategy to mitigate IRI is perhaps ischemic preconditioning (IPC) [220]. This involves preparing the organs for a prolonged ischemic period by subjecting them to mechanically or pharmacologically induced brief periods of ischemia/reperfusion [221-224]. IPC was shown to successfully reduce infarct size, preserve endothelial function, and circumvent cell dysfunction [225]. Ischemic post conditioning (IPost) involves preparing organs to cope better with IRI by subjecting them to brief periods of ischemia and reperfusion prior to extended periods of reperfusion [226-228]. IPost was shown to successfully reduce infarct size in dogs exposed to coronary IRI [4, 229].

Researchers have reported that both IPC and IPost operate by activating a common set of cytoprotective signaling cascades [4]. These “pro-survival” cascades are activated by reperfusion injury salvage kinases (RISK), namely, the phosphatidylinositol-3-OH kinase (IP3K)-Akt and the p42/p44 extracellular signal-regulated kinases (Erk1/2) [4, 185, 224, 230-232]. RISK inhibits cell death via apoptosis by inhibiting mitochondrial PTP opening [4, 185, 224, 230-232]. Briefly, IP3K and Erk1/2 increases intracellular NO levels by augmented phosphorylation of nitric oxide synthase-3 (NOS3) [4]. Increased NO levels activates soluble guanylyl cyclase (sGC) enzyme [4, 149]. This activated enzyme mediates the conversion of guanosine tri-phosphate (GTP) to cyclic guanosine 3’, 5’-monophosphate (cGMP) [149, 233, 234]. In turn, cGMP facilitates the phosphorylation of protein kinase G (PKG) to activate protein kinase C epsilon (PKCε) [4, 235, 236]. Due to open mitochondrial PTPs during ischemia/reperfusion, movement of PKCε into the mitochondria from the cytosol is unhindered [236]. PKCε is believed to exhibit vasorelaxation via opening of mitochondrial ATP sensitive potassium (K_ATP) channels [236-238]. K_ATP channels restore mitochondrial membrane potential and
therefore Ca$^{2+}$ ion and proton concentrations inside the mitochondria are normalized [4, 236-238]. As noted earlier, normalization of Ca$^{2+}$ ion concentration within the mitochondria thwart opening of PTPs [4, 185, 239].

A major disadvantage of mechanical IPC is the trauma and stress placed on major blood vessels and target organs [220]. A prior knowledge or prediction of ischemia is necessary in the case of IPC; whereas IPost can be induced at the outbreak of reperfusion injury during cardiac surgery or graft transplant [226, 227]. Both IPC and IPost are not only expensive but can also cause damages to important blood vessels and target organs [220].

1.2.1.2 Antioxidants

As discussed previously, high intracellular ROS levels aggravate IRI by inducing apoptosis, mitochondrial dysfunction, DNA degradation, and other oxidative tissue damages [4]. During long ischemic insults the endogenous detox system of the body consisting on enzymes such as SOD, glutathione peroxidase, and catalase is often neutralized by escalated ROS levels [4]. SOD converts toxic O$_2^-$ to H$_2$O$_2$ and O$_2$; while catalase and glutathione reduce H$_2$O$_2$ to H$_2$O [4]. Researchers have shown that intravenous injections of SOD and catalase were successful in improving contractility of "stunned" dog myocardium [240]. However, these antioxidants had no effect on myocardial infarct size in primate models [241]. Studies involving antioxidants such as lazaroids [242], tirilazad mesylate [243], and melatonin [244-246] have shown promising results with respect to attenuating oxidative tissue toxicity [4]. Further research needs to be conducted to establish the efficacy of antioxidants in treating IRI owing to inconclusive results from various animal studies and human clinical trials [4, 173, 247,
Interestingly, researchers believe that ROS in low concentrations are necessary for treatment strategies such as IPC to function as desired [185, 239, 249].

1.2.1.3 NO Releasing Agents and Inhalation-based Delivery of NO

The vaso-relaxation and anti-apoptotic properties of NO can be traced to cGMP. NO is produced in the endothelial cell layer from the enzymatic activities of nitric-oxide synthase (NOS) on L-arginine. In the endothelium, NO forms nitrosyl-heme complexes and activates sGC. This activated enzyme mediates the conversion of GTP to cGMP, a known vasodilator and inhibitor of apoptosis [149, 233, 234]. The anti-inflammatory responses of NO have been associated with its suppression of NF-κB expression thereby leading to inhibition of pro-inflammatory entities such as cytokines (i.e. IL-1, TNF-α), chemokines, adhesion molecules (i.e. E-Selectin, VCAM, and ICAM-1) and enzymes (i.e. cyclooxygenase-2) [129, 130]. NO-releasing drugs have been shown to possess enhanced anti-proliferative properties. It is believed that NO inhibits vascular smooth muscle cell (SMC) proliferation by the suppression of ornithine decarboxylase (ODC), an enzyme essential for cell growth [250, 251].

The benign properties of NO have been harnessed by researchers to design different NO-based therapeutics to mitigate IRI [4]. NO releasing compounds such as nitroglycerin (NTG) and sodium nitrite have been used to treat IRI [4]. Studies have underlined the vasorelaxing properties of NTG, brought about by actuating the sGC – cGMP – PKG – K_{ATP} cascade, during severe angina and acute myocardial infarction (AMI) [4, 252-254]. Duranski et al. has shown that sodium nitrite infusions lessen reperfusion injuries in ischemic mice hearts and livers [255]. Other compounds such as sildenafil [256-260] and BAY 58–2667 [261-263] have also exhibited cytoprotection against reperfusion injuries via the cGMP mediated vasorelaxation pathway [4].

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However, at high doses these compounds elicit excessive vasodilation and systemic hypotension [4, 264, 265].

Inhalation-based delivery of NO to ischemic tissues has been successfully used to treat IRI [162]. The advantageous effects of inhaled NO are brought about by both cGMP-dependent and cGMP-independent pathways [4, 266-270]. Inhalation-based NO delivery to tissues affected with IRI exhibited beneficial effects such as infarct size reduction [271], cardio-protection [227, 272-274], improvements in vascular perfusion [275], and reversal of pulmonary hypertension [268, 276-278]. Frostell et al. reported that lambs with constricted pulmonary circulation subjected to 80 ppm NO for 3 hours showed improved vasorelaxation and reversal of pulmonary constriction, but did not show significant changes in metHb levels and lung histology [268]. However, this approach delivers NO systemically throughout the body and more studies need to be conducted to confirm that the metHb levels remain unaffected post NO inhalation.

1.2.2 Physiologically Relevant Therapeutic Gases

IRI is a complex phenomenon consisting of several dependant and independent symptoms [46]. IRI has been recognized to elicit oxidative stress induced vasoconstriction, cell apoptosis, vascular inflammation and endothelial damage [4, 95, 168]. Vasoconstriction leads to a marked drop in the intravascular perfusion pressure causing ischemia [77]. Several bioactive gaseous ligands such as CO, NO, H₂S, and HNO have proven cytoprotective properties brought about by the various mechanistic pathways described in the literature. The protective cascades involved in promoting the benign influences of NO have been discussed in Section 1.2.1.3 of this manuscript. In this section we will review the ameliorative properties of other therapeutic gaseous ligands such as CO, H₂S, and HNO in an ischemia/reperfusion setting. Figure 1.3, used
with permission from Nature Publishing Group (NPG), a division of Macmillan Publishers Ltd., illustrates the origin and activities of these benign gaseous ligands involved in providing relief from reperfusion injuries [279].

**Figure 1.3:** Endogenous therapeutic gaseous ligands such as CO, NO and H$_2$S mitigate reperfusion injuries via their inherent vasorelaxing, anti-apoptotic and anti-inflammatory properties. Reprinted by permission from Nature Publishing Group, Macmillan Publishers Ltd: Nature Medicine, 17(11), Holger.K.Eltzschig & Tobias Eckle, Ischemia and reperfusion – from mechanism to translation, p. 1391–401, Copyright (2011) [279].

**Carbon Monoxide (CO)** – Heme Oxygenase (HO) suppression during ischemia creates a shortage of endothelial CO. CO has been recognized to possess vasodilating, anti-apoptotic, anti-inflammatory and anti-proliferative properties [158, 160, 161, 280]. CO is responsible for maintaining cyclic cGMP levels in SMCs. cGMP is an intrinsic vaso-regulator [281]. cGMP lowers the intracellular Ca$^{2+}$ ion concentrations to promote vasodilation either aided by cGMP-dependent protein kinases such as protein kinase G (PKG) or by its direct interaction with the Ca$^{2+}$-activated ATP-ases of the cell membrane [149]. High intracellular [Ca$^{2+}$] induces malfunctioning of the mitochondria and also
results in smooth muscle constriction, eliciting vasoconstriction and systemic hypertension. Two different signaling pathways mediate the anti-apoptotic properties manifested by CO in vascular endothelial cells and SMCs [158]. In endothelial cells, CO inhibits tumor necrosis factor-α (TNF-α) mediated apoptosis by activation of the p38 mitogen-activated protein kinase (p38 MAPK) pathway [161]. It has been previously reported in the literature that the p38 MAPK signaling transduction pathway inhibits apoptosis [161, 282]. In SMCs, CO activates sGC leading to increased intracellular cGMP levels [158]. The anti-apoptotic properties of cGMP have been noted in the literature [233, 234]. The anti-inflammatory responses of CO also involve activation of the p38 MAPK signaling pathway [283]. CO releasing molecules (CORM-1, CORM-2, and CORM-3) exhibit anti-inflammatory properties by various mechanisms such as prevention of neutrophil attachment on endothelial cells [284], downregulation of matrix metalloproteinases 1 and 2, and suppression of nuclear factor-κB (NF-κB) activation [130, 285]. The anti-proliferative properties of CO are brought about by intracellular accumulation of cGMP and the suppression of mitogens such as endothelin-1 (ET-1) and platelet-derived growth factor B (PDGF-B) [286, 287].

**Hydrogen Sulphide (H₂S)** – Inhalation based H₂S therapies and H₂S releasing compounds have been successful in alleviating IRI by stabilizing intravascular perfusion pressure, and generating hypothermia resulting in suspended-animation-like state in small animals [279, 288-290]. In mammals H₂S is synthesized from L-cysteine via four separate pathways. In vascular SMCs, L-cysteine undergoes dimerization to form L-cystine, which is subsequently converted to thiocysteine by cystathionine γ-lyase (CSE). CSE further catalyzes the reaction of thiocysteine with other thiol compounds such as glutathione and cysteine to yield H₂S. It is predicted that NO up-regulates the production
of H₂S in vascular SMCs by enhancing CSE activity. H₂S is believed to exhibit vasorelaxation via opening of the ATP sensitive potassium (K_{ATP}) channels present in the SMCs [291]. However, the effect of H₂S is temporary as it is rapidly scavenged by heme, disulphide containing proteins, and other metallo-proteins [291]. H₂S releasing molecules (derivatives of diclofenac and mesalamine) have been shown to possess enhanced anti-inflammatory properties such as inhibition of pro-inflammatory cytokines (IL-1 and TNF-α) and limiting leukocyte tissue infiltration [292, 293]. The anti-inflammatory responses of H₂S are believed to originate from its ability to suppress NF-κB activation [130].

**Nitroxyl (HNO) —** The beneficial effects of nitroxy (HNO/NO⁻) came to light recently when nitroxy donors were used to treat heart failure [164, 294]. Nitroxy is believed to cause vasodilation through a different pathway involving increased cytosol levels of calcitonin gene-related peptide (CGRP) and not cGMP [166]. Additionally, nitroxy donors enhance the contractility of cardiac muscles in the post-ischemic phase without affecting the vascular tone [165, 166, 295]. Since HNO is a highly reactive compound, it should be generated in situ. Angeli’s salt and Piloty’s acid are the two most commonly used nitroxy donors, however these donors exhibit unwanted side-effects such as hypotension, headache and gastrointestinal symptoms [294].

We believe delivery of these potent vasodilators to ischemic tissues can potentially elevate perfusion pressure to baseline levels and thereby mitigating vasoconstriction mediated systemic hypertension. Reperfusion results in deregulated systemic inflammation which can lead to severe organ damage [128, 129, 133, 134, 154, 206, 282, 292, 296, 297], and the role of apoptosis in IRI is well established [46, 90, 148, 158, 161]. In light of the proven anti-inflammatory and anti-apoptotic responses of these
gaseous ligands in various biological processes, we hypothesize that exogenous supply of these ligands will be successful in preventing apoptosis and vascular inflammation during the ischemic and reperfusion phases. Indeed a study conducted by Cabrales et al. showed that sudden oxygenation of ischemic tissues and subsequent reperfusion injuries could be avert via transfusion of CO saturated RBCs as opposed to their oxygenated counterparts [168]. Therefore, RBCs seem like a logical carrier for \textit{in vivo} delivery of exogenous NO and CO. However, in order to circumvent potential issues associated with RBC transfusion (i.e. short storage shelf life (42 days), limited supply and possibility of transmission of yet to be identified pathogens), hemoglobin (Hb)-based O$_2$ carriers (HBOCs) have been used to deliver exogenous CO to ischemic tissues in rat models with considerable success [280, 298]. In the following section we analyze the potential issues associated with using RBCs to deliver therapeutic gaseous ligands to ischemic tissues. The drawbacks linked to RBC transfusions motivated us to explore the possibility of using Hb based O$_2$ carriers (HBOCs) as transporters of NO and CO prior to O$_2$ for mitigating IRI.

\subsection*{1.2.3 Drawbacks of using RBCs as ligand carriers}

To treat hemorrhagic shock, clinicians normally stop the bleeding and transfuse the patient with RBCs in order to restore the lost blood volume and replenish intracellular O$_2$ supply. However RBC transfusions are plagued with issues such as short storage shelf life (42 days), blood type mismatch, limited supply and possibility of transmission of yet to be identified pathogens. This section describes these issues associated with allogenic transfusions.

In the US, more than 44,000 units of RBCs are used daily [299]. According to the 2009 National Blood Collection and Utilization Survey (NBCUS) conducted by the
American Association of Blood Banks (AABB), in 2008, ca. 17 million units of RBCs and whole blood were donated [300]. Although the figures do not suggest an immediate supply deficit in the US, blood supply is quite dire in several Asian and African countries. Blood donations in these countries are thwarted by a shortage of voluntary donors, lack of proper infrastructure to store, type and screen blood, and lack of well-trained staff [301]. In 2011, the WHO estimated a yearly demand of 100 million units of blood worldwide. However, according to the 2011 Global Database on Blood Safety (GDBS) report commissioned by the WHO, approximately 92 million units of blood were collected in 2011 [302, 303]. Therefore in 2011, there was an 8 million unit deficit of blood worldwide. In the US alone, there will be a shortage of 3-4 million units of blood by 2020 [302, 304].

In addition to projected shortages in supply, blood banks have to combat issues such as seasonal blood shortages [305-307], the short shelf-life of stored RBCs [308, 309], and the RBC storage lesion [310-312]. Allogenic transfusions are also plagued with transfusion-related acute lung injuries (TRALI) and bacterial infections resulting from contaminated blood transfusions [50, 313-320]. Additionally, blood group antigens attached on RBC surfaces limit donated blood transfusions to patients with same surface antigens [50, 318-322]. Transfusing a patient with incompatible blood type can result in complications such as clumping of blood cells, hemolysis, and may even have fatal consequences [50, 318-322]. These factors combined with the acute need for large quantities of blood in emergency situations such as wars and terrorist attacks motivate the need to develop artificial RBC substitutes that are universally compatible, pathogen free and are just as safe and efficacious as native RBCs. To avert the aforementioned side-effects associated with RBC transfusions we hypothesized to use artificial RBC
substitutes, specifically HBOCs, to deliver therapeutic gaseous ligands to ischemic tissues in order to mitigate IRI.

1.3 Artificial O₂ Carriers

As noted before, to circumvent the side-effects associated with allogenic RBC transfusions, researchers have attempted to develop artificial O₂ carriers or RBC substitutes to store and transport O₂ [4, 50, 302, 318-320, 323-335]. Artificial O₂ carriers are expected to have favorable properties such as O₂-carrying abilities similar to whole blood/RBCs, little or no renal toxicity, universal compatibility, longer shelf-lives (as compared to donated blood), ideal size for longer circulation half-lives (should be larger than pores lining the inner-walls of the blood vessels, but should not be large enough to block fine capillaries and/or get captured by macrophages) [50, 336]. Additionally, these O₂ carriers should not extravasate, should not elicit vasoconstriction mediated hypertension, and should not exhibit other side-effects [50, 336]. Two major approaches taken by researchers to design artificial RBC substitutes are namely, Hb-based O₂ carriers (HBOCs) and Perfluorocarbon (PFC)-based O₂ carriers (PFCOCs) [334, 337].

**HBOCs** – These O₂ carriers are synthesized from Hb purified from human, animal and recombinant sources [337-343]. HBOCs are further categorized into *acellular* and *cellular* HBOCs depending on the absence or presence of protective membranes encapsulating the Hb molecules [50, 328, 344-347]. Cell-free Hb transfusions are plagued with issues such as extravasation through pores lining the inner-walls of the vasculature [331, 348, 349], NO scavenging [350-352], ROS generation/oxidative tissue toxicity [48, 177, 353, 354], and vasoconstriction mediated systemic hypertension [45, 46, 48, 355]. To circumvent the issues associated with Hb transfusions researchers have
polymerized and cross-linked Hb to increase its size/molecular mass in order to prevent HBOC extravasation into the tissue space [51, 68, 356-359]. These types of HBOCs are known as *acellular* HBOCs. Another approach to avoid extravasation and prolong circulation half-lives is to encapsulate Hb molecules within lipid or polymer vesicles [346, 360-365]. These HBOCs are referred to as *cellular* HBOCs. It is believed that the external lipid/polymer membrane protects the encapsulated Hb from extravasation and NO scavenging [366]. Further, poly(ethylene glycol) (PEG) molecules attached onto the vesicle surfaces impart stealth property; thereby prolonging their *in vivo* circulation half-lives [367-369].

**PFCOCs** – These are chemically inert compounds made up of fluorinated hydrocarbons capable of dissolving considerable quantities of ionic gases such as O\textsubscript{2} and CO\textsubscript{2} [370-373]. The gases are held in the intermolecular spaces within PFC suspensions [374]. Therefore, the amount of O\textsubscript{2} carried by PFCOCs is directly proportional to the available pO\textsubscript{2} [374]. Studies have shown that PFCOCs are more efficient when alveolar pO\textsubscript{2} reaches ~400 mm Hg which is ~4 times higher than the physiological pO\textsubscript{2} levels in the alveoli [374, 375]. Thus, supplemental O\textsubscript{2} is usually required to increase the efficiencies of these O\textsubscript{2} carriers [374, 375]. Further, these suspensions are immiscible with aqueous solutions and need to be emulsified before transfusion *in vivo* [376-379]. Additionally, drawbacks of commercial PFCOCs such as instability issues leading to compromised O\textsubscript{2} delivery, activation of phagocytosis cascades, and increased vulnerability to ischemic stroke led to untimely termination of their clinical trials [380, 381]. It is for these reasons, in this study, we chose HBOCs over PFCOCs to deliver therapeutic gases to ischemic tissues.
1.4 HBOCs

1.4.1 Acellular HBOCs

Hb is the major protein present within the RBCs. It is primarily responsible for \( O_2 \) storage and transport in the systemic circulation. However, the presence of free tetrameric Hb in the blood stream is toxic and is usually associated with aged RBCs. The toxic effects of cell-free Hb in circulation is counteracted by an intrinsic detoxification system comprising of the serum protein haptoglobin, heme-oxygenase (HO), and ferritin [149].

Tetrameric Hb in circulation tends to spontaneously dissociate into \( \alpha \beta \) dimers. The dimers oxidize briskly releasing toxic free-heme into the circulation. Free-heme binds to serum albumin disrupting intravascular oncotic pressure and leading to vasoconstriction [4]. Haptoglobin irreversibly binds Hb/Hb subunits preventing their oxidation [4]. HO degrades heme to yield CO, biliverdin and iron [149]. CO is a potent vasodilator and therefore the action of HO indirectly offers vasorelaxation [160, 382]. However, increased cell-free Hb (or dimer) concentration in circulation saturates this intrinsic detoxification system. Further, cell-free Hb in the circulation can extravasate into the interstitial space between the endothelial cell layer and the SMC layer. This results in scavenging of important gaseous messenger molecules such as NO and CO from the interstitial tissue space [344]. Scavenging of these gaseous ligands exacerbates vasoconstriction at the microcirculatory level and hypertension at the systemic level [350, 383].

Spontaneous autoxidation of extracellular Hb leads to the formation of the highly reactive \( O_2^- \) free radical and metHb [384]. The superoxide free radical can generate other harmful ROS such as the hydroxyl radical and hydrogen peroxide [44-46]. The
superoxide radical can further catalyze the autoxidation of Hb to metHb [355]. MetHb, in which the iron in the heme ring is in the oxidized Fe\textsuperscript{3+} state, is not capable of transporting O\textsubscript{2}. Additionally, the formation of metHb is accompanied by the release of highly cytotoxic free-heme, which can lead to severe tissue and organ damage [385, 386]. In general, the toxicity of free Hb is further amplified if it extravasates through the blood vessel wall into surrounding tissues. In this scenario, the Hb generated ROS and free heme are in direct contact with tissues which can oxidize cellular lipids, proteins and nucleic acids and eventually lead to cell damage and dysfunction [46, 47].

Unmodified Hb solutions had circulation half-lives of around 30 min and caused renal toxicity due to dimerization and extravasation [4]. Researchers have cross-linked, polymerized, and surface conjugated Hb to increase its stability and size in order to circumvent dimerization and extravasation [4]. These HBOCs, lacking protective membranes, are classified as acellular HBOCs. This subclass includes chemically (crosslinked, polymerized, surface conjugated Hbs) and genetically modified (recombinant) Hbs [4].

1.4.1.1 Hb Crosslinking – First Generation HBOCs

As noted before, the presence of cell-free tetrameric Hb in systemic circulation can lead to side-effects such as dissociation into αβ dimers, extravasation of these small molecules into the interstitial space between the endothelial cell layer and the SMC layer, and scavenging of important gaseous messenger molecules leading to severe vasoconstriction and systemic hypertension [344]. To counteract the side-effects associated with dissociation of Hb into dimers and monomers, researchers have suggested stabilizing the Hb tetramer by intramolecular crosslinking.
A variety of cross-linkers such as bis(3,5 dibromosalicyl) fumarate (DBSF), Diaspirin (DCLHb), dimethylpimelimidate, acyl phosphates, phosphinic acids, 2-nor-2-formylpyridoxal 5’-phosphate, and glutaraldehyde have been used to crosslink Hbs [4]. DBSF mediated crosslinking of deoxygenated human Hb (both A and S) introduced links between the Lysine99α1 and Lysine99α2 residues and yielded low-affinity, tense or T-state cross-linked Hb. High-affinity, relaxed or R-state stabilized crosslinked Hbs were obtained by DBSF driven crosslinking reaction between the Lysine82β1 and Lysine82β2 residues of oxygenated Hb [4, 326, 387-393]. Though DBSF crosslinking successfully eradicated Hb dissociation into αβ dimers, studies have suggested that these crosslinked Hbs extravasated through the pores lining the inner-walls of the vasculature and caused NO scavenging related vasoconstriction [352, 394-400]. Further, clinical trials carried out on Diaspirin crosslinked (α99) Hb (DCLHb) developed by Baxter Healthcare showed that in-spite of augmented stability these potential acellular HBOCs were plagued by issues such as cytotoxicity, activation of cytoprotective cascades due to aggravated NO scavenging, and even led to myocardial lesions in small animals [4, 401-403]. Researchers (Olsen et al. and Jones et al.) have shown that introducing multiple crosslinks between Hbs yielded proteins with both high and low oxygen affinities [4, 404, 405]. Additionally, these “multi-linked” Hbs possessed superior cooperativities and thermal stabilities as compared to singly-crosslinked Hbs [4, 404, 405]. Other studies have attempted to crosslink Hbs using acyl phosphates [406, 407]. However, Kluger et al. noted that the disulphide bonds introduced by acyl phosphate crosslinkers could be reduced by reducing agents such as β-mercaptoethanol and dithiothreitol [4, 408]. Derivatives of phosphinic acid have been used to crosslink Hbs [4, 409, 410]. These crosslinked Hbs had oxygen affinities comparable to cell-free Hb; however, they did not exhibit sufficient cooperativities [4, 409, 410].
14.1.2 Hb Polymerization – Second Generation HBOCs

High MW Hb polymers synthesized using various methods such as “in-situ Schiff base generation, sulphydryl-maleimide click reaction, and “zero-length” crosslinking” have been reviewed in detail by Olsen and Tarasov in the book titled ‘Chemistry and Biochemistry of Oxygen Therapeutics’ edited by Mozzarelli and Bettati [4, 411, 412]. Both sulphydryl-maleimide click reaction method [4, 326, 413] and “zero-length” Hb crosslinking method [329, 331, 414-416] have yielded large Hb polymers; however, these techniques are still in their formative stages and have not been subjected to the extensive rigors of clinical trials [4, 411-413]. Three major ‘second generation’ HBOCs which made it to different phases of clinical trials are O-raffinose crosslinked polymerized hHb, Hemolink® (Hemosol Inc., Toronto, Canada), glutaraldehyde crosslinked and polymerized Hb, Hemopure® (HBOC-201®) (BioPure, Cambridge, MA), and glutaraldehyde polymerized pyridoxal 5’-phosphate treated human Hb, PolyHeme® (Northfield Laboratories Inc., Evanston, IL) [4, 412, 417, 418]. These preparations involved the generation of a Schiff base between the amino groups of Hbs and the aldehyde groups of the crosslinker [4]. The Schiff base was eventually reduced using reducing agents such as sodium borohydride (NaBH₄) (HBOC-201® and PolyHeme®) and dimethylamine borane (Hemolink®) [4].

Inter- and intramolecular crosslinking of Hb was observed in the case of Hemolink® [4]. However, different extents of polymerization led to the synthesis of different sized Hb polymers (128 kDa – 600 kDa). Additionally, these polydisperse HBOC solutions had short circulatory half-lives of around 14 – 20 h in humans. More importantly, phase III clinical trials of these HBOCs were aborted due to reports of myocardial infarctions and injuries to cardiac myocytes [4, 419, 420]. Polymerized Hb solutions such as Hemopure® and PolyHeme® were synthesized via glutaraldehyde
crosslinking [4]. It was noted that in alkaline pHs glutaraldehyde polymerizes to yield a polymer with multiple reactive sites which form longer crosslinks between Hb molecules [4, 421]. Longer crosslinks are deemed to be fragile and unstable as they tend to dissociate to release the Hb molecules [4, 422]. Further, the stability of glutaraldehyde crosslinked polymers is determined by the efficacy of the Schiff base reduction method [4, 423]. Incomplete Schiff base reductions can result in PolyHbs with compromised stability and high metHb levels [4, 422]. Hemopure® was shown to elicit NO scavenging mediated vasoconstriction and increased metHb levels upon transfusion [359, 424-431]. PolyHeme® transfusions accounted for more patient deaths in phase III clinical trials than regular blood transfusions [4].

The second generation acellular HBOCs were plagued by two major issues namely, NO scavenging related vasoconstriction and heme oxidation mediated ROS generation [4]. It has been proposed that these blood substitutes circulate closer to the blood vessel wall due to their smaller size compared to RBCs [4]. This increases extravasation tendencies and leads to the capture of endothelial NO [4]. NO scavenging results in vasoconstriction and increases mean arterial pressure [4, 432]. To circumvent the extravasation issues, strategies such as PEG attachment onto Hb molecules [433, 434], purification of larger polymers from glutaraldehyde crosslinked PolyHb solutions (diameter > 500 kDa) [51, 435-437], attachment of Hb onto polysaccharide containing nanoparticles [438], naturally occurring giant Hb molecules [439], and recombinant Hb oligomers [342, 440-443] are being explored by researchers around the world. It was also shown that cross-linked Hbs had higher autoxidation rates as compared to HbA [4, 444]. Autoxidation of acellular HBOCs not only increases metHb levels but also generates harmful ROS [4, 445, 446]. ROS generation by acellular HBOCs can potentially aggravate IRI and result in cell dysfunction and death [4, 447]. To counteract
the hazards presented by ROS generating acellular cross-linked Hbs, researchers have suggested the use of SOD and/or catalase conjugated glutaraldehyde polymerized Hb [4, 448-452]. Though the SOD/catalase conjugated PolyHbs were successful at mitigating oxidative tissue toxicity further experiments need to be performed to ascertain their efficacy [4].

1.4.2 Cellular HBOCs

It is hypothesized that vasoconstriction and hypertension observed upon administration of acellular HBOCs in vivo, is a direct consequence of extravasation of these molecules into the subendothelial space [453-455]. NO and CO, two vasodilators produced in the endothelium possess very high affinities for Hb as compared to O$_2$ [344]. Therefore, acellular HBOCs in the subendothelial space will readily scavenge endothelium derived NO and CO, and elicit severe vasoconstriction, systemic hypertension and oxidative tissue toxicity [344, 354, 456-461]. According to a second theory, vasoconstriction can also result from an ‘autoregulatory’ response by the SMCs constituting the blood vessel walls. In this case, the blood vessels constrict in order to counteract excessive O$_2$ delivery to the surrounding tissues by acellular HBOCs [149, 350, 462].

The side-effects associated with the use of acellular HBOCs emphasized the physiological significance of compartmentalizing Hb within the cellular structure of RBCs [4]. A strategy to attenuate the aforementioned side-effects involves encapsulating Hb inside particles larger in size compared to the pores lining the inner walls of blood vessels [463, 464]. Lipid vesicles encapsulating Hb molecules or liposome encapsulated Hbs (LEHs) have evolved as a leading HBOC candidate over the last few decades owing to their high Hb loading capacity and large molecular size [362, 367, 465-469].
The large size of these vesicles prevents them from extravasating through the blood vessel wall into the surrounding space. These vesicles also provide dual protection against NO and CO scavenging by virtue of the diffusion barriers afforded by the vesicle membrane and high encapsulated Hb concentration [344, 346]. In vivo these particles have been shown to be non-vasoactive just like RBCs. This may be due to their reduced NO dioxygenation rate constants and comparable oxygen off-loading rates [344, 470]. The idea behind the development of LEHs was to design a HBOC in which the Hb was encapsulated in a lipid bilayer shell, thereby, mimicking the structure of RBCs [344]. In earlier studies involving LEHs, it was observed that they possessed short circulatory half-lives [471, 472], and were prone to fuse or agglomerate after several days in storage [473]. It has been shown that these issues can be averted by surface conjugating LEHs with PEG, a US Food and Drug Administration (FDA) approved hydrophilic polymer, extensively used in drug delivery systems [464, 473, 474]. It has been shown previously that PEG surface conjugated LEHs (PEG-LEHs) possess numerous advantages over RBCs, such as the absence of blood-borne pathogens and blood group antigens, longer shelf-life, and high resistance to shear stress and oxidative damage [473, 475, 476].

Another strategy to avoid the side-effects observed in acellular HBOCs is to encapsulate Hb inside polymer vesicles or polymersomes [345, 347]. Polymersomes result from the self-assembly of amphiphilic diblock co-polymers in aqueous solutions [364]. Currently, researchers are exploring the possibility of using polymersomes as a potential drug delivery vehicle [363, 364]. As compared to LEHs, polymersome encapsulated Hbs (PEHs) are much larger in size and are equipped with more mechanically robust membranes which explain their augmented mechanical stability and longer circulation half-lives in vivo [345, 365]. Additionally, amphiphilic diblock co-
polymers of varying lengths/sizes offer better control over the hydrophobic membrane thickness and size of the hydrophilic PEG corona of these vesicles [365, 477, 478]. Previously, both biodegradable and non-biodegradable polymersomes have been shown to encapsulate Hb and bind O₂, and hence can act as safe and efficacious RBC substitutes [345, 347].

1.5 Scope and Outline of this Dissertation

Major menaces of IRI include apoptosis, vasoconstriction, ROS generation and cell inflammation [4]. We believe that supplying oxygenated RBCs to ischemic tissues in order to normalize perfusion and treat hemorrhagic shock will aggravate reperfusion injuries. The most common treatment for IRI, ischemic preconditioning (IPC), is not only expensive but can also cause damage to important blood vessels and target organs [220]. Therefore, we deemed it necessary to propose an economical and simple strategy to counteract IRI. The therapeutic potentials of intrinsic gaseous molecules such as CO and NO (reviewed earlier in this chapter) motivated us to exogenously supply these gases to ischemic tissues in order to circumvent reperfusion injuries [162-168, 479]. We hypothesize that, delivering these gases with proven anti-apoptotic, anti-inflammatory, and vasodilating properties to ischemic tissues prior to replenishing their O₂ supplies will alleviate reperfusion injuries significantly.

Previously, inhalation-based delivery of NO to ischemic tissues has been successfully used in treating IRI [162]. However, this approach can potentially increase metHb levels in circulation and can deliver NO systemically throughout the body. Another treatment strategy prevented sudden oxygenation of ischemic tissues, which was accomplished via transfusion of CO saturated RBCs to reduce IRI [168]. Therefore,
RBCs seem like a logical carrier for in vivo delivery of NO and CO. However, in order to circumvent potential issues associated with RBC transfusion (i.e. short storage shelf life (42 days), limited supply and possibility of transmission of yet to be identified pathogens), hemoglobin (Hb)-based O₂ carriers (HBOCs) have been used to deliver exogenous CO to ischemic tissues in rat models [280, 298]. Contemporary research has shown that HBOCs can be used as viable and effective RBC substitutes [51, 326, 357, 362-365, 367, 412, 436, 465-468, 471, 477, 480-486]. HBOCs offer major advantages over RBCs such as universal compatibility, no supply shortages, longer shelf-lives, pathogen-free, comparable oxygen affinities, devoid of side-effects, and can often be stored at room temperatures [50, 51, 302, 330, 332-335, 337-339, 487-491]. Additionally, researchers have suggested the use of HBOCs in reperfusion of ischemic tissues in view of their high O₂ affinities which allow them to deliver O₂ at moderate rates to ischemic tissues and thereby circumvent oxidative tissue toxicity [4, 492-494]. Further, attractive properties of HBOCs such as low viscosity and smaller size (as compared to RBCs) are expected to improve microcirculation upon reperfusion [4]. Indeed, contemporary research has shown that the use of HBOCs to deliver O₂ to ischemic tissues have been successful in reducing infarct size and improving myocardial oxygenation [4, 495-498]. The promising attributes of HBOCs motivated us to investigate these RBC substitutes as potential carriers of beneficial gaseous molecules to ischemic tissues. Thus, in this study, we hypothesize that HBOCs can be used to target delivery of potent gaseous ligands such as CO and NO to ischemic tissues in order to treat IRIs encountered during resuscitation from hemorrhagic shock.

Hb-based carriers were synthesized, characterized, and loaded with gaseous ligands (CO and NO) using techniques developed in our lab. We hypothesize that initially these blood substitutes will supply therapeutic gaseous molecules to ischemic...
tissues in order to mitigate reperfusion injuries such as tissue inflammation, cell apoptosis and cell proliferation. Transfusion of these particles will also help in restoring blood volume and shear stress, thereby promoting increased endothelial NO synthesis [168, 499-501]. After the beneficial gaseous ligands dissociate, these Hb-based carriers are expected to store and transport O₂. Thus the Hb-based carriers will perform multiple functions at different stages of the proposed treatment.

This dissertation presents economically feasible, large-scale methods for the production of Hb-based ligand carriers. The methods described in this study have been developed in our lab and do not involve the use of expensive machinery. Moreover, the methods described herein can be easily scaled to produce industrial sized batches. Human Hb for encapsulation inside lipid/polymer vesicles and bovine Hb for polymerization were purified and characterized by techniques developed by Palmer et al. [502]. This method produces ultra-pure HPLC grade Hb [502-504]. Polymerized bHb were synthesized by methods developed in our lab by Zhou and Palmer [51]; whereas the cellular HBOCs were produced by methods outlined by Rameez and Palmer [50]. Once synthesized these HBOCs were characterized in terms of their O₂-equilibria (i.e. P₅₀ and cooperativity coefficient (n)), size, Hb encapsulation, kinetics of gaseous ligand binding/release, and metHb levels. The Hb-based carriers were then loaded with gaseous ligands using degassing and gassing techniques developed in our lab. UV Vis spectroscopy and spectral deconvolution techniques were then used to confirm the ligand bound state of these HBOCs. The proposed research is both compelling and innovative as it attempts to develop a novel therapeutic to reduce/eliminate IRI by transfusion of HBOCs saturated with beneficial gaseous ligands. Therefore, state-of-the-art biophysical techniques were used to develop a safe and efficacious RBC substitute.
1.5.1 Outline of this dissertation

Initially, we decided to investigate cellular HBOCs as potential ligand carriers due to their known advantages over acellular HBOCs such as presence of protective membrane, long-term storage stability, larger size, and longer circulation times. Furthermore, in view of their potential advantages such as more robust membrane, larger size, and tunable membrane thicknesses, we explored polymersome encapsulated Hbs (PEHs) in chapter 2. In this study, biocompatible and biodegradable amphiphilic diblock copolymer poly(ethylene oxide-\(b\)-\(\varepsilon\)-caprolactone) (PEO-\(b\)-\(\varepsilon\)-PCL) of two different MWs were used to produce PEHs. Post synthesis, these potential ligand carriers were subjected to an array of biophysical characterizations such as \(O_2\)-PEH equilibria, concentration of Hb in PEH dispersions, metHb level inside PEHs, and gaseous ligand binding/release kinetics. Unfortunately, we observed that, though the PEHs had large size and retarded NO scavenging significantly, they encapsulated negligible amounts of Hb. Attempts to increase intra-PEH Hb concentrations by altering fabrication techniques and by using different MW polymers were largely unsuccessful.

In chapter 3, as the next logical step in our quest to synthesize Hb-based carriers capable of delivering therapeutic gaseous ligands to ischemic tissues we proposed to fabricate poly(ethylene glycol) (PEG) surface conjugated LEH (i.e. PEG-LEH) particles in order to improve upon the low Hb encapsulations observed with PEHs. Lipids distearoyl-phosphatidylcholine (DSPC), cholesterol, and poly(ethylene glycol)\(_{5000}\)-distearoyl-phosphatidylethanolamine (PEG\(_{5000}\)-DSPE) were used to formulate PEG-LEHs using techniques developed in our lab [50]. Analogous to PEHs, the PEG-LEHs were also subjected to thorough biophysical characterizations. Interestingly, though PEG-LEHs had much smaller diameters as compared to PEHs, the Hb encapsulations within the
lipid vesicles were almost 60 folds higher than within polymersomes. In fact, Hb concentrations of the PEG-LEH suspensions were comparable to that of whole blood, and Hb concentrations inside PEG-LEH particles were significantly higher than in RBCs. However, though we successfully synthesized PEG-LEH particles encapsulating HbCO, we were unable to synthesize PEG-LEHs encapsulating 100% HbNO. We believe that complete nitrosylation of Hb encapsulated within PEG-LEHs was thwarted by diffusion barriers, which prevented the penetration of NO to the core of the PEG-LEH particles.

In chapter 4, we explore glutaraldehyde crosslinked polymerized bovine Hb (polybHb) molecules as an alternate strategy to develop HBOCs capable of delivering beneficial NO gas to ischemic tissues. We reasoned that the absence of protective membranes will enable us to synthesize NO loaded PolyHb molecules. Bovine Hb was polymerized, separately in both deoxygenated and oxygenated states to yield tense (T) and relaxed (R) state PolybHb solutions respectively, by methods developed in our lab [51]. Similar to cellular HBOCs, these acellular HBOCs were also subjected to comprehensive biophysical characterizations. UV-Vis spectroscopy and spectral deconvolution studies confirmed the synthesis of over 99% pure CO and NO loaded T- and R-state PolybHb solutions. Our results have led us to believe that CO and NO loaded PolybHb molecules can potentially deliver these therapeutic gases to ischemic tissues upon introduction to systemic circulation.

Finally, chapter 5 outlines our suggestions and future plans for further development of these Hb-based ligand carriers designed to mitigate IRI. Altogether, the novel therapeutic proposed in this study can be an effective and economical alternative for current IRI treatment strategies.
2.1 Introduction

2.1.1 HBOCs as a treatment strategy for IRI

Stagnation or blockage of arterial blood flow to an organ or tissue for an extended period leads to ischemia. Ischemia is characterized by limited oxygen (O$_2$) transport to tissues coupled with an accumulation of tissue excretory products, such as carbon dioxide (CO$_2$), lactate, nicotinamide adenine dinucleotide (NADH), long chain acyl-coenzyme A, K$^+$ and H$^+$ ions [75-80]. Ischemia leads to rapid loss in cellular functionality and eventually causes cell death via necrosis. Therefore in order to treat ischemia, RBCs or plasma expanders are traditionally used to restore the blood volume and tissue oxygenation. This normalization of blood flow is referred as reperfusion [95]. Counterintuitively, reperfusion itself induces cell apoptosis, tissue inflammation, infarct formation and has been shown to elicit multiple organ dysfunction by activating cytotoxic injury processes such as reactive O$_2$ species (ROS) generation, neutrophil-endothelium interactions, and hypercontracture (i.e. intracellular overloading of cardiomyocytes with Ca$^{2+}$ ions) [46, 95, 96]. Reperfusion injury plays a critical role in aggravating patient conditions in clinical scenarios such as acute myocardial infarction, resuscitation from hemorrhagic shock, and organ transplants [4, 168, 169, 266, 505, 506]. Abundant ROS
and reactive nitrogen species (RNS) generated during reperfusion of ischemic tissues can cause further damage to cellular lipids and proteins in the post-ischemic phase [4]. The collective set of side-effects that occur during the ischemic and reperfusion phases is classified as ischemia reperfusion injury (IRI).

Previously, inhalation-based delivery of NO to ischemic tissues has been successfully used in treating IRI [162, 266]. However, this approach increases the methemoglobin (metHb) level in the blood and delivers the NO systemically throughout the body. Another treatment strategy prevents sudden oxygenation of ischemic tissues, which is accomplished via transfusion of CO saturated RBCs to reduce IRI [168]. Therefore, RBCs seem like a logical carrier for in vivo delivery of NO and CO. However, in order to circumvent potential issues associated with RBC transfusion (i.e. short storage shelf life (42 days), limited supply and possibility of transmission of yet to be identified pathogens), hemoglobin (Hb)-based O₂ carriers (HBOCs) have been used to deliver exogenous CO to ischemic tissues in rat models [280, 298].

Though Hb is the most readily found protein in blood (12 – 15 g/dL), its release from the protective RBC environment into the blood stream has been shown to have catastrophic effects [4]. The toxicity manifested by the presence of cell-free Hb in the circulation include Hb autoxidation and subsequent metHb formation, unfolding of the globin chain leading to the release of cytotoxic free-heme, renal toxicity, dissociation of tetrameric Hb into αβ dimers, and NO scavenging leading to vasoconstriction and systemic hypertension [351, 432, 446, 456, 457]. In general, the toxicity of free Hb is further amplified if it extravasates through the blood vessel wall into surrounding tissues. In this scenario, the Hb generated ROS, RNS and free heme are in direct contact with tissues which can oxidize cellular lipids, proteins and nucleic acids and eventually lead to cell damage and dysfunction [46, 47].
To avert the side-effects associated with cell-free Hb transfusions, researchers have synthesized chemically modified acellular HBOCs in order to increase HBOC size/molecular mass and thereby prevent their extravasation into tissue space. However, clinical studies on these chemically altered acellular HBOCs such as polymerized Hb, intra-molecularly crosslinked Hb, and polymer-conjugated Hb evoked vasoconstriction and hypertension \textit{in vivo}, suggesting HBOC extravasation into the tissue space [356, 359, 507]. The side-effects associated with the use of acellular HBOCs emphasized the physiological significance of compartmentalizing Hb within the cellular structure of RBCs [4]. Thus, a viable strategy to attenuate the aforementioned side-effects involves encapsulating Hb inside particles (Hb vesicles or HbVs) larger in size compared to the pores lining the inner walls of blood vessels and thereby mimic RBC functionalities [463, 464].

\subsection*{2.1.2 Advantages of Cellular HBOCs}

Liposome encapsulated Hb (LEHs) have evolved as a leading cellular HBOC candidate over the last few decades owing to their high Hb loading capacity and large molecular size [362, 367, 465-469]. The large size of these vesicles prevents them from extravasating through the blood vessel wall into the surrounding tissue space [50]. Hunt et al. synthesized LEHs by encapsulating Hb in the aqueous core of lipid vesicles, thereby, mimicking the structure of RBCs [50, 508]. In earlier studies involving LEHs, it was observed that they possessed short circulatory half-lives [471, 472], and were prone to fuse or agglomerate after several days in storage [473]. It has been shown that these issues can be averted by surface conjugating LEHs with poly(ethylene glycol) (PEG), a US Food and Drug Administration (FDA) approved hydrophilic polymer, extensively used in drug delivery systems [464, 473, 474]. It has been also shown that PEG surface
conjugated LEHs (PEG-LEHs) possess numerous advantages over RBCs, such as the absence of blood-borne pathogens and blood group antigens, longer shelf-life, and high resistance to shear stress and oxidative damage [473, 475, 476]. Surface conjugation of biocompatible PEG molecules onto LEH particles were carried out in order to recreate the hydration layer offered by the carbohydrates found on RBC membranes [50, 362, 464, 469, 473]. Surface PEGylation of LEHs with ~ 7 – 10 mol% PEG was successful in extending the circulation half-life of LEHs from 4 h to 12 h by delaying their eventual capture by the reticuloendothelial system (RES) resulting from their “reduced recognition by the circulating opsonins” [50, 473, 509-514]. Attempts to improve upon the circulation half-life of LEHs, by facilitating the crowding of PEG lipids conjugated to the outer surface of liposomes, were unsuccessful due to steric hindrance between adjacent PEG molecules at PEG concentrations above 11 mol% [50, 515]. In efforts to increase the circulation half-life of PEG-LEHs, researchers attempted to circumvent the PEG lipid overcrowding and steric hindrance related LEH stability issue by increasing the MW of the conjugated PEG lipids [469]. However, this approach was unsuccessful as it led to the formation of micelles [50, 515, 516]. This can be explained by the fact that the increase in the MW of PEG lipid resulted from an increase in the hydrophilic chain length only, the size of hydrophobic head group did not change significantly. It was also suggested that a high MW, long chain PEG molecule once conjugated onto the LEH outer surface, will restrict the approach of other PEG molecules and will thereby reduce overall PEGylation [515, 516]. Therefore, as noted by Rameez, optimizations of the circulation half-life of PEG-LEHs are plagued by problems originating from the various structural inhibitions of the PEG lipids [50]. The circulation half-life of HbV ranges from 48 h to a few days and is significantly shorter when compared to RBCs [50, 469, 482].
Thus, a considerable infusion of PEG-LEHs can potentially overburden the RES and cause toxicity due to heme degradation and iron overloading of tissues [482].

Another potential disadvantage of using PEG-LEHs as carriers of beneficial gaseous ligands to ischemic tissues arise from the curbed mechanical strength of these vesicles owing to their moderate membrane thicknesses (3 – 5 nm) [364, 478, 517]. To improve upon the mechanical stability of LEH membranes researchers have suggested the use of ‘polymerized liposomes’ [513, 518-521]. These liposomes were ‘stabilized by polymerizing unsaturated phospholipids in the bilayer membrane using combined redox type initiators and UV-irradiation’ [513, 518-521]. In other studies, liposomes were polymer coated to increase their mechanical stability without the introduction of ‘permanent covalent linkages between the hydrophobic lipid tails’ [513, 522-527]. Li et al. introduced actin matrix inside the aqueous core of lipid vesicles to amplify their mechanical stability [513]. To overcome the aforementioned side-effects of liposomes such as unsatisfactory circulation half-life and compromised mechanical stability, polymer vesicles composed of amphiphilic diblock copolymers (polymersomes) were used to encapsulate Hb [50, 328, 345, 347]. Thus, polymersomes appeared as an excellent candidate for delivering therapeutic gaseous ligands to ischemic tissues.

2.1.3 Polymersomes vs. Liposomes

Self-assembly of amphiphilic diblock copolymers, where one block is hydrophilic while the other is hydrophobic, is a thermodynamically favorable process as it reduces the total free energy of the system [364, 485, 528]. Polymersomes, resulting from the self-assembly of amphiphilic diblock copolymers, are being extensively developed as therapeutic drug carriers [328, 529-535]. Distinctive molecular properties of synthetic polymers used in polymersome synthesis such as molecular weight (MW) and polymer
chain length allow fine-tuning of carrier properties [364]. The use of polymersome encapsulated Hb (PEHs) for mitigating IRI was proposed to attenuate the side-effects associated with the transfusion of acellular HBOCs, which include short circulatory half-lives, extravasation through the blood vessel wall (owing to their comparatively smaller size), and NO scavenging leading to vasoconstriction and systemic hypertension [328, 345, 347, 351, 432, 446, 457]. Additionally, PEHs are touted to possess superior mechanical strength and circulation half-lives when compared to PEG-LEHs [50, 345].

Discher noted that "the polymers used for fabricating polymersomes are amphiphiles and are functionally very similar to the lipids used to synthesize liposomes [364]. Upon hydration, the polymer molecules self-assemble into polymersomes as long as a suitable hydrophilic to hydrophobic ratio is maintained [364, 516]. Analogous to lipids, the hydrophobic blocks of these copolymers, associate with each other in order to minimize contact with the hydrating solution and constitute the core of the polymersome membrane; while the hydrophilic moieties interact with the hydrating solution and form the inner and outer interfaces of the bilayer membrane [364]. The typical MWs of lipids are less than 1 kDa while the MWs of the synthetic copolymers are an order of magnitude higher (~10 kDa) [57, 364]. Amphiphiles differ substantially in their hydrophilic 'head group', however they are characterized by one or more hydrophobic chains composed of repeating ethylene units\((-\text{CH}_2 - \text{CH}_2-)_n\) \((n \sim 5 - 18)\) [364, 536]. Self-assembly of amphiphiles is a thermodynamically controlled process and aggregate stability is characterized in terms of \(n\) and its relationship with the critical micelle concentration (CMC) as shown in the following equation [364, 536].

\[
\text{CMC} \sim \exp\left(\frac{-n\varepsilon_R}{k_BT}\right)
\]
Here $\varepsilon_h$ is the interaction energy of the monomer with the aqueous solution, and $k_b T$ ($k_b$ is Boltzmann’s constant and $T$ is absolute temperature) is the thermal energy [364, 536]. It is well-known that vesicles form at surfactant concentrations $>\text{CMC}$, and at physiological temperatures for ethylene groups, $\varepsilon_h$ ranges from $1 - 2 k_b T_{\text{physiological}}$ (~$4 - 8$ pN nm) [364, 536]. Since polymer-based amphiphiles have larger hydrophobic MWs (i.e., have higher $n$) as compared to lipids, $[\text{CMC}]_{\text{lipids}} \gg [\text{CMC}]_{\text{polymers}}$ [364, 536]. Thus, upon interaction with an aqueous solution, the amphiphilic diblock copolymers potentially form very stable structures as opposed to lipids [364, 536]. This theory is further supported by the observation that inter-aggregate amphiphile exchange rates are directly proportional to $C_{\text{CMC}}$ [364].

Mechanical strength of vesicles can be correlated to their bilayer membrane thickness [364, 517, 529]. Liposome membrane thickness has been reported to be pretty consistent, ranging between $3 - 5$ nm [537, 538]. In comparison, experimental evidence shows that polymersome membrane core thickness ($d$) increases from 8 to 21 nm with increasing MWs of the copolymer hydrophobic block (2000 – 20000 kDa) [477, 528, 537]. Previous studies have established a mathematical correspondence between polymersome membrane thickness ($d$) and MW of the hydrophobic moiety of the copolymer ($MW_h$) using experimental techniques and molecular dynamics simulations as shown below [364, 539].

$$d \sim MW_h^{0.55}$$

It has also been shown that the lengths of the PEG chains (equivalent to PEO) attached on the polymersome surface can be tailored from 1.2 to 30 kDa by varying the MW of the copolymer hydrophilic moiety [516, 537]. Longer PEG chains on the polymersome
surface imply longer intravascular circulation half-lives and a superior degree of biocompatibility [364, 365, 478, 485, 516, 517, 528, 529, 537, 539].

Discher et al. showed that polymersomes, having membrane thicknesses ~ 8 nm, fabricated using poly (ethylene oxide-\textit{b}-\textit{ε}-ethylethylene) (PEO-PEE) were an order of magnitude tougher and roughly 10 times less permeable to water, as opposed to liposomes [50, 477, 485]. The thick polymersome membranes greatly impede transmembrane exchange of water molecules unlike the water permeable phospholipid membranes [364, 540]. Additionally, lateral diffusivity [541] and apparent membrane viscosity measurements [542, 543] have confirmed that vesicle membrane fluidity is inversely proportional to MW of the monomers [364]. Thus, membrane solidity of polymer vesicles is considerably more than lipid vesicles due to the comparatively higher MWs of the copolymer monomers [364]. The superior mechanical strength of polymersomes (in comparison with liposomes) was further illustrated by measuring bending rigidities of these two types of vesicles [50]. The bending elastic constants ($K_b$) of liposomes have been reported to range from 13.3 – 21.4 $k_bT$ (where $k_b$ is Boltzmann’s constant and $T$ is absolute temperature) [50, 485, 544]; while Ahmed et al. reported that polymersomes of membrane thicknesses 8 nm and 14.8 nm had bending rigidities around 33.3 and 465.5 $k_bT$ respectively [50, 478]. Previously, it has been shown that polymer vesicles preserve their shape, maintain a homogeneous size distribution, and do not compromise their contents when stored for a month or longer in isotonic solutions (such as saline) [477, 516, 528]. However, according to Lee et al., at room temperatures polymersomes were stable for at least 5 days when suspended in blood plasma under ‘turbid conditions’ [50, 477]. Another drawback, allegedly linked to the inferior mechanical strength of liposomal membranes, is that they passively leak
vesicle contents into the bulk solution thereby causing a loss in encapsulated drug concentrations [517, 545]. Liposomes fabricated using 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were shown to exhibit thermal transition (transformations of lamellar surface and expansion of membrane surface area caused by increases in temperature) at temperatures as low as ~ 23 – 25 ºC [50, 471, 477]. Interestingly, no such thermal effects were observed in the case of polymersomes for temperatures up to 60 ºC [50, 477]. These surface transition effects, if prominent at or around the vesicle preparation/storage temperatures, can lead to leakage of vesicle encapsulated contents such as drugs or as in the case of LEHs - toxic free Hb [50]. As noted before, the release of free Hb into the circulation can cause many deleterious outcomes and can eventually lead to severe vasoconstriction and systemic hypertension [50, 351, 432, 446, 456, 457].

Taken together, the findings of these physicochemical studies suggest that while lipid membranes have evolved to be more fluid-like at the expense of stability, polymer membranes can be tuned to optimize their mechanical strength, robustness, and solidity [364].

Unlike PEGylated-liposomes it is possible to obtain 100% PEGylation for polymersomes, further; the polymersomes possess comparatively longer PEG chains [50, 485, 515]. Due to steric interferences, the long PEG chains on polymersome surfaces have been shown to adopt “mushroom-like” or “brush-like” conformations [50, 360, 546, 547]. It has been reported that these conformational changes to the PEG chains of polymersomes enable them to elude phagocytosis and suppress activation of the human complement system when transfused in vivo [50, 360, 368, 546, 547]. Therefore, polymersomes present themselves as an excellent system for delivering therapeutics such as Hb bound to benign gaseous ligands to ischemic tissues due to their abilities to circumvent the human complement system and exhibit longer circulation
Polymersomes fabricated using diblock copolymers constituting of PEG chains ~1200 – 3680 Da (vesicle diameter ~100 nm and membrane thickness ~9.6 – 14.8 nm) were shown to have twofold longer circulation half-lives as compared to similar sized PEGylated-liposomes [50, 516]. The same study further concluded that the circulatory half-life of polymersomes increased with increasing MW of the PEG chains (hydrophilic moiety) and was independent of the hydrophobic membrane thickness (essentially MW of the hydrophobic moiety), due to effective coating of the hydrophobic membrane by the dense labyrinth created by the PEG chains [50, 516]. Thus, altering the hydrophobic head group of the copolymers does not adversely affect the circulation half-lives of the polymersomes [50, 364].

Discher et al. observed accumulations of polymersomes in the liver and spleen of rats (300 – 400 g) upon administering a dose of 5 mg polymersomes; however no toxic effects were induced in these animals over a period of 8 weeks [50, 516]. Also, no vesicle-cell adhesion effects were reported by researchers when polymersomes were incubated with blood plasma at room temperature under thorough mixing for a period of atleast 5 days [50, 477, 516]. Upon incubation with granulocytes, a key circulating phagocyte, polymersomes remained inert and did not exhibit any vesicle-phagocyte adhesive interaction [477]. Furthermore, no vesicle-RBC adhesion was observed for 2 – 3 h when polymersomes were mixed with whole blood in the absence of continuous stirring [50, 516]. Polymersomes are cleared in vivo by a mechanism which involves the attachment of an opsonizing protein (such as plasma C3b) on the vesicle surface; this protein flags the vesicle for clearance by the phagocytes of the liver and spleen [50, 477]. The disallowance of phagocytic ligand binding on PEO/PEG coated particles is well-established [369, 545], and thus the lack of polymersome recognition by the phagocytes is an important factor in lengthening circulatory half-lives of these vesicles.
upon transfusion in vivo [477]. In light of these promising attributes of polymersomes and considering their plausible advantages over PEG-LEHs and acellular HBOCs, we hypothesize that PEHs can be used as safe and efficacious carriers of therapeutic gaseous ligands to counteract IRI. In this study we used a biocompatible and biodegradable diblock copolymer, poly(ethylene oxide-\textit{b}-\varepsilon-caprolactone) (PEO-\textit{b}-\varepsilon-PCL), to fabricate PEHs. The synthesized polymersomes were subjected to a wide array of biophysical characterizations such as $O_2$-PEH equilibria, concentration of Hb inside PEH particles, metHb level inside PEHs, and gaseous ligand binding/release kinetics in order to test their efficacy as RBC substitutes.

Discher et al. generalized that the hydrophilic fraction ($f$) (ratio of hydrophilic to total mass) of diblock copolymers determine their time-averaged shape such as cylinders ($f \approx 0.35 \pm 0.1$), cones ($f > 0.45$), and wedges ($f < 0.25$). These molecular shapes in turn govern the formation of polymersomes, micelles and inverted microstructures respectively [364, 539]. Different copolymers are expected to differ in their $f$ ranges [548]. Additionally, it was shown that polycaprolactone (PCL) based copolymers formed vesicles between $f$ values 0.16 – 0.23 [548, 549], thus the polymers used in the current study were chosen on the basis on their $f$ value ($\approx 0.18$). In this study polyethyleneoxide (PEO) (equivalent of PEG) was selected as the hydrophilic block because it is widely acknowledged to impart biocompatibility and longer circulation half-lives to polymer vesicles [365, 550-552]. PCL was chosen to serve as the hydrophobic membrane because it has been shown that PCL can be degraded in the body by the hydrolysis of ester linkages [550, 553-556]. Additionally, as Ghoroghchian et al. noted, “PCL has the following advantages over other biodegradable polyesters 1) high permeability to small drug molecules; 2) maintains a neutral pH upon degradation; 3)
easily blends with other polymers; and 4) slow erosion kinetics as compared to polylactide (PLA), polyglycolide (PGA), and polylactic-co-glycolic acid (PLGA)” [550, 553]. Furthermore, Ghoroghchian et al. reported that the use of biocompatible PCL as “an implantable biomaterial in various drug delivery devices, adhesion barriers, bioresorbable sutures, and as scaffolds in tissue engineering for injury repair” is well known and widespread [550, 553-556]. Therefore, the inclusion of PCL will ensure a safe and complete degradation of the resulting vesicles in vivo [550].
2.2 Materials and Methods

2.2.1 Materials

The two different polymer vesicles investigated in the current study were synthesized using two biocompatible amphiphilic diblock copolymers constructed of poly(ethylene oxide-b-ε-caprolactone) (PEO-b-ε-PCL). Table 2.1 outlines the physical properties of the two diblock copolymers purchased from Polymer Source Inc. (Dorval QC, Canada) [557]. The chemical structure of PEO-b-ε-PCL, obtained from the company website is shown in Figure 2.1.

2.2.2 Purification of Human Hb from RBCs

Human Hb for encapsulation inside PEHs were purified and characterized by techniques developed by Palmer et al. [502]. Expired leuko-reduced human RBCs were procured from the American Red Cross (Columbus, OH). 4 bags of RBCs were pooled to a total volume of 2L and washed by centrifugation. The RBCs were packed by centrifuging them for 1 h at 3716g at 4ºC. The supernatant was discarded and the packed cells were further washed 3 times with 0.9% (w/v) isotonic saline solution (Fisher Scientific, Pittsburgh, PA) to remove cell free-Hb and other plasma proteins. The washed RBCs were eventually lysed overnight at 4ºC using phosphate buffer (PB) (3.75mM, pH 7.4). To obtain HPLC grade Hb, the RBC lysate was passed through a glass column packed with glass wool followed by filtration through a series of hollow fiber ultrafiltration (HFUF) cartridges; 50nm, 500kDa, and 100kDa (MWCO) (Spectrum Labs, Rancho Dominguez, CA); to remove shed RBC membrane, cell debris and other unwanted cellular proteins. The final solution was concentrated to [Hb] > 350 mg/mL on the 100 kDa hollow fiber cartridge (Spectrum labs) [502]. Hb concentration was determined using the Winterbourne Equation as described elsewhere [558].
Table 2.1: Number-averaged MW ($M_n$), polydispersity index (PDI), PEG length, hydrophilic mass fraction ($f_{\text{hydrophilic}}$), and vesicle membrane thickness ($d$) of the PEO-$b$-$\varepsilon$-PCL copolymers used in this study [557].

<table>
<thead>
<tr>
<th>Diblock Copolymer</th>
<th>$M_n$ (Da)</th>
<th>$M_w/M_n$ (PDI)</th>
<th>PEG length (Da)</th>
<th>$f_{\text{hydrophilic}}$</th>
<th>$d$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO(550)-$b$-$\varepsilon$-PCL(2500)</td>
<td>3050</td>
<td>1.13</td>
<td>550</td>
<td>0.18</td>
<td>7.1</td>
</tr>
<tr>
<td>PEO(2000)-$b$-$\varepsilon$-PCL(9000)</td>
<td>11000</td>
<td>1.3</td>
<td>2000</td>
<td>0.18</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Figure 2.1: General chemical structure of PEO-$b$-$\varepsilon$-PCL obtained from the website of Polymer Source Inc. (Dorval QC, Canada) [557].

2.2.3 PEH Synthesis

Prior to encapsulation, it was necessary to convert the Hb into its carbonyl (HbCO) form in order to ensure its oxidative stability during the PEH fabrication process (HbCO is approximately 200 times more stable than HbO$_2$ against autoxidation) [280]. Complete conversion of all Hb to HbCO was achieved by taking 100 mL concentrated Hb solution in a glass bottle and passing CO gas (99.9%) over it, in absence of light, and under gentle stirring for 4-5 hours. The complete conversion Hb to HbCO was confirmed via UV-Vis spectroscopy.

The PEH suspensions were prepared by the method of Rameez et al. [345]. Briefly, 4-5 g of PEO-$b$-$\varepsilon$-PCL was completely dissolved in 75-90 mL chloroform by
manual shaking. The chloroform was then evaporated using a rotary evaporator to yield a colorless dry polymer film. The film was vacuum-dried for an additional 48 hours followed by hydration with 80-100 mL of HbCO ([Hb] > 300 mg/mL) suspended in phosphate buffered saline (PBS, 0.1 M, pH 7.4) in a 1L round bottom flask. The polymer/Hb solution was thoroughly mixed using a shaker/incubator (Excella E25, New Brunswick Scientific, Hamburg, Germany) at 25 ºC for 14 h in a CO saturated atmosphere to further aid the formation of PEHs by self-assembly of the polymer molecules. Post hydration and mixing, a polydisperse PEH suspension (average diameter (d) ~ 2200 nm) was obtained. At this stage various size-reduction/homogenization techniques were adopted in order to optimize the Hb encapsulation efficiency of these polymer vesicles. These were as follows:

1. The PEH dispersions were incubated in a water-bath sonicator (Branson Sonifier 450, VWR Scientific, West Chester, PA) for either 30 min or 1 hr followed by further mixing of the contents of the flask at room temperature for 15-30 min. The diameter of the PEH particles was then measured using dynamic light scattering (DLS) (Malvern Instruments Ltd., Worcestershire, United Kingdom).

2. The PEH suspensions were passed through a cell-disrupter (Constant Systems, Daventry, UK) 7-8 times in batch mode with a pressure-head of 15,000 psi to form unilamellar vesicles. After each pass, the diameter of PEH particles was measured using DLS.

Following homogenization, unencapsulated Hb was separated from the PEH suspensions by diafiltration (25ºC) using a 500 kDa HFUF cartridge (Spectrum Laboratories, Rancho Dominguez, CA) using PBS (0.1 M, pH 7.4) as the diluting buffer. This process was repeated 8-9 times to ensure complete removal of cell-free Hb using a
1:10 (v/v) ratio of PEH:PBS until the filtrate appeared colorless. The filtrate from the 9th diafiltration cycle was collected and assessed via UV-vis spectroscopy to verify the complete removal of cell-free Hb. The remaining PEH suspension was further concentrated using a mini 500 kDa HFUF cartridge (Spectrum Labs, Rancho Dominguez, CA). Sterile lab supplies were used for all experiments. All tubing, glassware, and filters were decontaminated by immersing them overnight in 1 M NaOH and then rinsing them thoroughly with double distilled de-ionized water.

2.2.4 PEH Size Distribution

PEH diameter and size distribution were recorded at 37 ºC using a Zetasizer Nano DLS spectrometer (Malvern Instruments Ltd., Worcestershire, United Kingdom).

2.2.5 Hb and MetHb Concentration inside PEH Particles

The concentration of Hb and metHb (autoxidized Hb, in which the iron in the heme ring is in the oxidized Fe$^{3+}$ state, not capable of transporting O$_2$) in PEH dispersions were measured after lysing these vesicles with Triton X-100 as described before by Rameez and Palmer [328, 344]. Briefly, 100 - 250 μL of PEH solution was diluted with 650 - 800 μL of PBS (0.1 M, pH 7.4). This diluted solution was then heated to about 5 – 10 ºC above the phase transition temperature of PEO-PCL (61 ºC) for around 15 – 20 minutes. 100 μL of a 10% v/v solution of Trion X-100 was added to the heated vesicle solution and mixed thoroughly for 1-2 minutes. Immediately afterwards, this solution was centrifuged at 20,000g for 5 – 10 minutes. Post-centrifugation, the supernatant was collected and assayed for total released Hb concentration and percent metHb by UV-vis spectroscopy and analyzed using the Winterbourn equation [558].
2.2.6 \( \text{O}_2\)-PEH Equilibria Measurements

The CO bound Hb, encapsulated in PEH particles, were photolysed via irradiation with visible light in an \( \text{O}_2 \) saturated atmosphere. Briefly, 2-3 mL of the concentrated PEH solution was diluted with PBS (0.1 M, pH 7.4) in a 1:2 (v/v) ratio, and placed in a sealed serum bottle. A long needle was used to de-gas the solution for about 10-15 minutes. Photolysis was carried out in the presence of visible light (GE Edison SP 10° 90W) in an \( \text{O}_2 \) saturated atmosphere for 15-20 minutes. The serum bottle was placed in an ice-bath and care was taken to maintain the temperature at 4ºC throughout the process in order to minimize metHb formation. The conversion of carbonyl Hb (HbCO) to oxy-Hb (HbO\(_2\)) was verified by measuring the absorption spectra of lysed Hb from the PEH suspensions by UV-vis spectroscopy [558]. The \( \text{O}_2\)-PEH equilibrium binding curves were generated using a Hemox Analyzer (TCS Scientific Corp., New Hope, PA) at 37 ºC (physiological temperature) [85, 358, 360, 361, 559-561]. This instrument synchronously measures the oxygen saturation of Hb (expressed as a percentage) using dual-wavelength spectrophotometry, and the dissolved oxygen concentration (expressed as pO\(_2\), mm Hg) using a Clark oxygen electrode [561]. Briefly, 0.75 – 1 mL PEH solution (depending on encapsulated [Hb]) was diluted with 4 – 4.25 mL Hemox buffer in a plastic vial (TCS Scientific Corp.). Further, 20 \( \mu \)L of Additive-A, 10 \( \mu \)L of Additive-B and 10 \( \mu \)L of antifoaming agent (TCS Scientific Corp.) were added to the mixture. Excess compressed air was bubbled through the PEH solution to saturate it to a pO\(_2\) of 147 ± 1 mm Hg. This step was carried out with care for 45 – 60 min to allow for the \( \text{O}_2 \) to diffuse across the thick PEO-PCL membrane. Post saturation, the air stream was replaced by pure \( \text{N}_2 \) to deoxygenate the PEH solution. The respective absorbances of HbO\(_2\) and deoxy-Hb were recorded via dual-wavelength
spectrophotometry and were used to compute the oxygen saturation (%) of Hb encapsulated in the PEH particles. Hb O₂-saturation was plotted as function of pO₂ to yield the O₂ equilibrium curves (OECs) using a computer program (TCS Scientific Corp.). Hill equation (Eq. 2.1) was used to fit the OECs obtained for PEHs and control Hb.

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

Eq. 2.1

Where Abs is measured absorbance, A₀ and A_∞ are absorbances at 0 mm Hg and at maximum saturation respectively, P₅₀ is the partial pressure of O₂ at which 50% of the Hb present is saturated and n is the cooperative coefficient [502, 562, 563].

2.2.7 Rapid Kinetic Measurements of PEH dispersions

PEH gaseous ligand binding/release kinetics were assessed using an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer (Applied Photophysics Ltd., Surrey, United Kingdom). Prior to any stopped flow measurements, all Hb encapsulated in the PEH particles were converted to the oxy-state by the photodissociation technique described above. Rapid kinetic measurements were performed using protocols previously described by Rameez and Palmer [344-346]. For all stopped flow measurements, a control of human Hb was used to ensure the authenticity of the results.

Oxygenated PEH solutions having an overall heme concentration of 15 μM were promptly mixed with a 1.5 mg/mL sodium dithionite solution (Sigma-Aldrich, St. Louis, MO), to measure the O₂ off-loading rate. Both solutions were prepared in PBS (0.1 M, pH 7.4). The absorbance for this deoxygenation reaction was monitored at 437.5 nm at 20 °C.
CO association rates were measured by rapidly mixing deoxygenated PEH solutions (15 μM on heme basis) with different concentrations of saturated CO solution. Both deoxygenated PEH and saturated CO solutions were prepared in PBS (0.1 M, pH 7.4) in the presence of 1.5 mg/mL sodium dithionite. This reaction was also monitored at 437.5 nm at 20 ºC. CO-binding to Hb is a second order reaction and hence computing its rate constant is complicated. To simplify analysis the pseudo unimolecular assumption was made. The reaction was carried out separately using two very high concentrations of CO (232 and 464 μM) as compared to the heme concentration (15μM). The two apparent first order reaction rates obtained from these measurements were then plotted against the corresponding CO concentrations, the slope of this fit yielded the second order rate constant for CO association.

The NO dioxygenation reaction involves the conversion of ferrous oxy-Hb to ferric Hb. This is a very fast reaction (reaction rates ~ 10^7 M⁻¹s⁻¹), thus certain precautions were taken to effectively monitor this reaction. A very dilute solution of oxygenated PEH (1 μM on heme basis) was reacted with low concentrations of NO stock solution (12.5 and 25 μM). NO dioxygenation of the control (human Hb) was monitored for the shortest time scale the instrument could measure (0.0125 s). For PEH particles, the dioxygenation reaction was considerably lengthened by the trans-membrane and the intra-cellular diffusion barriers; hence a longer time scale (1-2 s) was used. The dioxygenation reactions were monitored at 420 nm at 20 ºC. Like CO association, NO dioxygenation is also a second order reaction and thus two different dilutions of the NO stock solution were used. The NO stock solution was prepared by bubbling NO gas through a deoxygenated solution of 0.1 M phosphate buffer (PB), pH 7.4 as outlined by
Rameez and Palmer [346]. PBS (0.1 M, pH 7.4) was used as the reaction buffer for all kinetic measurements.

2.3 Results and Discussion

2.3.1 PEH Size Distribution

HBOC size plays a crucial role in determining the safety and efficacy of RBC substitutes [456, 457]. The small size of commercial acellular HBOCs are largely responsible for the observed vascular side-effects when these HBOCs are administered in vivo [149]. HBOC size impacts its biodistribution, capture by the reticuloendothelial system (RES), and circulation half-lives in the blood stream [549, 564-566]. To avert these side-effects, researchers have developed HBOCs, such as PEHs and PEG-LEHs, which are larger than the size of pores lining the inner walls of the vasculature [328, 345, 347, 463, 464].

The biophysical properties of the polymersomes prepared by the various methods explored in the present study are listed in Table 2.2. The PEHs synthesized in this study had average diameters ranging from ~ 600 – 1500 nm. These sizes are larger than the ideal particle size (~ 80 – 150 nm) for optimal circulation persistence and proper biodistribution of HbVs when administered in vivo [367, 567, 568]. Circulation persistence of vesicles in the blood stream varies inversely with particle size and directly with particle surface area to volume ratio. Vesicles having diameters larger than 1 µm not only have minimal circulation persistence but can also block fine capillaries [569-571]. Thus, nanometer-scale vesicles are crucial for adequate biodistribution and prolonged circulation in the blood stream [549]. A variety of techniques such as sonication for a longer period of time, increasing no. of passes through the cell-disrupter,
and using a higher pressure-head in the cell-disrupter were unsuccessful in achieving the desired size reduction for these vesicles.

The MW of the PEO blocks of the diblock copolymers considered in this study were 550 Da and 2000 Da keeping in mind that substantial aggregations of polymersomes are predicted above a critical PEO MW of ~ 4000 Da. However aggregation of 2 to 3 polymersome particles have been reported for polymers having PEO blocks with MW as low as 600 Da. PEO is known to disturb the polymersome membrane assembly and instigate aggregation [572]. The large size of the PEH particles generated in this study can be a result of this aggregation. In future, the membrane morphology of these vesicles needs to be studied using techniques such as Transmission Electron Microscopy (TEM), optical microscopy, and fluorescence Confocal Laser Scanning Microscopy (CLSM).

Based on experimental data and molecular dynamics simulations, a mathematical correspondence between polymersome membrane thickness \((d)\) and MW of the hydrophobic moiety of the copolymer \((MW_h)\) has been proposed by Srinivas et al. [364, 539]. The membrane thicknesses of the PEHs synthesized in the current study are listed in Table 2.1, and were calculated using the correlation presented by Srinivas et al. [539]. The membrane core thicknesses for the PEHs synthesized in this study ranged from 7.1 – 19.6 nm. All in all, though the thick PEH membranes provide greater protection against NO and CO scavenging, their large size makes them unsuitable for unhindered passage through fine capillaries and effective biodistribution of these HBOCs when administered in vivo [567, 568].
### Table 2.2: Biophysical properties of PEHs

<table>
<thead>
<tr>
<th>Property</th>
<th>Sonication (30 min)</th>
<th>Sonication (1 h)</th>
<th>Cell Disrupter (15000 psi, 8 passes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
<td>PEO(550)-b-ε-PCL(2500)</td>
<td>PEO(550)-b-ε-PCL(2500)</td>
<td>PEO(550)-b-ε-PCL(2500)</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>1520.8</td>
<td>1336.5</td>
<td>603.1</td>
</tr>
<tr>
<td>[Hb] in suspension (g/dL)</td>
<td>0.22</td>
<td>0.32</td>
<td>0.21</td>
</tr>
<tr>
<td>MetHb (%)</td>
<td>0.54</td>
<td>0.80</td>
<td>0.57</td>
</tr>
<tr>
<td>P50 (mm Hg)</td>
<td>15.64</td>
<td>7.65</td>
<td>10.68</td>
</tr>
<tr>
<td>Cooperativity</td>
<td>1.48</td>
<td>2.04</td>
<td>0.84</td>
</tr>
<tr>
<td>koff, O2 (s⁻¹)</td>
<td>3.82</td>
<td>3.75</td>
<td>14.61</td>
</tr>
<tr>
<td>kon, CO (μM⁻¹s⁻¹)</td>
<td>0.16</td>
<td>0.08</td>
<td>0.11</td>
</tr>
<tr>
<td>kox, NO (μM⁻¹s⁻¹)</td>
<td>1.50</td>
<td>0.58</td>
<td>1.03</td>
</tr>
</tbody>
</table>

#### 2.3.2 Hb and MetHb Concentration inside PEH Particles

The objective of this study was to synthesize PEH dispersions with a solution [Hb] comparable to that of whole blood (15.7 g/dL for men and 13.8 g/dL for women) [573]. Thus it was critical to measure the [Hb] and % metHb inside the synthesized PEH particles. This was achieved by lysing these vesicles using Triton X-100 as noted before [328, 344, 347]. Table 2.2 compares the [Hb] inside the various polymersome vesicles prepared in this study. It is evident from the table that the polymersomes encapsulated
insignificant amounts (~ 0.23 g/dL) of Hb in comparison with RBCs (31 - 37 g/dL) [574, 575]. Efforts to improve upon the low Hb encapsulation in PEHs by modifying the fabrication technique and by using different MW polymers were largely unsuccessful. This low encapsulation can in part be explained by the two contributing factors discussed below.

Amphiphilic diblock copolymers are structurally very similar to lipids, however they have significantly larger MWs [360]. In aqueous solutions, the hydrophobic moieties of these copolymers get together forming the core of the polymersome membrane while the hydrophilic blocks interact with the hydrating solution and form the inner and outer interfaces of the bilayer membrane. The high MW hydrophobic moieties of the diblock copolymers used in this study led to the formation of PEHs with thick membranes (7 – 19 nm) which resulted in a loss of vesicle core volume available for Hb encapsulation. The polymersome core volume was further compromised by the hydrophilic PEO chains constituting the inner face of the bilayer membrane. Thus, the benefits of additional mechanical stability afforded by the thick PEH membranes are negated by the greatly reduced vesicle core volume; this severely affects the Hb encapsulating capacities of PEHs. To counteract this problem Rameez et al. has suggested the use of amphiphilic triblock copolymers to synthesize PEHs with asymmetric membranes [345, 576]. The outer leaflet of the asymmetric membrane will be constructed using an amphiphilic copolymer with long PEO chains, while the inner leaflet will constitute an amphiphilic copolymer with short PEO chains [345, 576]. This will allow for a substantial increase in the polymersome core volume without compromising the beneficial effects brought about by the PEO chains attached to the outer surface of the vesicle [345].
The isoelectric point (pI) of human Hb is ~ 7.2 [577], and in this study PBS (0.1 M, pH 7.4) was used as the dispersion buffer. Thus, the Hb acquired a slight negative charge at pH 7.4. It is well-known that the PEO blocks of the polymersome bilayer membrane are also negatively charged [578]. Therefore, Hb encapsulations in these vesicles are further hindered by the electrostatic repulsions between the negatively charged bilayer membrane and Hb [50].

**Table 2.2** also reports the metHb levels of the PEHs synthesized in this study. Prior to PEH fabrication the % metHb of the starting material (stock HbCO solution) was also measured by UV-vis spectroscopy and analyzed using the Winterbourn equation [558]. The metHb levels of the stock HbCO solution were below detectable limits whereas the metHb levels of the PEH dispersions ranged from 0.54 – 1.06 %. 3 of the 4 formulations explored in this study yielded PEHs with metHb levels <1% (normal metHb % in RBCs *in vivo* [579, 580]). Thus, we conclude that a considerable protection against autoxidation of Hb and formation of metHb was provided by the thick polymersome membrane. Additionally, as opposed to lipid vesicles, polymer vesicles do not facilitate Hb autoxidation, because the Hb molecules in polymersomes are intercalated in the PEG chains which do not partake in any oxidative reaction [360, 361, 581]. Further, reducing agents such as ascorbate can be co-encapsulated with Hb to synthesize polymersomes with augmented resistance to Hb autoxidation. Recent studies have highlighted the role of ascorbic acid/ascorbate in suppressing Hb autoxidation *in vivo* [353, 582].

### 2.3.3 O₂-PEH Equilibria Measurements

The O₂ equilibrium data were fit to the Hill equation (Eq. 2.1) to determine the $P_{50}$ and cooperativity coefficient ($n$) as noted in section 2.2.6. **Table 2.2** lists the $P_{50}$ and $n$ for
the PEH dispersions synthesized in this study. The $P_{50}$ values for the PEHs ranged from 8 – 16 mm Hg which are significantly lower than the values obtained for RBCs ($p<0.05$). However, these values are comparable to those of cell-free Hb ($P_{50} \sim 11 – 13$ mm Hg) [502].

Sakai et al. has previously shown that in ischemic tissues, where $pO_2 < 10$ mm Hg, phospholipid vesicles with low $P_{50}$ ($\sim 8$ mm Hg) were the primary source of $O_2$, surpassing RBCs [481]. The small size of these vesicles ($\sim 250$ nm) allow them to perfuse through partially blocked capillaries which otherwise obstruct the passage of much larger sized RBCs ($\sim 8000$ nm) [492]. Moreover, the local $pO_2$ levels are greatly reduced as blood flow through these vessels are extremely slow. RBCs with higher $P_{50}$ (lower oxygen affinity) release most of their $O_2$ in transit, before reaching the targeted ischemic tissues [481, 494, 583]. Thus, the low $P_{50}$ of the polymersomes synthesized in this study makes them ideal for oxygenation of ischemic targets [481, 584].

The absence of 2, 3-diphosphoglycerate (2, 3-DPG), an important allosteric modulator of $O_2$ binding to Hb [62], can explain the observed low $P_{50}$ values for the PEHs encapsulating human Hb [50]. 2, 3-DPG is removed during purification and processing of the Hb used in this study [502]. $P_{50}$ values of the PEHs can be controlled by co-encapsulating 2, 3-DPG during PEH fabrication. However, 2, 3-DPG is expensive and is chemically unstable [585], thus its phosphate analogues such as pyridoxal 5’-phosphate (PLP), and inositol hexaphosphate (IHP) can be co-encapsulated to regulate the $P_{50}$ of PEHs [481, 483, 497, 585-589].

The cooperativity coefficients of the PEHs are in the range of 0.48 – 2.04 and are significantly lower than the values obtained for both RBCs and Hb ($p<0.05$). Figure 2.2 compares typical oxygen equilibria curves of RBCs, cell-free-Hb and PEH particles. The shape of the equilibrium $O_2$ binding curves obtained for the PEH dispersions are not as
prominently sigmoidal as those obtained for RBCs and cell-free Hb. This indicates that encapsulation of Hb inside PEO-PCL vesicles adversely affected the cooperative binding of O$_2$ to Hb and thus explains the low cooperativities obtained for the PEHs. This presents itself as a potential problem with regards to the efficacy of PEH particles to deliver therapeutic gaseous ligands to tissues affected by IRI.

![Oxygen Equilibrium Curve](image)

Figure 2.2: Comparison of O$_2$ equilibria of RBC, cell free-Hb and PEH particles.

### 2.3.4 Rapid Kinetic Measurements of PEH dispersions

Rapid kinetic measurements were done on the PEHs formulated in the current study to compare their ligand binding/release kinetics to that of RBCs. These rates are important to test the ability of these particles to store and transport important gaseous ligands such as CO and NO *in vivo* [590]. It is a well-known fact that the cellular structure of Hb vesicles retard gaseous ligand binding/release kinetics when compared to stroma free-Hb [344, 345, 463, 470, 591-593]. Multiple theories have been proposed to explain this retardation in kinetics but no conclusion has yet been reached [594-597]. NO dioxygenation kinetics can be directly related to the NO scavenging mechanism for the development of vasoconstriction and hypertension, and the O$_2$ dissociation
measurements can be related to the autoregulation theory for the development of vasoconstriction and hypertension. Thus, rapid kinetic measurements were deemed significant in assessing the ability of the polymersomes to elicit vasoconstriction and hypertension.

2.3.4.1 Reactions with O2

The $k_{\text{off},O_2}$ values (Table 2.2) obtained for the PEH particles formulated in this study ranged from 1.9 – 14.6 s$^{-1}$, and are not significantly different from the values obtained for RBCs ($p>0.05$). However, the $k_{\text{off},O_2}$ rates for both PEHs and RBCs are significantly lower ($p<0.05$) than the values obtained for the control, human Hb (34-41 s$^{-1}$). Figure 2.3 compares typical kinetic time courses of O$_2$ dissociation for PEHs, RBCs and cell-free Hb measured using an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer.

The $k_{\text{off},O_2}$ rates obtained for the PEHs in this study are 3 – 18 folds lower than those obtained for cell-free Hb. RBCs and PEG-conjugated liposome encapsulated Hbs (PEG-LEHs) have also shown similar retardations in their O$_2$ offloading rates when compared to cell-free Hb [345]. These observations underline the fact that encapsulation of Hb inside the aqueous core of Hb carriers such as RBCs, PEG-LEHs, and PEHs play a major role in controlling the delivery of O$_2$ and other physiological gases to the tissues. Thicker membranes of the PEHs (7 – 19 nm) as compared to RBCs (3 – 4 nm) allow them to manifest superior levels of retardation in O$_2$ offloading [50]. In fact, a decrease in O$_2$ offloading rates was observed when the membrane thickness (d) of polymersomes was increased from 7.1 nm (for PEHs prepared using PEO (550)-b-ε-PCL (2500)) to 19.6 nm (for PEHs prepared using PEO (2000)-b-ε-PCL (9000)). Therefore, increasing the hydrophobic membrane thickness of PEHs thwarts trans-membrane O$_2$ diffusion,
thereby slowing down $O_2$ release from the encapsulated Hb. High offloading rates of $O_2$ from Hb forms the basis of the ‘autoregulation theory’ of development of vasoconstriction and systemic hypertension [598-600]. According to the autoregulation theory an increase in $O_2$ delivery by diffusion, decreases $O_2$ acceptance by tissues because of vasoconstriction [149, 462]. Thus, moderate $O_2$ release rates are critical in determining HBOC efficacy. The PEHs synthesized in this study can potentially deliver $O_2$ to ischemic tissues at regulated rates avoiding vasoconstriction due to oversupply of $O_2$.

Figure 2.3: Time courses for deoxygenation of human (A) Hb (B) RBCs and (C) PEHs in presence of 1.5 mg/mL sodium dithionite. The experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 437.5 nm at 20 °C. PBS (0.1M, pH 7.4) was used as the reaction buffer.
2.3.4.2 Reactions with CO

Figure 2.4 shows characteristic CO association kinetic time courses for deoxygenated (A) Hb, (B) RBCs, and (C) PEHs upon reaction with CO stock solution (464 µM). Figure 2.4 (D) plots the dependence of the apparent rates on CO concentration for RBCs, stroma free-Hb and PEHs. Additionally, the $k_{\text{on,CO}}$ values obtained for the polymersomes synthesized in this study are listed in Table 2.2.

Figure 2.4: Time courses for CO (464 µM) association reaction of deoxygenated (A) Hb, (B) RBCs, and (C) PEHs. The deoxygenation was carried out in presence of 1.5 mg/mL sodium dithionite. Experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 437.5 nm at 20 °C and PBS (0.1M, pH 7.4) was used as the reaction buffer. (D) Comparison of CO association rates of human Hb, RBCs and PEHs.
The $k_{\text{on,CO}}$ values obtained for the PEHs formulated in this study varied from 0.07 – 0.16 μM$^{-1}$s$^{-1}$ and are not significantly different from the values obtained for RBCs which ranged between 0.07 – 0.15 μM$^{-1}$s$^{-1}$ ($p>0.05$). However, the CO binding rate constants for both PEHs and RBCs are significantly lower ($p<0.05$) than the values obtained for cell-free Hb (0.19 – 0.24 μM$^{-1}$s$^{-1}$). Thus, analogous to O$_2$ offloading, the CO binding to Hb is retarded by encapsulation of Hb inside the aqueous core of RBCs and polymersomes. Additionally, PEHs synthesized using PEO (2000)-$b$-$\varepsilon$-PCL (9000) yielded a lower $k_{\text{on,CO}}$ value (0.07 μM$^{-1}$s$^{-1}$) as compared to the PEHs formulated using PEO (550)-$b$-$\varepsilon$-PCL (2500) ($k_{\text{on,CO}} \sim 0.11$ μM$^{-1}$s$^{-1}$). Thus, increasing the hydrophobic membrane thickness of PEHs lowered trans-membrane CO diffusion and resulted in lower CO association rates. Though the CO dissociation rates of PEHs have not been measured in the current study, we can logically conclude that the thick PEH membranes will impede the release of CO from encapsulated Hb, as was observed in the case of O$_2$ offloading from these vesicles. Thus the PEHs can potentially deliver exogenous CO to ischemic tissues at moderate rates in order to circumvent reperfusion injury resulting from ROS generation and oxidative tissue toxicity [147, 163, 168, 479]. In the future, experiments will be designed to measure the $k_{\text{off,CO}}$ rates of PEHs in order to determine their CO offloading abilities.

2.3.4.3 Reactions with NO

**Figure 2.5** shows characteristic NO dioxygenation kinetic time courses for oxygenated (A) Hb, (B) RBCs, and (C) PEHs upon reaction with NO stock solution (25 μM). **Figure 2.5 (D)** shows dependence of the observed rates on NO concentration for Hb, RBCs and PEHs. Furthermore, the values for the NO dioxygenation rate constants ($k_{\text{ox,NO}}$) obtained for the polymersomes synthesized in this study are reported in Table
2.2. The low HbO$_2$ concentrations (~ 0.5 µM on heme basis) used in these experiments led to subtle absorbance changes; hence an average of 10-15 kinetic traces were considered for each time course in order to enhance signal-to-noise ratio.

![Graphs showing NO dioxygenation rates for Hb, RBCs, and PEHs](image)

Figure 2.5: Time courses for NO (25 µM) dioxygenation reaction of oxygenated (A) Hb, (B) RBCs, and (C) PEHs. Experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 420 nm at 20 °C and PBS (0.1M, pH 7.4) was used as the reaction buffer. (D) Comparison of NO dioxygenation rates of human Hb, RBCs and PEHs.

The NO dioxygenation rates obtained for the PEHs (0.42 – 1.50 µM$^{-1}$s$^{-1}$) were significantly higher (p<0.05) than those obtained for RBCs (0.04 – 1.12 µM$^{-1}$s$^{-1}$). However, the $k_{ox,NO}$ values for both PEHs and RBCs were significantly lower (p<0.05)
than the values obtained for cell-free Hb (24.89 – 50.42 μM⁻¹s⁻¹). The NO dioxygenation rate constants, obtained in this study, for cell-free Hb are comparable to the values reported in the literature (20 – 34 μM⁻¹s⁻¹) [50, 345, 601, 602]. Approximately, 650 fold reduction in NO dioxygenation rates of RBCs as compared to cell-free Hb has been reported in the literature [50, 603]. In the present study, a similar retardation (50 – 625 folds) was observed for RBCs. The intracellular diffusion barrier afforded by the large RBC size and high intra-RBC Hb encapsulation promotes this retardation in NO binding [463]. Rameez et al. reported a 19 fold reduction (as compared to cell-free Hb) in NO binding rates for PEHs formulated using poly (butadiene-b-ethylene oxide) copolymer. Interestingly, compared to cell-free Hb, a 39 fold reduction in NO dioxygenation rate constants was observed for PEHs synthesized in this study. Moreover, the $k_{\text{ox,NO}}$ values of the PEHs are 19 – 21 folds lower than acellular HBOCs, and 10 folds less than the values reported for PEG-LEHs in the literature [366, 436, 604]. Increasing the hydrophobic membrane thickness of polymersomes led to a considerable decrease in NO binding rates. The $k_{\text{ox,NO}}$ values decreased from 1.03 μM⁻¹s⁻¹ for PEHs prepared using PEO (550)-b-ε-PCL (2500) to 0.42 μM⁻¹s⁻¹ for PEHs synthesized using PEO (2000)-b-ε-PCL (9000)). Thus, analogous to $O_2$ dissociation and CO association, the hydrophobic membrane thickness of PEHs has a noticeable effect in reducing trans-membrane NO diffusion and thereby suppressing NO scavenging by these vesicles. In fact, the thick hydrophobic membranes are the main reason for the superior reduction in NO dioxygenation rates observed in the case of PEHs as compared to acellular HBOCs. Since the polymersomes encapsulated low quantities of Hb, the resistance to NO diffusion by intra-PEH Hb concentration is less pronounced in these vesicles. Overall, the particles synthesized in the current study are likely to induce very little NO
scavenging mediated vasoconstriction and hypertension once administered in vivo, owing to their thick hydrophobic membranes (d ~ 7 – 19 nm). This property makes PEHs a viable candidate for the delivery of therapeutic gaseous ligands to ischemic tissues in order to avoid reperfusion injury.

Monitoring the reaction kinetics of cell-free Hb, RBCs and PEHs with physiologically relevant gaseous ligands (O₂, CO and NO) provided useful discernments regarding the roles of the trans-membrane and the intracellular diffusion barriers in regulating the transport of gaseous ligands in circulation. These kinetic experiments were deemed important in order to test the efficacy of PEHs as potential carriers of therapeutic gaseous ligands such as CO and NO. The results from these rapid kinetic measurements showed that when administered in vivo, the PEH particles can potentially deliver O₂ to ischemic tissues at regulated rates, thereby averting vasoconstriction due to oversupply of O₂. The PEHs can also deliver benign CO to ischemic tissues at controlled rates in order to circumvent reperfusion injury resulting from ROS generation and oxidative tissue toxicity. Additionally, low NO binding rates of these vesicles enable them to limit scavenging of endothelial NO, thus repressing NO scavenging mediated vasoconstriction and hypertension. The results of the current work further showed that the PEHs significantly retarded ligand binding/release kinetics (when compared to cell-free Hb), a characteristic comparable to RBCs. Moreover, tuning the hydrophobic membrane thicknesses of PEHs by appropriate selection of copolymers can regulate their O₂ offloading, CO association and NO dioxygenation rates. It is possible to control the gaseous ligand binding/release kinetics of these vesicles; this can potentially counteract the side-effects associated with acellular HBOCs. Therefore, from a kinetic
perspective, the polymersomes synthesized in this study seem suitable candidates for delivering therapeutic gaseous ligands to mitigate ischemia reperfusion injury.

2.4 Conclusions

In the current study, we used biocompatible and biodegradable diblock copolymer, (PEO-b-ε-PCL) of two different MWs to fabricate PEHs. In order to test the efficacy of these polymer vesicles in transporting therapeutic gaseous ligands to ischemic tissues, we subjected these vesicles to a series of biophysical characterizations such as O₂–PEH equilibria, concentration of Hb in PEH dispersions, metHb level inside PEHs, and gaseous ligand binding/release kinetics. Our results indicate that PEHs despite having clear advantages such as large size and low O₂ off-loading, CO association, and NO dioxygenation rates, encapsulated negligible amounts of Hb. Attempts to increase Hb encapsulation in PEHs by altering the fabrication technique and by using different MW polymers were not successful. As a next step in our quest to formulate HBOCs to deliver therapeutic gaseous ligands to ischemic tissues we proposed to fabricate PEG-LEH particles in order to improve upon the low Hb encapsulations observed with the PEHs. However, to our knowledge this is the first study which has explored the possibility of encapsulating Hb inside vesicles fabricated using PEO-b-ε-PCL.
CHAPTER 3  Liposome Encapsulated Hemoglobin (LEHs)

3.1 Introduction

3.1.1 Why Liposomes?

In order to prevent the onset of deleterious reperfusion injuries in ischemic tissues this study aimed at synthesizing HBOCs capable of delivering therapeutic gaseous ligands such as CO and NO to ischemic tissues. We hypothesized that these HBOCs loaded with gaseous ligands of known anti-apoptotic, anti-inflammatory, and vasorelaxing properties [162-168, 479] once transfused in vivo will thwart the emergence of reperfusion injuries in ischemic tissues by reducing tissue inflammation, cell apoptosis and cell proliferation. Transfusion of these particles will also help in restoring blood volume and shear stress, thereby promoting increased endothelial NO synthesis. After the beneficial gaseous ligands dissociate from the HBOCs, the HBOCs are expected to store and transport O₂. Thus the HBOCs will perform multiple functions at different stages of the proposed treatment.

Contemporary research has shown that side-effects such as short circulatory half-lives, extravasation through the pores lining the inner walls of the vasculature, and NO scavenging leading to vasoconstriction and systemic hypertension observed in the
case of acellular HBOCs were alleviated by encapsulating Hb inside vesicular structures [323, 324, 330, 345, 490, 605-607]. The side-effects associated with the use of acellular HBOCs emphasized the physiological significance of compartmentalizing Hb within the cellular structure of RBCs. Thus, a viable strategy to attenuate the aforementioned side-effects involves encapsulating Hb inside particles (Hb vesicles or HbVs) larger in size compared to the pores lining the inner walls of blood vessels [4, 463, 464]. The large size of these vesicles prevent their extravasation through the walls of blood vessels and the robustness of the vesicle membrane promotes the stability of these particles in vivo [4, 365, 473, 476, 482, 608]. It is for these beneficial properties (large size, robust membrane, storage stability, and extended half-life); HbVs were chosen to transport pharmacologically important gaseous ligands to ischemic tissues in the current study.

Amphiphilic diblock co-polymer (poly(ethylene oxide)-poly(caprolactone), PEO-PCL) was used to fabricate polymersome encapsulated Hbs (PEHs), whereas poly(ethylene glycol) (PEG) conjugated phospholipid distearoyl-phosphatidylcholine (DSPC) in combination with cholesterol were used to prepare PEGylated liposome encapsulated Hbs (PEG-LEHs). These two types of cellular HBOCs were characterized in terms of their O2-equilibria (i.e. $P_{50}$ and cooperativity coefficient ($n$)), Hb encapsulation, kinetics of gaseous ligand binding/release, and metHb levels. PEHs, despite having clear advantages over PEG-LEHs such as larger molecular size, thicker and more robust membrane, retarded NO binding and O2 off-loading kinetics, encapsulated negligible amounts of Hb. Attempts to increase Hb encapsulation in PEHs by altering fabrication technique and by using different MW polymers were largely unsuccessful (as discussed in detail in Chapter 2). As an alternate strategy to formulate HBOCs to deliver therapeutic gaseous ligands to ischemic tissues we proposed to fabricate PEG-LEH particles in order to improve upon the low Hb encapsulations observed with the PEHs.
LEHs have evolved as leading HBOC candidates over the last few decades owing to their high Hb loading capacity and large molecular size [362, 367, 465-469]. These vesicles provide dual protection against NO and CO scavenging by virtue of the diffusion barriers afforded by the vesicle membrane and high intracellular Hb concentration [344, 346]. *In vivo* these particles have been shown to be non-vasoactive just like RBCs. This may be due to their reduced NO dioxygenation rate constants and comparable oxygen off-loading rates [344, 470]. The idea behind the development of LEHs was to design a HBOC in which the Hb was encapsulated in a lipid bilayer shell.
thereby, mimicking the structure of a RBC [344]. A schematic representation of the PEG-LEH structure is presented in Figure 3.1.

3.1.2 Structural components of PEG-LEH particles

3.1.2.1 Poly (ethylene glycol) - PEG

Earlier LEH preparations were plagued with drawbacks such as short circulatory half-lives [471, 472] and agglomeration issues after several days in storage [473]. These limitations were alleviated by surface conjugating LEHs with PEG, a US Food and Drug Administration (FDA) approved hydrophilic polymer, extensively used in drug delivery systems [464, 473, 474]. PEG is well-known for reducing non-specific binding of proteins and this property of PEG has led to extensive research for its use in drug delivery systems over the past 3 decades [464, 473, 474, 609-626]. However, the scientific communities share a divided opinion about PEG’s role in blocking immune responses in the body [613-616, 627-630]. PEG is a linear or branched, neutral polyether that is nontoxic and is both hydrophilic and lipophilic [627, 631]. PEG is available in a variety of molecular weights (MWs), at MWs below 1000 g/mol PEGs are viscous, colorless liquids while at higher MWs they are waxy, white solids. Generally polyols having MWs <20,000 g/mol are referred to as PEGs and those >20,000 g/mol are classified as poly (ethylene oxide) (PEO) [631]. The molecular formula of PEG is depicted below,

\[ \text{OH} - (\text{CH}_2\text{CH}_2\text{O})_n - \text{CH}_2\text{CH}_2\text{OH} \]

As noted before PEG is amphiphilic and is soluble in both water and organic solvents; however its behavior in aqueous solutions is of prime importance in explaining its lowering of non-specific protein binding and non-immunogenicity [631]. In aqueous solutions an envelope of water molecules develop around the PEG molecule owing to its
hydrophilic nature [632]. Each ethylene glycol subunit binds to 2-3 water molecules causing the PEG molecule to swell up and assume a larger size compared to other polymers in solution [627, 631, 632]. This phenomenon coupled with high mobilities of the PEG chain in solution [633] enables it to effectively exclude other polymers from its presence in an aqueous environment [631]. The lowering of protein binding by PEG can be explained by virtue of these properties; since the penetration of the water envelope by protein in order to effect PEG-protein interactions is an energetically unfavorable process. Additionally, compression of the PEG molecule upon approach of a protein molecule (steric repulsion) can further prevent PEG-protein interactions. Other reasons for low protein binding exhibited by PEG include entropic penalty on protein adhesion [634-637] and blockage of underlying adsorption sites [637, 638]. Thus, PEG/PEO thwarts protein binding to vesicle surfaces and thereby prevents clearance of HbVs by the immune system. Surface conjugation of LEHs with PEG significantly increases their circulation half-lives and alleviates immunogenicity and antigenicity [50, 473, 509-514]. It has been also shown that PEG surface conjugated LEHs (PEG-LEHs) possess numerous advantages over RBCs, such as the absence of blood-borne pathogens and blood group antigens, longer shelf-life, and high resistance to shear stress and oxidative damage [473, 475, 476].

3.1.2.2 Cholesterol

Another integral lipid used to formulate the PEG-LEH membrane is cholesterol. Cholesterol imparts rigidity to the PEG-LEH membranes and accounts for approximately 45% (on mole basis) of total lipids constituting the PEG-LEH membranes [50, 346, 362, 367, 474]. It is well-known that cholesterol helps in strengthening LEH membranes by inserting themselves between adjacent phospholipid molecules in the lipid membrane,
and thus has been used in the various PEG-LEH fabrication procedures described in the literature [50, 346, 362, 367, 474, 639]. Researchers further believe that ‘the addition of cholesterol to the lipid make-up of liposomes enhances their resistance to biophysical shocks and damages’ [50, 346, 473, 475].

3.1.2.3 Distearoyl-phosphatidylcholine – DSPC

A variety of phospholipids have been used to formulate liposomes in the past [50, 346]. In order to optimize intra-LEH Hb concentrations and boost circulatory half-lives of these vesicles, researchers fabricated LEHs using anionic phospholipids such as dimyrstoyl-phosphatidylglycerol and dipalmitoyl-phosphatidylglycerol [640, 641]. However, LEHs synthesized using anionic phospholipids manifested drawbacks such as vasoconstriction and systemic hypertension, short circulatory half-lives, and platelet count depletion [50, 640, 641]. The neutral DSPC phospholipids used in this study can potentially avert the side-effects associated with LEHs prepared using anionic phospholipids [50, 346].

Valenzuela et al. suggested the incorporation of tocopherols in order to minimize membrane lipid oxidation observed in LEHs synthesized using unsaturated phospholipids [642]. However, Rameez et al. pointed out that the problem of membrane lipid oxidation can be avoided by simply using saturated phospholipids such as DSPC to constitute LEH membranes [50, 346]. Previous studies have used dipalmitoyl-phosphatidylcholine (DPPC) as one of the major lipid components in LEH preparations [473, 643]. However, it is well known that DPPC has a phase transition temperature of 41°C which is extremely close to the core body temperature (37°C). Thus, transfusion of LEHs containing DPPC may lead to increased PEG-LEH membrane fluidity, membrane destabilization and release of free Hb into the circulation. The saturated DSPC
phospholipids used in our formulations have a much higher phase transition temperature at 55°C [346] and should not encounter these issues.

### 3.1.3 Common PEG-LEH formulation techniques

In order to match intra-RBC Hb concentration and thereby mimic the oxygen carrying capabilities of RBCs, most PEG-LEH formulation techniques described in the literature aim at synthesizing vesicles with high Hb to lipid ratios [50, 346]. To optimize Hb to lipid ratio in LEHs it is critical to reduce vesicle size/diameter and decrease the number of bilayers constituting the vesicle membrane (lamellarity) [50, 508, 568, 644-646]. Furthermore, reducing LEH diameters also prevent these vesicles from blocking fine capillaries once they are transfused in vivo [50, 346]. A wide range of techniques such as detergent dialysis, reverse phase evaporation, microfluidization, and membrane extrusion has been used in the past to achieve size reduction in LEHs [50]. However, most techniques for preparing PEG-LEHs described in the literature are complex, time-consuming and expensive. Techniques using detergent dialysis and reverse phase evaporation involve the use of detergents and organic solvents. These can prove harmful to the structural stability and activity of Hb and may denature or chemically modify the encapsulated Hb [50, 346, 471]. Membrane extrusion has been used to decrease the lamellarity of liposomes formed after hydration. However, the high rates of membrane fouling and low permeation rates of the lipid/Hb suspension through the extruder makes membrane extrusion a difficult technique to scale-up [471, 647]. The cell-disrupter used in the current study can be used to homogenize large batches of liposomes in a fast and efficient manner.

Some studies have purified Hb for encapsulation within LEHs using dichloromethane [644], while others have heat pasteurized Hb at 60°C [568]. The use of
organic solvents and heat can potentially denature Hb [50, 346]. Thus, in this study Hb was purified using tangential flow filtration, an efficient technique to yield HPLC-grade Hb without the use of heat or organic solvents [502]. As noted before, PEG has been widely used in prior studies to impart ‘stealth’ properties to LEHs, thereby delaying their capture by the reticuloendothelial system (RES) and hence prolonging their circulatory half-lives [368, 470, 481, 483, 484, 515, 644]. Most PEG-LEH formulation techniques reported in the literature yielded vesicles incorporating expensive PEG on both inner and outer surfaces of the vesicle membrane [362]. The presence of large PEG molecules on the inner surface of the bilayer membrane decreases vesicle core volume and adversely affects Hb encapsulation within PEG-LEHs. In the current study, PEG-lipid is post-inserted only onto the outer surface of the bilayer membrane after the homogenization step [50, 346]. We feel that this aids in boosting the Hb encapsulation within the PEG-LEH particles. Additionally, post-insertion avoids unnecessary PEGylation of the inner surface of the bilayer membrane and also helps in lowering the overall production cost [50, 346]. A majority of the PEG-LEH synthesis techniques reported in the literature used ultracentrifugation to concentrate the PEG-LEH suspension and remove unencapsulated free Hb [464, 470, 473, 475, 481, 483, 484, 585, 604, 607, 608, 644, 648-650]. However, these processes will be difficult to scale up and would require the use of large ultracentrifuges which will greatly increase the cost of LEH production [50, 346]. The diafiltration technique, using 500 kDa HF cartridges developed by Rameez and Palmer, adopted in the present study is easy to scale up, efficient, and a cost effective alternative [50, 346].

In summary, to improve upon the poor Hb encapsulation observed in the case of PEHs we decided to synthesize PEG-LEH dispersions with solution [Hb] comparable to that of whole blood. The PEG-LEHs were synthesized, using an efficient and cost
effective method developed by Rameez et al. [50, 346], in order to deliver therapeutic gaseous ligands to ischemic tissues.
3.2 Materials and Methods

3.2.1 Materials

Three different lipids, namely, distearoyl-phosphatidylcholine (DSPC), cholesterol, and poly(ethylene glycol)_{5000} - distearoyl-phosphatidylethanolamine (PEG_{5000}-DSPE) were used to synthesize the liposomes investigated in this study. DSPC was purchased from Avanti Polar Lipids (Alabaster, AL), while PEG_{5000}-DSPE and cholesterol were procured from Laysan Bio Inc. (Arab, AL) and Sigma-Aldrich (St. Louis, MO) respectively. The hollow fiber (HF) filter modules (part number: M1-500S-360-01S, rated pore size: 500 kDa and surface area: 1050 cm^2) used for diafiltration and separation of free-Hb from PEG-LEH suspensions were purchased from Spectrum Laboratories (Rancho Dominguez, CA). All other chemicals needed were obtained from Sigma-Aldrich (St. Louis, MO). Figure 3.2 depicts the chemical structures of DSPC (A), PEG_{5000}-DSPE (B) [651], and (C) Cholesterol [652]. Expired leuko-reduced human RBCs were procured from the American Red Cross (Columbus, OH).

3.2.2 Purification of Human Hb from RBCs

Human Hb for encapsulation inside LEHs were purified by the method of tangential flow filtration (TFF) developed by Palmer et al. [502]. The specifics of the TFF method was previously described under the subheading Purification of human Hb from RBCs in the Materials and Methods section of Chapter 2 (section 2.2.2). Hb concentration and metHb levels were determined using the Winterbourne Equation as described elsewhere [558].

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Prior to encapsulation in lipid vesicles, it was necessary to convert the Hb into its carbonyl (HbCO) form in order to ensure its oxidative stability during the LEH fabrication process (HbCO is approximately 200 times more stable than HbO₂ against autoxidation) [280]. Complete conversion of all Hb to HbCO was achieved by taking 100 mL concentrated Hb solution in a glass bottle and passing CO gas (99.3%) over it, in
absence of light, and under gentle stirring for 4-5 hours. The complete conversion Hb to HbCO was confirmed via UV-Vis spectroscopy.

PEG-LEH suspensions were prepared by the method of Rameez and Palmer [346]. Briefly, DSPC and cholesterol in 1:1 molar ratio were completely dissolved in chloroform by manual shaking. The chloroform was then evaporated using a rotary evaporator to yield a white dry lipid film. The film was vacuum-dried for an additional 48 hours followed by hydration with 100 mL of HbCO ([Hb] > 350 mg/mL) suspended in phosphate buffered saline (PBS, 0.1 M, pH 7.4) in a 1L round bottom flask. The lipid/Hb solution was thoroughly mixed at 25 °C for 14 h in a CO saturated atmosphere to further aid the formation of LEHs by self-assembly of the lipid molecules. The LEH suspension was then passed through a cell-disrupter (Constant Systems, Daventry, UK) 2-3 times in batch mode with a pressure-head of 10,000 psi to form unilamellar vesicles. After each pass, the diameter of LEH particles was measured using dynamic light scattering (DLS). This homogenization process was stopped, once the LEHs reached a diameter < 300 nm. Homogenization was followed by PEGylation. PEG_{5000}^-DSPE (1% w/v) suspended in PBS (0.1 M, pH 7.4) was added to the homogenized LEH/Hb solution. 1 mL of PEG solution was added to every 2 mL of LEH/Hb suspension to keep the PEG concentration below its critical micelle concentration (CMC). The PEGylation reaction was allowed to proceed at 55 – 60 °C for 1 hr under a CO saturated atmosphere. The solution was gently stirred to promote the insertion of the PEG-lipid onto the outer vesicle surface.

Unencapsulated Hb was separated from the PEG-LEH suspension by diafiltration (25 °C) using a 500 kDa hollow fiber ultrafiltration (HF) cartridge using PBS as the diluting buffer. This process was repeated 8-9 times to ensure complete removal of cell-free Hb using a 1:10 (v/v) ratio of PEG-LEH:PBS until the filtrate appeared colorless. The filtrate from the 9th diafiltration cycle was collected and assessed via UV-vis
spectroscopy to verify complete removal of cell-free Hb. The washed PEG-LEH suspension was further concentrated using mini 500 kDa cartridges (Spectrum Labs, Rancho Dominguez, CA). Sterile lab supplies were used for all experiments. All tubing, glassware, and filters were decontaminated by immersing them overnight in 1 M NaOH and then rinsing them thoroughly with double distilled de-ionized water.

### 3.2.4 PEG-LEH Size Distribution

An Eclipse® asymmetric flow field-flow fractionator (A4F) (Wyatt Technology Corp., Santa Barbara, CA) connected in series to an 18-angle Dawn Heleos® multi-angle static light scattering (MASLS) photometer (Wyatt Technology Corp., Santa Barbara, CA) was used to measure the size distribution of PEG-LEH particles. The MASLS photometer comes equipped with a 30 mW GaAs laser operating at a wavelength of 658 nm. ASTRA 5.3 software (Wyatt Technology Corp., Santa Barbara, CA) was used to analyze the light scattering spectra and estimate the PEG-LEH size distribution. 0.1 M PBS, pH 7.4 was used as the elution buffer. Additionally, PEG-LEH hydrodynamic diameter was recorded at 37°C using a Zetasizer Nano DLS spectrometer (Malvern Instruments Ltd., Worcestershire, United Kingdom).

### 3.2.5 Hb and MetHb Concentration inside PEG-LEH Particles

The concentration of Hb and metHb (autoxidized Hb, in which the iron in the heme ring is in the oxidized Fe$^{3+}$ state, not capable of transporting $O_2$) in PEG-LEH dispersions were measured after lysing these vesicles with Triton X-100 as described before by Rameez and Palmer [328, 344]. Briefly, about 100 μL of PEG-LEH solution was diluted with 800 μL of PBS. This diluted solution was then heated to about 5 – 10 °C above the phase transition temperature of DSPC (55 °C) for around 20 – 25 minutes.
100 μL of a 10% v/v solution of Trion X-100 was added to the heated vesicle solution and mixed thoroughly for 1-2 minutes. Immediately afterwards, this solution was centrifuged at 20,000g for 5 – 10 minutes. Post-centrifugation, the supernatant was collected and assayed for total released Hb concentration and percent metHb by UV-vis spectroscopy and analyzed using the Winterbourn equation [558].

3.2.6 O₂-PEG-LEH Equilibria Measurements

The CO bound Hb, encapsulated in PEG-LEH particles, were photolysed via irradiation with visible light in an O₂ saturated atmosphere. Briefly, 2-3 mL of the concentrated PEG-LEH solution was diluted with PBS (0.1 M, pH 7.4) in a 1:2 (v/v) ratio, and placed in a sealed serum bottle. A long needle was used to de-gas the solution for about 10-15 minutes. Photolysis was then carried out in the presence of visible light (GE Edison SP 10° 90W) in an O₂ saturated atmosphere for 15-20 minutes. The serum bottle was placed in an ice-bath and care was taken to maintain the temperature at 4°C throughout the process in order to minimize metHb formation. The conversion of carbonyl Hb (HbCO) to oxy-Hb (HbO₂) was verified by measuring the absorption spectra of lysed Hb from the PEH suspensions by UV-vis spectroscopy [558]. The O₂-PEG-LEH equilibrium binding curves were generated using a Hemox Analyzer (TCS Scientific Corp., New Hope, PA) at 37 ºC (physiological temperature) [85, 358, 360, 361, 559-561]. The instrument synchronously measures the oxygen saturation of Hb (expressed as a percentage) using dual-wavelength spectrophotometry, and the dissolved oxygen concentration (expressed as pO₂, mm Hg) using a Clark oxygen electrode [561]. Briefly, 200 – 300 μL PEG-LEH solution was diluted with 4.7 – 4.8 mL Hemox buffer (TCS Scientific Corp.) in a plastic vial. Further, 20 μL of Additive-A, 10 μL of Additive-B and 10 μL of antifoaming agent (TCS Scientific Corp.) were added to the mixture. Excess
compressed air was bubbled through the PEG-LEH solution to saturate it to a pO$_2$ of 147 ± 1 mm Hg. This step was carried out carefully for 25 – 35 min to allow the O$_2$ to diffuse across the lipid membrane and saturate the encapsulated Hb. Post saturation; the air stream was replaced by pure N$_2$ to deoxygenate the PEG-LEH solution. The respective absorbances of HbO$_2$ and deoxy-Hb were recorded via dual-wavelength spectrophotometry and were used to compute the oxygen saturation (%) of Hb encapsulated in the PEG-LEH particles. Hb O$_2$-saturation was plotted as function of pO$_2$ to yield the O$_2$ equilibrium curves (OECs). Hill equation (Eq. 2.1) was used to fit the OECs obtained for PEG-LEHs and control Hb [360, 361, 559]. The P$_{50}$ (partial pressure of O$_2$ at which 50% of the Hb present is saturated) and the cooperative coefficient (n) of PEG-LEH particles were recorded and compared to corresponding values obtained for free-Hb and RBCs.

3.2.7 Rapid Kinetic Measurements of PEG-LEH dispersions

PEG-LEH gaseous ligand binding/release kinetics were assessed using an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer (Applied Photophysics Ltd., Surrey, United Kingdom). Prior to any stopped flow measurements, all Hb encapsulated in the PEG-LEH particles were converted to the oxy-state by the photodissociation technique described above. Rapid kinetic measurements were performed using protocols previously described by Rameez and Palmer [344-346] and outlined in detail under the subheading Rapid Kinetic Measurements of PEH dispersions in the Materials and Methods section of Chapter 2 (section 2.2.7). For all stopped flow measurements, a control of human Hb was used to ensure the authenticity of the results.
3.2.8 Lipocrit of PEG-LEH dispersions

The PEG-LEH dispersions were diluted 10× in PBS (0.1 M, pH 7.4) and were subjected to ultracentrifugation (L90K, Beckman Coulter Inc., Brea, CA) at 100,000g for 1 h. The lipocrit or the percentage of packed PEG-LEH particles were then computed using the following equation:

\[
\% \text{ Lipocrit} = \frac{V_1 - V_2}{V_1} \times D \times 100
\]  

Eq. 3.1

\(V_1\) = initial volume of PEG-LEH solution

\(V_2\) = final supernatant volume after ultracentrifugation

D = dilution factor

The lipocrit was used to calculate the number of PEG-LEH particles per mL of suspension \((N)\) as shown below:

\[
N = \frac{\Phi \times \text{Lipocrit}}{V_{\text{PEG-LEH}}}
\]  

Eq. 3.2

Where \(\Phi\) = packing fraction and \(V_{\text{PEG-LEH}}\) = volume of an individual PEH-LEH particle which was computed from the PEG-LEH diameter obtained from DLS or A4F.

Similarly, the number of Hb molecules encapsulated per particle was computed from the volume of an individual PEG-LEH particle by simply using the known volume of a Hb molecule. The diameter of a Hb molecule has been previously reported in literature to be 5.5 nm [653].
3.2.9 Long Term Storage Stability of PEG-LEH Particles

PEG-LEH particles, stored at 4°C for 30 days, were diluted 10× in PBS (0.1 M, pH 7.4) and ultracentrifuged (L90K, Beckman Coulter Inc., Brea, CA) at 100,000g for 1 h. The supernatant was carefully removed and its volume was recorded. The supernatant was assayed for total Hb released via UV-vis spectroscopy and analyzed using the Winterbourn equation \[558\]. The packed PEG-LEHs were then re-suspended in 1 mL PBS (0.1 M, pH 7.4) and the total volume of the re-suspended vesicles was recorded. The Hb concentration inside the re-suspended PEG-LEH particles were obtained by lysing the vesicles with Triton X-100 using techniques described above (section 3.2.5). The total degree of lysis in these PEG-LEHs over their storage period was estimated using the following equations:

\[
\% \text{ Lysis} = \left( \frac{\text{Wt. of } Hb \text{ in Supernatant}}{\text{Wt. of } Hb \text{ in Supernatant} + \text{Wt. of } Hb \text{ inside } PEG \text{ – } LEHs} \right) \times 100
\]

Eq. 3.3

Where,

\[
\text{Wt. of } Hb \text{ in Supernatant} = \text{Supernatant } [Hb] \times (\text{Vol. of Supernatant}) \quad \text{Eq. 3.4}
\]

and,

\[
\text{Wt. of } Hb \text{ inside } PEG \text{ – } LEHs = [Hb] \text{ in } PEG \text{ – } LEHs \times (\text{Vol. of re – suspended vesicles}) \quad \text{Eq. 3.5}
\]

3.2.10 PEG-LEHs encapsulating HbCO and HbNO

A major objective of this study was to encapsulate CO and NO bound Hb inside lipid vesicles. During preparation of PEG-LEHs, it was necessary to convert the Hb into the HbCO form (described earlier) in order to ensure its oxidative stability (HbCO is
approximately 200 times more stable than HbO\textsubscript{2} against autoxidation) [280]. Post PEG-LEH synthesis, the CO bound state of encapsulated Hb was confirmed via UV-vis spectroscopy after lysing the PEG-LEH particles to yield cell-free Hb using techniques described earlier. Since the affinity of Hb for NO is \(~1000\)-fold greater than its affinity for CO [590], deoxygenated PEG-LEH particles encapsulating HbCO were treated with 99.9% pure NO gas (bubbled through 5M NaOH to remove nitrite impurities) for 3 – 6 h in a sealed environment and in absence of light to convert the encapsulated Hb to its NO bound state. The PEG-LEH vesicles were then lysed by techniques described earlier and absorption spectra of the lysed Hb were obtained by UV-vis spectroscopy. We attempted a direct displacement reaction to form HbNO encapsulated PEG-LEH particles by treating PEG-LEHs encapsulating HbCO with ultra-pure NO gas. The reaction scheme is as follows,

\[ HbCO + NO \rightarrow HbNO + CO \]

Cell-free Hb was converted to its HbCO and HbNO forms to serve as controls in this experiment. Briefly, 5 – 10 mL concentrated Hb solution was taken in a sealed serum bottle and a long needle was used to de-gas the Hb solution for 20-30 min. The de-gassing was achieved with alternate cycles of vacuum and inert N\textsubscript{2} gas. Samples were taken at regular intervals to measure the dissolved pO\textsubscript{2} using a Blood Gas Analyzer (Siemens Rapidlab 248, Diamond Diagnostics, Holliston, MA). The de-gassing process was continued till the pO\textsubscript{2} dropped to 0 mm Hg. Ultra-pure (99.3%) CO gas was then passed through the serum bottle for 1 – 2 h, care was taken to not bubble any gas through the protein solution as that would lead to froth formation. The CO bound state of the Hb was then confirmed via UV-vis spectroscopy.
Due to the greater affinity of Hb for NO as compared to CO, we hypothesized that NO could replace CO from HbCO to form HbNO. To test this hypothesis, 5 – 8 mL concentrated HbCO solution was taken in a sealed serum bottle and was treated with ultra-pure (99.9%) NO gas (bubbled through 5M NaOH to remove nitrite impurities) for 1 – 2 h in absence of light. The NO bound state of the Hb was then confirmed via UV-vis spectroscopy. All gassing and de-gassing experiments were carried out in an ice-bath to prevent the formation of metHb.

3.2.11 Statistical Analysis

Anova/2 sided t-tests were performed to check the statistical significance of the various results obtained for control Hbs, RBCs and PEG-LEHs. In all analyses, p < 0.05 was considered as the level of statistical significance. Statistical analyses were performed using JMP version 9 (SAS Institute Inc., Cary, NC). JMP v9 was downloaded at no cost from the Office of the Chief Information Officer (OCIO) website at the Ohio State University.

3.3 Results and Discussion

It was important to study size, Hb encapsulation, O₂ carrying capacity, gaseous ligand binding/release kinetics, lipocrit, and long term storage stability of the fabricated PEG-LEH particles in order to judge their suitability as potential gaseous ligand carriers for treating IRI. The biophysical properties of the liposomes synthesized in the present study are listed in Table 3.1.

3.3.1 PEG-LEH Size Distribution

HBOC size plays a crucial role in determining the safety and efficacy of RBC substitutes [456, 457]. The small size of commercial acellular HBOCs are largely
responsible for the observed vascular side-effects when these HBOCs are administered in vivo [149]. HBOC size impacts its biodistribution, capture by the reticuloendothelial system (RES), and circulation half-lives in the blood stream [549, 564-566]. To avert these side-effects, researchers have developed HBOCs, such as PEHs and PEG-LEHs, which are larger than the size of pores lining the inner walls of the vasculature [328, 345, 347, 463, 464].

<table>
<thead>
<tr>
<th>Table 3.1: Biophysical properties of PEG-LEHs</th>
</tr>
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<tbody>
<tr>
<td><strong>Property</strong></td>
</tr>
<tr>
<td><strong>Diameter (nm)</strong></td>
</tr>
<tr>
<td>[Hb] in suspension (g/dL)</td>
</tr>
<tr>
<td>MetHb (%)</td>
</tr>
<tr>
<td>k_{off, O2} (s^{-1})</td>
</tr>
<tr>
<td>k_{on, CO} (μM^{-1}s^{-1})</td>
</tr>
<tr>
<td>k_{ox, NO} (μM^{-1}s^{-1})</td>
</tr>
<tr>
<td>P_{50} (mm Hg)</td>
</tr>
<tr>
<td>Cooperativity</td>
</tr>
<tr>
<td>Lipocrit/Hematocrit</td>
</tr>
<tr>
<td>[Hb] inside (g/dL)</td>
</tr>
<tr>
<td>No. of PEG-LEH particles/mL dispersion (x 10^{13})</td>
</tr>
<tr>
<td>% Lysis after 30 days</td>
</tr>
<tr>
<td><em>(n=8)</em></td>
</tr>
<tr>
<td>No. of molecules of gas transported by a PEG-LEH particle (x 10^{3})</td>
</tr>
<tr>
<td>No. of Hb molecules/PEG-LEH particle (x 10^{3})</td>
</tr>
</tbody>
</table>
Size distribution of PEG-LEH particles formulated in this study was measured using an Eclipse® asymmetric flow field-flow fractionator (A4F) connected in series to an 18-angle Dawn Heleos® multi-angle static light scattering (MASLS) photometer. The light scattering spectra thereby obtained was analyzed using ASTRA 5.3 software and yielded an average diameter of 253.28 ± 27.25 nm (comparable to 255.83 ± 20.81 nm measured using DLS) for PEG-LEH particles. Additionally, from the light scattering spectra, PEG-LEH particle size distribution was computed. Figure 3.3 shows a typical PEG-LEH size distribution plot where 96% of the total population had diameters between 200-360 nm.

Figure 3.3: A typical PEG-LEH size distribution plot generated using light scattering data obtained from a Dawn Heleos® MASLS photometer. Particle fractionation was achieved using an Eclipse® A4F.
As noted above, the average hydrodynamic diameter obtained for the PEG-LEH particles synthesized in this study was estimated to be $\sim 255.83 \pm 20.81$ nm (DLS measurement). These sizes are slightly larger than the ideal particle size ($\sim 160 \text{–} 220$ nm) required for optimal circulation persistence and proper biodistribution of these nanoparticles [367, 567, 568]. Circulation persistence of vesicles in the blood stream varies inversely with particle diameter and directly with particle surface area to volume ratio [569-571]. In our PEG-LEH fabrication method, the hydrodynamic diameter of the PEG-LEH particles can be easily regulated by increasing the number of passes through the cell-disrupter (Constant Systems, Daventry, UK) and/or using a higher pressure-head (15,000/20,000 psi instead of 10,000 psi) during the homogenization step. We were able to synthesize PEG-LEH particles having diameters ranging from 150 – 180 nm by increasing the number of passes and/or using a higher pressure-head. However, this adversely affected the Hb encapsulation within these vesicles ([Hb] $\sim 6.5$ g/dL). We believe that this loss in Hb encapsulation can be attributed to the rupture of some of these vesicles upon exposure to longer extrusion periods (achieved by increasing the number of passes through the cell-disrupter). A major goal of this study was to synthesize PEG-LEH dispersions with a solution [Hb] comparable to that of whole blood (15.7 g/dL for men and 13.8 g/dL for women) [573]. Thus, we deemed it necessary to strike a balance between the particle size (governed by the number of passes through the cell-disrupter) and the Hb encapsulation. Therefore, we decided to pass the LEH suspension through the cell-disrupter 2-3 times in batch mode with a pressure-head of 10,000 psi during the fabrication process in order to optimize the Hb encapsulation at the expense of a slightly larger particle size. As is evident from Table 3.1 and also discussed later on, we were able to synthesize PEG-LEH particles having solution [Hb] comparable to that of whole blood.
The hydrodynamic diameters obtained for the PEG-LEHs synthesized in this study are significantly smaller (p<0.05) than those obtained for the PEHs (Chapter 2), while they are significantly larger (p<0.05) than the diameters reported in the literature for acellular HBOCs (<100 nm) [51, 149, 436, 648, 654, 655]. Therefore, unlike PEHs, the PEG-LEH particles are suitable for unhindered passage through fine capillaries and concurrently these particles will also abstain from eliciting vasoconstriction and systemic hypertension observed upon the administration of acellular HBOCs in vivo [50, 149].

Interestingly, no precipitation was observed when the PEG-LEHs were stored at 4ºC for extended periods of time. Surface modification of LEHs with PEG is believed to prevent particle aggregation and is also responsible for the colloidal stability of these vesicles [50, 473]. Previous studies have shown that unmodified LEHs aggregate within a period of just 3 days [50, 473]. In future, we plan to study the membrane morphology of these vesicles using techniques such as Transmission Electron Microscopy (TEM), optical microscopy, and fluorescence Confocal Laser Scanning Microscopy (CLSM).

### 3.3.2 Hb and MetHb Concentrations inside PEG-LEH Particles

A major objective of this study was to synthesize PEG-LEH dispersions with a solution Hb concentration comparable to that of whole blood (15.7 g/dL for men and 13.8 g/dL for women) [573]. Thus, it was critical to measure the [Hb] and % metHb inside the synthesized PEG-LEH particles. Table 3.1 compares the [Hb] observed in PEG-LEH suspensions, RBCs and cell-free Hb. The PEG-LEH dispersions prepared in this study had [Hb] around 12.01 ± 1.29 g/dL. Indeed, in this study we successfully prepared PEG-LEH suspensions having [Hb] comparable to that of whole blood (15.7 g/dL for men and 13.8 g/dL for women) [573]. Compared to PEG-LEHs, RBC suspensions had significantly higher (p<0.05) [Hb] at around 22.51 ± 5.91 g/dL. However, the intra-PEG-
LEH Hb concentration, estimated using the lipocrit of the PEG-LEH dispersions prepared in this study (26.1 ± 2.7%), ranges from 46.3 ± 5.3 g/dL and is significantly higher (p<0.05) than the estimated intra-RBC Hb concentration (32.16 ± 8.44 g/dL). This observation can be attributed to the smaller size of the PEG-LEH particles as compared to RBCs (250 nm vs. 8000 nm).

Table 3.1 also reports the metHb levels of the PEG-LEHs synthesized in this study. Prior to PEG-LEH fabrication the % metHb of the starting material (stock HbCO solution) was also measured by UV-vis spectroscopy and analyzed using the Winterbourn equation [558]. The metHb levels of the stock HbCO solution were below detectable limits whereas the metHb levels of the PEG-LEH dispersions ranged from 0.91 ± 0.1%. The low metHb levels observed in the PEG-LEHs can be attributed to the fact that all Hb was converted to its more stable HbCO form prior to encapsulation within the lipid vesicles (HbCO is approximately 200 times more stable than HbO₂ against autoxidation) [280]. Further, reducing agents such as ascorbate can be co-encapsulated with Hb to synthesize liposomes with improved resistance to Hb autoxidation. Recent studies have highlighted the role of ascorbic acid/ascorbate in suppressing Hb autoxidation in vivo [353, 582].

3.3.3 O₂-PEG-LEH Equilibria Measurements

The O₂-PEG-LEH equilibrium data were fit to the Hill equation (Eq. 2.1) to determine the $P_{50}$ and cooperativity coefficient ($n$) as noted earlier. Table 3.1 lists the $P_{50}$ and $n$ of the PEG-LEH suspensions prepared in this study. The shape of the equilibrium O₂ binding curves obtained for the PEG-LEH dispersions are sigmoidal and are similar to those obtained for RBCs and cell-free Hb. This indicates that the cooperative binding of O₂ to Hb was not compromised by the fabrication method used in the present study.
Both the $P_{50}$ and $n$ were slightly lower ($p<0.05$) for PEG-LEHs than RBCs. Similar findings have been reported in the literature and can be attributed to the fact that the tight packing of the Hb molecules in smaller sized PEG-LEH particles discourages structural changes in the Hb tetramer affecting its O$_2$ binding equilibria [344, 656]. These observations are illustrated by Figure 3.4 which compares typical oxygen equilibria curves of RBCs, cell free-Hb and PEG-LEH particles.

![Image of oxygen equilibrium curve]

Figure 3.4: Comparison of O$_2$ equilibria curves of RBC, cell free-Hb and PEG-LEH particles.

The volume of a Hb molecule has been shown to increase very slightly by 0.2% (0.002 cm$^3$/g) during its transition from the deoxy to the oxy state [657]. The binding of O$_2$ to Hb is a steric effect. We think that the decrease in cooperativity coefficient of PEG-LEHs compared to RBCs is due to the higher intracellular concentration of Hb inside the vesicles (46 g/dL in PEG-LEHs as compared to 32 g/dL in RBCs). This leads to ‘crowding’ of encapsulated Hb within the vesicles and thus reduces/hinders the transmission of the quaternary structural changes between neighboring globin chains.
The quaternary conformational changes observed in a Hb molecule during its transition from the deoxy, T-state to the oxy, R-state involve rotation of the two symmetrical αβ dimers by 15º relative to each other and a translation of 0.1 nm along the rotation axis [84]. This rotation about the axis is perhaps hindered by close packing of Hb molecules encapsulated inside a PEG-LEH particle [346], therefore resulting in low cooperativities.

As listed in Table 3.1, the $P_{50}$ values for the PEG-LEHs range between 17.60 ± 1.72 mm Hg and are significantly lower than the values obtained for RBCs (27.16 ± 4.45 mm Hg). Sakai et al. has previously shown that in ischemic tissues, where $pO_2 < 10$ mm Hg, phospholipid vesicles with low $P_{50}$ (~ 8 mm Hg) were the primary source of $O_2$, surpassing RBCs [481]. The small size of these vesicles (~ 250 nm) allow them to perfuse through partially blocked capillaries which otherwise obstruct the passage of much larger sized RBCs (~ 8000 nm) [492]. Moreover, the local $pO_2$ levels are greatly reduced as blood flow through these vessels are extremely slow. RBCs with higher $P_{50}$ (lower oxygen affinity) release most of their $O_2$ in transit, before reaching the targeted ischemic tissues [481, 494, 583]. Therefore, the PEG-LEHs synthesized in this study with moderate $P_{50}$ values are suitable candidates for reperfusing targeted ischemic tissues [481, 584].

When compared to unencapsulated cell-free Hbs, the PEG-LEHs exhibited significantly higher (p<0.05) $P_{50}$ values but the cooperativity coefficients obtained for the PEG-LEHs are significantly lower (p<0.05). The low cooperativities observed can be linked to the encapsulation of the Hbs inside the aqueous core of the lipid vesicles and the tight packing of the Hb molecules within the vesicle. However, chemical modifications to the Hb molecules encapsulated within the PEG-LEH particles might also adversely affect the cooperativities. Thus, in the future, we plan to perform matrix-assisted laser desorption/ionization (MALDI) spectral analysis of the PEG-LEHs to rule
out chemical modifications to encapsulated Hbs as a probable cause for the low cooperativities observed in the case of these vesicles [50].

The absence of 2, 3-diphosphoglycerate (2, 3-DPG), an important allosteric modulator of O$_2$ binding to Hb [62], can explain the lower $P_{50}$ values (as compared to RBCs) observed for the PEG-LEHs encapsulating human Hb [50]. 2, 3-DPG is removed during purification and processing of the Hb used in this study [502]. $P_{50}$ values of the PEG-LEHs can be controlled by co-encapsulating 2, 3-DPG during PEG-LEH fabrication. However, 2, 3-DPG is expensive and is chemically unstable [585], thus researchers have co-encapsulated its phosphate analogues such as pyridoxal 5’-phosphate (PLP), and inositol hexaphosphate (IHP) to regulate the $P_{50}$ of Hb vesicles [481, 483, 497, 585-589]. However, co-encapsulation of IHP (in 1:1 molar ratio with Hb) led to drastic increases in the $P_{50}$ values ($P_{50}$ ~ 60 – 65 mm Hg) and only 70-80% of the encapsulated Hb could be oxygenated at physiologically high pO$_2$ of around 100 mm Hg. Also, PLP needs to be co-encapsulated at much higher molar ratios (atleast 3:1) in order to regulate the $P_{50}$ values of encapsulated Hb. Due to these potential problems we decided against co-encapsulating 2, 3-DPG or its analogues to boost the $P_{50}$ values of the PEG-LEHs synthesized in the current study.

3.3.4 Rapid Kinetic Measurements of PEG-LEH Dispersions

Rapid kinetic measurements were done on the PEG-LEHs formulated in the current study to compare their ligand binding/release kinetics to that of RBCs. These rates are important to test the ability of these particles to store and transport important gaseous ligands such as CO and NO. It is well-known that the cellular structure of Hb vesicles impede gaseous ligand binding/release kinetics when compared to stroma free-Hb [344, 345, 463, 470, 591-593]. Multiple theories have been proposed to explain this
retardation in kinetics but no conclusion has yet been reached [594-597]. NO dioxygenation kinetics can be directly related to the NO scavenging mechanism for the development of vasoconstriction and hypertension, and the O\textsubscript{2} dissociation measurements can be related to the autoregulation theory for the development of vasoconstriction and hypertension. Thus, rapid kinetic measurements were deemed significant in assessing the ability of these particles to elicit vasoconstriction and hypertension.

3.3.4.1 Reactions with O\textsubscript{2}

The \( k_{\text{off}, \text{O}_2} \) values (Table 3.1) obtained for the PEG-LEH particles formulated in this study ranged between 11.18 ± 1.82 s\(^{-1}\), and are slightly higher (p<0.05) than the values obtained for RBCs (8.61 ± 2.55 s\(^{-1}\)). However, the \( k_{\text{off}, \text{O}_2} \) rates for both PEG-LEHs and RBCs are significantly lower (p<0.05) than the values obtained for the control, human Hb (34-41 s\(^{-1}\)). Figure 3.5 compares typical kinetic time courses of O\textsubscript{2} dissociation for PEG-LEHs, RBCs and cell-free Hb measured using an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer.

The \( k_{\text{off}, \text{O}_2} \) rates obtained for the PEG-LEH particles synthesized in this study are 3 – 4 folds lower than the rates obtained for cell-free Hb. Comparable retardations in \( k_{\text{off}, \text{O}_2} \) rates are observed for RBCs (3 – 7 folds) when compared to cell-free Hb. Similar retardations in O\textsubscript{2} offloading rates have been previously reported in the literature for both PEG-LEHs and RBCs [50, 344, 346, 658]. Additionally, Chapter 2 of this manuscript reports that the \( k_{\text{off}, \text{O}_2} \) rates obtained for the PEHs in this study are 3 – 18 folds lower than those obtained for cell-free Hb. All in all, these observations underline the fact that encapsulation of Hb inside the aqueous core of Hb carriers such as RBCs, PEG-LEHs,
and PEHs play a major role in controlling the delivery of O\textsubscript{2} and other physiological gases to the tissues.

![Image](image1)

![Image](image2)

Figure 3.5: Time courses for deoxygenation of human (A) Hb, (B) RBCs, and (C) PEG-LEHs in presence of 1.5 mg/mL sodium dithionite. The experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 437.5 nm at 20 °C. PBS (0.1M, pH 7.4) was used as the reaction buffer.

The $k_{\text{off,O}_2}$ rates for the PEG-LEHs synthesized in this study are slightly higher than the rates observed for RBCs. RBCs and PEG-LEHs have similar membrane thicknesses (3 – 4 nm), and therefore, are expected to provide comparable trans-
membrane diffusion barriers to impede the diffusion of $O_2$ [50, 149, 346]. However, the PEG-LEHs are much smaller in size as compared to the RBCs (diameters of 255 nm vs. 8000 nm) [344, 346]. Thus, the RBCs provide a larger intracellular diffusion barrier to oxygen offloading [346]. According to Rameez et al. the intracellular diffusion barrier increases with the size of the particle as well as the intracellular Hb concentration [346]. Though the intracellular [Hb] in PEG-LEHs (46.3 ± 5.3 g/dL) is considerably higher than what is observed in RBCs (32.16 ± 8.44 g/dL), we believe that the ~ 32 fold decrease in particle size of PEG-LEHs, as compared to the RBCs, offsets the effect of the higher intracellular [Hb]. Therefore, comparatively lower $k_{off,O_2}$ rates observed in the case of PEG-LEHs can be explained by their smaller size and thereby compromised intracellular diffusion barrier compared to RBCs.

High offloading rates of $O_2$ from Hb forms the basis of the ‘autoregulation theory’ of development of vasoconstriction and systemic hypertension [598-600]. According to the autoregulation theory an increase in $O_2$ delivery by diffusion, decreases $O_2$ acceptance by tissues because of vasoconstriction [149, 462]. Thus, moderate $O_2$ release rates are critical in determining HBOC efficacy. The PEG-LEHs synthesized in the present study considerably retard $O_2$ offloading as compared to acellular HBOCs [51, 366, 436, 648, 654] and cell-free Hb. Furthermore, their $k_{off,O_2}$ rates are comparable to that of RBCs. Thus, the PEG-LEHs synthesized in this study can potentially deliver $O_2$ to ischemic tissues at regulated rates avoiding vasoconstriction due to oversupply of $O_2$.

### 3.3.4.2 Reactions with CO

Figure 3.6 shows characteristic CO association kinetic time courses for deoxygenated (A) Hb, (B) RBCs, and (C) PEG-LEHs upon reaction with CO stock solution (464 μM). The time courses were similar for Hb, RBCs and PEG-LEHs. Figure
3.6 (D) plots the dependence of the apparent rates on CO concentrations for RBCs, stroma free-Hb and PEG-LEHs. Therefore, the slopes of the linear fits in Figure 3.6 (D) indicate the second order CO binding rate constants of the different species. Additionally, the $k_{\text{on,CO}}$ values obtained for the liposomes synthesized in this study are listed in Table 3.1.

The $k_{\text{on,CO}}$ values obtained for the PEG-LEHs synthesized in this study ranged between $0.17 \pm 0.01 \, \mu\text{M}^{-1}\text{s}^{-1}$ and are significantly higher than the CO association rate constants obtained for RBCs ($0.09 \pm 0.03 \, \mu\text{M}^{-1}\text{s}^{-1}$) ($p<0.05$). This observation can be explained by the larger intracellular diffusion barrier offered by RBCs owing to their much larger diameter as compared to the PEG-LEHs (8000 nm vs. 250 nm) [463].

The $k_{\text{on,CO}}$ rates obtained for the cell-free Hb tested in this study ranged between $0.20 \pm 0.01 \, \mu\text{M}^{-1}\text{s}^{-1}$. These values are significantly higher ($p<0.05$) than the values obtained for RBCs, however, no significant difference ($p>0.05$) is observed when these values are compared to the $k_{\text{on,CO}}$ rates obtained for the PEG-LEHs. Therefore, we conclude that encapsulation inside lipid membranes did not affect the CO association kinetics. Sakai et al. has reported similar observations for CO association rates of phospholipid vesicles using a different PEG-LEH formulation technique [463]. These observations can be explained by the fact that the magnitudes of the CO binding rate constants are much smaller than those of O$_2$ and NO binding [50, 346, 590, 659]. The O$_2$ binding rate constants of cell-free Hb has been reported as $3.3 \, \mu\text{M}^{-1}\text{s}^{-1}$ (20ºC, pH 7) [590, 660], whereas the NO association rate constants of Hb has been estimated to be $\sim 35 \, \mu\text{M}^{-1}\text{s}^{-1}$ (20ºC, pH 9.1) [590, 661]. Therefore, the CO association rate constants are 10 folds and 100 folds smaller in magnitude as compared to the O$_2$ and NO association rate constants respectively. Thus, subtle differences in the low magnitude CO binding
rate constants are often not captured by the kinetic measurements performed in this study.

Figure 3.6: Time courses for CO (464 μM) association reaction of deoxygenated (A) Hb, (B) RBCs, and (C) PEG-LEHs. The deoxygenation was carried out in presence of 1.5 mg/mL sodium dithionite. Experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 437.5 nm at 20 °C and PBS (0.1M, pH 7.4) was used as the reaction buffer. (D) Comparison of CO association rates of human Hb, RBCs and PEG-LEHs.

Dissociation rate constants of HbCO has been previously reported in the literature [590]. Gibson et al. estimated the $k_{\text{off,CO}}$ rates of sheep blood to be $\sim 0.012 \text{ s}^{-1}$ (23.5°C, pH 9.1) [662]. We believe that, analogous to CO association, the PEG-LEHs
will release CO quicker than RBCs due to their smaller intracellular diffusion barrier (size difference, 250 nm vs. 8000 nm). Thus, we conclude that the PEG-LEHs can potentially deliver exogenous CO to ischemic tissues at slow to moderate rates in order to circumvent reperfusion injury resulting from ROS generation and oxidative tissue toxicity [147, 163, 168, 479]. In the future, experiments will be designed to measure the $k_{\text{off,CO}}$ rates of PEG-LEHs in order to determine their CO offloading abilities.
3.3.4.3 Reactions with NO

Figure 3.7 shows characteristic NO dioxygenation kinetic time courses for oxygenated (A) Hb, (B) RBCs, and (C) PEG-LEHs upon reaction with NO stock solution (25 µM). Figure 3.7 (D) shows dependence of the observed rates on NO concentration for Hb, RBCs and PEG-LEHs. Furthermore, the values for the NO dioxygenation rate constants ($k_{\text{ox,NO}}$) obtained for the liposomes synthesized in this study are reported in Table 3.1.

![Figure 3.7](image)

**Figure 3.7:** Time courses for NO (25 µM) dioxygenation reaction of oxygenated (A) Hb, (B) RBCs, and (C) PEG-LEHs. Experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 420 nm at 20 ºC and PBS (0.1M, pH 7.4) was used as the reaction buffer. (D) Comparison of NO dioxygenation rates of human Hb, RBCs and PEG-LEHs.
The low HbO$_2$ concentrations (~ 0.5 µM on heme basis) used in these experiments led to subtle absorbance changes; hence an average of 10-15 kinetic traces were considered for each time course in order to enhance signal-to-noise ratio.

The NO dioxygenation rates obtained for the PEG-LEHs (2.14 ± 0.76 µM$^{-1}$s$^{-1}$) were significantly higher (p<0.05) than those obtained for RBCs (0.33 ± 0.32 µM$^{-1}$s$^{-1}$). However, the $k_{ox,NO}$ values for both PEG-LEHs and RBCs were significantly lower (p<0.05) than the values obtained for cell-free Hb (34.99 ± 7.46 µM$^{-1}$s$^{-1}$). The NO dioxygenation rate constants, obtained in this study, for cell-free Hb are comparable to the values reported in the literature (20 – 34 µM$^{-1}$s$^{-1}$) [50, 345, 601, 602]. Approximately, 650 fold reduction in NO dioxygenation rates of RBCs as compared to cell-free Hb has been reported in the literature [50, 603]. In the present study, a similar retardation (50 – 625 folds) was observed for RBCs. The intracellular diffusion barrier afforded by the large RBC size and high intra-RBC Hb encapsulation promotes this retardation in NO binding [463]. The $k_{ox,NO}$ values of the PEG-LEH particles synthesized in this study are 10 – 17 folds lower than the rates observed for cell-free Hb. Furthermore, the NO dioxygenation rate constants for the PEG-LEHs are 8 – 9 folds lower than commercial acellular HBOCs, and about 4 folds less than the values reported for PEG-LEHs in the literature [366, 436, 604]. However, the $k_{ox,NO}$ values for RBCs are ~ 7 folds lower than that of the PEG-LEHs formulated in this study. This is an expected observation due to the smaller size (250 nm vs. 8000 nm) of the PEG-LEHs leading to a smaller intracellular diffusion barrier in the case of the PEG-LEHs as compared to the RBCs. Interestingly, it has been shown that PEG-LEHs possess similar $k_{ox,NO}$ values and exhibit comparable retardation to NO diffusion as RBCs when extrapolated to the size of RBCs (8000nm) via computer simulations [50, 346, 598]. All in all, the PEG-LEHs synthesized in the
current study are likely to induce very little NO scavenging mediated vasoconstriction and hypertension once administered in vivo, owing to their hydrophobic phospholipid membranes. This property makes PEG-LEHs a viable candidate for the delivery of therapeutic gaseous ligands to ischemic tissues in order to avoid reperfusion injury.

Monitoring the reaction kinetics of cell-free Hb, RBCs and PEG-LEHs with physiologically relevant gaseous ligands (O\textsubscript{2}, CO and NO) were deemed important in order to test the efficacy of PEG-LEHs as potential carriers of therapeutic gaseous ligands such as CO and NO. The results from these rapid kinetic measurements showed that when administered in vivo, the PEG-LEH particles can potentially deliver O\textsubscript{2} to ischemic tissues at regulated rates, thereby averting vasoconstriction due to oversupply of O\textsubscript{2}. The PEG-LEHs are also capable of delivering benign CO to ischemic tissues at controlled rates in order to circumvent reperfusion injury resulting from ROS generation and oxidative tissue toxicity. Additionally, low NO binding rates of these vesicles enable them to limit scavenging of endothelial NO, thus repressing NO scavenging mediated vasoconstriction and hypertension. The results of the current work further showed that the PEG-LEHs significantly retarded ligand binding/release kinetics (when compared to cell-free Hb), a characteristic comparable to RBCs. Therefore, from a kinetic perspective, the liposomes synthesized in this study seem suitable candidates for delivering therapeutic gaseous ligands to mitigate ischemia reperfusion injury.

3.3.5 Lipocrit of PEG-LEH Dispersions and Related Calculations

Lipocrit or the fraction of the volume of PEG-LEH dispersions that is made up of packed PEG-LEH particles is conceptually identical to hematocrit which is the fraction of the volume of whole blood that is occupied by packed RBCs [663]. Typical hematocrit observed in men ranges from 40 – 54%; and in women from 36 – 48% [664]. In
comparison, the lipocrit obtained for the PEG-LEHs synthesized in this study varied between 26.1 ± 2.7%. A typical calculation for estimating the lipocrit of these vesicles has been shown below.

\[ V_1 = \text{initial volume of PEG-LEH solution} = 1000 \, \mu\text{L} \]

\[ V_2 = \text{final supernatant volume after ultracentrifugation} = 975 \, \mu\text{L} \]

\[ D = \text{dilution factor} = 10 \]

From Equation 3.1,

\[ \% \text{ Lipocrit} = \frac{V_1 - V_2}{V_1} \times D \times 100 \]

\[ \therefore \text{ Lipocrit} = \frac{1000\mu\text{L} - 975\mu\text{L}}{1000\mu\text{L}} \times 10 \times 100 = 25\% \]

The lipocrit was used to calculate the number of PEG-LEH particles per mL of suspension \((N)\) as shown below:

\[ N = \frac{(\Phi \times \text{Lipocrit})}{V_{\text{PEG-LEH}}} \]

Where \(\Phi = \text{atomic packing fraction} = 0.74\) for close packing [665].

\[ d_{\text{PEG-LEH}} = \text{diameter of PEG-LEH from DLS} = 221.7 \, \text{nm} \]

\[ V_{\text{PEG-LEH}} = \text{volume of an individual PEH-LEH particle} = \frac{4\pi}{3} \left( \frac{d_{\text{PEG-LEH}}}{2} \right)^3 \]

\[ = 5.7 \times 10^6 \, \text{nm}^3 \]

Therefore,

\[ N = \frac{(\Phi \times \text{Lipocrit})}{V_{\text{PEG-LEH}}} = \frac{0.74 \times 0.25 \, \text{mL}}{5.7 \times 10^{-15} \, \text{cm}^3} = 3.2 \times 10^{13} \, \text{particles/mL suspension} \]

Similarly, the number of Hb molecules encapsulated per particle was computed from the volume of an individual PEG-LEH particle by simply using the known volume of a Hb molecule. The diameter of a Hb molecule has been previously reported in literature to be 5.5 nm [653].
\[
Volume \ of \ a \ Hb \ molecule = \frac{4\pi}{3} \left(\frac{5.5}{2}\right)^3 = 87.07 \text{ nm}^3
\]

\[
\therefore \text{No. of Hb molecules per PEG – LEH molecule} = 0.74 \times \left(\frac{5.7 \times 10^6}{87.07}\right)
\]

= 48,466 molecules

3.3.6 Long Term Storage Stability of PEG-LEH Particles

FDA mandates a total lysis of <1\% during the 42-day storage period of transfusable RBCs [666, 667]. The total degree of lysis in the PEG-LEHs over their 30 day storage period was observed to be around 0.37 ± 0.30\%. Surface conjugation with PEG and storage of these vesicles in their CO form account for the low Hb leakages (%) observed over their storage period. In the future, we plan to monitor % lysis in PEG-LEH particles, stored at 4ºC for 0, 2, 4, 6, 8, and 12 months.
3.3.7 PEG-LEHs Encapsulating HbCO and HbNO

Figure 3.8 compares the Soret band (A) and the Q bands (B) of CO (red) and NO (blue) bound cell free-Hb.

Figure 3.8: (A) Soret and (B) Q Bands of NO and CO bound cell free-Hb.
Pure spectra of HbCO were obtained using UV-vis spectroscopy after treating cell-free Hb with ultra-pure CO gas. The pure spectra for HbCO had a Soret peak at 419nm (Figure 3.8A) and the Q bands were at 540nm and 569nm (Figure 3.8B). These peaks were in agreement with those reported in the literature for pure HbCO [590]. Pure spectra of HbNO were obtained using UV-vis spectroscopy following treatment of HbCO with ultra-pure NO gas. The pure spectra for HbNO had a Soret peak at 418nm (Figure 3.8A) and the Q bands were at 545nm and 575nm (Figure 3.8B). The spectra obtained for HbNO in this study were also in agreement with the UV-spectra for pure HbNO reported in the literature [590].

Figure 3.9: UV-vis spectra of lysed PEG-LEH particles.

Figure 3.9 compares the UV-vis absorption spectra of lysed PEG-LEH vesicles encapsulating HbCO (red) and a mixture of HbCO and HbNO (blue). The spectra
obtained after lysing the PEG-LEH particles encapsulating HbCO had Soret peak at 419nm and Q bands at 540nm and 569nm (Figure 3.9, red). Thus, this observation confirmed that the Hb encapsulated in the PEG-LEH particles were in the HbCO form. However, the UV-spectra obtained after lysing these vesicles following treatment with ultra-pure NO gas were deemed to be a mixture of HbCO and HbNO (Figure 3.9, blue). These spectra neither corresponded to pure HbCO nor to pure HbNO spectra. Characteristic of pure HbNO, a maximum at 418nm in the Soret region was observed and the minimum between the two Q bands in the visible region was not as pronounced as that of HbCO. However the two Q bands, though right-shifted, did not correspond to the Q band maxima for pure HbNO (540nm and 572nm as opposed to 545nm and 575nm observed for pure HbNO) [590]. Thus we predict that the lysed Hb obtained from PEG-LEH particles treated with NO gas is a mixture of HbCO and HbNO. We believe that a complete conversion of HbCO to HbNO in PEG-LEHs was thwarted by the trans-membrane and the intra-cellular diffusion barriers, which prevented the penetration of NO to the core of the PEG-LEH particles. In the future, we plan to use spectral deconvolution techniques to determine the individual percentages of these species encapsulated in the PEG-LEH particles. Alternate techniques such as hydrating the dried lipid film with HbNO to form NO loaded PEG-LEH particles were not successful as HbNO has a high rate of dissociation and is unstable in presence of O2. Partial conversion of the encapsulated HbCO to HbNO in PEG-LEHs, and their overall failure in encapsulating and thereby transporting pure HbNO presents itself as a major drawback with regards to the efficacy of these particles in delivering NO as a therapeutic gaseous ligand to tissues affected by IRI. However, a mixture of HbCO and HbNO can also prove to be a viable therapeutic for IRI as it brings together the beneficial properties of both gases. Though the PEG-LEHs synthesized in this study are capable of delivering CO
exogenously, this study was unable to synthesize PEG-LEHs delivering pure HbNO to tissues affected by IRI.
3.4 Conclusions

In this study, we successfully synthesized PEG-LEH particles with a solution [Hb] = 10-14 g/dL, a value comparable to that of whole blood (~15 g/dL). The PEG-LEHs were subjected to a wide range of biophysical characterizations in order to determine their potency of transporting therapeutic gaseous ligands to tissues affected by ischemia. Our results indicated that the liposomes synthesized in the current study were able to overcome the issue of low Hb encapsulation observed in the case of PEHs (Chapter 2). The Hb concentration measured in the PEG-LEH dispersions were ~60 folds higher than those observed for the polymersomes. The difference in the relative concentrations of the PEH and PEG-LEH dispersions are evident in Figure 3.10, where PEG-LEH (B) suspensions appear more dense and viscous as compared to PEH (A) dispersions. Furthermore, the PEG-LEHs synthesized had ideal sizes for prolonged in vivo circulation (~255 nm) and effectively retarded O₂-dissociation and NO dioxygenation kinetics.

In our quest to deliver therapeutic gaseous ligands to ischemic tissues, we successfully encapsulated CO bound Hb within PEG-LEH particles. When transfused in vivo these CO loaded vesicles will potentially deliver anti-apoptotic, anti-inflammatory, and vaso-relaxing CO gas at regulated concentrations to ischemic tissues in order to prevent reperfusion injury. However, our efforts to synthesize NO loaded PEG-LEHs were partially successful. We were able to synthesize PEG-LEHs encapsulating a mixture of HbCO and HbNO. We believe that a complete conversion of HbCO to HbNO in PEG-LEHs was thwarted by the trans-membrane and the intra-cellular diffusion barriers, which prevented the penetration of NO to the core of the PEG-LEH particles. As an alternate strategy to develop HBOCs capable of delivering beneficial NO gas to
ischemic tissues we proposed to synthesize polymerized bovine Hb (polybHb) bound to NO. We felt that the absence of a protective membrane will enable us to synthesize 100% pure polyHb-NO.

Figure 3.10: Relative concentrations of (A) PEH dispersions with [Hb] ~ 0.2 g/dL and (B) PEG-LEH dispersions with [Hb] ~ 12g/dL. The PEG-LEH solutions appear dense and viscous.
CHAPTER 4  Polymerized Bovine Hemoglobin (PolybHb)

4.1 Introduction

4.1.1 Why acellular HBOCs?

The main objective of this study was to engineer HBOCs capable of delivering therapeutic gaseous ligands such as Carbon Monoxide (CO) and Nitric Oxide (NO) to ischemic tissues. We hypothesized that HBOCs loaded with benign gaseous ligands of proven anti-apoptotic, anti-inflammatory, and vasorelaxing properties [162-168, 479] will potentially avert reperfusion injuries commonly observed in ischemic tissues by minimizing tissue inflammation, cell apoptosis and cell proliferation.

Initially, we explored cellular HBOCs (PEHs – Chapter 2 and PEG-LEHs – Chapter 3) for their clear advantages over acellular HBOCs such as absence of vasoactivity, long-term storage stability, larger size (hence no extravasation through the blood vessel wall), longer circulation times and reduced oxidative stress [324, 346, 347, 469, 606]. However, we encountered different problems with both these types of cellular HBOCs. PEHs encapsulated negligible amounts of Hb. The problem of Hb encapsulation was completely averted with the PEG-LEHs; these lipid vesicles encapsulated Hb comparable to RBCs (Chapter 3). Additionally, we successfully synthesized PEG-LEHs encapsulating HbCO. We believe that these CO-loaded lipid
vesicles can potentially mitigate reperfusion injuries in ischemic tissues upon introduction to the systemic circulation during resuscitation from hemorrhagic shock. However, we were unable to synthesize PEG-LEHs encapsulating pure HbNO. Since the affinity of Hb for NO is much higher than its affinity for CO [590]. We attempted a direct displacement reaction to form HbNO encapsulated PEG-LEH particles by treating PEG-LEHs encapsulating HbCO with ultra-pure NO gas, according to the following reaction scheme:

\[ HbCO + NO \rightarrow HbNO + CO \]

However, upon lysing the vesicles and obtaining the UV-vis spectra of the encapsulated Hb, we observed a partial conversion of the encapsulated HbCO to HbNO. Increasing the NO-treatment time to ~6h did not change the spectra. We believe that a complete displacement of CO with NO was thwarted by the trans-membrane and intra-cellular diffusion barriers. Figure 4.1 shows a schematic of these two diffusion barriers commonly encountered in RBCs and cellular HBOCs [463].

Therefore, we predict that the partial NO loading of PEG-LEHs observed in our experiments could be due to,

1. High affinity of NO to Hb and rapid formation of HbNO induces a NO sink from the inner surface of the bilayer membrane to the core of the vesicles thereby hindering further NO diffusion and binding.
2. Lower gas diffusivities in highly viscous and concentrated Hb solutions.
3. Large particles (~250 nm) leading to longer diffusion distances to the core.
4. Resistance offered by the PEG-LEH membrane perhaps slows down entry of NO molecules into the vesicles.
Thus, as an alternate strategy to synthesize HBOCs capable of carrying beneficial NO and CO molecules to ischemic tissues we decided to explore acellular HBOCs lacking cell membranes. We hypothesized that the absence of protective membranes will enable us to formulate pure and stable acellular HBOCs loaded with NO and CO.

Figure 4.1: Diffusion barriers commonly encountered in cellular HBOCs and RBCs. (A) Unstirred layer near the outer surface of RBCs and cellular HBOCs. (B) Trans-membrane diffusion barrier originating from the protective membranes of RBCs and cellular HBOCs and the Intracellular diffusion barrier due to high intra-RBC and intra-vesicle Hb concentrations. This figure was originally published in the Journal of Biological Chemistry. Hiromi Sakai, Atsushi Sato, Kaoru Masuda, Shinji Takeoka, Eishun Tsuchida. Encapsulation of Concentrated Hemoglobin Solution in Phospholipid Vesicles Retards the Reaction with NO, but Not CO, by Intracellular Diffusion Barrier. J Biol Chem. 2008; 283(3): p. 1508-17. © The American Society for Biochemistry and Molecular Biology. The Copyright holder of this journal allows non-commercial reuse of any published material [463].
4.1.2 Glutaraldehyde Crosslinked Polymerized bHb

Contemporary research has shown that Hb polymerization mediated by glutaraldehyde crosslinking can lead to the formation of large and high MW Hb polymers (PolyHbs) [51]. Glutaraldehyde is a bifunctional 5-carbon dialdehyde; it non-specifically binds to multiple amino acid residues on Hb surface such as lysines, cysteines, histidines and tyrosines [4, 51, 668, 669]. It has been shown that PolyHbs were successful in mitigating the side-effects associated with HbA₀ and crosslinked Hb transfusions such as short circulatory half-lives, dissociation into αβ dimers, extravasation through the pores lining the inner walls of the vasculature, metHb formation, and ROS generation [68, 398, 412, 462, 670, 671].

Hemopure®, synthesized using the glutaraldehyde crosslinking method and developed by BioPure (Cambridge, MA), is the lone commercialized PolyHb product approved for clinical use (in South Africa only) [418]. Olsen et al. noted that “Hemopure® had a MW distribution ranging between 130 – 500 kDa and was essentially a mixture of different sized polymers resulting from different extents of the polymerization reaction” [4, 672]. Hemopure® has been shown to successfully deliver O₂ in some animal models yet other studies involving both animals and humans have reported severe vasoconstriction upon the introduction of this acellular HBOC [4, 359, 425-430, 488, 673-675]. Researchers have attributed the observed vasoconstriction to factors such as expedited NO (a potent vasorelaxing agent) scavenging, augmented O₂ delivery resulting in an “autoregulatory” response by the blood vessels to regulate O₂ delivery to the tissues, and oxidative side reactions on “the surface of the endothelial layer” and surrounding tissue space [51, 431]. Hemopure® is essentially a polydisperse mixture of different sized PolyHb molecules [672]. Also, the size reported for the largest PolyHb
was barely greater than 500 kDa [672]. This led researchers to believe that low to moderate rates of PolyHb extravasation can be expected with these acellular HBOCs [51].

In light of unsuccessful clinical trials, researchers proposed to increase the size and MW of glutaraldehyde polymerized Hbs to successfully avert the side-effects associated with most ‘second generation’ HBOCs such as HBOC-201® and PolyHeme® [676, 677]. It was further suggested that, not only should PolyHb solutions be devoid of any cell-free tetrameric Hb but they should also have diameters larger than 7 nm in order to successfully circumvent extravasation through pores lining the inner walls of the vasculature [677-679].

We have successfully synthesized glutaraldehyde crosslinked high MW polymerized Hbs in our lab [51, 435-437]. We were able to produce PolyHbs exhibiting different degrees of O₂ affinity achieved by polymerizing Hb completely locked in either oxygenated (relaxed or R-state PolyHb) or deoxygenated (tense or T-state PolyHb) state at different crosslinking ratios [51, 436]. Additionally, Zhou et al. from our lab has shown that the absolute MW distributions of PolyHbs can be tailored by regulating the glutaraldehyde (crosslinker) ratio [51, 436]. In another study, Cabrales et al. successfully separated fractions of high (>500 kDa) and low MW (<500kDa) PolyHbs from a heterogeneous mix of T-state PolyHbs. Furthermore, it was shown that the high (>500kDa) MW fraction having a 50:1 crosslink density caused no vasoconstriction and did not increase the mean arterial pressure drastically when transfused in vivo [437]. These results further confirm that larger HBOCs are ideal to prevent vasoconstriction and systemic hypertension [51, 437, 457]. However, viscous PolyHb solutions with high crosslink ratios can potentially cause hypertension upon transfusions at high concentrations [51, 437]. Therefore, in order to synthesize a better flowing and
comparatively less viscous PolyHb solution we chose a 30:1 crosslink ratio to polymerize Hb.

All in all, in light of the aforementioned advantages, we chose glutaraldehyde crosslinked high MW PolyHbs to deliver vasorelaxing and anti-apoptotic gaseous ligands such as NO and CO to ischemic tissues. Previously, both T- and R-state PolyHbs synthesized in our lab were shown to mediate nitrite reduction and NO formation driven vasodilation [435]. Furthermore, we decided to use polymerized bovine Hb (PolybHb) as opposed to polymerized human Hb (PolyhHb) due to the known advantages of bovine Hb (bHb) such as no supply shortages and since allosteric modulators are not required to enhance oxygen affinity of bHb (Cl− ions abundantly present in humans are allosteric modulators of bHb) [4].

4.2 Materials and Methods

4.2.1 Materials

Fresh bovine blood stored in 3.8% sodium citrate solution adjusted to a final concentration of 90:10 v/v (bovine blood : sodium citrate solution) was purchased from Quad Five (Ryegate, MT). Glutaraldehyde (50-70%), NaCl, KCl, NaOH, Na2S2O4, CaCl2·2H2O, Na-Lactate, N-acetyl-L-cysteine, NaCNBH3, and NaBH4 were procured from Sigma-Aldrich (St. Louis, MO). The hollow fiber (HF) filter modules (rated pore sizes: 50nm, 100 kDa and 500 kDa) used for both tangential flow filtration (TFF) and separation of unreacted free-Hb from PolybHb solutions were purchased from Spectrum Laboratories (Rancho Dominguez, CA). K3FeCN6, KCN and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).
4.2.2 Purification of Hb from bovine blood

Bovine Hb for polymerization were purified by the method of tangential flow filtration (TFF) developed by Palmer et al. [502]. Briefly, 2 bottles of fresh bovine blood in citrate were pooled to a total volume of 2L and washed by centrifugation. The bovine RBCs were packed by centrifuging the whole blood for 1 h at 3716g at 4ºC. The supernatant was discarded and the packed cells were further washed 3 times with 0.9% (w/v) isotonic saline solution to remove cell-free Hb, white blood cells, and other plasma proteins. The washed RBCs were eventually lysed overnight at 4ºC using phosphate buffer (PB) (3.75mM, pH 7.4). To obtain HPLC grade Hb, the RBC lysate was passed through a glass column packed with glass wool followed by filtration through a series of hollow fiber ultrafiltration (HFUF) cartridges, 50nm, 500kDa, and 100kDa (MWCO) (Spectrum Labs, Rancho Dominguez, CA); to remove shed RBC membrane, cell debris and other unwanted cellular proteins. The final solution was concentrated to [Hb] > 350 mg/mL on the 100 kDa hollow fiber cartridge [502]. Hb concentration was determined using the Winterbourne Equation as described elsewhere [558].

4.2.3 Polymerization of Hb – PolybHb synthesis

Both deoxy- and oxy- bovine Hb were polymerized using the method developed by Palmer et al. [51, 357, 437] to yield tense (T) and relaxed (R) state PolybHbs respectively. To prepare T-state (deoxygenated) PolybHb, it was critical that the bHb solution was devoid of any dissolved O₂ prior to and during the polymerization reaction. Presence of even minute quantities of dissolved O₂ can potentially lead to the formation of a mixture of T- and R-state PolybHbs. In order to synthesize R-state (oxygenated) PolybHb however, it was critical that the Hb solution was fully saturated with O₂ prior to
and after the polymerization reaction. A mixture of R- and T-state PolybHb can result in this case if the Hb is not completely saturated with O₂ [357].

Briefly, to generate T-state bHb, 30-33 grams freshly thawed bHb was diluted in ice-cold PB (20 mM, pH 8.0) to yield a total volume of 1200 mL [51]. Diluted bHb solution was placed inside a sealed, air-tight glass bottle connected to a Schlenk line. The glass bottle in turn was placed on an ice-bath to maintain the temperature of the bHb solution below 4°C (in order to minimize metHb formation). One manifold of the Schlenk line was connected to a tank of purified N₂ (inert) gas while the other manifold was connected to a vacuum-pump. The bHb solution was degassed by subjecting it to alternate cycles of N₂ and vacuum by operating the stopcock on the dual manifold Schlenk line. After 4-5 hours of degassing, samples were drawn from the bottle using a long needle to measure the pO₂ of the bHb solution using a Rapidlab 248 (Siemens, Malvern, PA) blood gas analyzer. If the pO₂ of the bHb solution was > 20 mm Hg, the deoxygenation process was continued till the pO₂ fell below 20 mm Hg. When the measured pO₂ of the bHb solution dropped to < 20 mm Hg, 300mL ice-cold Na₂S₂O₄ solution (1.6 mg/mL) was titrated into the bHb solution using a syringe pump (Razel Scientific, St. Albans, VT) to fully deoxygenate the bHb solution and achieve a pO₂ value of 0 mm Hg. To confirm this, the pO₂ of the bHb solution was measured periodically during the titration process [51]. In contrast, R-state bHb was prepared by saturating 1500 mL, 20 mg/mL bHb solution with pure O₂ gas for a period of 2-3 hours on an ice-bath. Complete O₂-saturation of the bHb solution was confirmed by measuring the pO₂ of the bHb solution (pO₂ > 749 mm Hg) [51].

The T- or R-state bHb were then polymerized using a 30:1 molar ratio of glutaraldehyde to bHb [51]. The glutaraldehyde solution was prepared by diluting the necessary volume of glutaraldehyde with 5 - 10 mL degassed PB (20 mM, pH 8.0). A
30mL syringe was used to add the glutaraldehyde solution dropwise to T- or R-state bHb in the sealed bottle under continuous stirring. The polymerization reaction was allowed to proceed at 37°C for 2 hours in the absence of light under N₂ (for T-state) or O₂ (for R-state) atmosphere [51]. In this study we tested the suitability of T- and R-state 30:1 PolybHb solutions as potential carriers of therapeutic gaseous ligands such as CO and NO to ischemic tissues.

In order to reduce the Schiff base which may result from a reversible reaction between lysine residues and free aldehyde and also to reduce the metHb level of PolybHb solutions, 6 – 8 mL of 6 – 8 M NaCNBH₃ in PB (20 mM, pH 8.0) was added to the reaction mixture at the end of the 2 hour reaction. The PolybHb solution was allowed to sit on an ice-bath under continuous stirring for 30 min. Finally, 20 mL of 2 M freshly made NaBH₄ was injected into the reaction mixture to quench the polymerization reaction. Both NaBH₄ and NaCNBH₃ were used in this method as together they reduce both the Schiff base and free aldehyde in solution [51, 357]. The pO₂ of the bHb solution was monitored before and after polymerization and also after quenching the reaction to ensure that polymerization was carried out with the bHb in the desired quaternary state (T- or R-state).

4.2.4 Clarification and Diafiltration of PolybHb Solutions

The PolybHb solutions were purified by the method developed by Palmer and Zhou [51]. Briefly, the crude PolybHb solutions were first clarified by passing them through a glass column packed with glass wool (autoclaved at 250°C for 30 min) to remove any large particles [51, 680]. The clarified PolybHb solution was then diluted to a total volume of 2L using ice-cold modified Ringer’s lactate buffer. We deemed it necessary to re-suspend the PolybHb molecules in isotonic modified Ringer’s lactate
buffer (NaCl – 115 mmol/L, KCl – 4 mmol/L, CaCl₂·2H₂O – 1.4 mmol/L, NaOH – 13 mmol/L, Na-Lactate – 27 mmol/L, and N-acetyl-L-cysteine – 2 g/L) in order to administer and assess these acellular HBOCs in animal models in the future. This buffer exchange, from PB (20mM, pH 8.0) to modified Ringer’s lactate, was achieved by subjecting the PolybHb solutions through 7-8 stages of diafiltration (4°C) using a 500 kDa HF cartridge (Spectrum Labs, Rancho Dominguez, CA). Additionally, this process ensured complete removal of unreacted Hb using a 1:10 (v/v) ratio of PolybHb to modified Ringer’s lactate until the filtrate appeared colorless. The filtrate from the 8th diafiltration cycle was collected and assessed via UV-vis spectroscopy to verify complete removal of free Hb. The buffer-exchanged PolybHb suspensions were further concentrated using mini 500 kDa cartridges (Spectrum Labs, Rancho Dominguez, CA). The resulting concentrated PolybHb solutions were stored at -80°C for future use. Sterile lab supplies were used for all experiments. All tubing, glassware, and filters were de-contaminated by immersing them overnight in 1 M NaOH and then rinsing them thoroughly with double distilled de-ionized water.

4.2.5 Hb and MetHb Concentration of PolybHb Solutions

The cyanomethemoglobin method described in the literature was used to measure the Hb concentration and % metHb of bHb/PolybHb solutions [51, 681].

4.2.6 Hydrodynamic Diameter and Zeta Potential of PolybHb Solutions

As described by Palmer and Zhou, a Zetasizer Nano DLS spectrometer (Malvern Instruments Ltd., Worcestershire, United Kingdom) was used to measure the hydrodynamic molecular diameter and zeta (ζ) potential of bHb and PolyHb solutions at 37°C [51]. The bHb/PolybHb solutions were diluted to a final concentration
of ~ 2 mg/mL using PBS (0.1M, pH 7.4) [51]. An internal heating system and temperature controller fixed the sample temperature at 37°C. The hydrodynamic diameter was determined using Dynamic Light Scattering (DLS), whereas \( \zeta \) potential was measured using Doppler velocimetry and Phase Analysis Light Scattering (PALS) [51].

### 4.2.7 \( \text{O}_2 \)-PolybHb Equilibria Measurements

The \( \text{O}_2 \)-PolybHb equilibrium binding curves were generated using a Hemox Analyzer (TCS Scientific Corp., New Hope, PA) at 37 °C (physiological temperature) [85, 358, 360, 361, 559-561]. The instrument synchronously measures the oxygen saturation of Hb (expressed as a percentage) using dual-wavelength spectrophotometry, and the dissolved oxygen concentration (expressed as \( \text{pO}_2 \), mm Hg) using a Clark oxygen electrode [561].

Briefly, 100µL PolybHb solution was diluted with 4.9 mL Hemox buffer (TCS Scientific Corp.) in a plastic vial. Further, 20 µL of Additive-A, 10 µL of Additive-B and 10 µL of antifoaming agent (TCS Scientific Corp.) were added to the mixture. Excess compressed air was bubbled through the PolybHb solution to saturate it to a \( \text{pO}_2 \) of 147 ± 1 mm Hg. This step was carried out carefully for ~30 min to allow the \( \text{O}_2 \) to completely saturate the PolybHb solution. Post saturation; the air stream was replaced by pure \( \text{N}_2 \) to deoxygenate the PolybHb solution. The respective absorbances of \( \text{HbO}_2 \) and deoxy-Hb were recorded via dual-wavelength spectrophotometry and were used to compute the oxygen saturation (%) of PolybHb molecules. PolybHb \( \text{O}_2 \)-saturation was plotted as function of \( \text{pO}_2 \) to yield the \( \text{O}_2 \) equilibrium curves (OECs). Hill equation (Eq. 2.1) was used to fit the OECs obtained for PolybHbs and control Hb [360, 361, 559]. The \( \text{P}_{50} \) (partial pressure of \( \text{O}_2 \) at which 50% of the polybHb present is saturated) and the
cooperative coefficient (n) of PolybHb molecules were recorded and compared to corresponding values obtained for free-Hb and RBCs.

4.2.8 Rapid Kinetic Measurements of PolybHb solutions

PolybHb gaseous ligand binding/release kinetics were assessed using an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer (Applied Photophysics Ltd., Surrey, United Kingdom). Rapid kinetic measurements were performed using protocols previously described by Rameez and Palmer [344-346]. For all stopped flow measurements, a control of bovine Hb was used to ensure the authenticity of the results.

Oxygenated PolybHb solutions having an overall heme concentration of 15 μM were promptly mixed with a 1.5 mg/mL sodium dithionite solution (Sigma-Aldrich, St. Louis, MO), to measure the O₂ off-loading rate. Both solutions were prepared in PBS (0.1 M, pH 7.4). The absorbance for this deoxygenation reaction was monitored at 437.5 nm at 20 ºC.

CO association rates were measured by rapidly mixing deoxygenated PolybHb solutions (15 μM on heme basis) with different concentrations of saturated CO solution. Both deoxygenated PolybHb and saturated CO solutions were prepared in PBS (0.1 M, pH 7.4) in the presence of 1.5 mg/mL sodium dithionite. This reaction was also monitored at 437.5 nm at 20 ºC. CO-binding to Hb is a second order reaction and hence computing its rate constant is complicated. To simplify analysis the pseudo unimolecular assumption was made. The reaction was carried out separately using two very high concentrations of CO (232 and 464 μM) as compared to the heme concentration (15μM). The two apparent first order reaction rates obtained from these measurements were
then plotted against the corresponding CO concentrations, the slope of this fit yielded the second order rate constant for CO association.

The NO dioxygenation reaction involves the conversion of ferrous oxy-Hb to ferric Hb. This is a very fast reaction (reaction rates \( \sim 10^7 \text{ M}^{-1}\text{s}^{-1} \)), thus certain precautions were taken to effectively monitor this reaction. A very dilute solution of oxygenated PolybHb (1 \( \mu \text{M} \) on heme basis) was reacted with low concentrations of NO stock solution (12.5 and 25 \( \mu \text{M} \)). NO dioxygenation of the control (bovine Hb) was monitored for the shortest time scale the instrument could measure (0.0125 s). The dioxygenation reactions were monitored at 420 nm at 20 °C. Analogous to CO association, NO dioxygenation is also a second order reaction and thus two different dilutions of the NO stock solution were used. The NO stock solution was prepared by bubbling NO gas through a deoxygenated solution of 0.1 M phosphate buffer (PB), pH 7.4 as outlined by Rameez and Palmer [346]. PBS (0.1 M, pH 7.4) was used as the reaction buffer for all kinetic measurements.

### 4.2.9 PolybHb molecules as carriers of CO and NO

In light of the anti-apoptotic, anti-inflammatory and vasorelaxing properties of CO and NO, we hypothesize that their presence in the systemic circulation can potentially inhibit the release of cytotoxic molecules during reperfusion [158, 161, 162, 168]. Therefore, in this study we attempted to use cellular and acellular HBOCs to deliver these therapeutic gaseous ligands to ischemic tissues in order to mitigate reperfusion injuries.

CO bound T- and R-state PolybHb solutions were synthesized by treating deoxygenated PolybHb solutions with ultra-pure CO gas (99.3%). Briefly, 15-20 mL PolybHb solution was taken in a sealed serum bottle and a long needle was used to de-
gas the solution for 30-40 min under continuous stirring. The de-gassing was achieved with alternate cycles of vacuum and inert N₂ gas. Samples were taken at regular intervals to measure the dissolved pO₂ using a Blood Gas Analyzer (Siemens Rapidlab 248, Diamond Diagnostics, Holliston, MA). The de-gassing process was continued till the pO₂ dropped to 0 mm Hg. At this stage the PolybHb solution inside the serum bottle was devoid of any dissolved O₂; however, the degassing process might be partially unsuccessful in removing some of the O₂ bound to the PolybHb molecules. Since CO binds almost 200 times more strongly to Hb as compared to O₂, CO molecules can successfully displace O₂ molecules bound to PolybHb according to the following chemical reaction [590]:

\[ \text{PolybHb - O}_2 + \text{CO} \rightarrow \text{PolybHb - CO} + \text{O}_2 \]

Ultra-pure (99.3%) CO gas was passed through the serum bottle for 1 – 2 h, care was taken to not bubble any gas through the protein solution as that would lead to froth formation. Also, the serum bottle was wrapped in aluminium foil and care was taken to avoid direct light sources as HbCO tends to photolyse. The CO bound state of polybHb was then confirmed via UV-vis spectroscopy.

Since the affinity of Hb for NO is ~1000-fold greater than its affinity for CO [590], we hypothesized that NO could replace CO from PolybHb-CO to form PolybHb-NO. The reaction scheme is as follows,

\[ \text{PolybHb - CO + NO} \rightarrow \text{PolybHb - NO} + \text{CO} \]

Thus, 15-20 mL PolybHb-CO solution was taken in a sealed serum bottle and was treated with ultra-pure (99.9%) NO gas (bubbled through 5M NaOH to remove nitrite impurities) for 1 – 2 hours in the absence of light. The NO bound state of the PolybHb
was then confirmed via UV-vis spectroscopy. All gassing and de-gassing experiments were carried out in an ice-bath to prevent metHb formation.

4.2.10 Spectral Deconvolution and Stability of Liganded PolybHb

Spectral deconvolution studies were performed to test the purity and stability of PolybHb-CO and PolybHb-NO solutions. Percentages of CO and NO bound PolybHb were measured as a function of time by spectral deconvolution using reference spectra for pure deoxyHb, oxyHb, nitrosylHb, metHb, and carboxyHb as previously described [435, 682-687]. Visible spectra between 450 – 700 nm were collected for PolybHb-CO and PolybHb-NO solutions separately [435, 685, 686]. The percentages of these species in respective solutions were estimated by fitting the obtained spectra versus previously obtained reference spectra using a least squares method previously described in the literature [435, 682-687]. The spectral deconvolution protocols and MS Excel macros used in our lab to determine percentages of liganded PolybHbs were developed using guidelines and suggestions provided by Dr. Rakesh Patel’s lab at the Department of Pathology, Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA. Briefly, to generate the reference spectra, pure Hb species were synthesized using methods described below.

4.2.10.1 DeoxyHb

Roughly 50 µM PolybHb solution (on heme basis) was taken in a sealed serum bottle and degassed by alternate cycles of vacuum and N₂ gas using a long needle. The pO₂ of the solution was checked periodically using a Blood Gas Analyzer (Siemens Rapidlab 248, Diamond Diagnostics, Holliston, MA). When the pO₂ of the solution dropped below 20 mm Hg, 1.5 mg/mL sodium dithionite was added to the PolybHb solution to achieve complete deoxygenation. The pO₂ of the solution was verified to be 0
mm Hg. Reference spectra of deoxyHb were then obtained between 450 – 700 nm using a UV-Vis spectrometer. Deoxygenated PBS (0.1M, pH 7.4) containing 1.5 mg/mL sodium dithionite was used to reference the spectrometer. The peaks of the Soret and Q bands for deoxyHb were compared to the values reported in the literature [590]. The spectral measurements were done using sealed quartz cuvettes to maintain strict anaerobic conditions. Transfer of solutions from serum/gas bottles to cuvettes were carried out anaerobically.

4.2.10.2 OxyHb

Previously synthesized deoxyHb solution was treated with ultra-pure O₂ gas for 20-30 min under continuous stirring. The experiment was carried out in an ice-bath maintained at 4°C to avoid metHb formation. The pO₂ of the solution was periodically monitored using a Blood Gas Analyzer. When the pO₂ of the solution was >749 mm Hg, pure spectra of oxyHb were obtained between 450 – 700 nm using a UV-Vis spectrometer. The peaks of the Soret and Q bands for HbO₂ were compared to the values reported in the literature [590].

4.2.10.3 CarboxyHb

Previously synthesized deoxyHb solution was treated with ultra-pure CO gas for 20-30 min under continuous stirring. The experiment was carried out in an ice-bath maintained at 4°C to avoid metHb formation. Pure spectra of carboxyHb were obtained between 450 – 700 nm using a UV-Vis spectrometer. The peaks of the Soret and Q bands for HbCO were compared to the values reported in the literature [590].
4.2.10.4 NitrosylHb

Previously synthesized carboxyHb solution was further degassed by alternate cycles of vacuum and N₂ gas using a long needle to remove traces of dissolved O₂ from the solution. The pO₂ of the solution was checked periodically using a Blood Gas Analyzer (Siemens Rapidlab 248, Diamond Diagnostics, Holliston, MA). When the pO₂ dropped below 0 mm Hg; the HbCO solution was treated with ultra-pure NO gas for 30-40 min under continuous stirring. Sodium dithionite was not used in this reaction as it is known to react with NO [590]. The experiment was carried out in an ice-bath maintained at 4°C to avoid metHb formation. Pure spectra of nitrosylHb were obtained between 450 – 700 nm using a UV-Vis spectrometer. The peaks of the Soret and Q bands for HbNO were compared to the values reported in the literature [590].

4.2.10.5 MetHb

Previously synthesized deoxyHb solution was treated with excess potassium ferricyanide (K₃FeCN₆). The resulting mixture was then desalted by passing through a GE™ Healthcare Hi-Prep™ 26/10 desalting column (Fischer Scientific) pre-packed with Sephadex™ G-25 fine media in order to remove excess K₃FeCN₆ and obtain pure metHb solution. Pure spectra of metHb were then obtained between 450 – 700 nm using a UV-Vis spectrometer. The peaks of the Soret and Q bands for metHb were matched with values reported in the literature [590].

CO and NO bound PolybHb solutions were synthesized using methods described earlier (Section 4.2.9). Ligand bound PolybHb solutions were appropriately diluted and their visible spectra between 450 – 700 nm were acquired using a UV-Vis spectrometer. To confirm the synthesis of pure CO and NO bound PolybHb, the spectra obtained from these solutions were analyzed using a spectral deconvolution technique described in the
literature [435, 682, 684-688]. Additionally, the deconvolution technique was also used to monitor the stability of these ligand loaded PolybHbs over a period of 72 h.

4.3 Results and Discussions

It was important to study size, zeta potential, Hb and metHb concentration, O₂ equilibria, and gaseous ligand binding/release kinetics of the fabricated PolybHb solutions in order to judge their suitability as potential gaseous ligand carriers for treating IRI.

4.3.1 Hb and MetHb Concentration of PolybHb Solutions

The Hb concentrations measured for the 30:1 PolybHb solutions synthesized in our lab ranged between 11.6 – 12.3 g/dL for T-State, and between 13.2 – 14.5 g/dL for R-state PolybHb. These concentrations are comparable to the [Hb] of whole blood (15.7 g/dL for men and 13.8 g/dL for women) [573]. Buehler et al. proposed that molecular interactions between individual PolybHb molecules intensify with increasing PolybHb concentrations; therefore concentrated PolybHb solutions (~10 g/dL) have been shown to have high viscosities [436]. In their study, Buehler et al. reported that at high concentrations (~10 g/dL), the PolybHb solutions were more viscous than cell-free Hb (~1.6 cp) and blood (~3 cp) [436, 689]. It is well-known that blood viscosity strongly impacts blood vessel diameter owing to the shear stress interactions with the endothelium [690]; moreover decreases in blood viscosity leads to vasoconstriction and systemic hypertension [436, 690, 691]. Buehler et al. also noted that concentrated PolybHb solutions when transfused in vivo can potentially augment NO production via mechanotransduction of the endothelium [436, 692-697]. NO is a known vasodilator and inhibitor of apoptosis [130, 149, 162, 266, 698], thus NO generation caused by
transfusion of viscous PolybHb solutions can lead to vasorelaxation and can attenuate reperfusion injuries [436]. Additionally, high PolybHb concentrations also imply that a larger number of therapeutic gaseous molecules (CO and NO) can be supplied to the ischemic tissues in order to circumvent reperfusion injuries. Thus, PolybHb concentration can be used as a controlling factor to deliver the required dosage of these beneficial gases to the needy tissues.

The metHb levels observed for the T- and R-state PolybHb solutions were <4%, these values are lower than the metHb levels reported for other HBOC preparations in the literature [436, 670, 699]. In order to reduce metHb formation we added NaCNBH₃ solution to the reaction mixture post polymerization [436]. Additionally, the modified lactated Ringer’s buffer used for the diafiltration step contained N-acetyl-L-Cysteine, a known antioxidant which mitigates heme oxidation and reduces metHb formation [700]. The deoxygenation process for T-state, the oxygenation process for R-state, and the diafiltration step were all carried out on ice-baths maintained at 4ºC to further prevent metHb formation [51, 436].

4.3.2 Hydrodynamic Diameter and Zeta Potential of PolybHb Solutions

The hydrodynamic diameters of the 30:1 T-state PolybHb ranged between 34 – 45 nm while the diameters of R-state PolybHb ranged between 62 – 68 nm. These diameters are considerably larger than the diameters reported in the literature for cell-free bHb (~7 nm) [701]. Therefore, these acellular HBOCs can avoid the side-effects associated with transfusions of cell-free Hb such as Hb autoxidation and subsequent metHb formation, unfolding of the globin chain leading to the release of cytotoxic free-heme, renal toxicity, dissociation of tetrameric Hb into αβ dimers, extravasation through the blood vessel walls into the surrounding tissue space, and scavenging endothelial NO
leading to severe vasoconstriction and systemic hypertension [351, 432, 446, 456, 457].

It is however noteworthy that PolybHbs are considerably smaller in size as compared to
the cellular HBOCs explored in this study. The PEHs with average diameters ~1100 nm
are 15 – 25 folds larger than the PolybHbs, whereas the PEG-LEHs with average
diameters ~255 nm are 3 – 5 folds larger.

The ζ potentials of the T-state PolybHbs were approximately -14 ± 1.2 mV, while
the ζ potential values for the R-state PolybHbs ranged between -12 ± 1.1 mV. The
absolute ζ potential values of the PolybHbs are comparatively higher than the values
obtained for cell-free bHb (-3.7 ± 0.6 mV). The ζ or electrokinetic potential, an “interface
characteristic potential”, is the electrical potential at the shear plane when a particle is
dispersed in a bulk solution [702]. The ζ potential is a measure of particle surface charge
[703], and is thus “an indicator of the surface electrostatic repulsive force of the particle”
[51]. The ζ potential changes with pH and ionic makeup of the surrounding bulk solution.
Therefore, in this study we used PBS (0.1M, pH 7.4) to evaluate the ζ potential of the
PolybHb solutions, since PBS (0.1M, pH 7.4) is iso-osmolar relative to plasma [51]. The
ζ potential of PolybHbs can also forecast their tendencies to form aggregates with other
colloidal particles in systemic circulation upon transfusion [704]. Therefore, higher the
absolute value of the ζ potential, lesser the possibility of colloidal aggregation and hence
greater the colloidal stability of these PolybHb molecules [51]. Moreover, to prevent
undesirable particle aggregation, HBOCs should have absolute ζ potential values equal
or higher than the values reported for RBCs [51]. The isoelectric point of bHb is 6.8
[705]; thus when suspended in PBS (0.1M, pH 7.4), the bHbs and PolybHbs acquired
negative charges [51]. As noted by Zhou et al., the ζ potential values obtained for the
30:1 T- and R-state PolybHbs synthesized in our lab are “within the limited flocculation
range |5| - |15| mV, which implies a low aggregation potential of these acellular HBOCs
when transfused *in vivo*" [51, 704]. Additionally, the ζ potential values obtained for the 30:1 T- and R-state PolybHbs are comparable to the value reported for RBCs (-16.8 mV [588]), further suggesting the colloidal stability of the PolybHb solutions in systemic circulation [51].

### 4.3.3 O₂-PolybHb Equilibria Measurements

The O₂-PolybHb equilibrium data were fit to the Hill equation (*Eq. 2.1*) to determine the $P_{50}$ and cooperativity coefficient ($n$) as noted earlier. Unlike RBCs and cell-free bHb, the shape of the equilibrium O₂ binding curves obtained for PolybHbs are not sigmoidal. This indicates a significant loss in cooperative binding of O₂ to Hb in PolybHbs when compared to RBCs and cell-free Hb. These observations are illustrated in *Figure 4.2*, which compares typical oxygen equilibria curves of RBCs, cell-free bHb, T- and R-state PolybHb solutions.

Lower O₂ affinities ($P_{50} \sim 33.8$ mm Hg) were observed for 30:1 T-state PolybHbs when compared to cell-free bHb ($P_{50} \sim 25 – 28$ mm Hg) and RBCs ($P_{50} \sim 23 – 27$ mm Hg). Zhou et al. suggested that this may be because polymerization of bHb in the deoxy-state limits the consequent T-state PolybHbs to a “more deoxy-conformation as compared to cell-free bHb and RBCs” [51]. As noted by Zhou, “the $P_{50}$ of the 30:1 T-state PolybHbs synthesized in our lab are comparable to the $P_{50}$ values reported in the literature for commercial Hb-based O₂ carrier, HBOC 201® (38 mm Hg)” [417, 418]; “however, our values are lower as compared to another commercial HBOC, O-raffinose crosslinked polymerized hHb (O-R-PolyHbA₀), Hemolink® (Hemosol Inc, Toronto, Canada) [51, 655, 706, 707]. Thus, the 30:1 T-state PolybHbs have higher O₂ affinities as compared to O-R-PolyHbA₀ but their O₂ affinities are comparable to HBOC 201®” [51, 417, 418, 655, 706, 707].
As compared to cell-free bHb and RBCs, higher \(O_2\) affinities (\(P_{50} \sim 0.94\) mm Hg) were observed for 30:1 R-state PolybHbs. Zhou et al. reported similar findings and suggested that this may be because ‘polymerization of bHb in its \(O_2\)-saturated state limits the consequent R-state PolybHbs to a more oxy-conformation as compared to cell-free bHb and RBCs’ [51]. Interestingly, Zhou et al. noted that the 30:1 R-state PolybHbs synthesized in our lab had “higher \(O_2\) affinities (lower \(P_{50}\) values) when compared to commercially available amide bond polymerized bHb, Oxyvita\textsuperscript{®} (6.4 mm Hg)” [51, 414, 415]. Furthermore, Zhou proposed that glutaraldehyde crosslinking in our PolybHbs had a greater effect on \(O_2\) affinity as compared to amide bond facilitated crosslinking in Oxyvita\textsuperscript{®} [51].

![Oxygen Equilibrium Curve](image)

**Figure 4.2:** Comparison of \(O_2\) equilibria curves of cell-free bHb, RBCs, R- and T-state PolybHb.

Both 30:1 T- and R-state PolybHbs had lower cooperativity coefficients \((n) (\leq 1)\) as compared to cell-free bHb \((n \sim 2.6 – 2.8)\) and RBCs \((n \sim 2.2 – 2.5)\). The inter- and
intra-molecular crosslinks affected by the glutaraldehyde molecules limits the structural flexibility of the PolybHbs, thereby inhibiting the transmission of quaternary structural changes between neighboring globin chains of the individual bHb units in a PolybHb molecule [51]. The quaternary conformational changes observed in a Hb molecule during its transition from the deoxy- to the oxy-state involve rotation of the two symmetrical αβ dimers by 15º relative to each other and a translation of 0.1 nm along the rotation axis [708]. This rotation about the axis is perhaps hindered by the inter- and intra-molecular glutaraldehyde crosslinks in PolybHbs, thus resulting in the observed low cooperativities [51].

### 4.3.4 Rapid Kinetic Measurements of PolybHb Solutions

Rapid kinetic measurements were done on the PolybHbs to compare their ligand binding/release kinetics to that of cell-free Hb and RBCs. These rates are important to test the ability of these particles to store and transport important gaseous ligands such as CO and NO. NO dioxygenation kinetics can be directly related to the NO scavenging mechanism for the development of vasoconstriction and systemic hypertension, and the O₂ dissociation measurements can be related to the autoregulation theory for the development of vasoconstriction and systemic hypertension. Thus, rapid kinetic measurements were deemed significant in assessing the ability of these particles to elicit vasoconstriction and hypertension in systemic circulation.

#### 4.3.4.1 Reactions with O₂

The $k_{\text{off},O_2}$ values obtained for 30:1 T-state PolybHbs were ~18 s⁻¹, whereas for R-state PolybHbs the rates were ~8.5 s⁻¹. The $k_{\text{off},O_2}$ rates obtained for the T-state are higher than the values obtained for RBCs (8.61 ± 2.55 s⁻¹), while the rates for R-state are
similar to the ones obtained for RBCs. However, the $k_{off,O_2}$ rates for T- and R-state PolybHbs are both lower than the values obtained for the control, bovine Hb (34 – 39 s$^{-1}$). R-state PolybHbs possessed lower $k_{off,O_2}$ rates as compared to T-state PolybHbs; this is an expected behavior given the higher O$_2$ affinities observed for the R-state PolybHbs [51]. **Figure 4.3** compares typical kinetic time courses of O$_2$ dissociation for RBCs, cell-free Hb, T- and R-state PolybHbs. The O$_2$-offloading reaction was monitored for 4s for RBCs, T- and R-state PolybHbs; while a 0.25s time-scale was used to monitor the reaction for cell-free Hb.

![Figure 4.3: Time courses for deoxygenation of (A) bovine Hb, (B) RBCs, (C) T-state PolybHb, and (D) R-state PolybHb in presence of 1.5 mg/mL sodium dithionite. The experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 437.5 nm 20 ºC. PBS (0.1M, pH 7.4) was used as the reaction buffer.](image-url)
The $k_{\text{off,}O_2}$ rates obtained for the T-state PolybHbs are ~2 folds lower than the rates obtained for bHb. Interestingly, these $O_2$ off-loading rates are only slightly higher than the rates observed for PEG-LEHs, a cellular HBOC (Chapter 3). Further, the $k_{\text{off,}O_2}$ rates obtained for the R-state PolybHbs are ~4 folds lower than the rates obtained for bHb. Additionally, these $O_2$ off-loading rates are comparable to the rates obtained for PEHs, a cellular HBOC equipped with a thick and robust polymer membrane (Chapter 2). Therefore, in-spite of lacking a protective membrane (unlike PEG-LEHs and PEHs) in order to provide a physical barrier to thwart trans-membrane $O_2$ diffusion, the T- and R-state PolybHbs retard $O_2$ off-loading considerably.

High offloading rates of $O_2$ from Hb forms the basis of the ‘autoregulation theory’ of development of vasoconstriction and systemic hypertension [598-600]. According to the autoregulation theory an increase in $O_2$ delivery by diffusion, decreases $O_2$ acceptance by tissues because of vasoconstriction [149, 462]. Thus, moderate $O_2$ release rates are critical in determining HBOC efficacy. The PolybHbs synthesized in the present study considerably retard $O_2$ offloading as compared to cell-free Hb [51, 366, 436, 648, 654]. Furthermore, their $k_{\text{off,}O_2}$ rates are comparable to that of PEG-LEHs and PEHs. Thus, the PolybHbs synthesized in our lab can potentially deliver $O_2$ to ischemic tissues at regulated rates avoiding vasoconstriction due to oversupply of $O_2$.

4.3.4.2 Reactions with CO

Figure 4.4 shows characteristic CO association kinetic time courses for deoxygenated (A) bHb, (B) RBCs, (C) T-state PolybHbs, and (D) R-state PolybHbs upon reaction with CO stock solution (464 μM). The time courses were similar for Hb, RBCs and PEG-LEHs (0.5 s). Figure 4.5 plots the dependence of the pseudo first order rates on CO concentrations for RBCs, stroma free-Hb, T- and R-state PolybHbs. Therefore,
the slopes of the linear fits in Figure 4.5 indicate the second order CO binding rate constants of the different species.

The $k_{on,CO}$ rates obtained for cell-free Hb tested in this study ranged between $0.20 \pm 0.01 \, \mu M^{-1}s^{-1}$. These values are significantly higher ($p<0.05$) than the values obtained for RBCs ($0.10 \pm 0.04 \, \mu M^{-1}s^{-1}$). This is because the high intra-RBC Hb concentration and RBC membrane both act as physical barriers to retard CO binding to Hb. Due to the absence of protective membranes; $k_{on,CO}$ rates obtained for the T-state PolybHbs ($\sim 0.14 \, \mu M^{-1}s^{-1}$) are higher than those obtained for RBCs. However, the T-state PolybHbs had lower CO association rates as compared to cell-free bHb. Similar observations were made by Zhou et al., and it was suggested that polymerization of Hb in the T-state limits “accessibility to the heme pockets” available for CO binding [51]. Interestingly, T-state PolybHbs had lower CO association rates as compared to PEG-LEHs (Chapter 3) even though the latter possesses protective membranes to act as physical barriers to retard CO binding. We conclude, that in this case the inaccessibility of heme pockets in T-state PolybHbs make up for the absence of a protective membrane in successfully retarding CO association. R-state PolybHbs had much higher $k_{on,CO}$ rates ($\sim 0.22 \, \mu M^{-1}s^{-1}$) when compared to RBCs; however, their CO binding rates were very slightly higher than those obtained for cell-free bHb. Zhou et al. proposed that “polymerization of Hb in the oxygenated state resulted in R-state PolybHbs acquiring more open conformations leading to higher availabilities of heme pockets for CO association” [51].

Dissociation rate constants of HbCO has been previously reported in the literature [590]. Gibson et al. estimated the $k_{off,CO}$ rates of sheep blood to be $\sim 0.012 \, s^{-1}$ $(23.5^\circ C, \, pH \, 9.1)$ [662]. We believe that, analogous to $O_2$ off-loading, PolybHbs will
release CO quicker than RBCs as they lack protective membranes. Thus, we conclude that PolybHbs can potentially deliver exogenous CO to ischemic tissues at moderate rates in order to circumvent reperfusion injury resulting from ROS generation and oxidative tissue toxicity [147, 163, 168, 479]. In the future, experiments will be designed to measure the $k_{off,CO}$ rates of PolybHbs in order to determine their CO offloading abilities.

Figure 4.4: Time courses for CO (464 µM) association reaction of deoxygenated (A) bovine Hb, (B) RBCs, (C) T-state PolybHb, and (D) R-state PolybHb. The deoxygenation was carried out in presence of 1.5 mg/mL sodium dithionite. Experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 437.5 nm at 20 °C and PBS (0.1M, pH 7.4) was used as the reaction buffer.
Figure 4.5: Comparison of CO association rates of cell-free Hb, RBCs, T- and R-state PolybHbs.

4.3.4.3 Reactions with NO

Figure 4.6 shows characteristic NO dioxygenation kinetic time courses for oxygenated (A) bHb, (B) RBCs, (C) T-state PolybHbs, (D) R-state PolybHbs upon reaction with NO stock solution (25 µM). Figure 4.7 plots the dependence of the pseudo first order rates on NO concentrations for RBCs, stroma free-Hb, T- and R-state PolybHbs. Therefore, the slopes of the linear fits in Figure 4.7 indicate the second order NO dioxygenation rate constants of the different species.

The NO dioxygenation rates obtained for the T-state PolybHbs (~16.6 µM⁻¹s⁻¹) were comparable to the rates obtained for R-state PolybHbs (~14.5 µM⁻¹s⁻¹). Similar \( k_{\text{ox,NO}} \) values were reported by Zhou et al. [51]. Previous studies have also reported that different types of PolyHbs cause similar levels of \( \text{in vivo} \) NO scavenging, thus the similarities in their NO dioxygenation rates can be anticipated [51, 709]. The \( k_{\text{ox,NO}} \) values obtained for the PolybHbs were significantly higher (\( p<0.05 \)) than those obtained
for RBCs (0.33 ± 0.32 μM⁻¹s⁻¹). However, the NO dioxygenation rates observed for the PolybHbs were almost 2 folds lower than the rates obtained for cell-free Hb (34.99 ± 7.46 μM⁻¹s⁻¹).

Figure 4.6: Time courses for NO (25 μM) dioxygenation reaction of oxygenated (A) bHb, (B) RBCs, (C) T-state PolybHb, and (D) R-state PolybHb. Experimental data shows an average of 10-15 kinetic traces. The reactions were monitored at 420 nm at 20 °C and PBS (0.1M, pH 7.4) was used as the reaction buffer.

The NO dioxygenation rate constants, obtained in this study, for cell-free Hb are comparable to the values reported in the literature (20 – 34 μM⁻¹s⁻¹) [50, 345, 601, 602]. The relatively higher NO dioxygenation rates obtained for the PolybHbs as compared to
PEHs (0.42 – 1.50 μM⁻¹s⁻¹, Chapter 2) and PEG-LEHs (2.14 ± 0.76 μM⁻¹s⁻¹, Chapter 3) can be explained by the absence of protective membranes in these acellular HBOCs. We believe that transfusions of NO saturated PolybHbs will not only avoid reperfusion injury in ischemic tissues but will also alleviate any NO scavenging related vasoconstriction previously observed during in vivo transfusion studies of these acellular HBOCs.

![Figure 4.7: Comparison of NO dioxygenation rates of human Hb, RBCs, T- and R-state PolybHb.](image)

The results from these rapid kinetic measurements showed that when administered in vivo, R-state PolybHbs can potentially deliver O₂ to ischemic tissues at slow to moderate rates, thereby averting vasoconstriction due to oversupply of O₂. The PolybHbs are also capable of delivering benign CO to ischemic tissues at controlled rates in order to circumvent reperfusion injury resulting from ROS generation and oxidative tissue toxicity. Though PolybHbs have comparatively higher NO dioxygenation
rates than RBCs, PEG-LEHs, and PEHs; introduction of PolybHbs saturated with NO in the systemic circulation can potentially attenuate the side-effects (NO scavenging and vasoconstriction) observed in prior in vivo studies involving these acellular HBOCs.

4.3.5 PolybHb Molecules as Carriers of CO and NO

Figure 4.8A compares the Soret bands of CO bound bHb, T- and R-state PolybHb; while Figure 4.8B compares their Q bands. The pure spectra for HbCO had a Soret peak at 419nm (Figure 4.8A) and the Q bands had peaks at 540nm and 569nm (Figure 4.8B). These peaks were in agreement with those reported in the literature for pure HbCO [590]. CO bound states of T- and R-state PolybHb were confirmed by examining their respective Soret and Q band peaks. Both PolybHbs had Soret (Figure 4.8A) and Q band peaks (Figure 4.8B) unique to HbCO (i.e. Soret band peak at 419nm and Q band peaks at 540nm and 569nm; [590]).

Figure 4.9A compares the Soret bands of NO bound bHb, T- and R-state PolybHb; while Figure 4.9B compares their Q bands. The pure spectra for HbNO had a Soret peak at 418nm (Figure 4.9A) and the Q bands had peaks at 545nm and 575nm (Figure 4.9B). These peaks were in agreement with those reported in the literature for pure HbNO [590]. NO bound states of T- and R-state PolybHb were confirmed by examining their respective Soret and Q band peaks. Both PolybHbs had Soret (Figure 4.9A) and Q band peaks (Figure 4.9B) unique to HbNO (i.e. Soret band peak at 418nm and Q band peaks at 545nm and 575nm; [590]). Additionally, characteristic of HbNO spectra reported in the literature, the minimum between the two Q bands in the visible region (of the PolybHb-NO spectra) was not as pronounced as observed in the case of HbCO spectra.
Figure 4.8: Comparison of (A) Soret band and (B) Q bands of CO bound cell-free bHb (red), R-state (blue), and T-state PolybHb (green).

Figure 4.9: Comparison of (A) Soret band and (B) Q bands of NO bound cell-free bHb (red), R-state (blue), and T-state PolybHb (green).
### 4.3.6 Spectral Deconvolution and Stability of Ligand Bound PolybHb

**Figure 4.10A** shows the relevant reference spectra of the species (deoxy-, oxy-, carboxy-, met-, and nitrosyl-) expected to be involved in the synthesis of T-state stabilized NO and CO bound PolybHbs. **Figure 4.10B** shows the spectral deconvolution results of the starting material (T-PolybHb), CO bound T-PolybHb immediately after synthesis, and T-PolybHb-CO 72 h post synthesis. From our deconvolution results we confirmed that the starting material was primarily a mixture of oxy- and deoxy-PolybHb (91.5% PolybHb-\(O_2\) and 8.2% deoxy-PolybHb). Deconvolution results confirmed the formation of pure PolybHb-CO (99.99%) after degassing and treating the starting material with ultra-pure CO gas as described earlier. HbCO is approximately 200 times more stable than HbO\(_2\) against autoxidation [280]; our deconvolution results indicated that 72 h post synthesis 92% of the T-PolybHb was in its CO bound state. However, we observed a significant amount (~8%) of met-PolybHb at this time point. This may be due to different reasons such as stress exerted on the PolybHb-CO molecules due to periodic sampling, improper sealing of the serum bottle, exposure to light leading to lysis of HbCO, and other experimental errors. The CO bound T-PolybHbs are expected to deliver most of the beneficial CO gaseous ligands to needy ischemic tissues much before the 72 h time point introduction to systemic circulation. Additionally, most PolybHb preparations reported in the literature have circulation half-lives ranging between 15 – 20 h [417, 418, 710], thus we expect that by the 72 h time point most of these acellular HBOCs will be cleared from the circulation by the Reticulo Endothelial System (RES). **Figure 4.10C** shows the spectral deconvolution results of NO bound T-state stabilized PolybHb: 0h, 24h, 48h, and 72h post transfusion. Our results confirmed the synthesis of pure and stable PolybHb-NO. Though the deconvolution results
indicated traces of T-PolybHb-O₂ (2.4%) at the 0h time-point, our results confirmed 99.99% PolybHb-NO at subsequent time-points. Therefore, our results confirmed earlier observations that under anaerobic conditions HbNO is highly stable as the affinity of Hb for NO is more than 1000-fold greater than its affinity for CO [590]. Furthermore, these results indicate that we successfully synthesized ligand loaded PolybHb solutions capable of delivering these beneficial gases to ischemic tissues upon transfusion in vivo.

Figure 4.10: (A) Pure species reference spectra for deconvolution of T-state stabilized PolybHb (30:1). The expected species in this case are nitrosyl (red), oxy (green), carboxy (blue), deoxy (yellow), and metHb (brown). (B) Purity and stability (at 25°C) of CO bound T-state PolybHb. (C) Percent and stability (at 4°C) of NO bound T-PolybHb at 0h, 24h, 48h, and 72h post synthesis.
Figure 4.11A shows the relevant reference spectra of the species (deoxy-, oxy-, carboxy-, met-, and nitrosyl-) expected to be involved in the synthesis of R-state stabilized NO and CO bound PolybHbs.

Figure 4.11: (A) Pure species reference spectra for deconvolution of R-state stabilized PolybHb (30:1). The expected species in this case are nitrosyl (red), oxy (green), carboxy (blue), deoxy (yellow), and metHb (brown). (B) Purity and stability (at 25°C) of CO bound R-state PolybHb. (C) Percent and stability (at 4°C) of NO bound R-PolybHb at 0h, 24h, 48h, and 72h post synthesis.
Figure 4.1B shows the spectral deconvolution results of the starting material (R-PolyHb), CO bound R-PolyHb immediately after synthesis, and R-PolyHb-CO 72 h post synthesis. Expectedly, deconvolution results indicated that 99.9% of our starting material was R-PolyHb bound to O₂. Deconvolution results further confirmed the successful conversion of all R-PolyHb-O₂ to yield pure PolyHb-CO (99.99%) after degassing and treating the starting material with ultra-pure CO gas as described earlier. Interestingly, 72 h post synthesis, 99.99% R-PolyHb was still bound to CO. Further, at this time-point, percent of met-PolyHb in our solutions were negligible or below detection limits. Figure 4.1C shows the spectral deconvolution results of NO bound R-state stabilized PolyHb: 0h, 24h, 48h, and 72h post transfusion. Our results confirmed the synthesis of pure and stable R-PolyHb-NO. Deconvolution results confirmed the formation of 99.99% pure R-PolyHb-NO immediately after treatment of R-PolyHb-CO with ultra-pure NO gas. However, our deconvolution results indicated traces (around 1-2%) of PolyHb-O₂, PolyHb-CO, deoxy-PolyHb, and met-PolyHb at subsequent time-points. Nonetheless, at all time points more than 90% of the PolyHb were bound to NO. These results suggest that we successfully synthesized R-state stabilized PolyHb solutions capable of delivering beneficial NO and CO gases to ischemic tissues upon introduction to systemic circulation.
4.4 Conclusions

In order to synthesize HBOCs capable of transporting and delivering therapeutic gaseous ligands (CO and NO) to ischemic tissues we explored glutaraldehyde crosslinked T- and R-state stabilized PolybHbs in this chapter. As described previously in Chapter 3, we were successful in synthesizing CO loaded PEG-LEH particles. However, we could achieve only partial NO loading in PEG-LEHs, a cellular HBOC. We believe that the protective bilayer membrane in the case of PEG-LEHs prevented complete NO saturation of the encapsulated Hbs. In order to alleviate the issue of partial NO loading, we decided to explore acellular HBOCs such as glutaraldehyde crosslinked PolybHbs as potential carriers of therapeutic gases.

The PolybHb solutions were prepared using a method devised by Zhou and Palmer in our lab [51, 436]. We successfully synthesized 99% pure CO and NO loaded T- and R-state PolybHb solutions. The ligand bound states of the PolybHb molecules were verified by UV-Vis spectroscopy and their purity was ascertained by spectral deconvolution techniques. Our results have led us to believe that CO and NO loaded PolybHb molecules can potentially deliver these beneficial gases to ischemic tissues upon introduction to systemic circulation. Transfusion of these particles will also help in restoring blood volume and shear stress, thereby promoting increased endothelial NO synthesis. Additionally, after the beneficial gaseous ligands dissociate from the PolybHb molecules, these acellular HBOCs are expected to store and transport O₂. Thus the ligand loaded PolybHb solutions will perform multiple functions at different stages of the proposed treatment.
CHAPTER 5  Future Work

The goal of the current study was to develop safe and efficacious Hb-based ligand carriers capable of delivering therapeutic gaseous ligands to ischemic tissues in order to circumvent reperfusion injuries encountered during resuscitation from hemorrhagic shock. We successfully developed acellular (PolybHb) and cellular (PEG-LEH) HBOCs capable of storing and transporting benign gaseous ligands such as CO and NO. These carriers will initially deliver vasorelaxing, anti-apoptotic, and anti-inflammatory gaseous ligands to ischemic tissues to avoid reperfusion injuries. Once the beneficial gases dissociate from the carriers, these blood substitutes are expected to store and transport O₂, normalize perfusion pressure, improve microcirculation, and restore blood volume and shear stress. All in all, the novel therapeutic proposed in this study can be an inexpensive and viable alternative to present IRI treatment strategies. However, further improvements and assessments are necessary to establish ligand loaded HBOCs as a first-line therapy to mitigate IRI in clinical practices.

5.1 Delivery of H₂S and HNO to Ischemic Tissues

5.1.1 PEG-LEHs as carriers of H₂S

As noted in Chapter 1, H₂S is generated endogenously in the body and functions as a neuromodulator and a smooth muscle cell relaxant [711-713]. In light of the vasorelaxing [714], anti-inflammatory [715], and anti-apoptotic properties [716] of H₂S,
we propose to supply HBOC bound H$_2$S gaseous molecules to tissues affected by IRI. Though H$_2$S is known to reduce metHb, it has an inhibitory effect on cytochrome-c oxidase and it does not bind to mammalian Hb [717].

The clam *Lucina Pectinata* has three distinct types of Hb molecules. The monomeric HbI has been shown to bind and transport H$_2$S, while HbII and HbIII are O$_2$ reactive [711, 717]. The clam is found in sulphide-rich environments and the nutritional needs of this mollusc are derived from a symbiotic relationship with sulfideoxidizing bacteria. The molecular weight of HbI has been reported to be 16900 Da [718, 719]. The high affinity of HbI for H$_2$S is explained by a very high association rate ($k_{on} = 2.3 \times 10^5$ M$^{-1}$ s$^{-1}$) coupled with an extremely slow off-rate ($k_{off} = 0.22 \times 10^{-3}$ s$^{-1}$) [719-721]. Structural studies of the binding site of HbI have revealed that the HbI-H$_2$S conjugate is stabilized by a glutamine (Gln) residue at the distal E7 position instead of the usual histidine (His) found in mammals [711, 718-724]. Additionally, researchers have reported that, HbI has phenylalanine (Phe) residues at the B10, E11 and CD1 distal positions forming a “Phe-cage” which further stabilize H$_2$S binding by hydrogen bonding [711, 718-724].

Purification of HbI can be carried out by the detailed methods outlined by Kraus et al. [720]. Briefly, “25g frozen clam gills will be pulverized under liquid nitrogen in a porcelain mortar. The powered gills will be suspended in 100ml CO saturated solution containing Hepes buffer (10 mM, pH 7.5), EDTA (5 mM, pH 7.5), and dithiothreitol (1 mM, pH 7.5). The solution will be allowed to thaw under continuous CO sparging. The slurry will then be pH adjusted to 7.4 and centrifuged at 12000g for 20 min. The red supernatant will be carefully filtered from the cell debris by vacuum filtration. The supernatant will then be passed through a Sephadex® G75 column, at 4°C, at a flowrate of ~25 ml/h with CO saturated PBS (50 mM, pH 7.4). The eluting fractions will be concentrated using YM-10 membrane filters (EMD Millipore). Yellow, low MW fractions...
along with the colorless cysteine rich proteins will be suspended in triethanolamine (10mM, pH 8.3) and further purified by passing through a DEAE-Sepharose® Cl-6B column using a 60 ml/h flow rate. The fractions obtained can be suspended in PBS (50 mM, pH 7.4) and stored at -80°C. Using this method Kraus et al. were able purify Hbl having concentrations of about 10 mg/ml [720]. However, such low Hb concentrations are not suitable for our PEG-LEH fabrication method. Thus we need to scale up the purification process in order to attain Hbl concentrations of ~250-300 mg/ml.

Starting with a raw material of concentrated Hbl solution our usual PEG-LEH fabrication techniques (described in Chapter 3) can be followed to obtain PEG-LEH particles encapsulating Hbl. Finally, the Hbl vesicles will be treated with ultrapure H2S gas (99.9%) bubbled through 5 M NaOH to form Hbl-H2S encapsulated PEG-LEHs.

5.1.2 PEG-LEHs as carriers of HNO

Nitroxyl (HNO/NO\(^{-}\)) donors have been shown to enhance the contractility of cardiac muscles in the post-ischemic phase without affecting the vascular tone [165, 166, 295]. Nitroxyl is believed to cause vasodilation by increasing cytosolic levels of calcitonin gene-related peptide (CGRP) [166]. Since HNO is a highly reactive compound, it should be generated in situ. Angeli’s salt and Piloty’s acid are the two most commonly used nitroxyldonors, however these donors exhibit unwanted side-effects such as hypotension, headache and gastrointestinal symptoms [294]. Thus, to avert these side-effects, we propose to systemically supply PEG-LEHs encapsulating Hb bound HNO to the ischemic tissues. Recently it has been shown that deoxygenated Hb and Mb can trap free HNO to form stable Hb-HNO and Mb-HNO adducts [725-727]. Due to its short half-life, HNO will be synthesized in situ from the decomposition of HNO-precursors such as Piloty’s acid (PA) (phenylsulfonylhydroxylamic acid) and methylsulfonylhydroxylamic
acid (MSHA) (Cayman Chemicals, Ann Arbor, MI) [725, 728]. Alternately Hb-HNO adducts can also be generated by the reduction of nitrosyl Hb (HbNO) by 4, 4-dimethyl-1, 1-trimethylene-2, 2-dipyridinium (DTDP)/Zn-Hg amalgam (Sigma Aldrich, St. Louis, MO) [725].

5.1.2.1 Decomposition of HNO donors

Human Hb (hHb) will be purified and characterized by techniques developed in our lab [502-504]. The Hb will be re-suspended in PBS (50 mM, pH 9.4) as the decomposition of HNO-precursors requires an alkaline environment. 100 mL concentrated Hb solution (>300 mg/mL) will be de-gassed and deoxygenated by alternate cycles of vacuum and N₂. Samples will be taken at regular intervals and complete deoxygenation of Hb will be verified by UV-vis spectroscopy [558]. Additionally, pO₂ will be measured to ensure the removal of any dissolved O₂. Hb-HNO adducts will be prepared by the method of Kumar et al. [725]. Briefly, anaerobic stock solutions of HNO-precursors will be freshly prepared in deoxygenated PBS (0.1M, pH 7.4) under an inert atmosphere in a glovebox using the molar ratios of PA and MSHA reported by Kumar et al. [725]. However trial runs with different ratios of PA and MSHA will be carried out to optimize the concentrations of these HNO-precursors. As described by Kumar et al., 20 mL, 82.8 mM PA or 20 mL, 214 mM MSHA will be added to 4.65 mM 100 mL deoxyHb. The solution will be allowed to mix for 45 min – 1 h with gentle stirring and then eluted through a Sephadex® G25 column with PBS (0.1M, pH 7.4). The resultant Hb-HNO solutions are known to be quite stable when stored anaerobically and protected from irradiation with light [725, 729].
5.1.2.2 Reduction of HbNO by DTDP

HbNO will be synthesized in our lab using techniques described in Chapters 3 and 4 of this dissertation. As described by Kumar et al., 0.24g DTDP in 20 mL deoxygenated PBS (50mM, pH 9.4) will be reduced with 200 mg Zn-Hg amalgam [725]. 1 mL of the reduced DTDP solution will be reacted with 100 mL HbNO in an anaerobic glove box [725]. The resulting Hb-HNO solution will be filtered through glass wool and run through a Sephadex® G25 column using PBS (50 mM, pH 7.4) [725]. Once synthesized the Hb-HNO solutions will be concentrated using YM-10 membrane filters (EMD Millipore) [725]. UV-vis spectra of the Hb-HNO adducts will be recorded to confirm the formation of Hb-HNO. A characteristic Soret band at 420 nm and the Q bands (not resolved) at 544 nm is expected [590]. Further confirmation may be obtained by Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR) spectroscopy.

It has been shown that Hb-HNO has a half-life of more than 6 months at 4°C under anaerobic conditions in absence of light while its stability in presence of light and O₂ are still being explored [729]. Thus, we plan to carry out the fabrication of PEG-LEH particles encapsulating Hb-HNO in a glovebox avoiding direct irradiation with light. Additionally, we believe that once Hb-HNO is encapsulated inside PEG-LEHs its stability will not be an issue and the robust LEH membrane will prevent the autoxidation of Hb-HNO. Methods previously developed in our lab to synthesize PEG-LEHs (described in Chapter 3) will be used with slight modifications. Instead of a cell-disrupter, a bench-top extruder will be used to obtain unilamellar vesicles; thereby, the extrusion process can be carried out in a glovebox under anaerobic conditions.
5.2 Offloading of Gaseous Ligands from Hb-Based Carriers

The efficacy of the treatment proposed in this study is largely dependent on the ability of Hb-based carriers to release the therapeutic gaseous ligands to ischemic tissues. Both HbCO and HbNO are very stable compounds and exhibit extremely low dissociation rates, 0.012s\(^{-1}\) and 0.00009s\(^{-1}\) respectively [590]. We have not yet measured CO and NO offloading rates for PolyHbs and PEG-LEHs in our lab; however, we can predict that these off-rates will be even slower than their free Hb counterparts.

The ability of light to dissociate HbCO and HbNO is well recognized [590]. Photodissociation with a high quantum yield (Φ) (the ratio of the number of photons effective in the dissociation of a ligand to the total number of photons absorbed) is an inherent property of all CO-heme compounds [590]. Thus, an irradiation of the animal heart with higher wavelength light will be carried out post transfusion to photo-dissociate HbCO. Additionally, in a separate study, the \textit{in vitro} CO release from Hb vesicles (HbV) was measured after rapidly mixing CO-HbV with \(\text{O}_2\)-RBCs [280]. Briefly, a suspension of CO-HbV ([Hb] = 10 g/dL, 0.5 mL) was mixed with a suspension \(\text{O}_2\)-RBCs ([Hb] = 10g/dL, 4.5 mL) using a vortex mixer. The mixture was aliquoted and centrifuged (3000g) at different intervals. The supernatant containing the HbVs was collected and the percent of HbCO remaining inside the HbVs was estimated using UV-vis spectroscopy. The results indicated that after 5 min mixing, more than 80% of the CO was released from the vesicles. We propose to repeat this study developed by Sakai et al. using ligand loaded HBOCs and human RBCs. We expect to observe similar magnitudes of ligand unloading with CO, NO, \(\text{H}_2\text{S}\), and HNO saturated HBOCs.

Also, it has been shown that shining red to near infra-red light (R/NIR) between 630 and 830 nm generated by low-energy lasers or LEDs can release NO from
nitrosylated heme. Additionally, NIR has been shown to reduce infarct size, protect neurons from methanol toxicity, offer cytoprotection after IRI, and stimulate angiogenesis [730]. Specifically, it has been shown in another study that NIR at 670 nm releases NO from HbNO and this released NO was successful in infarct-size reduction in ischemic rabbit hearts [731]. Thus to facilitate the dissociation of HbNO and release of NO from Hb-based carriers we propose to irradiate the animal heart with NIR (670 nm, 60 mW/cm²) post transfusion.

5.3 Animal Studies

Ligand loaded Hb-based carriers synthesized in this study will be transfused in animals using hemorrhagic shock resuscitation protocols in order to evaluate their efficacy at mitigating IRI. The in vivo studies will help us ascertain the ability of these Hb-based carriers to systemically deliver therapeutic gaseous ligands to ischemic tissues. These studies will also allow us to determine the magnitude of vasorelaxation and cytoprotection afforded by these benign gases upon reperfusion. Furthermore, the in vivo studies will provide valuable insights into the capabilities of these ligand carriers to systemically deliver O₂ and ameliorate microcirculation.

5.3.1 Hamster Window Chamber Model

Gaseous ligand loaded Hb-based carriers will be administered to Golden Syrian Hamsters outfitted with a window chamber [168, 732]. Animal handling will be done following procedures described in the “Guide for the care and use of laboratory animals” (National Institutes of Health publication no. 85-23, revised 1996).

Briefly, the window chamber will be implanted using a 50 mg/kg dosage of sodium pentobarbital (Sigma-Aldrich, St. Louis, MO). The chamber window comprises of
a 15 mm circular window and two identical titanium frames. One frame of the chamber will be placed on the dorsal side of the animal after lifting the dorsal skin flap. The skinfold will be removed from one side with the help of a stereomicroscope and backlighting until only a thin layer of retractor muscle and hypodermis on the other side remain. Saline and then a cover slip will be applied on the side that the skinfold has been removed, and will be held in place by the second frame of the chamber. At this point, arterial and venous catheters (PE-50) containing heparinized saline solution (30 IU/mL) will be introduced into the carotid artery and jugular vein. The catheters will run underneath the skin and emerge at the dorsal side of the neck, where they will be attached to the window chamber frame positioned on the dorsal side (Figure 5.1, used with permission from Lippincott Williams and Wolters Kluwer Health: Shock Injury, Inflammation and Sepsis, 22(5), P. Cabrales, A. Tsai, and M. Intaglietta, Hyperosmotic-Hyperoncotic Versus Hyperosmotic-Hyperviscous: Small Volume Resuscitation in Hemorrhagic Shock, Copyright (2004)) [732].

4-5 days after this operation, the animals will undergo measurements of heart rate (HR), mean systemic arterial blood pressure (MAP), hematocrit (Hct), and arterial pO₂. Animals meeting certain pre-established threshold values for these parameters will be included for experiments. These threshold values, as specified by Cabrales et al., are HR >340 beats/min, MAP >80 and <125 mm Hg, systemic Hct >45%, and pO₂ > 50 mm Hg [733].

5.3.2 Hemorrhagic Shock and Resuscitation Protocol

The total blood volume of a hamster is usually assumed to be ca. 7% of their body weight [168]. Hemorrhagic shock will be induced by the removal of 50% of the total blood volume at a rate of 1 mL/min from the carotid artery catheter. After allowing 1 hr for ischemia to set in, animals will be transfused with ligand loaded Hb-based carriers at the same rate; 1 mL/min. The volume replenished will be identical to the volume withdrawn.

5.3.3 Proposed Measurements

To observe the effects of the ligand loaded HBOCs on ischemia reperfusion, blood samples will be drawn from animals at different time intervals before and after transfusion: -2h, 0h, 3h, 6h, 12h, 24h and 48h. The following tests/characterization will be done at each time point.

5.3.3.1 Systemic Parameters Before and After Resuscitation

pH, partial pressures of oxygen (pO₂) and carbon dioxide (pCO₂) of the drawn blood samples will be measured using a blood gas analyzer (Rapidlab 248 Blood Gas Analyzer, Siemens, Deerfield, IL) immediately after sampling. Hct of the collected blood samples will be measured using heparinized thin glass capillary tubes and a micro-
hematocrit centrifuge (Ample Scientific). Viscosity of plasma and whole blood will be measured at a shear rate of ~160 s\(^{-1}\) at 37 °C using a DV-II plus viscometer (Brookfield Engineering Laboratories, Middleboro, MA). Colloid osmotic pressure of plasma will be measured using a 420 Colloid osmometer (Wescor, Logan, UT) after centrifugation (5000g, 2 min) of the collected blood samples. Whole blood lactate concentration will be measured from a 25 μL sample using a YSI 1500 SPORT, lactate analyzer (YSI Incorporated, Yellow Springs, OH). Additionally, HR and MAP will be recorded using a MP 150 (Biopac system, Santa Barbara, CA) [734]. Alternatively, the systolic and diastolic pressures will be recorded and the MAP will be calculated using the following equation [735]:

\[
MAP = P_{\text{diastolic}} + \frac{1}{3}(P_{\text{systolic}} - P_{\text{diastolic}})
\]

In a separate study carried out by Sakai et al. [280], PEG-LEH particles encapsulating HbCO was used for hemorrhagic shock resuscitation in male Wistar rats. The study compared the abilities of CO saturated Hb-Vesicles, O\(_2\) saturated Hb-Vesicles, O\(_2\) saturated RBCs, CO saturated RBCs, and empty lipid vesicles as resuscitative fluids. The results obtained by Sakai et al. suggested no concrete differences in the systemic parameters between CO and O\(_2\) saturated resuscitative fluids [280]. However, as compared to Sakai’s study we propose a much simpler PEG-LEH fabrication technique not only yielding more stable PEG-LEH particles but also transporting other potent gaseous ligands such as NO, H\(_2\)S and HNO. Additionally, we propose to transfuse ligand loaded PolyHb solutions as resuscitative fluids in order to mitigate reperfusion injury. Therefore, we expect to obtain significant differences in the
5.3.3.2 Spectroscopic Measurements

UV-vis spectroscopy and the Winterbourn equation will be used to monitor the concentration of various ligand bound Hb (CO, NO, H$_2$S and HNO) molecules in PolyHb solutions and also inside PEG-LEH particles.

5.3.3.3 Micro-Hemodynamic Properties

Various micro-hemodynamic properties will be recorded using techniques previously described in the literature by Cabrales et al. [732]. Briefly, intricate mappings of the chamber vasculature will be constructed in order to track the same blood vessels studied at baseline (2h prior to hemorrhagic shock) throughout the length of the experiment. Functional capillaries or capillary segments that see a transit of at least 2 RBCs (or HBOCs)/min will be assessed in 10 consecutive microscopic fields, totalling an area of approximately 0.5 mm$^2$. The microscopic fields will be observed systematically by displacing the field of view by 200 microns/step in the lateral direction. This will be ensured by observing each step using a video monitor. The first field will be chosen by a distinctive anatomic landmark (i.e., large blood vessel bifurcation) so that the same fields and blood vessels are observed at each time point. Care will be taken to ensure that each field has about 2-5 functional capillaries. The ratio of the total length of RBC (or HBOC)-perfused capillaries to the total area of the microscopic field of view is known as the functional capillary density (FCD, cm$^{-1}$). FCD will be measured by recording and totalling the length of the capillaries which will see RBC/HBOC transit in the field of view. A change in FCD values from the baseline is indicative of the extent of capillary perfusion [168, 732].
Additionally, blood flow will be calculated using the following formula [168];

\[
\text{Blood Flow} = \frac{\pi}{4} * V * D^2
\]

Where \( V \) is the mean blood velocity and \( D \) is the diameter of the blood vessel. The mean blood velocity will be calculated for arteries and veins using the photodiode cross correlation technique described in the literature [168, 736]. Wall shear stress (WSS) defined as a product of wall shear rate (WSR) and \( \eta \) (blood viscosity) will also be computed [168, 732]. WSR will be obtained from the following relationship [732];

\[
\text{Wall Shear Rate} = 8 * V * \frac{1}{D}
\]

In the study carried out by Cabrales et al. [168], CO saturated RBCs (CO-RBCs) were compared with unsaturated RBCs (O\(_2\)-RBCs) using a hemorrhagic shock resuscitation protocol. Figure 5.2 is reprinted from Resuscitation, 72(2), P. Cabrales, A.G. Tsai, M. Intaglietta, Hemorrhagic shock resuscitation with carbon monoxide saturated blood, p. 306-318, Copyright (2007), with permission from Elsevier. Figure 5.2 compares FCDs of CO-RBCs and O\(_2\)-RBCs over the course of the experiment conducted in the aforementioned study [168]. From the figure, it is evident that resuscitation with RBCs reperfused various capillary networks; thereby normalizing perfusion pressure and restarting various physiological pathways involved in processing metabolic waste [168]. It is interesting to note that CO-saturation of RBCs did not affect this normalization process adversely [168]. We anticipate similar results with CO, NO, H\(_2\)S, and HNO saturated HBOCs.
Figure 5.2: Changes in Functional Capillary Density (FCD) over time in hamsters subjected to hemorrhagic shock and either reperfused with O\textsubscript{2} saturated RBCs (O\textsubscript{2}RBC), or CO saturated RBCs (CORBC), or receiving no resuscitation (NR). CO-RBCs did not inhibit normalization of capillary perfusion. Reprinted from Resuscitation, 72(2), Pedro Cabrales, Amy G. Tsai, Marcos Intaglietta, Hemorrhagic shock resuscitation with carbon monoxide saturated blood, p. 306-318, Copyright (2007), with permission from Elsevier.

5.3.3.4 \textit{In Vivo} Cell Death Visualization

A variety of techniques have been established to monitor cellular apoptosis. Most techniques were performed \textit{in vitro} with tissue cross-sections and wholemounts while others were performed \textit{in vivo} providing valuable insights about apoptosis including its kinetics [737]. The assay proposed in the current study was developed by Yang et al. and involves the use of annexin V and propidium iodide (PI) labelling [737]. Annexin V binds to phosphatidylserine, a membrane phospholipid which translocates from the inner to the outer leaflet of the cell membrane at the onset of apoptosis [737]. PI on the other hand is a dye that binds to the nuclei of necrotic or late apoptotic cells having increased
membrane permeability [737]. Cells labelled with fluorescently tagged annexin V or PI can be visualized with intravital fluorescence microscopy [168, 737].

Experiments for visualizing in vivo cell death will be carried by methods outlined by Cabrales et al. [168]. Briefly, equal volumes of annexin V (Alexafluor 488 conjugate, Molecular Probes, Eugene, OR) and PI (0.2 mg/ml, Molecular Probes) will be mixed, and about 150 μL of the mixture will be injected into the anterior chamber of the hamster 30 min before visualization by intravital microscopy. Imaging of labelled cells will be performed at 6h, 12h, 24h, and 48h after resuscitation with ligand loaded HBOCs. Microscopic images will be captured with a low light video camera (ORCA 9247, Hamamatsu, Tokyo, Japan) and recorded at 5 frames per second at a resolution of 1344x1024 pixels per frame. Single and double staining of the cells in the skin fold window and the percentages of cells labelled with Annexin V and/or PI will be estimated at different time points. Data will be expressed as an average cell count in about 30 – 40 selected microscopic fields for the tissues and the endothelial walls separately [168]. There is no known reaction of CO/NO/H$_2$S/HNO with annexin V or PI [168, 737].

As mentioned before, Cabrales et al. [168] compared CO-RBCs with O$_2$-RBCs using a hemorrhagic shock resuscitation protocol. Based on their findings, Cabrales et al. concluded that resuscitation with CO saturated RBCs successfully averted apoptosis upon reperfusion. Figure 5.3 is reprinted from Resuscitation, 72(2), P. Cabrales, A.G. Tsai, M. Intaglietta, Hemorrhagic shock resuscitation with carbon monoxide saturated blood, p. 306-318, Copyright (2007), with permission from Elsevier. Figure 5.3 compares the number of apoptotic and necrotic cells 8h after resuscitation in the study carried out by Cabrales et al. [168]. The anti-apoptotic properties of CO are manifested here as the number of apoptotic cells for CO-RBCs were significantly lower than O$_2$-RBCs (p<0.05) [168]. It is evident from Cabrales’ results that resuscitation with CO-
RBCs also reduced necrosis [168]. We anticipate similar results for CO, NO, H₂S, and HNO saturated HBOCs proposed in this study.

Figure 5.3: A comparison of the number of apoptotic and necrotic cells in 40 microscopic fields, 8h after resuscitation with O₂ RBCs, CO-RBCs or no resuscitation (NR). Reprinted from Resuscitation, 72(2), Pedro Cabrales, Amy G. Tsai, Marcos Intaglietta, Hemorrhagic shock resuscitation with carbon monoxide saturated blood, p. 306-318, Copyright (2007), with permission from Elsevier.

5.3.3.5 TUNEL Tissue Viability

To confirm that the cells labelled with annexin V and PI were apoptotic and/or necrotic, programmed cell death will be monitored using the terminal transferase mediated dUTP nick end-labelling (TUNEL) assay (In Situ Cell Death Detection Kit, TMR fluorescein, Roche Applied Sciences, Alameda, CA) as described in the literature [168]. Briefly, the hamsters will be sacrificed and the window tissue recovered. Sections of the tissue will be washed with 0.1 M citrate buffer and micro-waved. After rapid cooling, these sections will be treated with terminal deoxynucleotidyl transferase (TdT) for 1h at 37 °C in the absence of light. After an hour, the reaction will be terminated by repeated
washes with PBS (0.1 M, pH 7.4) and the sections will be secured by a cover-slip. The sections of labelled cells can then be observed through a fluorescence microscope (excitation $\lambda = 450$-500 nm and detection $\lambda = 515$-565 nm) to verify and cross-check the tissue viability results with the in vivo cell death measurements effected by Annexin V and PI labelling.

**5.3.3.6 Histology**

Organs will be resected from euthanized animals for histopathologic examination according to methods previously described in the literature [280]. Immediately following resection, organs will be fixed in 10% formalin neutral buffer and the paraffin sections will be stained with eosin/hematoxylin [280]. All tissues will be analyzed for 3-nitrotyrosine, a direct indicator of oxidative damage [738-740]. 4-μm-thick paraffin sections will be treated with 0.3% H$_2$O$_2$ in ethanol for 20 min [280]. The paraffin sections will then be treated with an antibody diluent (S2022; DakoCytomation, Glostrup, Denmark) to block nonspecific protein binding [280]. Subsequently, the sections will be incubated overnight at 4°C with hamster monoclonal antibody against 3-nitrotyrosine (10x dilution of NITT12-A; Alpha Diagnostic International, Inc., San Antonio, TX) [280]. The sections will be further incubated for 45 min at 25°C with goat antibodies against hamster immunoglobulins conjugated to the amino acid polymer (Histofine Simple Stain MAX-PO(M); Nichirei Corp, Tokyo, Japan) [280]. Absence of NITT12-A, the primary antibody, will serve as a negative control [280]. Color will be developed using 3, 3-diaminobenzidine (16.7%, Sigma-Aldrich Corp., St. Louis, MO) in 0.05 M Tris-HCl, pH 7.4, containing 0.4% H$_2$O$_2$[280]. Hematoxylin will be used to stain the nuclei [280].
5.4 Cytotoxicity Assays

IRI is a complex phenomenon consisting of several dependant and independent symptoms [46]. IRI has been recognized to elicit oxidative stress induced vasoconstriction, cell apoptosis, vascular inflammation and endothelial damage [95, 168]. Vasoconstriction leads to a marked drop in the intravascular perfusion pressure causing ischemia [77]. Reperfusion results in deregulated systemic inflammation which can lead to severe organ damage [168], and cell death via apoptotic/necrotic pathways [46]. In light of the proven vasodilating, anti-inflammatory and anti-apoptotic responses of CO, NO, H₂S, and HNO in various biological processes, we hypothesize that exogenous supply of these ligands transported by Hb-based carriers will be successful in preventing vasoconstriction, apoptosis, and vascular inflammation during the ischemic and reperfusion phases.

In vivo these gaseous ligand loaded HBOCs are expected to primarily interact with the endothelial cells lining the walls of the blood vessels, macrophages present in blood, and the vascular smooth muscle cells (VSMCs). Thus to study the efficacy of this therapeutic intervention, it is of prime importance to assess if these ligand carriers exhibit cytotoxic behaviour once infused in vivo. Cytotoxicity caused by HBOCs can be due to various reasons such as particle aggregation leading to blockage of fine capillaries, lysis/disintegration resulting in the release of free Hb into the systemic circulation causing severe vasoconstriction and systemic hypertension, and rapid HBOC uptake by the reticuloendothelial system (RES) before successful delivery of the therapeutic treatment [28, 68, 179, 302, 337, 445, 700, 741]. The Hb-based ligand carriers formulated in the current study have attributes to potentially nullify these drawbacks and offer cytoprotection to the tissues affected by IRI. To test their efficacy as
non-cytotoxic therapeutics we propose to conduct certain in vivo cytotoxicity assays at different time intervals before and after transfusion: -2h, 6h, 12h, 24h and 48h. Since IRI is known to induce cell apoptosis/necrosis and our treatment prevents apoptosis/necrosis we plan to use an in vivo cell death visualization technique (as noted before) using annexin V and PI staining developed by Yang et al. [737] and corroborate the results with suitable ex vivo tissue viability assays [95, 168]

The use of annexin V and PI labelling to visualize in vivo cell death will enable us to avoid the common procedures involved in other in vitro cytotoxicity assays such as fixation, sectioning, dissection and sequential treatment with multiple reagents [737]. Thus, the method of choice proposed in the current study provides a simple, economic and direct visualization technique to monitor the effect of our therapeutic intervention on apoptosis and necrosis. However, one problem associated with the proposed assay is that the apoptotic cells will initially stain with annexin V but will subsequently be necrotic and display nuclear staining with PI. Thus it is not possible to ascertain if the cells which stain with PI were previously annexin V⁺.

To assess the potential of gaseous ligand loaded HBOCs in inducing cytotoxicity; we need to understand the mechanistic aspects of the health hazards presented by the nanoparticles within the body. It is well known that the cell viability decreases in human body upon increase of oxidative stress due to overproduction of partially reduced oxygen (reactive oxygen species, ROS) [28, 29, 31, 179, 741]. Many investigators have convincingly demonstrated that cellular homeostasis seeks for a balance between the rate of ROS production and the rate of oxidant elimination; the pathogenic outcome of ROS [28, 741]. Thus, cellular oxidative stress is supposedly one of the most important factors explaining the genesis of in-vivo and in-vitro cytotoxicity. Incidentally, in our case we attempt to synthesize gaseous ligand loaded HBOCs with spherulitic morphologies.
The molecular characteristics as exhibited by nanoparticles will not be prominent in this case. Hence, it is surmised that the average reactive electron charge density will be rather low and due to the favorable spherulitic shape there will be no specific region of intensity active electron configuration. There is therefore sufficient ground for us to hypothesize that our particles will not be severely prone to overproduce ROS and hence would bear least potential to induce cytotoxicity. Nevertheless, we must contemplate the design of appropriate experiments to determine the potential of these ligand carriers in inducing cytotoxicity. This will be done as follows:

1. Study of the shape and size of the particles with techniques such as high resolution Transmission Electron Microscopy (TEM), and Field Emission Scanning Electron Microscopy (FESEM). Atom probe tomography will be employed to know the position of different types of atoms in the proposed nanosystem.

2. ROS measurement will be conducted by the usual technique of measuring fluorescence at a predetermined excitation level in a fluorescent reader [742, 743]. This study will enable us to know if particle size dependent ROS production is a concern in the proposed research.

3. Notwithstanding the origin of cytotoxicity, the cell viability study will be carried out initially by the proposed in vivo cell death visualization technique using annexin V and PI staining.

4. If at all we get high cell death data, which in our argument, is of remote possibility, we will look for more elaborate assay methods. We propose to use clinically approved cell viability assays such as XTT/PMS reduction, Alamar Blue assay, dye exclusion by Trypan Blue, and MTS/PMS calorimetric assay [744-749]. Longo-Sorbello et al. compares the different commercially available cytotoxicity and cell proliferation assay methods in great detail in their chapter titled “Cytotoxicity and Cell Growth
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