OXYGEN TRANSPORT WITHIN A HEPATIC HOLLOW FIBER BIOREACTOR

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by

Jesse P. Sullivan, B.S., M.S.

Andre F. Palmer, Director

Graduate Program in Chemical and Biomolecular Engineering

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Abstract

by

Jesse P. Sullivan

Hepatic hollow fiber bioreactors are considered a promising class of bioartificial liver assist device (BLAD). Unfortunately, the development of this type of device is currently hindered by oxygen limited transport to cultured hepatocytes, due to the low solubility of oxygen in aqueous media. A priori knowledge of the dissolved oxygen concentration (pO2) profile within a hepatic hollow fiber bioreactor is important in designing an effective BLAD. In designing BLADs for clinical use, it is important to note that hepatocytes in vivo experience a spectrum of oxygen tensions (pO2 ranging from 25 – 70 mmHg). This pO2 gradient in the liver sinusoid is extremely important for the development of proper differentiated function (zonation) of hepatocyte phenotypes. In order to provide an in vivo-like oxygen spectrum to cultured hepatocytes housed within a hollow fiber bioreactor, several different engineering approaches were explored. These included: supplementing the circulating media stream of the hollow fiber bioreactor with a hemoglobin-based oxygen carrier (HBOC) with defined oxygen binding and release kinetics; inoculating different types of hepatocytes; operating the bioreactor with the extracapillary space (ECS) ports open and closed; and varying several other operating parameters of the bioreactor system. We hypothesize that these parameters can be manipulated to improve hepatocyte oxygenation and attain the desired in vivo pO2
spectrum. Provision of proper oxygen conditions should create a fully functional BLAD that could potentially help thousands of liver failure patients.
DEDICATION

To Merideth.
The love of my life.
I ask of you.
Will you be my wife?
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CHAPTER 1:
INTRODUCTION

1.1 Motivation

Development of a fully functional hepatic hollow fiber bioreactor that is capable of mimicking the oxygen microenvironment of the liver would have a great impact on the treatment options available to patients suffering from acute liver failure. Hollow fiber bioreactors housing hepatocytes (parenchymal cells which make up a majority of \textit{in vivo} liver mass) are currently the most promising type of bioartificial liver assist device (BLAD) being developed to date. Hepatic hollow fiber bioreactor reduce the level of shear stress normally placed on cultured cells within other types of BLADs, and provides a biomimetic three-dimensional environment \cite{1}. However, development of this type of BLAD is hampered by poor oxygen transport to the cultured cells \cite{2, 3}. Inadequate oxygen transport is primarily due to the low solubility of oxygen in aqueous media (0.22 mM at 1 atm and 37°C) \cite{4} and the long diffusion path lengths through the hollow fiber membrane. This oxygen transport limitation is especially problematic with hepatocytes due to their characteristically high oxygen demand \cite{5-7}.

Hepatocytes cultured in specified oxygenation conditions exhibit known sets of characteristic functions and these phenotypic differentiated functions must be maintained in an extracorporeal liver assist device. Inlet oxygen concentrations can not be arbitrarily raised, since a hyperoxic (overly oxygenated) environment can be as detrimental to the hepatocyte culture as hypoxic (poorly oxygenated) environment, due to the formation of
reactive oxygen species [8-10]. Therefore, increasing and specifically targeting the oxygen spectrum experienced by hepatocytes in vivo is vital in order to maintain the retention of differentiated function [11]. Provision of proper oxygenation within a hepatic hollow fiber bioreactor is the primary focus of this dissertation. We hypothesize that with optimal operating conditions, and the supplementation of an oxygen carrier, hepatocytes can be cultured in a designated oxygen environment, leading to a viable BLAD and/or in vitro liver model.

1.2 Acute Liver Failure

Liver failure is a leading cause of death within the United States, especially for the middle aged population [12]. End-stage liver disease in the United States costs approximately nine billion dollars each year, taking 26,000 lives [13]. Acute liver failure (ALF) is one form of end-stage liver disease with high rates of morbidity and mortality [14, 15]. ALF is characterized by a sudden and rapid necrosis of liver cells, resulting in loss of hepatic function, in a patient with no underlying chronic liver damage [16-18]. Approximately 2,000 patients are diagnosed with ALF each year in the United States alone [19]. Advances in surgery have made orthotopic liver transplantation a promising treatment option; however, it is severely constrained by a shortage of donor organs and patient health [20].

The liver, unlike most normally quiescent tissues in the body, is able to regenerate in response to cell death [21], and in approximately 25% of ALF cases the patient retains the capability for native liver regeneration to a fully functional capacity [22, 23]. However, no test currently exists to determine whether or not a patient’s liver retains the
potential for native recovery [24]. Therefore, a support device that can maintain an ALF patient would provide sufficient time during diagnosis to determine if native liver regeneration will occur or, if necessary, until a suitable donor organ can be found and transplanted [19, 25, 26]. A fully functional BLAD would also be useful pre- and post-OLT surgery to stabilize the ALF patient and aid in the recovery process. Such a device could support an ALF patient suffering with as much as 70 – 80% hepatic necrosis [21]. Hence, there is a clear need for a robust and global supportive liver therapy for liver failure patients, which hopefully can be met with a hepatic hollow fiber bioreactor.

1.3 Liver Structure, Function and Zonation

The liver is a very unique organ, performing many important functions including a major role in the body’s synthetic, metabolic, biotransformation, digestive, storage, and detoxification activities [1, 27, 28]. Along with being the largest and one of the most complex internal organs, the liver is the only organ fed by a vein [28]. In addition to being fed by the highly oxygenated hepatic artery, the liver is also fed via the portal vein, which collects blood from capillaries surrounding the stomach and small intestines. Therefore, all the nutrients, toxins, drugs, and everything else that is orally ingested and absorbed by the gastrointestinal tract is first sent through the liver before entering the rest of the body. The liver is a compartmentalized organ, broken down into discrete lobules (containing multiple acini) which contain many specialized capillary structures known as sinusoids. Figure 1.1 is a schematic of the liver sinusoid, the functional subunit of the liver, where the mixed input traverses a single-cell plate of hepatocytes. Note that the endothelial lining of the sinusoid is fenestrated to allow for efficient mass transport to the
hepatocytes. After exiting the sinusoids the blood is collected in the hepatic vein to be returned to the heart [28]. Mimicking the structure, function, and oxygen spectrum of the liver sinusoid presented in Figure 1.1 is a primary aim of the work presented within this dissertation.
Figure 1.1 Schematic of the general structure, oxygenation zones, and functional hepatic zonation of an individual liver sinusoid.
The liver is a highly oxygen intensive organ, consuming over a fifth of the body’s oxygen supply [6, 7, 29]. Oxygen consumption by hepatocytes establishes an oxygen gradient along the length of the liver sinusoid from approximately 70 down to 25 mmHg. This oxygen gradient, along with hormonal and extracellular matrix variations, is the principal regulator of differentiated hepatocyte function (hepatic zonation) within the liver sinusoid [30-32]. Maintaining a specific oxygen spectrum in a mixed phenotype hepatocyte culture facilitates their transformation into homogeneous (zone specific) hepatocytes [33]. These hepatocyte zones within the liver sinusoid differ in enzyme content and subcellular structure, thus each phenotype (periportal, pericentral, and perivenous) possess different capabilities.

As can be seen in Figure 1.1, the entrance region of the liver sinusoid, the periportal (afferent) zone, experiences the highest oxygen tensions (pO₂ = 60 – 70 mmHg). The periportal zone's predominant activities include oxidative energy metabolism, amino acid catabolism, ureagenesis, gluconeogenesis, cholesterol synthesis, bile formation, and protective metabolism [34]. The periportal zone contains the highest hormone levels (insulin, glucagons, etc.) and feeds into the pericentral zone, where periportal functions (and hormone concentrations) taper and perivenous functions are first observed. The pericentral zone (pO₂ = 35 – 60 mmHg) contains a localization of the Cytochrome P₄₅₀ subfamily IIB (CYP2B) enzyme that is used in certain biotransformation reactions [30]. Lastly, the perivenous (efferent) zone, the least oxygen rich section of the sinusoid (pO₂ = 25 – 35 mmHg), is known to be the primary site of
glycolysis, glycogen synthesis from glucose, liponeogenesis, ketogenesis, glutamine formation, and xenobiotic metabolism [34].

The zonation of hepatocytes into differentiated phenotypes in vivo plays a major role in the homeostasis of several compounds within the body. Maintaining homeostasis is a vital liver function that is generally accomplished by one zone of hepatocyte phenotypes metabolizing and another synthesizing a particular substance. For example, glucose can be synthesized in the periportal zone via gluconeogenesis and metabolized in the perivenous zone via glycolysis. This method of chemical homeostasis is also important for glutathione, cysteine and cholesterol [35, 36]. All three zone specific hepatocyte phenotypes must be present in a BLAD to replace the majority of liver functions. Hence, the provision of a controlled oxygen gradient ranging from ~25 – 70 mmHg within a BLAD is expected to create functional hepatocyte zonation similar to what is observed in vivo, which is believed to be required for the realization of a practical BLAD [30].

1.4 Replacement Liver Therapies

Researchers have attempted to create a liver assist device for over 40 years [37], and this venture is currently receiving renewed attention [1, 23, 38]. Many different strategies for liver replacement/support therapies have been attempted over the years. These have included allogeneic liver transplantation, artificial liver devices, biological liver supports, and bioartificial devices. Each of these various strategies has met with varying degrees of success, discussed below.
1.4.1 Orthotopic Liver Transplantation

As mentioned earlier, orthotopic liver transplantation is currently the best treatment option for an ALF patient. With advancement in surgical procedures, an allogeneic liver transplant can raise a patient’s one year survival rate to over 80% [16, 39]. However, this therapy is very expensive ($75,000 – 240,000) and donor organs are in short supply [2, 14, 18]. Furthermore, many patients are not eligible for a liver transplant as a result of alcohol or drug abuse, infection, metastatic cancer, or cardiovascular disease [40]. Approximately 23 – 27% of ALF patients die while waiting for an organ [16, 41] and the shortage of donor organs is expected to increase three-fold over the next decade [42]. Due to this critical organ shortage many different extracorporeal replacement liver therapies have been explored, with the aim of sustaining and prolonging the life of patients suffering from acute liver failure.

1.4.2 Liver Support Devices

Initial attempts to develop a liver assist device included the construction of fully artificial devices to replace one or a few of the liver’s functions, usually consisting of removing toxins (generally of low molecular weight, such as ammonia) from the bloodstream [43]. Hemoperfusion over charcoal [44, 45], hemoadsorption [46, 47], hemodialysis [48, 49], plasmapheresis [40], plasma exchange [50], and other methods of blood detoxification [51-54] have all been explored, but met with limited success due to their narrow focus in light of the many varied functions of the liver [55, 56]. In clinical trials, wholly artificial systems were ineffective [23, 57], showing insignificant improvement in ALF survival rates [58-60].
Another entirely different approach sought to provide a purely biological replacement of the liver by implantation or external filtering through an animal organ, these two strategies are referred to as xenotransplantation [61-63] and xenographic cross-circulation [46, 64], respectively. Hypothetically, this provides global therapy that allows for the realization of many of the liver’s functions. Improvements were observed when compared to purely artificial devices, but these methods suffer from immunological concerns, compatibility issues, insufficient treatment time, and/or other logistical problems [37]. However, the improvements observed with biological devices, in addition to the success of transplantation, suggest that a biological component must be present within an liver assist device for there to be successful treatment [2, 65]. Consequently, it was proposed to develop a bioartificial liver assist device (BLAD), containing a biological component (hepatocytes) maintained within a bioreactor to function in place of a patient’s damaged liver [57, 66, 67].

There are four main types of bioartificial devices that are currently being studied, each with inherent advantages and disadvantages: flat plate/monolayer, perfused beds/scaffolds, beds with encapsulated/suspended cells, and hollow fiber bioreactors [68]. Monolayer hepatocyte cultures on a flat plate bioreactor have been utilized in many early experimental studies due to their uniform cell distribution and easily controllable microenvironment [30]. These devices suffer from scalability issues, the potential for a large dead volume, shear stress on the cells, a low surface area to volume ratio, and difficult long term maintenance [54, 69, 70]. The perfused bed or scaffolds provide good mass transport to cells, a three-dimensional structure, and are scalable. However, they suffer from non uniform perfusion, clogging, and shear stress on the cells [41, 71-74].
Encapsulated and suspended cell systems have a uniform microenvironment and scale easily. However, encapsulation matrices degrade over time and are a barrier to mass transport, cell suspensions lead to poor cell stability, and both options expose cells to shear forces [75-79]. Hollow fiber bioreactors, first described by Knazek [80], provide an attachment surface for cells that allows for immunoisolation and protection from shear forces [23]. Additionally, hollow fiber bioreactors provide a high surface area to volume ratio, are scalable, and contain a three-dimensional architecture that replicates the \textit{in vivo} liver sinusoid environment [81-83]. However, this device suffers from non-uniform cell distribution and the membrane is a barrier to mass transport [22, 37]. The benefits of a hollow fiber bioreactor system outweigh the deficits, making it the most attractive option for a BLAD [68, 83, 84]. Therefore, all of the experimental studies described in this dissertation utilized a hepatic hollow fiber bioreactor system.

1.4.3 Hepatic Hollow Fiber Bioreactors

The bioartificial liver assist device that our group has focused on is a hollow fiber bioreactor housing hepatocytes. A hollow fiber bioreactor, or artificial capillary system as our module is called, is composed of a bundle of hollow fibers contained within a larger cylinder, thus, creating two separated spaces. These separated volumes are the space within the hollow fibers (the lumen) where the media flows, and the space outside of the hollow fibers (the ECS) where the hepatocytes are inoculated and cultured. Via the circulating media stream, nutrients and oxygen are transported to the cells, through the membrane, and toxins and cellular products are taken away. A picture of one of the experimental hollow fiber bioreactors is presented in Figure 1.2 with a schematic of an axial cross-section of the device, depicting the lumen (white tubes) and ECS (shaded...
region). As noted above, hollow fiber bioreactors contain many inherent advantages over contemporary bioreactors including the biomimetic cell environment which allows for cell-cell interaction, improving viability and function [68]. These devices usually operate with hepatocytes cultured in the stagnant ECS. Hence, the hepatocytes attach to the outside surface of the hollow fibers, while the media flows through the lumen of the fibers [70, 85]. The main disadvantage of this type of bioreactor is the membrane barrier to oxygen transport and alleviating this problem is a focus of this dissertation.
Figure 1.2 Picture of one of the hollow fiber bioreactors utilized in the experimental trial described in Chapter 6 with a schematic of the cross-section of the bioreactor (the white area of the schematic represents the lumen with media flowing from left to right, while the green shaded area represents the hepatocytes in the ECS).
1.4.4 Hepatocyte Cell Types

The type of hepatocyte cells cultured within a BLAD can affect its function. Currently, several cell types have been investigated, including: primary hepatocytes, stem cells, and cell lines. Each of these cell types have distinct advantages and disadvantages for use within a BLAD [86]. Primary cells are cells that are removed directly from a living organism. Primary hepatocytes can come from many different sources, human cells would be ideal but they are in short supply [58], so most research is focused on porcine, rabbit, canine, or rodent cells. Liver-specific function and zonal differentiation can only be retained for only a short period of time in primary hepatocytes [87, 88]. Primary hepatocytes can proliferate *in vivo*, but usually do not do so in culture; several research groups are continuing to address this critical flaw [2, 89]. Lack of proliferation has lead to the development of hepatic cell lines that show improved growth and maintain various differentiated functions.

Many different cell lines have been developed including: immortalized rat [90], porcine [91] and human cells [92-97], and human tumor derived hepatoblastomas [98-100]. Each of these cell lines has been examined for differentiated hepatocyte function and proliferation capability with varying degrees of success. Several of the hepatoma cell lines are currently being cultured in liver support devices [101, 102]. Finally, stem cells have been considered for this application. Potential sources include transdifferentiated nonhepatic, adult liver progenitor, and embryonic stem cells [103-105]. The use of stem cells is promising but still in its infancy and may be more applicable in future devices. Currently, limited success has been achieved in differentiating stem cells to hepatocytes [106-110].
For the experiments presented in this dissertation, both primary rat hepatocytes and C3A hepatoma cells were utilized. These cell types were cultured because each provides unique advantages within the experimental hollow fiber bioreactor systems. The C3A hepatoma cells provide some liver specific function and proliferation potential, while primary cells maintain lower oxygen consumption rates, and retain full differentiated function in the short-term [100, 111, 112]. Pictures were taken of each of the cell types utilized within this dissertation and are presented in Figure 1.3. The left picture (A) represents C3A hepatoma cells cultured in Chapter 3 (the same cells were also cultured in Chapter 6), and the right picture (B) represents primary rat hepatocytes cultured in Chapter 5. Both primary and immortalized hepatocytes maintain similar size and morphology in a flat plate culture as is evident in Figure 1.3. Additionally, these cells were cultured in our studies, since they are readily available, C3A cells are commercially sold and the primary rat hepatocytes were isolated by colleagues at Wayne State University.

![Figure 1.3 Brightfield images taken of (A) C3A hepatoma cells that were cultured in Chapter 3 and (B) primary rat hepatocytes cultured in Chapter 6.](image)

Figure 1.3 Brightfield images taken of (A) C3A hepatoma cells that were cultured in Chapter 3 and (B) primary rat hepatocytes cultured in Chapter 6.
1.5 Design Criteria of Hepatic Hollow Fiber Bioreactors

The construction of the hollow fiber bioreactor, bioreactor operating conditions, and media make-up were all selected to fulfill the specific oxygen demands of hepatocytes. Additionally, the BLAD must last on the order of days to weeks, be easy to use, maintain sterility within a hospital setting, and consist of 10 – 40% of the \textit{in vivo} liver cell mass [37, 40, 113, 114]. To be clinically relevant, a BLAD must also have adequate bidirectional mass transport, maintain cell viability and function, and have the potential for scale-up [37]. Further, the bioreactor’s hollow fiber membrane needs to be chosen to exclude large proteins such as hemoglobin (this is important given that free hemoglobin is known to be toxic [115-118]) and permeable to smaller proteins such as albumin (so they can be used within the system and not allowed to build up concentration in the hollow fiber bioreactor ECS).

Improving oxygen provision to the hepatocytes cultured within the hollow fiber bioreactor was the main focus of the studies discussed within this dissertation. The amount of oxygen delivered, spectrum of oxygen tensions experienced by cultured hepatocytes, and average oxygen concentration within the hollow fiber bioreactor ECS were all evaluated. The aim of these studies is to increase oxygen transport to cultured hepatocytes and replicate \textit{in vivo}-like oxygenation; with oxygen tensions ranging from 25 to 70 mmHg and an average oxygen concentration of \approx 44 mmHg [119-121]. Providing this oxygenation will hopefully create a fully functional hepatic hollow fiber bioreactor for potential use as a BLAD, or for drug discovery and fundamental liver physiology.
1.6 Scope of this Study

As previously mentioned, hepatocytes require large amounts of oxygen and the provision of a narrowly defined spectrum of oxygen concentrations to maintain their differentiated function. Inadequate oxygen transport has limited the efficacy of BLADs for use in ALF patients. Previous studies have indicated that hepatic hollow fiber bioreactors are still the most promising BLAD alternative, even with their inherent oxygen transport limitations [37]. We aim to both enhance oxygenation and provide the \textit{in vivo} range of oxygen tensions experienced in the liver sinusoid to hepatocytes housed within hollow fiber bioreactors, in order to create a hepatocyte culture with maximum differentiated function. We hypothesize that supplementation of the circulating media stream of a hepatic hollow fiber bioreactor with an appropriate hemoglobin-based oxygen carrier and the optimal selection of key bioreactor operating parameters (i.e., flow rates and inlet pO$_2$s) will both enhance oxygenation and provide a physiological spectrum of oxygen tensions to culture hepatocytes within the bioreactor.

In Chapter 2, a novel oxygen transport model was developed in order to calculate the oxygen concentration profile through an individual hollow fiber within the hollow fiber bioreactor system. The rationale for the development of this oxygen transport model was to design a hepatic hollow fiber bioreactor with the appropriate hemoglobin-based oxygen carrier and optimal bioreactor operating parameters that would replicate the three oxygenation zones observed \textit{in vivo}. The oxygen transport model was based upon previous oxygen transport models with several key additions (i.e., Michaelis-Menten oxygen consumption kinetics and radial oxygen carrier and velocity profiles). The oxygen transport model was compared to similar analytical oxygen transport models that
utilize the asymptotic limits of the Michaelis-Menten oxygen consumption kinetics implemented in the previously described numerical oxygen transport model. In subsequent chapters the complete mathematical oxygen transport model was validated via comparison to experimental data, and further simulated to predict optimal bioreactor operating conditions that replicate the three major oxygenation zones within the liver sinusoid. The basic model is derived in Chapter 2, and any further improvements are noted in subsequent chapters.

In Chapter 3, C3A hepatocytes were cultured in two experimental hollow fiber bioreactors. A control bioreactor system with no oxygen carrier was cultured and an experimental system supplemented with bovine red blood cells (hemoglobin-based oxygen carrier) was cultured. From the experimental systems, metabolic, synthetic, and oxygen consumption data were measured. The oxygen transport model developed in Chapter 2 was simulated with the actual dimensions of an individual fiber within the bioreactor, along with various inlet flow rates, inlet pO₂s, and included the presence of bRBCs as an oxygen carrier. The oxygen transport model was simulated to calculate the hollow fiber bioreactor exiting pO₂ and compared to experimentally measured outlet pO₂ data in order to validate the model. Oxygen transport simulations were conducted to calculate the oxygen concentration profile along the length of individual hollow fibers within the bioreactor under various experimental conditions and to predict enhanced bioreactor operating conditions for future studies.

In Chapter 4, the oxygen transport model was further improved to include separately calculated hemoglobin-based oxygen carrier concentration and velocity profiles within the lumen of the hollow fiber bioreactor. Additionally, oxygen
binding/release kinetics of seven different hemoglobin-based oxygen carriers of varying oxygen affinity were studied within the context of the oxygen transport model to predict their ability to replicate the three \textit{in vivo} oxygenation zones within the liver sinusoid. Oxygen transport simulations examined the effect of media flow rate, concentration of hemoglobin-based oxygen carrier, kinetics of oxygen binding/release, and inlet dissolved oxygen concentration on oxygen transport to cultured hepatocytes. For these operating conditions the \( pO_2 \) exiting the bioreactor, total amount of oxygen transported, spectrum of oxygen experienced within the bioreactor ECS, and average bioreactor ECS oxygen concentration was determined.

In Chapter 5, a similar experimental study as the one presented in Chapter 3 was conducted, except with primary rat hepatocytes, instead of the C3A cell line, cultured within the ECS of the hollow fiber bioreactor. In this case, the oxygen transport model was updated so that the model parameters accurately reflect the oxygen consumption of primary cells, an ECS fractional void volume, and the new dimensions of the bioreactor. The oxygen transport model was subsequently modified to reflect an additional set of more clinically relevant operating conditions (increased hepatocyte density, human red blood cell supplementation, and physiological inlet \( pO_2 \)). Oxygen transport simulations identified promising bioreactor operating conditions for cultures of primary hepatocytes.

In Chapter 6, another experimental study involving the culture of C3A hepatocytes within a hollow fiber bioreactor was performed. In this study, convection through the extracapillary space (ECS) was examined via operating the hollow fiber bioreactor with the ECS port valves open. Three systems were operated simultaneously: (1) a control case with ECS ports closed and no oxygen carrier, (2) a case with
hemoglobin-based oxygen carrier supplementation and the ECS ports closed, and (3) a convection enhanced case with the ECS port valves open and no oxygen carrier present. Significant improvement in oxygen provision and differentiated hepatocyte function were observed for both the hemoglobin-based oxygen carrier supplemented system and the convection enhanced system (open ECS ports) compared to the control case.
CHAPTER 2:
MATHEMATICAL OXYGEN TRANSPORT MODEL

2.1 Introduction

The motivation behind the work in this dissertation is to determine engineering strategies for improving oxygen transport and providing a physiological spectrum of oxygen tensions to hepatocytes housed within hollow fiber bioreactor systems, as well as to determine the effect of increased oxygenation and the provision of a physiological spectrum of oxygen tensions on the differentiated function of cultured hepatocytes. The experimental studies are expensive, time consuming, and must be very carefully conducted. This limits the number of experimental studies that can be performed, necessitating a strategy to narrow the number of potential experimental trials. By developing an oxygen transport model that simulates the experimental hollow fiber bioreactor system, different types of HBOCs and bioreactor operating conditions can be evaluated in silico. Therefore, the oxygen transport model could focus the experimental studies only on the most promising HBOCs and bioreactor operating conditions. The development of a novel oxygen transport model to simulate the experimental hepatic hollow fiber bioreactor system is described in this chapter.
2.1.1 Krogh Tissue Model

The basis for oxygen concentration profile calculations in the oxygen transport model developed in this dissertation originate from a model developed by August Krogh in 1919 [122]. Krogh’s paper sought to explain the supply of oxygen from capillaries to skeletal muscle via an analytical oxygen transport model. The Krogh tissue model, as it became known, assumes an idealized bed of evenly spaced capillaries, where a single capillary blood vessel is surrounded by an annulus of tissue that is representative of the muscle. Details of the derivation of the Krogh tissue model, and it’s assumptions can be found in the literature [123]. This basic model has been continually improved upon, but still contains shortcomings [124-126]. Additional calculations in the oxygen transport model developed here require a numerical integration method [123, 127].

2.1.2 Finite Element Method

To numerically solve the coupled set of partial differential equations that compromise the oxygen transport model developed in this chapter, the finite element method was utilized. The finite element method is a numerical method that discretizes a surface into finite elements, simplifies the partial differential equations into ordinary differential equations, and then solves for each element individually; keeping the boundaries of adjacent elements continuous [128]. For the computer simulations presented in this dissertation the Chemical Engineering module within the multiphysics software package COMSOL 3.3 (Burlington, MA) was utilized. The precision of the solution obtained from the finite element method calculations depends on the number of finite elements and the meshing of the finite elements within the modeled structure; more elements leads to increased accuracy but longer computational time. The mesh and
meshing technique were optimized for this current application via trial and error to obtain a minimum element quality of 0.1 (a heuristic provided by COMSOL) and a reasonable solution time.

2.2 Oxygen Transport Model Development

The oxygen transport model developed in this chapter is a representative single fiber model where a two-dimensional axisymmetric cross-section of the oxygen concentration profile is presented as the solution. The model is derived from the Krogh tissue model, which contains a flowing vessel surrounded by an annulus of tissue. However, the oxygen transport model presented here contains an additional domain (the middle rectangle region in Figure 2.1) to represent the hollow fiber membrane that is present in the individual hollow fibers. Figure 2.1 is a schematic of an individual hollow fiber, where the bolded black boxes represent the three modeled domains of the simulations: lumen, membrane, and extracapillary space (ECS). Each of these domains contains its own set of governing equations and parameters. It is important to note that the flow of the media through the lumen is considered from left to right for all of the simulations.
Figure 2.1 Individual hollow fiber, with the black boxes representing a typical axisymmetric cross-section utilized in the oxygen transport simulations.

The upper black rectangle of the cross-section in Figure 2.1 represents the lumen of the hollow fiber and includes a velocity flow profile, convection–diffusion of oxygen, and (when applicable) one-step reaction kinetics with a variable reaction rate to describe oxygen binding/release from a HBOC. The middle black rectangle represents the membrane of the hollow fiber, and is considered to be a fully hydrated homogeneous layer, where no oxygen is produced or consumed and the only mechanism of oxygen mass transport is via radial diffusion. The membrane pore size is sufficiently small to exclude any of the HBOCs considered in this study, thus confining them to the lumen space. The lower black rectangle is an annulus of representative ECS volume surrounding a single fiber. The representative ECS domain is a continuum of hepatocytes; where mass transport of oxygen is via radial diffusion and Michaelis–Menten (MM) kinetics model hepatic oxygen consumption.
2.2.1 Flow Profile through the Lumen

The first part of the oxygen transport model to be specified is the velocity profile within the lumen of the modeled space. For the control case of no oxygen carriers present in the lumen, the velocity flow profile can be considered Poiseuille flow throughout the length of the tube (equation 2.1). The Reynolds number was calculated to be much less than 100 for all of the flow rates simulated (well within the laminar flow regime), which is ideal since in vivo the liver sinusoid is considered not well-mixed [129]. Initially, the full Navier-Stokes equation was implemented to define the velocity profile in the hollow fiber lumen. However, no significant difference was found compared to a Poiseuille flow profile. Hence, this simplified flow profile was utilized in all simulations. In equation 2.1, \( u(r) \) is the radially varying velocity profile in the lumen of the hollow fiber bioreactor, \( u_{\text{max}} \) is the maximum fluid velocity at the centerline of the lumen, and \( r_L \) is the distance from the centerline of the lumen to the inner surface of the hollow fiber membrane. Entrance effects were considered to be negligible since the profile evolution length was found to be much less than the length of the encapsulated end of the fiber for all flow rates examined, according to a formula developed by Kay and Nedderman [130]. No radial flow was considered because the trans-membrane pressure is considered negligible with the ECS ports closed at the flow rates examined [25, 131]. Additionally, the hollow fiber bioreactor was operated at confluency, where the hepatocytes grow in a dense layer attached to the outer surface of the membrane, further inhibiting any possible cross-membrane flow.

\[
\begin{align*}
  u(r) &= u_{\text{max}} \left[ 1 - \left( \frac{r}{r_L} \right)^2 \right] \\
  \text{...........................................................................................................(2.1)}
\end{align*}
\]
The addition of an oxygen carrier to the circulating media disrupts the flow pattern, and can produce alterations in the velocity and HBOC concentration profiles within the lumen. The Fahraeus effect alters the axial HBOC concentration profile or can elicit a lower HBOC concentration to be present in the lumen compared to that in the media reservoir bottle [132]. However, due to the large radius of the lumen ignoring this effect does not significantly alter the results of any the studies presented in this dissertation [133, 134]. Additionally, a radial HBOC concentration profile can develop due to the radial shear stress gradient resulting in aggregation of flowing particles towards the centerline of the cylindrical tube, referred to as the Fahraeus-Lindqvist Effect [135]. At low HBOC concentrations, it was determined that this effect is not significant due to the large radius of the lumen compared to the size of the HBOCs [134]. At higher concentrations of HBOC, it was determined that an HBOC concentration profile can develop within this size vessel. The characteristic length ($L_C$) for the HBOC concentration profile to evolve was calculated for each of the HBOCs (equation 2.2) to determine if a concentration profile will develop within the length of the hollow fiber bioreactor. The constant in equation 2.2 ($k_C$), was measured experimentally and depends on the radius of the supplemented HBOC ($a$) and volume fraction of the flowing HBOC particles present in the tube [136]. The induction length ($L_C$) was calculated to be of similar magnitude to the length of the hollow fiber bioreactor only for a small number of the simulated cases.

$$L_C \approx k_C \cdot \frac{r_i^3}{a^2} \quad \text{.................................................................(2.2)}$$

The inlet radial HBOC concentration profile is assumed to be homogenous due to mixing associated with fluid flow from one large vessel (silicone tubing of $\sim 0.32$ cm
inner diameter) dispersed into many smaller hollow fibers (330 µm inner diameter). The inlet velocity profile is considered to follow Poiseuille flow (discussed above). The inlet profiles then evolve into the steady state HBOC concentration and velocity profiles that have been published in the literature [137, 138], and are presented in Chapters 3 and 4. The detailed calculation methods, and Matlab (MathWorks Natick, MA) code utilized in this oxygen transport model, are provided in the Appendix.

2.2.2 Oxygen Transport and Kinetics

The full convection-diffusion equation (equation 2.3) is solved in each of the defined domains of the oxygen transport model (lumen, membrane, and ECS), where each domain has a defined oxygen diffusivity coefficient ($D_{O_2}$), reaction term ($R$), and velocity profile ($u$). The domain interfaces are defined as continuous concentration and continuous flux boundary conditions, the entrance to the lumen is at a specified constant HBOC concentration, the pO$_2$ profile at the exit of the lumen is calculated from the simulation and the remaining outer boundaries of the modeled area are defined by no flux symmetry/insulation boundary conditions.

$$\nabla \cdot \left(D_{O_2} \nabla pO_2\right) = R - u \cdot \nabla pO_2 \tag{2.3}$$

The reaction term ($R$) in equation 2.3, in the ECS domain, is utilized to describe oxygen consumption by the cultured hepatocytes. Some early simulations considered a constant oxygen consumption rate (OCR), zero-order kinetics, by the hepatocytes within the ECS [139]. However, most recent models are now solved numerically and allow for the inclusion of Michaelis-Menten (MM) kinetics (equation 2.4), which has previously been shown to be more accurate for describing hepatocyte oxygen consumption [140,
Many literature sources have published values of $V_{\text{Max}}$ (maximum reaction velocity) and $K_M$ (Michaelis constant) for different types of hepatocytes [38, 113, 140]. Hepatocytes exposed to different oxygen gradients can produce different MM kinetic values [11], and therefore these oxygen consumption parameters were continually verified with experimental data.

$$R = \frac{V_{\text{Max}} \cdot pO_2}{K_M + pO_2}$$

(2.4)

The reaction term ($R$) in the membrane domain of the oxygen transport model is always zero since oxygen is neither produced nor consumed in this domain. In the lumen domain, the value of $R$ depends on if there are any HBOCs present in the circulating media stream. Only with an oxygen carrier present will the reaction term need to be utilized. Enough information is now provided to solve the oxygen transport model for the control case (of no HBOC supplementation in the circulating culture media), or to simulate the case of any plasma perfused BLAD [127]. However, it has been found that in the case of no HBOC the system does not provide a sufficient amount of oxygen required for proper hepatocyte culture [137, 138]. If the circulating media stream is supplemented with HBOCs, the reaction term in equation 2.3 ($R$) has to be included in the model.

2.2.3 Oxygen Carrier Transport and Oxygen Binding/Release Kinetics

In the oxygen transport model, HBOCs are constrained to only exist in the lumen. The hollow fiber membrane is impermeable to the HBOCs utilized, and therefore the HBOCs are not present in the hollow fiber membrane domain or ECS domain. The transport of oxygenated HBOC in the lumen is described by the addition of a second
coupled convection-diffusion equation to the lumen space (equation 2.5); where $S$ is the fractional saturation of the oxygen carrier with oxygen, $Hb_T$ is the total amount of oxygen binding sites on the HBOCs, and $R_{oxyHBOC}$ is the rate of formation of oxygenated HBOC (oxyHBOC). In equation 2.5, the diffusivity of the HBOC ($D_{HBOC}$) is determined by the Stokes-Einstein equation (equation 2.6), where $k$ is the Boltzmann constant, $T$ is the absolute bioreactor temperature, and $\eta$ is the circulating media viscosity.

$$\nabla \cdot \left( D_{HBOC} \nabla (S \cdot Hb_T) \right) = R_{oxyHBOC} - u \cdot \nabla (S \cdot Hb_T) \tag{2.5}$$

$$D_{HBOC} = \frac{k \cdot T}{6\pi \eta a} \tag{2.6}$$

The reaction describing the rate of formation of oxygenated HBOC ($R_{oxyHBOC}$) is found from a one-step reversible chemical reaction between oxygen and a single oxygen binding site on the HBOC (equation 2.7). The reaction rate of HBOC binding with oxygen is equal and opposite to oxygen production in the lumen ($R = -R_{oxyHBOC}$), and this is how equation 2.3 and 2.5 are coupled.

$$O_2 + Hb \xrightleftharpoons[k^-][k^+] HbO_2 \tag{2.7}$$

The derived rate law with constant reaction rate coefficients ($k^+$ and $k^-$) is written as equation 2.8. The variable rate coefficient model originally proposed by Moll [142] is implemented, where the dissociation reaction rate coefficient ($k^-$) remains a constant while the association reaction rate coefficient ($k^+$) is variable (according to equation 2.9). Equation 2.9 is derived from the requirement that at equilibrium (where $S = S_{eq}$) the rate of formation of oxyHBOC is zero ($R_{oxyHBOC} = 0$).

$$R_{oxyHBOC} = k^+ \cdot pO_2 \cdot (1 - S) \cdot Hb_T - k^- \cdot S \cdot Hb_T \tag{2.8}$$
\[ k^+ = \frac{k^- \cdot S_{eq}}{pO_2 \cdot (1 - S_{eq})} \] .................................................................................................................(2.9)

Substituting the association reaction rate coefficient (equation 2.9) into the rate law describing the rate of formation of oxyHBOC (equation 2.8) yields the rate law describing oxygen binding/release to/from HBOC utilized in the reaction rate term in the oxygen transport model (equation 2.10). This type of analysis has been successfully utilized for the development of several oxygen transport models [143, 144].

\[ R_{\text{oxyHBOC}} = k^- \cdot Hb \left[ \frac{S_{eq}}{1 - S_{eq}} (1 - S) \right] \] .................................................................................................................(2.10)

2.2.4 Oxygen-Hemoglobin Equilibrium

The equilibrium fractional saturation of HBOC with oxygen molecules (\( S_{eq} \)) that is contained within the reaction rate law describing the rate of formation of oxyHBOC (equation 2.10) can be determined by utilizing a relationship that links the saturation of oxyHBOC in equilibrium with dissolved oxygen; i.e. \( S_{eq} = f(pO_2) \). Under constant conditions of stable temperature, pH, and carbon dioxide partial pressure, sigmoidal oxygen-HBOC equilibrium curves (OHECs) were observed for all of the HBOCs examined. Two different models that mathematically describe the form of the OHECs were studied. The Hill equation [145] can be used to fit experimentally measured OHECs to regress the half saturation constant (\( P_{50} \)) and Hill coefficient (\( n \)) (equation 2.11, derived in the Appendix). \( P_{50} \) is a measure of HBOC oxygen affinity, defined as the \( pO_2 \) at half saturation of the available oxygen binding sites on a HBOC. A high \( P_{50} \) value indicates a low oxygen affinity HBOC, while a low \( P_{50} \) value indicates a high oxygen affinity HBOC. Hence, fitting experimental OHECs for various HBOCs to the Hill
equation permits us to obtain a simple measure of the oxygen affinity of the oxygen carrier. Nonetheless, the Hill equation is not used in the oxygen transport model to describe the experimental OHEC. This is because at low and high pO$_2$ values significant deviations between predicted and experimentally measured oxygen carrier saturations are observed [137], presented in Chapter 3. For the oxygen transport model, the Adair equation [146] was implemented to more accurately represent the behavior of the OHECs for the various HBOCs (equation 2.12). The Adair parameters ($a_1$-$a_4$) take into account the four oxygen binding sites on a tetrameric hemoglobin molecule; this is also called the intermediate compound hypothesis. The Adair equation representation of the equilibrium fractional saturation of hemoglobin ($S_{eq}$) as a function of the local pO$_2$ value is connected to the oxygen transport model through the rate law describing the rate of formation of oxyHBOC ($R_{oxyHBOC}$) by substituting equation 2.12 into equation 2.10.

\[
S_{eq} = \frac{pO_2^n}{P_{50}^n + pO_2^n} 
\]

\[
S_{eq} = \frac{a_1 pO_2 + 2a_2 pO_2^2 + 3a_3 pO_2^3 + 4a_4 pO_2^4}{4 \left(1 + a_1 pO_2 + a_2 pO_2^2 + a_3 pO_2^3 + a_4 pO_2^4\right)}
\]

The presented equations form the framework of the oxygen transport model developed in this dissertation. Each of the following chapters contains specifications and biophysical parameters for different experimental studies and minor alterations to the derived model. This oxygen transport model has been utilized with great success in each of the studies performed in the following chapters.
2.3 Asymptotic Limits of Oxygen Consumption

The Michaelis-Menten oxygen consumption kinetics implemented to describe hepatocyte oxygen consumption within the ECS is a major reason why the oxygen transport model must be solved via numerical solver. Analytical solutions can be found for a simplified model that contains the asymptotic limits of Michaelis-Menten oxygen consumption kinetics, equation 2.4. At high oxygen concentrations, where the concentration of oxygen is much greater than the Michaelis constant \( pO_2 >> KM \), zero-order kinetics dominate and equation 2.13 can be implemented. At low oxygen concentrations, where the Michaelis constant is much larger than the oxygen concentration \( KM >> pO_2 \), first-order kinetics are found, equation 2.14.

\[
R = V_{Max} \tag{2.13}
\]

\[
R = \frac{V_{Max} \cdot pO_2}{KM} \tag{2.14}
\]

Figure 2.2 shows the hepatocyte oxygen consumption rates within the bioreactor ECS for zero-order, first-order, and MM kinetics, utilizing the MM parameters \( V_{Max} \) and \( KM \) that were experimentally determined in Chapter 3. It can be seen in Figure 2.2 that at low oxygen concentrations \( pO_2 < 5 \text{ mmHg} \) first-order kinetics would be accurate and that at high oxygen concentrations \( pO_2 > 120 \text{ mmHg} \) zero-order kinetics may be used. However, over the central \( pO_2 \) region, where most of the simulations were conducted, only the MM kinetic model does a good job describing hepatocyte oxygen consumption when compared to the zero-order and first-order kinetics models. Additionally, it has been experimentally determined that Michaelis-Menten kinetics more realistically describe the oxygen consumption of hepatocytes [11, 141]. Figure 2.2 indicates that the range of oxygen tensions experienced during experimental operation of the hollow fiber
bioreactors falls between the asymptotic limits of either zero-order or first-order kinetics. Therefore, Michaelis-Menten oxygen consumption kinetics was assumed for all of the numerical oxygen transport simulations. However, the analytical models, for zero-order and first-order kinetics, were derived and examined to confirm the use of Michaelis-Menten kinetics.
Figure 2.2 The calculated oxygen consumption rates for zero-order, first-order, and Michaelis-Menten type kinetics over the possible range of pO$_2$ experienced by the hepatocytes in the hollow fiber bioreactor system utilized in the experimental studies described in Chapter 3.
Analytical models for oxygen transport within a diffusion-limited isotropic hollow fiber bioreactor have been derived with additional assumptions [147]. The assumptions made for the aforementioned numerical oxygen transport model are utilized in the analytical model; i.e., a steady state Krogh type model with axially parallel hollow fibers, homogeneous membrane and ECS. The analytical oxygen transport models also require the additional simplification that no radial dissolved oxygen concentration profile exists in the lumen. Two analytical models that contain the asymptotic limits of MM oxygen consumption kinetics (equations 2.14 and 2.15) are derived in the Appendix (A.3 Willaert Oxygen Transport Model).

As predicted, inclusion of zero-order reaction kinetics in the oxygen transport model over predicts the amount of oxygen consumed by the cells and calculates that the dissolved oxygen concentration decreases to zero within the first centimeter of the bioreactor (less than a tenth of the total axial hollow fiber length). Additionally, inclusion of first-order kinetics in the oxygen transport model severely under predicts the amount of oxygen consumed by the hepatocytes, and leads to large hyperoxic regions within the cell space. The analytical solutions with zero- and first-order oxygen consumption kinetics were found to be inferior to the numerical model at the most critical simulated dissolved oxygen concentrations. Therefore, this analytical solution cannot be utilized to correctly model oxygen transport within the hollow fiber bioreactor system. Nonetheless, valuable information can still be gained from comparison to this type of analytical model.
2.4 Other Oxygen Transport Models

Several groups have published oxygen transport models to simulate the mass transport of oxygen within a hepatic hollow fiber bioreactor. All of these previously developed oxygen transport models (including the one presented in this text) are based on the Krogh tissue model, with many of the same assumptions [11, 38, 148]. The implementation of numerical solvers has allowed more accurate calculations of the dissolved oxygen concentration profile within the hollow fiber bioreactor. Initially, the OHEC was assumed to be linear over the narrow pO₂ region modeled [149]. Subsequently, the Margaria equation was implemented to more accurately describe the OHEC [11], and Kelman’s equations have also been used with limited success to represent hemoglobin’s OHEC [150, 151]. The Hill equation, which is still commonly utilized to describe the OHECs was included in several studies [38, 152, 153]. Finally, in the oxygen transport model developed here, the well accepted Adair equation was utilized [138]. With careful analysis it can be shown that the Adair equation is more accurate than the Hill equation at high and low pO₂ values [137], presented in Chapter 3. Therefore, for all of the simulations presented in this dissertation the Adair equation was employed to mathematically represent the OHECs for all the HBOCs.

2.5 Further Model Development

The framework for the complete oxygen transport model has been developed in this chapter. The oxygen transport model was continually updated with each new experimental study, and these updates are noted in the chapter where they are first utilized. Also, the set of simulation parameters for each of the experimental systems is
presented within that particular chapter. In addition to the formulas presented above an ECS fractional cell volume is added to the oxygen transport model in Chapter 5. This factor impacts a few of the equations, as noted in the text of Chapter 5. The collected data from the experimental oxygen consumption studies presented in this dissertation supported and validated the developed oxygen transport model, which was in turn utilized to predict improved bioreactor operating conditions for future experimental studies.
CHAPTER 3:
SIMULATION OF OXYGEN CARRIER MEDIATED OXYGEN TRANSPORT TO C3A HEPATOMA CELLS HOUSED WITHIN A HOLLOW FIBER BIOREACTOR

3.1 Introduction

As discussed in the first chapter of this dissertation, oxygen (O₂) provision to hepatocytes housed within a hollow fiber (HF) bioreactor is limiting. In order to provide additional oxygen to the cultured hepatocytes we have supplemented the circulating media with whole, intact bovine red blood cells (bRBC). This study compares a control of no red blood cell supplementation to an experimental system with 5% of the human red blood cell concentration present in the circulating media. Key metabolic and synthetic functions were tracked during the experiment to indicate overall culture health and dissolved oxygen measurements were taken to assess the impact of the bRBC supplementation.

For the first experimental study we choose to inoculate C3A hepatoma cells into the hollow fiber bioreactor cartridges. As explained in Chapter 1.4.4, the C3A cell line has many advantages that impact this study. C3A cells are a clonal derivative of the HepG2 hepatoblastoma cells line that is currently being utilized in the Extracorporeal Liver Assist Device (ELAD® Vital Therapies) that is in clinical trials [65, 113], and in modeling studies shows inadequate oxygen provision [11]. The C3A cell line is an attractive option for culture in this type of BLAD due to their known nutrient
requirements, commercial availability, previous characterization, and ability to proliferate [58, 86]. C3A cells are known to maintain many differentiated function including producing high levels of albumin and exhibiting a functioning urea cycle [154, 155]. Preventing the accumulation of ammonia in the blood by a functioning urea cycle is a vital requirement for a fully functional BLAD. Additionally, the synthesis of albumin is important because albumin binds to and delivers toxins to hepatocytes, and helps maintain pH and osmotic balance in the blood. Although C3A cells do not retain all of the functions of a primary hepatocyte [86, 99], they provide the possibility for culture within a fully functional BLAD.

3.2 Materials and Methods

The experimental HF bioreactor that was modeled in this study is Spectrum Laboratories’ (Rancho Dominguez, CA) CellMax® artificial capillary system that consists of a HF cartridge (Spectrum Laboratories, Cat. #400-014) composed of ~81 polyethylene fibers, each containing symmetric pores possessing a 95% molecular weight cut off of 0.3 μm (Figure 3.1). This particular HF bioreactor was selected for its large pore size (allowing high molecular weight proteins, such as albumin, to diffuse across the HF membrane while retaining circulating bRBCs), and small ECS volume (allowing confluency to be attained within the bioreactor within a short time frame with minimal cell inoculation). Since the primary aim of this article is to model O2 transport within an individual fiber of a BLAD, the experimental operation of the HF bioreactor is not discussed in detail. For a detailed experimental description of the HF bioreactor, the reader is directed to a previously published article describing the operation of a similar
BLAD (using a different HF cartridge) with bRBC supplementation [156]. Briefly, the current HF bioreactor was inoculated with ~2x10⁶ C3A hepatocytes (ATCC, Manassas, VA, Cat. #CRL-10741) into the ECS and maintained within a Heraeus incubator (Kendro Laboratory Products, Hanau, Germany) at 37°C and 5% CO₂. Circulating media, consisting of 90% Eagle’s Minimum Essential Media (MEM, ATCC, Cat #30-2003), 10% Fetal Bovine Serum (ATCC, Cat #30-2020), and 0.2% Normocin (an antibiotic/antimycotic agent; Invivogen, San Diego, CA, Cat. #ant-nr-o), was pumped from a 250 ml reservoir bottle through ~2 m of silicon tubing (which facilitated O₂ transfer from the ambient atmosphere of the incubator to the culture medium) into the HF bioreactor. The circulating culture medium thus delivers O₂ and other nutrients to the hepatocyte culture, and then picks up metabolic waste products, which are transported to the reservoir bottle where they are subsequently diluted. The HF bioreactor was first operated with no bRBC supplementation of the circulating culture media (as a control) and after the required parameters were obtained (described below), the circulating medium was supplemented with bRBCs at ~5% hRBC concentration. Similar to the experiments described in our previous article [156], select metabolic (glucose consumption and lactate production) and synthetic (albumin production) functions were followed throughout the study to provide a gauge of cell health, and to allow for an estimation of the cell number at confluency.
Figure 3.1 Schematic of the experimental hepatic HF bioreactor system along with a cross-sectional view of a single hollow fiber. Solid rectangles within the enlarged fiber represent the modeled space.
The primary parameters obtained from our experimental study that were required for direct comparison with the modeling study included the bRBC concentration (maintained at ~5% hRBC concentration as determined by a hemacytometer), the circulating media flow rate, and the dissolved O₂ concentration at the inlet and outlet of the HF bioreactor. In order to measure the dissolved O₂ concentration within the circulating media, the experimental setup was outfitted with two inline O₂ probes (Lazar Research Laboratories, Los Angeles, CA) at the entrance and exit of the bioreactor. The probes were calibrated with water saturated with compressed air, zeroed using water saturated with sodium sulfite, and sterilized by flowing 70% ethanol through them for at least 1 hour. From the dissolved O₂ measurements, the O₂ bound to hemoglobin (Hb) was calculated using the Hill [145] and Adair [146] equation, and the total amount of O₂ (dissolved and bound to Hb) entering and exiting the HF bioreactor was determined. The difference between the total amount of O₂ (dissolved and bound to Hb) entering and exiting the HF bioreactor yielded the global bioreactor O₂ consumption. The Hill and Adair parameters that describe the O₂ binding properties of the bRBCs utilized in the study were regressed from a curve fit of the equations to the O₂-Hb equilibrium curve (OHEC) measured using a HEMOX Analyzer (TCS Scientific Corp., New Hope, PA).

3.3 Oxygen Transport Model Development

3.3.1 Velocity and Oxygen Carrier Concentration Profile Development

Blood is considered a non-Newtonian fluid, where the aggregation of RBCs is partially attributed to the presence of various plasma proteins, especially fibrinogen
We utilized washed bRBCs resuspended within our hepatocyte growth media. Therefore, the circulating media did not contain any plasma proteins. Consequently, the variation from Newtonian flow is less than it would be for whole blood. Nevertheless, the possibility that bRBC supplementation alters the circulating media’s velocity profile as a consequence of the formation of a bRBC concentration profile was examined for the various operating conditions of the hollow fiber bioreactor in this study. In tube flow, the Fahraeus effect can cause an axial bRBC concentration profile to develop [132]. However, the diameter of the fibers in this study is large enough such that the bRBC concentration in the media reservoir bottle accurately reflects the bRBC concentration within the bioreactor fibers [134]. Additionally, a radial bRBC concentration profile can be formed due to the Fahraeus–Lindqvist effect [135] in which bRBCs aggregate near the centerline of the flowing lumen due to the shear stress gradient. For most of the hollow fiber bioreactor operating conditions studied ($Hb_T < 85\%$ hRBC), the bRBCs are not expected to experience significant shear deformation [134], which can lead to a change in bRBC concentration and velocity profiles. The characteristic length for a bRBC concentration gradient to develop ($L_C$) was determined for all cases by equation 2.2 (as described in Chapter 2 and the Appendix). Only at 100% hRBC was $L_C$ on the same order of magnitude as the bioreactor length [136]. At 100% hRBC concentration, the bRBC concentration and velocity profiles are found from the shear stress profile within the lumen and are presented in Figure 3.2. These profiles were entered into the oxygen transport model, resulting in $<9\%$ overestimation of the final OCR. This minimal change in OCR indicates that assuming a homogenous bRBC concentration profile and Poiseuille flow profile is reasonable for the various situations explored.
Figure 3.2 Velocity profile (left axis) for bRBC concentration profiles (right axis) in the lumen of the HF bioreactor for constant bRBC concentration profile (lines) and theoretical bRBC altered concentration profile (dashed lines).
3.3.2 Characteristic Parameters

Table 3.1 lists the parameters that were entered into the O$_2$ transport simulations, and the literature sources from which were derived. The HF bioreactor used for the experimental studies provided the dimensions of the modeled area (Figure 3.1). The O$_2$ diffusivity for each of the sections of the individual HF was obtained from the literature. The diffusivity of O$_2$ in the lumen was assumed to be that of water at 37°C and the diffusivity of O$_2$ in the ECS was found from the literature. The membrane of the HF bioreactor is typically considered fully hydrated, and therefore the diffusivity of O$_2$ in the membrane was assumed to be the same as that within the lumen [11]. This is supported by the Renkin equation [158], which indicates that the diffusivity of O$_2$ in the lumen and membrane are essentially equal. The literature suggests that MM kinetics most accurately describe hepatic O$_2$ consumption, and a range of MM parameters have been reported. In general, $V_{Max}$ has been reported to range from 5 – 100 nmol/(cm$^3$-s) [11], with a majority of attention being focused at the lower end of this range for three-dimensional cultures. Similarly, the $K_M$ values reported within the literature range from 0.5 – 5.6 mmHg [11], with a focus on the middle of this range. The kinetics of O$_2$ binding and release to/from Hb are incorporated with a variable reaction rate model (as discussed in Chapter 2), where the forward reaction rate constant was found in the literature and the reverse reaction rate constant depends on a set of Adair parameters that were measured for the bRBCs (presented in the Results section). The user-controlled variables for the HF bioreactor were varied in order to predict optimum bioreactor operating conditions. The inlet O$_2$ tension ($p_{O_2,in}$) was examined between 5 – 135 mmHg, which fully encompasses the range of physiological O$_2$ tensions experienced
within the liver sinusoid. The maximum velocities utilized in equation 2.1 were calculated from measured circulating media flow rates. Finally, the concentration of bRBCs in the circulating media was varied from 0 – 100% hRBC concentration in order to determine to what extent O2 delivery is improved as bRBC concentration is increased.

TABLE 3.1
PARAMETERS OF THE HOLLOW FIBER BIOREACTOR UTILIZED IN THE OXYGEN TRANSPORT MODEL

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusivity of O2 in lumen, $D_l$</td>
<td>$3.0 \times 10^{-5}$</td>
<td>cm$^2$/s</td>
<td>[140, 148]</td>
</tr>
<tr>
<td>Diffusivity of O2 in membrane, $D_m$</td>
<td>$3.0 \times 10^{-5}$</td>
<td>cm$^2$/s</td>
<td>[11]</td>
</tr>
<tr>
<td>Diffusivity of O2 in ECS, $D_k$</td>
<td>$2.0 \times 10^{-5}$</td>
<td>cm$^2$/s</td>
<td>[148]</td>
</tr>
<tr>
<td>Bunsen solubility, $\alpha$</td>
<td>$4.31 \times 10^{-8}$</td>
<td>g/(cm$^3$-mmHg)</td>
<td>[159]</td>
</tr>
<tr>
<td>Maximum reaction rate, $V_{Max}$</td>
<td>25</td>
<td>nmol/(cm$^3$-s)</td>
<td>[11, 127]</td>
</tr>
<tr>
<td>Michaelis constant, $K_M$</td>
<td>3</td>
<td>mmHg</td>
<td>[127, 140]</td>
</tr>
<tr>
<td>Diffusivity of bRBC in lumen, $D_{RBC}$</td>
<td>$6.8 \times 10^{10}$</td>
<td>cm$^2$/s</td>
<td>[160]</td>
</tr>
<tr>
<td>O2-Hb dissociation rate coefficient, $k^-$</td>
<td>44</td>
<td>1/s</td>
<td>[161, 162]</td>
</tr>
<tr>
<td>Inlet partial pressure of O2, $p_{O2,in}$</td>
<td>5 – 135</td>
<td>mmHg</td>
<td>Measured</td>
</tr>
<tr>
<td>Maximum velocity in lumen, $u_{max}$</td>
<td>0.85 – 13.74</td>
<td>cm/s</td>
<td>Measured</td>
</tr>
<tr>
<td>bRBC concentration, $Hb_T$</td>
<td>0 – 0.15</td>
<td>g/cm$^3$</td>
<td>Measured</td>
</tr>
</tbody>
</table>

3.3.3 Oxygen-Hemoglobin Equilibrium Curves

The OHEC of bRBCs was measured using a HEMOX analyzer (Figure 3.3). The OHEC was fit to Hill’s equation (regressed parameters: $P_{50}$ and $n$) and Adair’s equation (regressed parameters: $a_1$-$a_4$) using Scientist (MicroMath Research, St. Louis, MO) to regress the parameters (Figure 3.3). The Hill equation was found to predict the experimentally measured OHEC well at moderate pO2 values (15 – 35 mmHg), but deviated significantly at high and low pO2 values. The Adair equation was found to yield...
a more accurate description of the experimentally measured OHEC, especially at the extremes of the fractional O₂ saturation of Hb. For most of the simulations carried out, the region of interest within the lumen included pO₂ values greater than 40 mmHg, and therefore the Adair equation was determined to yield the best description of the experimentally measured OHEC.
Figure 3.3 Experimentally measured oxygen-hemoglobin equilibrium curves for bovine red blood cells along with curve fits to the Hill and Adair equations.
3.4 Sensitivity Analysis

The input parameters for the oxygen transport model were tested with a standard one-at-a-time (OAT) sensitivity analysis, where the effect of changing a single input parameter value was evaluated by comparison with a standard set of parameters (Table 3.1), also known as ceteris paribus [163]. The entire list of inputs was reviewed to determine which of the parameters would need to be analyzed. The parameters that were varied in this paper were not considered in this analysis as their effects are already provided in the various sections (concentration of bRBC, flow rate, and pO\textsubscript{2,\text{in}}) of this article. Additionally, the experimentally determined parameters (Adair constants, \(a_{1-4}\), and Michaelis-Menten parameters, \(K_M\) and \(V_{Max}\)) were not included since their effects were observed when they were determined. The remaining parameters were varied to determine the overall sensitivity of the OCR to changes in input. Oxygen dissociation from hemoglobin (\(k^-\)) and the diffusivity of the bRBCs (\(D_{RBC}\)) were found to have almost no effect on the output of the simulation at ± 50% parameter deviation. Next, the Bunsen solubility was tested at ±1 and 2 standard deviations from the averaged value presented in Table 3.1. Again, this parameter was found to result in relatively small changes in the overall OCR (< 2.1% for all cases). Finally, the set of diffusivities throughout the various regions of the HF were changed by ± 10% and then 50%. The resulting OCRs all changed by < 3% and < 20% from the standard values in Table 3.1, respectively. In addition to changing each manually, the diffusivity in the lumen was set equal to the diffusivity in the membrane (\(D_l = D_m\)), as assumed previously, and the pair was tested at ± 50% parameter deviation. This linked sensitivity was less than that of the individual changes (< 7% for all tested values). From the set of resulting sensitivities determined,
we feel it is appropriate to conclude that the model is not overly sensitive to any of the parameter values. Also, the changes within each of the parameter ranges studied did not once cause any convergence issues with the simulations. Since all simulations converged properly, we may also conclude that the oxygen transport model is stable within the necessary parameter limits.

3.5 Results

The health and viability of the hepatocyte culture was assessed by monitoring select metabolic (glucose consumption and lactate production) and synthetic (albumin secretion) functions throughout the duration of the experimental study. From the measured metabolic and synthetic indicators, the number of hepatocytes present at confluence within the HF bioreactor was estimated to be between $8 \times 10^8$–$10^9$ hepatocytes. Throughout the experimental study, the bRBC concentration in the circulating media was maintained at about 5% hRBC concentration, $Hb_T \sim 0.008$ g/cm$^3$. bRBCs were also analyzed throughout the study for the formation of methemoglobin (metHb) via a modification of the cyanomethemoglobin method [164]. The metHb levels were found to never exceed 3%, allowing for metHb not to be considered within the formulation of the model described in this paper. The circulating media flow rate was found for each pump setting utilized within the experimental HF bioreactor study, in addition to the highest and lowest flow rate available for the HF bioreactor. These flow rates were determined both prior to and after each experiment, and were found to be relatively consistent. The select system flow rates measured were $Q = 1.77 \pm 0.18$, $4.72 \pm 0.18$, $8.35 \pm 0.30$,  

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16.8 (± 0.92), and 28.6 (± 0.73) ml/min. The maximum velocity through a single fiber was determined from these flow rates, and utilized in the simulations via equation 2.1.

3.5.1 Key Metabolic and Synthetic Data

During the experimental study described, metabolic (glucose) and synthetic (lactate and albumin) parameters were measured and the results are published in the literature [156]. These assays were conducted to monitor cell culture health and to indirectly indicate the improvement in oxygen provision to the hepatocytes. Briefly, samples were taken during each of the media changes and were assayed in at least duplicate and averaged. First the glucose consumption and the lactate production were measured; it was found that the glucose consumption was similar between the control case and bRBC supplemented case. The lactate production was higher in the control case for all of the measurements after an initial lag phase. The ratio of lactate production to glucose consumption gives a measure of the oxygenation of the hepatocyte culture. This is due to the fact that lactic acid is generally produced in hypoxic environments, where two moles of lactic acid are produced for every mole of glucose consumed. We can observe in Figure 3.4 (left-hand plot) that in the control case of no oxygen carrier supplementation the lactate production to glucose consumption ratio is higher. This indicates that in the bRBC supplemented case was better oxygenated since under well oxygenated cases glucose is fully broken down by the TCA cycle and no lactate is produced.
Figure 3.4 Ratio of lactic acid produced to glucose consumed (left) and albumin production over the course of the study (right). Standard errors are indicated by the error bars and significant difference from the control culture is indicated by an asterisk over the respective time period *(P<0.001). Legend: blue is the control system and red is the experimental system.
Albumin synthesis is another vital function for hepatocytes to maintain in culture within a BLAD. As mentioned above, albumin is an important protein in transport and regulation within the bloodstream. Additionally, albumin is a marker of confluency of the cell culture. The synthesis of albumin greatly increases when the culture reaches confluency as can be seen in Figure 3.4 (right-hand plot). Additionally, we observed that significantly more albumin is produced in the bRBC supplemented case, which indicates that more oxygen is available to the cultured hepatocytes.

3.5.2 Comparison to Experimental Data

Dissolved O$_2$ concentrations were measured at the inlet and exit of the HF bioreactor at three different pump speeds, for both the case of no bRBC supplementation and bRBC supplementation (Figure 3.5). The error bars presented on the figure denote the instrumentation error associated with the inline pO$_2$ probes. pO$_2$ values were recorded over the course of at least 2 hours to allow for steady state measurement of the pO$_2$. Figure 3.5 shows the changes in total (dissolved and bound to Hb) O$_2$ concentration ($\Delta$C$_{O_2}$) within the circulating media, with the flow rate and inlet pO$_2$ of each measurement noted. The $\Delta$C$_{O_2}$ values predicted by the O$_2$ transport model are displayed for comparison to the experimentally acquired data (Figure 3.5). For all cases considered, the model predicted the $\Delta$C$_{O_2}$ values within experimental error.
Figure 3.5 Comparison of the experimentally measured $\Delta C_{\text{O}_2}$ over the entire bioreactor and values calculated by the oxygen transport model at several circulating media flow rates and inlet pO$_2$ values.
3.5.3 Model Predictions

In order to evaluate the impact of bRBC concentration on O₂ delivery to the hepatocyte culture, the O₂ transport model was utilized to simulate bRBC supplementation conditions ranging from 0 – 100% hRBC concentration. The OCR was found by performing a mass balance on the total O₂ concentration over the entire bioreactor (ΔC₀₂), accounting for the volume of the ECS and the flow rate of the circulating media. Consequently, several flow rates were considered, including those for which experimental dissolved O₂ concentration measurements were obtained. The results of the modeling study are shown in Figure 3.6, which indicates that increasing the bRBC concentration and/or the flow rate, results in an increase in the O₂ provided to the hepatocytes maintained within the HF bioreactor. The model predicts that the OCR increases fastest from 0 – 20% hRBC concentration, and levels off at a constant rate of increase for the rest of the bRBC concentrations studied (20 – 100% hRBC concentration).

At several circulating media flow rates, simulations were conducted for inlet pO₂ values ranging from 5 – 135 mmHg, without bRBCs present in the media and at 25% hRBC concentration. Increasing the inlet pO₂ linearly increases the OCR without bRBCs present. Supplementing the circulating media with bRBCs results in an increase in the OCR, which reaches a maximum at pO₂ ~30 mmHg and then decreases. Figure 3.7 shows that the normalized OCR (OCR_{bRBC}/OCR_{No bRBC}) exhibits a similar trend to the bRBC supplemented case. The results of the simulations show a decrease in the normalized OCR with increasing circulating media flow rate (Figure 3.7). These results indicate that bRBCs are more effective in transporting O₂ to hepatocytes at slower flow
rates, and that for the conditions simulated a maximum OCR increase of about ~10 fold was observed.

The model was also utilized to simulate a two-dimensional axisymmetric cross-section of an individual HF and representative ECS space, which allows for visualization of the $O_2$ tension at any point throughout the HF. Figure 3.8 shows these cross-sections and, as can be seen, contour lines have been added to denote the oxygenation ranges of the previously defined hepatocyte phenotype zones. Simulations were conducted at five different flow rates (including the three where experimental data was collected) and at four different bRBC concentrations (0%, 5%, 25% and 100% hRBC concentration). The plots indicate that increasing the circulating media flow rate and bRBC concentration increases the $O_2$ spectrum experienced by the hepatocytes. In the control case of media not supplemented with bRBCs, the model predicts poor hepatocyte function and viability. Additionally, at the lower flow rates, hepatocytes are predicted not to experience enough oxygenation to function properly. The plotted results in Figure 3.8 show that the first layer of attached cells experience periportal oxygenation, and may also experience pericentral oxygenation at increased bRBC concentrations and circulating media flow rates. bRBC supplementation of the circulating media allows for a significant increase in the $O_2$ available to the hepatocytes. At lower flow rates ($Q = 1.77 \& 4.72$ ml/min) with no bRBCs present in the circulating media, significant anoxic regions ($pO_2 < 0.5$ mmHg) are present. Addition of bRBCs to the circulating media completely removed the presence of anoxic regions in the ECS, and increased the volume of hepatocytes in the perivenous zone by ~10%. At intermediate and high circulating media flow rates with bRBC supplementation pericentral oxygenation increased to ~5% ($Q = 4.72 \& 8.35$
ml/min) and ~10% (Q = 16.8 & 28.6 ml/min) compared to the control case of no bRBC supplementation. However, in all the simulated cases hepatocytes did not experience periportal oxygenation.
Figure 3.6 Predicted OCR values calculated from the oxygen transport model for bRBC concentrations ranging from 0 – 100% of the *in vivo* hRBC concentration at five different flow rates.
Figure 3.7 Predicted OCR values calculated from the oxygen transport model for inlet pO$_2$ values ranging from 5 – 135 mmHg at five different flow rates.
Figure 3.8 Oxygen transport model predicted oxygen concentration plots at five flow rates and four bRBC concentrations (Note: fiber axes are not equal).
3.6 Discussion

The O₂ transport model describes the steady-state O₂ concentration profile in an individual HF. The geometry of the modeled element dictates that evenly spaced fibers with a representative annulus of ECS adequately describe the bed of fibers within the HF bioreactor. The modeled space exhibits radial symmetry, and therefore solving the O₂ concentration profile as a two-dimensional cross-section will not affect the solution. When considering flow through the fibers, entrance and exit effects are neglected since the entrance length [130] is less than the thickness of the tube sheets in which the fiber ends are encapsulated and, therefore, fully developed flow is achieved before O₂ transfer begins. Within the model, each domain (lumen, membrane, and ECS) is considered a homogeneous continuum [11, 38], possessing a specific effective O₂ diffusivity. Additionally, O₂ transport is driven by hepatocyte O₂ consumption, and is limited by the supply of O₂ in the lumen. Many recent advances were incorporated into the Krogh tissue cylinder model to reduce its dependence on some of the less reliable assumptions. These improvements include a better description of the pO₂ distribution within the lumen space due to the addition of the circulating media velocity and full O₂ transport equations. Additionally, the inclusion of the Adair equation to describe O₂ dissociation from Hb provides a more accurate representation of the radial O₂ profile in the lumen. The inclusion of a membrane in the model is needed to describe the HF bioreactor accurately, whereas many earlier models (especially those solved analytically) did not contain this barrier to diffusive resistance. Within the ECS, hepatic O₂ consumption is described by MM type kinetics, which is an improvement over the constant O₂ consumption assumption that is often made (zero-order kinetics). The inclusion of these advances in
modeling O₂ transport within an individual HF allow for a more accurate prediction of the pO₂ profile within this device.

The possibility that bRBC supplementation alters the circulating media’s velocity profile as a consequence of the formation of a bRBC concentration profile was examined for the HF bioreactor in this study. In tube flow, the Fahraeus effect can cause an axial bRBC concentration profile to develop [132]. However, the diameter of the fibers in this study is large enough such that the bRBC concentration in the media reservoir bottle accurately reflects the bRBC concentration within the HF bioreactor fibers [134]. Of additional consideration, Marginal-Zone theory describes the Fahraeus-Lindqvist effect [135] in which bRBCs aggregate near the centerline of the flowing lumen due to the shear stress gradient. Under the existing HF bioreactor operating conditions, the bRBCs are not expected to experience significant deformation [134]. Since the bRBC profile is considered homogeneous, it was found that the velocity profile was unchanged for increasing bRBC concentration.

The precise O₂ binding parameters of the bRBCs utilized in these studies were required in order to accurately run the simulations and calculate the bioreactor OCR. The original Krogh model and its early permutations did not account for the cooperativity of Hb, but instead used a linear O₂ binding curve [123], which can only approximate a very small linear section of the OHEC. For this model, bRBC OHECs were fit with both the Hill and Adair equations. The OHEC represented with by the Hill equation is better than simply using a linear binding curve; however, it deviates from the experimentally measured OHEC at high and low pO₂ values. The Adair equation, which was used in
these simulations, accounts for the four O\textsubscript{2} binding sites on the Hb protein, and is therefore more accurate, especially at the extremes of the fractional O\textsubscript{2} saturation of Hb.

A range of MM constants have been utilized previously to describe the hepatic O\textsubscript{2} consumption rate, which reflects the varied hepatocyte cell types and culture conditions that have been studied [38]. Consequently, we determined the \( K_M \) and \( V_{\text{Max}} \) values that were most appropriate for the C3A hepatocyte cell line within our HF bioreactor by comparison of the O\textsubscript{2} transport simulations to the experimental data. From the experimental OCR results, we determined that at \( V_{\text{Max}} = 25 \text{ nmol/(cm}^3\text{-s)} \) and \( K_M = 3 \text{ mmHg} \), the O\textsubscript{2} transport simulations produced results in close agreement with the experimental values. The \( V_{\text{Max}} \) determined is within the range of values reported by other groups, but is higher than expected for the three-dimensional setup studied [38]. We believe that this discrepancy may be due to the limited oxygenation provided to the hepatocytes causing them to work in an inefficient manner. The predicted O\textsubscript{2} concentration profiles (Figure 3.8) indicate that only hepatocytes in the first (or in some cases second) adherent layer on the outside of the fiber receive proper oxygenation, and that the biomimetic three-dimensional arrangement that is theoretically achievable is not being fully utilized. With two-dimensional flat plate bioreactor configurations, \( V_{\text{Max}} \) has been observed to be an order of magnitude higher, and this may explain the increased \( V_{\text{Max}} \) observed experimentally. Additionally, the experimentally measured OCR appears to be more sensitive to changes in flow rate and bRBC concentration than the model predicts, indicating that \( V_{\text{Max}} \) decreases under better oxygenation conditions. We believe that the experimental OCR is affected more by media flow rate and bRBC concentration due to improved oxygenation of the hepatocytes leading to a more \textit{in vivo}-like
environment for the hepatocytes, which influences their metabolic capacity [165]. Hepatocytes in a better oxygenated environment work more efficiently, and thus a lower $V_{\text{Max}}$ would more accurately describe their OCR [141]. The $K_M$ value found is also within the range reported in the literature, indicating agreement with previously reported values [127].

Once the O$_2$ transport model and bioreactor operating parameters (Table 3.1) were verified by comparison to experimentally determined values, the user-controlled variables were varied in order to help optimize the experimental operating conditions for future studies. Supplementation of bRBCs into the circulating media increases the availability of O$_2$ within the lumen feed stream. As the bRBC concentration in the circulating media is raised, the OCR within the HF bioreactor would be expected to increase due to the increasing availability of O$_2$ allowing for an increasing driving force for diffusional transport of O$_2$ across the HF membrane. The bRBC concentration was only studied at human physiological concentrations and below, since at elevated bRBC concentrations the addition of bRBCs begins to dilute the concentration of other components in the media and is expected to increasingly contribute to fiber fouling [166]. Furthermore, increasing the circulating media flow rate through the HF cartridge is also predicted to result in increased hepatic O$_2$ consumption. However, increasing the circulating media flow rate could be harmful to the cultured hepatocytes due to the possibility of generating a considerable trans-membrane flow that could result in increased shear stress being placed on the hepatocytes.

We witnessed that at $pO_{2,\text{in}} = 95$ mmHg and $Q = 1.77$ ml/min, a three-fold increase in the circulating media flow rate with no bRBCs present is required to produce
an increase in OCR that is equivalent to that obtained by supplementing that circulating media with a bRBC concentration of only 5% hRBC concentration. Consequently, we believe that bRBC supplementation is a more effective means of improving O₂ delivery to a HF culture than simply increasing the circulating media flow rate.

Values have been published for the OCR of hepatocytes in various culturing environments. Nyberg et al. [111] reported that HepG2 cells cultured in a three-dimensional gel had an OCR of ~5.7 μM/s. Nyberg’s reported OCR is within the calculated values presented in Figure 3.6 for no bRBC supplementation (~2 – 7 μM/s) at the flow rates studied. The type of bioreactor and operating conditions used in between the studies are not identical, but the agreement indicates that the model’s predicted results compare well with the previously reported values. A more recent study of porcine hepatocytes in a HF device was also in good agreement with the OCR of the control case (no bRBCs present) data [167]. The mean OCR in their study is reported as 0.190 nmol/hr/million cells, while our experimental results found OCRs of 0.0804, 0.113, and 0.155 nmol/hr/million cells (for Q = 4.7, 8.3, and 16.8, respectively), whereas the bRBC supplemented bioreactor cases exhibited elevated OCRs of 0.182, 0.282, 0.418 nmol/hr/million cells, assuming that at confluency 8x10⁸ hepatocytes were present. Again, our experimental setup is not identical to those reported in the literature, but the values found experimentally and modeled here correspond to previously reported literature values. Additional literature sources have been reviewed with continued positive comparisons [168, 169].

The pO₂ entering the HF cartridge can be varied 3 ways: 1) altering the O₂ content in the atmosphere of the incubator, 2) adjusting the length of the silicone tubing, and 3)
altering the circulating media flow rate. The maximum OCR is attained by maintaining the inlet pO\(_2\) just above the maximum slope on the OHEC (Figure 3.3), which generally corresponds to the P\(_{50}\). For the bRBCs utilized in our experimental studies, this corresponds to a pO\(_2\) \(\sim\) 30 mmHg. Therefore, we believe that O\(_2\) delivery could be improved by either manipulating the inlet pO\(_2\) so that it is closer to the highly sloped region of the OHEC, or by possibly utilizing an O\(_2\) carrier with a shifted OHEC (in comparison to the normal bRBC OHEC). For the hepatocytes cultured, the inlet pO\(_2\) must remain elevated and therefore a right shifted OHEC might be useful in increasing the O\(_2\) delivered. Right-shifting the OHEC of bRBCs could be achieved by cross-linking bRBCs with glutaraldehyde in an anoxic environment (deoxyHb conformation) [170, 171]. Additionally, sucrose and other neutral solutes have been shown to reduce Hb-O\(_2\) affinity and right shift the OHEC [172, 173].

The maximum achievable OCR was observed to occur at an inlet pO\(_2\) close to the P\(_{50}\) of the O\(_2\) carrier. Therefore, we believe that an O\(_2\) carrier could potentially be engineered specifically for maximal O\(_2\) transport to a cell culture with known O\(_2\) consumption kinetics. There are many cell types that require specific pO\(_2\) environments for realization of optimized viability and differentiated function that could benefit from a specialized O\(_2\) carrier. For instance, stem cells typically prefer low O\(_2\) tensions when cultured [174, 175]. Hence, the use of an O\(_2\) carrier with an increased O\(_2\) affinity (such as bRBCs that are cross-linked while the internal Hb is in the oxyHb conformation) could be employed to allow for optimum O\(_2\) delivery at low O\(_2\) tensions. bRBC O\(_2\) carriers were the focus of this article, but other Hb-based O\(_2\) carriers, both cellular [176-180] and acellular [181-184], are currently being developed in our lab, and could potentially be
used as a media supplement. Given the OHEC for any of these O₂ carriers, the O₂ transport model can easily be altered to reflect its O₂ binding properties and provide bioreactor O₂ concentration profiles.

The O₂ concentration profiles generated by the O₂ transport model (Figure 3.8) describe the O₂ distribution throughout the HF bioreactor, which allows for visualization of hepatocyte oxygenation within the ECS and reinforces the idea that increased flow rate and bRBC concentration increases O₂ availability to cultured hepatocytes. The addition of bRBCs to the circulating media removes the anoxic region present in simulations run at the lower flow rate and reduces the hypoxic region in all cases. However, O₂ provision to the ECS indicates that hypoxic regions persist, and that only the first few layers of cells growing on the HF membrane surface experience proper oxygenation. The O₂ transport model suggests that the proper O₂ spectrum cannot be fully achieved with the current HF bioreactor. Consequently, it is our expectation that a cartridge containing a large number of fibers per cross-sectional area (resulting in a reduced Krogh radius) should be employed to improve O₂ delivery. Reducing the fiber spacing would improve O₂ delivery to most of the cultured hepatocytes, and would more closely resemble the in vivo situation, where generally only one hepatocyte layer separates the sinusoid vessels. However, a completely fenestrated HF arrangement would reduce the number of cells in the bioreactor and complicate scale up efforts. Therefore, the fiber spacing must be chosen carefully to ensure that the ECS is large enough for the maintenance of a practical number of hepatocytes, while the ECS surrounding each fiber must be small enough to provide sufficient oxygenation to the attached hepatocytes.
The HF bioreactor used in the experimental studies had a membrane thickness of 50 μm, which is on the same order of magnitude of many HF bioreactors. The model indicates that the HF membrane’s O₂ diffusive resistance is significant and we have seen that this additional diffusive resistance (not present *in vivo*) must be accounted for to achieve the desired oxygenation within the ECS. Therefore, to obtain higher pO₂ values within the ECS compared to what the model is currently predicting, the pO₂,in needs to be raised to above the physiological O₂ concentration. Raising the ambient pO₂ in the incubator environment and/or extending the length of silicone tubing (to increase the residence time for O₂ transfer into the circulating media) could possibly lead to the attainment of supraphysiological levels of O₂ within the HF bioreactor lumen that we believe is necessary to culture hepatocytes within the proper oxygenation spectrum experienced *in vivo*. Additionally, utilizing the minimum membrane thickness for individual hollow fibers that does not compromise the integrity of the fibers would be advantageous in increasing O₂ transport to hepatocytes.

In addition to optimizing the operating conditions of a single biomimetic BLAD, the O₂ transport model may also be useful in determining operating conditions for running atypical BLADs for more specific applications. Many of the liver’s zone specific ailments have been treated with only marginal success, and a specifically tailored BLAD could be used to treat patients suffering from perivenous necrosis or periportal lesions. Currently there is no practical technique to separate periportal and perivenous cells from isolated hepatocyte cultures [185], so a mixture of phenotypes must be used to seed a cell culture. The O₂ transport model could be used to determine optimal culture conditions to allow for the operation of a HF bioreactor within a predetermined ECS oxygenation
range that results in a BLAD dominant in a desired hepatocyte phenotype. In addition to O₂ provision, hepatocyte phenotype induction with glucagons (periportal) or insulin (perivenous) [186] could assist in creating a single zone BLAD. Such single zone BLADs could potentially be connected together to create a BLAD system capable of providing global therapy to ALF patients.

3.7 Conclusions

The oxygen transport model presented in this work demonstrates that supplementation of the circulating media of a HF bioreactor with an O₂ carrier, in our case bRBCs, resulted in increased O₂ delivery to housed hepatocytes. The simulated results were verified by experimental measurements that indicated that bRBC supplementation produced an increase in hepatocyte OCR and pO₂ spectrum within the HF bioreactor, which should allow for increased cellular activities. Furthermore, the O₂ transport model predicted that increasing in the concentration of supplemented bRBCs increased O₂ availability and consequently increased the OCR. We expect increased O₂ delivery to hepatocytes to result in improved hepatocyte viability and differentiated function. Additionally, a more in vivo-like pO₂ distribution can be created, further favoring hepatocyte viability and allowing for a full range of hepatic phenotypes to form within the bioreactor ECS. Hence, this model is intended to support future work aimed at optimizing the design and development of hepatic HF bioreactors for clinical use. We believe that this enabling technology can be applied to most mammalian cell cultures maintained within a HF bioreactor. The three-dimensional environment of the HF device provides the most in vivo-like arrangement of cultured cells available in a commercial
bioreactor. However, poor O₂ transport within these devices prohibits their widespread usage. We hypothesize, and prove both experimentally and theoretically, that supplementing the circulating media of a HF bioreactor with an O₂ carrier can improve oxygenation within the ESC that typically hinders cell growth and function. Additionally, we infer that by altering the O₂ binding properties of the O₂ carrier, a specialized O₂ carrier can be created for improving oxygenation to different cell types.
This chapter, in part or in full, is a reprint of the material as it appears in:


The dissertation author was the first author on this paper with the dissertation committee chair as the primary investigator.
CHAPTER 4:
TARGETED OXYGEN DELIVERY WITHIN HEPATIC HOLLOW FIBER
BIOREACTORS VIA SUPPLEMENTATION OF HEMGLOBIN-BASED OXYGEN
CARRIERS

4.1 Introduction

In the experimental work presented in Chapter 3, we observed that bovine red blood cell (bRBC) supplementation of the circulating media stream enhanced oxygenation of cultured C3A hepatoma cells compared to a culture with no O₂ carrier [156]. Despite this success, of increasing oxygenation and supporting the oxygen transport model developed in this dissertation, the cultured hepatocytes were not exposed to the desired in vivo O₂ spectrum (25 – 70 mmHg) [137]. In order to remedy this problem, we hypothesize that altering the kinetics of O₂ binding/release to/from hemoglobin-based O₂ carriers (HBOCs) could potentially target O₂ delivery to cell cultures. Seven different HBOCs were examined in this study to determine which would be best for future experimental work. High P₅₀ (low O₂ affinity) HBOCs preferentially targeted O₂ delivery at high inlet pO₂ values. Conversely, low P₅₀ (high O₂ affinity) HBOCs targeted O₂ delivery at low inlet pO₂ values. Additionally, inlet pO₂, flow rate, and HBOC concentration were varied to find optimal bioreactor operating conditions. HBOCs should be able to enhance the oxygen delivery to cultured hepatocytes, while
exposing them to *in vivo*-like O$_2$ tensions which is critical to create a fully functional BLAD.

4.2 Oxygen Transport Model Development

4.2.1 Velocity and Oxygen Carrier Concentration Profiles

The addition of an oxygen carrier to the flowing lumen can disrupt the flow profile, causing an altered velocity profile and oxygen carrier concentration profile to develop. As is described in Chapter 2 and 3, and given in detail in the Appendix, the velocity profile changes from a parabolic to a more blunted profile and the HBOC concentration profile changes from homogeneous to having an increased concentration near the center of the flowing tube (Figure 3.2). For the cases examine in this study, we found that the induction length ($L_C$) is of similar magnitude to the length of the hollow fiber bioreactor only for larger radii HBOCs (XLbRBC1, XLbRBC2, and bRBC) at the maximum concentrations simulated (100% of the human *in vivo* concentration of hemoglobin). Therefore, only in these select cases is the concentration profile calculated and implemented within the oxygen transport model.

The induction length for the profiles to develop in this sized bioreactor with the largest of the oxygen carriers examined is at $L_C = 2.54$ cm. Therefore, to accurately portray the profiles through the length of the entire lumen, we have started with initial profiles and exponentially evolved into the calculated profiles, seen in Figure 3.2. Figure 4.1 and Figure 4.2 indicate the axisymmetric HBOC concentration and velocity profiles
down the length of the hollow fiber lumen, respectively. The calculations to find these profiles are found from an iterative solution that is provided in the Appendix.

Figure 4.1 illustrates how the entrance oxygen carrier concentration profile evolves into the calculated oxygen carrier concentration profile at $L_C$, while maintaining a constant flow averaged oxygen carrier concentration throughout the length of the hollow fiber. The oxygen carrier concentration profile alters the velocity profile by changing the local viscosity of the circulating media. In Figure 4.2 the inlet velocity profile exhibits Poiseuille flow, and evolves into a more blunted velocity profile down the length of the tube. For the calculated steady state oxygen carrier concentration and velocity profiles, we assumed an exponential evolution from the initial profiles to the steady state profiles over the calculated characteristic length, Figure 4.1 and Figure 4.2 respectively. The exponential evolution of the initial profiles into the steady state profiles is assumed, since calculations for finding the exact transition profiles are cumbersome and do not significantly change the final solutions. Additional simulations to test this assumption were conducted for all initial cases.

Additional simulations to test this assumption were conducted and indicated insignificant error (average ECS pO$_2$ < 3.5% error, and the calculated oxygen consumption rate < 0.5% error) in the cases not utilizing the altered flow profiles, thus validating the assumption. The calculated profiles included in the oxygen transport model for the simulations of XLbRBC1, XLbRBC2 and bRBCs with 100% of the human \textit{in vivo} concentration of hemoglobin (100% hHb) were important in obtaining accurate solutions.
Figure 4.1 Radial HBOC concentration profile through the entire length of the lumen of a single hollow fiber at an initial HBOC concentration of 100% of the human in vivo concentration of hemoglobin (100% hHb).

Figure 4.2 Radial fluid velocity profile through the entire length of the lumen of a single hollow fiber at an initial HBOC concentration of 100% of the human in vivo concentration of hemoglobin (100% hHb).
4.2.2 Character Parameters

The oxygen transport model described in this dissertation was verified by comparison to experimental data measured in a similar bioreactor system in Chapter 3. The difference between the bioreactor dimensions presented in this chapter and the previous pertains to the ECS volume and the number of fibers within the device. For this study, a Spectrum Laboratories (Rancho Dominguez, CA) hollow fiber cartridge (Cat. #400-012) is modeled, which contains a 12 ml ECS and ~850 fibers. The device is assumed to have evenly spaced fibers and, therefore, only a single fiber is modeled to represent the oxygen concentration profile throughout the entire bioreactor. Dimensions of the individual hollow fiber that was modeled are presented in Figure 4.3. This cartridge was selected for its smaller ECS volume per fiber ratio that we determined is required to achieve the desired oxygenation spectrum (~25 – 70 mmHg) observed in vivo. In vivo, more than 75% of the hepatic sinusoids (whose structure we are attempting to mimic with individual hollow fibers) are surrounded by a single cell layer of hepatocytes, with the remainder not in direct contact with the vasculature [28, 187].
Table 4.1 lists the parameters that were utilized in the O₂ transport simulations, and the literature sources from where they were obtained. The O₂ diffusivity for each of the sections of the individual hollow fiber was obtained from the literature. The diffusivity of O₂ in the lumen was assumed to be that of water at 37°C, and the diffusivity of O₂ in the ECS was found from the literature. The membrane of the hollow fiber bioreactor is typically considered fully hydrated, and therefore the diffusivity of O₂ in the membrane was assumed to be the same as that within the lumen [11]. This is supported by the Renkin equation [158], which indicates that the diffusivity of O₂ in the lumen and
membrane are essentially equal. The literature suggests that MM kinetics most accurately describe hepatic O\textsubscript{2} consumption, and a range of MM parameters have been reported. In general, \( V_{\text{Max}} \) has been reported to range from 5 – 100 nmol/(cm\textsuperscript{3}-s) \[11\], with a majority of attention being focused at the lower end of this range for three-dimensional cultures. Similarly, \( K_M \) values reported in the literature range from 0.5 – 5.6 mmHg \[11\], with a focus on the middle of this range. The kinetics of O\textsubscript{2} binding/release to/from HBOCs are incorporated in the O\textsubscript{2} transport model with a \textit{variable reaction rate coefficient model} rate law (equation 2.10), where the reverse reaction rate constant was found from the literature and the forward reaction rate coefficient depends on the Adair equation (equation 2.12) parameters that were measured for the HBOCs (presented in 4.2.4 Hemoglobin-Based Oxygen Carriers). The user-controlled variables for the hollow fiber bioreactor were varied in order to predict optimum bioreactor operating conditions. The inlet O\textsubscript{2} tension (pO\textsubscript{2,in}) was examined between 0.25 and 155 mmHg, which fully encompasses the range of physiological O\textsubscript{2} tensions experienced within the liver sinusoid. The maximum circulating media velocities utilized in equation 2.1 were calculated from our experimentally measured circulating media flow rates. Finally, the concentration of HBOCs in the circulating media was varied from 0 to 100% hHb (percent of the human \textit{in vivo} hemoglobin concentration) in order to determine to the extent of O\textsubscript{2} delivery improvement as a function of HBOC concentration.
TABLE 4.1

BIOPHYSICAL PARAMETERS OF THE HOLLOW FIBER BIOREACTOR
UTILIZED IN THE OXYGEN TRANSPORT MODEL

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusivity of O2 in lumen, $D_l$</td>
<td>$3.0 \times 10^{-5}$</td>
<td>$\text{cm}^2/\text{s}$</td>
<td>[140, 148]</td>
</tr>
<tr>
<td>Diffusivity of O2 in membrane, $D_m$</td>
<td>$3.0 \times 10^{-5}$</td>
<td>$\text{cm}^2/\text{s}$</td>
<td>[11]</td>
</tr>
<tr>
<td>Diffusivity of O2 in ECS, $D_k$</td>
<td>$2.0 \times 10^{-5}$</td>
<td>$\text{cm}^2/\text{s}$</td>
<td>[148]</td>
</tr>
<tr>
<td>Bunsen solubility, $\alpha$</td>
<td>$4.31 \times 10^{-8}$</td>
<td>$\text{g}/(\text{cm}^3\cdot\text{mmHg})$</td>
<td>[159]</td>
</tr>
<tr>
<td>Maximum reaction rate, $V_{Max}$</td>
<td>25</td>
<td>nmol/(cm$^3$·s)</td>
<td>[11, 127]</td>
</tr>
<tr>
<td>Michaelis constant, $K_M$</td>
<td>3</td>
<td>mmHg</td>
<td>[127, 140]</td>
</tr>
<tr>
<td>$\text{O}_2$-HBOC dissociation rate coefficient, $k$</td>
<td>44</td>
<td>1/s</td>
<td>[161, 162]</td>
</tr>
<tr>
<td>Inlet partial pressure of $\text{O}<em>2$, $p</em>{\text{O}_2,\text{in}}$</td>
<td>$0.25 – 155$</td>
<td>mmHg</td>
<td>Varied</td>
</tr>
<tr>
<td>Overall bioreactor flow rate, $Q$</td>
<td>$1.77 – 28.6$</td>
<td>ml/min</td>
<td>Varied</td>
</tr>
<tr>
<td>Total HBOC concentration, $Hb_T$</td>
<td>$0 – 0.15$</td>
<td>$\text{g/cm}^3$</td>
<td>Varied</td>
</tr>
</tbody>
</table>

4.2.3 Hollow Fiber Characteristics

The hollow fibers within the bioreactor have been characterized by the manufacturer and are of known dimensions with a 95% molecular weight cut off at 0.3 μm. However, the porosity, symmetry, and pore arrangements were all identified experimentally. The fibers examined were found to contain symmetric pores with low tortuosity. A cross-section of the hollow fiber membrane shows parallel grooves that are not increasing in size, Figure 4.4 (top). These were observed all the way throughout the fiber and indicate low tortuosity through the fiber. Since the grooves do not appear to change in width, this indicates that the pores are symmetric and this is further indicated by viewing the outer surface of the fiber, which contains the same size pores as the interior Figure 4.4 (bottom). Images were taken with a field emission scanning electron microscope (Hitachi S-5700; Hitachi Instruments, Tokyo, Japan) after platinum
sputtering (Emitech K675X; Emitech Ltd, Ashford, Kent, UK) for 2 min at 375 mA to
make the surface of the hollow fiber conductive. Measurements on the wet and dry
weight of a set of fibers were obtained to approximate porosity (~80%) of the hollow
fiber membranes. The large porosity (void volume), along with low tortuosity and
symmetric pores, supports our assumption that the membrane can be considered to have
approximately the same diffusivity as the media that saturates it ($D_l = D_m$).
Figure 4.4 Representative scanning electron micrograph image of the cross-section (above) and surface (below) of a hollow fiber membrane taken from the hollow fiber bioreactor simulated in this article.
4.2.4 Hemoglobin-Based Oxygen Carriers

The equilibrium relationship between dissolved O$_2$ and oxyHBOC can be described mathematically by the Hill and Adair equations (as described in Chapter 2 and 3). The Adair equation was used in the O$_2$ transport model, since it provides a more accurate representation of the OHEC at all pO$_2$ values. For the HBOCs studied, the Hill ($P_{50}$ and $n$) and Adair ($a_{1-4}$) equilibrium parameters were obtained from the literature, and the remaining equilibrium parameters were measured and regressed using Scientist (MicroMath, Saint Louis, MO). The OHEC equilibrium parameters regressed from the Hill and Adair equations are presented in Table 4.2, and the resulting OHECs are plotted in Figure 4.5 indicating the variable O$_2$ affinities of the HBOCs examined. All HBOCs were synthesized from either bovine red blood cells (bRBCs) or outdated human red blood cells (hRBCs). In a recent article, we examined the performance of native intact bRBCs, and determined that these were not the most ideal HBOCs for our hepatic hollow fiber bioreactor, since they could not provide the correct amount or spectrum of O$_2$ required by the hepatocytes for proper zonation [137]. Therefore, in this current study we explore 7 HBOCs, including: 3 low $P_{50}$ and 3 high $P_{50}$ HBOCs, compared to the $P_{50}$ of intact bRBCs (Table 4.2), to determine the ideal HBOC for our hepatic hollow fiber bioreactor system.
<table>
<thead>
<tr>
<th>HBOC</th>
<th>Hill Parameters</th>
<th>Radius</th>
<th>Adair Parameters</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P_{50}$</td>
<td>$n$</td>
<td>$a$</td>
<td>$a_1$</td>
</tr>
<tr>
<td></td>
<td>[mmHg]</td>
<td>[nm]</td>
<td>[mmHg$^{-1}$]</td>
<td>[mmHg$^{-2}$]</td>
</tr>
<tr>
<td>MP4</td>
<td>5.35</td>
<td>1.25</td>
<td>9.3</td>
<td>0.579</td>
</tr>
<tr>
<td>XLbRBC1</td>
<td>9.98</td>
<td>1.92</td>
<td>3390</td>
<td>0.00113</td>
</tr>
<tr>
<td>XLbRBC2</td>
<td>18.62</td>
<td>2.07</td>
<td>3390</td>
<td>0.0877</td>
</tr>
<tr>
<td>bRBC</td>
<td>26.29</td>
<td>2.72</td>
<td>3390</td>
<td>0.0446</td>
</tr>
<tr>
<td>LEHb1</td>
<td>31.03</td>
<td>2.35</td>
<td>119</td>
<td>0.00200</td>
</tr>
<tr>
<td>LEHb2</td>
<td>38.25</td>
<td>2.06</td>
<td>125</td>
<td>0.00983</td>
</tr>
<tr>
<td>PolyBvHb</td>
<td>54.20</td>
<td>1.17</td>
<td>58.4</td>
<td>0.0437</td>
</tr>
</tbody>
</table>
The first HBOC examined was MP4 (Hemospan®, Sangart), a PEG conjugated human hemoglobin. MP4 has the lowest $P_{50}$ of the HBOCs we simulated [188], and has been shown to be an effective HBOC in recent clinical studies [193]. To engineer low $P_{50}$ (high $O_2$ affinity) HBOCs, bRBCs can be cross-linked/polymerized with glutaraldehyde (a difunctional aldehyde), which stabilizes the hemoglobin protein and alters its oxygen binding properties [170]. Reacting bRBCs with glutaraldehyde in an oxygenated environment cross-links hemoglobin in the relaxed (R) state resulting in a left-shifted OHEC. The extent of left-shift in the OHEC is determined by the extent of glutaraldehyde cross-linking. Two of these cross-linked bRBC solutions were chosen for our simulations (XLbRBC1 & 2) [191].

HBOCs with high $P_{50}$ values (low $O_2$ affinity) can be produced by several methods. The first two high $P_{50}$ HBOCs examined were comprised of liposome-encapsulated tetrameric hemoglobin (LEHb) dispersions. In this system, hemoglobin was encapsulated in the aqueous core of a lipid shell composed of dimyristoyl-phosphatidylcholine (DMPC), cholesterol, and dimyristoyl-phosphatidylglycerol (DMPG) to form LEHb1, which encapsulated ~146 mg/ml of hemoglobin [176]. In the second LEHb dispersion, hemoglobin was encapsulated in the aqueous core of a lipid shell composed of DMPC, cholesterol, dimyristoyl-phosphoethanolamine-polyethylene glycol, DMPG, and $\alpha$-tocopherol to form LEHb2, which encapsulated ~92.7 mg/ml of hemoglobin [178]. Both LEHbs (LEHb1 & 2) were extruded in PBS buffer, and exhibited significantly increased $P_{50}$ values (low oxygen affinity). Finally, the highest $P_{50}$ HBOC simulated was PolyBvHb, which is produced by polymerizing bovine hemoglobin.
PolyBvHb was most likely synthesized in an anoxic environment, which stabilizes the hemoglobin tetramer in the tense (T) state resulting in a right-shifted OHEC [171].

There are various other methods for engineering the O₂ affinity of HBOCs, and the previously mentioned examples indicate only a range of the possibilities. In our lab, we are developing new cellular [177, 179, 180, 194, 195] and acellular [181-184] based HBOCs. The shape of the HBOC’s OHEC may indicate a range of maximal O₂ delivery efficacy, usually within the most highly sloped region of the OHEC. Therefore, we can deduce from Figure 4.5 that low P₅₀ HBOCs (MP4, XLbRBC1 & 2) should yield optimal O₂ delivery at pO₂ values below 25 mmHg and high P₅₀ HBOCs (LEHb1 & 2, PolyBvHb) should yield optimal O₂ delivery at high pO₂ values.
Figure 4.5 Oxygen-HBOC equilibrium curves of HBOCs simulated in this study, listed in order of increasing $P_{50}$. 
4.2.5 Oxygenation of the Extracapillary Space

Oxygen transport simulations in the presence of HBOCs were conducted under a wide range of bioreactor operating conditions. For each simulation, the O$_2$ consumption rate (OCR) of the hepatocyte culture was directly calculated from the steady state simulation according to equation 4.1 [196]. Equation 4.1 represents a simple mass balance on the total amount of O$_2$ transported to the hepatocyte culture in the bioreactor, where the concentration of O$_2$ entering and leaving (C$_{O_2,\text{in}}$ and C$_{O_2,\text{out}}$) the bioreactor consists of both dissolved O$_2$ and oxyHBOC. The flow rate (Q) was determined experimentally, and the volume of the ECS (Vol$_{ECS}$) was provided by the bioreactor manufacturer (Spectrum Labs). The volume of the ECS is the basis for the calculated OCR, since an experimentally accurate cell count could not be attained. Furthermore, estimating the cell number from metabolic consumption data only gives a rough estimate of cell density. Normalizing the OCR to a control case of no HBOCs supplementation of the circulating media is more representative of the enhancement in O$_2$ delivered to the culture by the addition of a HBOC. In equation 4.2, each set of bioreactor operating conditions is compared to a corresponding simulation under the exact same conditions except with no HBOC supplementation of the circulating media stream to determine the normalized OCR.

\[
\text{OCR} = \frac{Q(C_{O_2,\text{in}} - C_{O_2,\text{out}})}{Vol_{ECS}} \tag{4.1}
\]

\[
\text{OCR}_{\text{Normalized}} = \frac{\text{OCR}_{\text{HBOC}}}{\text{OCR}_{\text{No HBOC}}} \tag{4.2}
\]

The supplementation of HBOCs to the circulating media stream of the hollow fiber bioreactor can improve O$_2$ delivery to cultured hepatocytes. In addition to
increasing the amount of O₂ made available to the hepatocyte culture, hepatocytes require exposure to a specific range of O₂ tensions (25 – 70 mmHg) within their environment to exhibit proper differentiated function. Culturing hepatocytes with proper differentiated function is one of the main goals of current BLAD research [197, 198]. Therefore, we must evaluate the oxygenation spectrum within the hollow fiber bioreactor ECS to select the optimal HBOC and operating conditions for proper hepatocyte culture. The representative ECS of a hollow fiber bioreactor can be separated into six pO₂ zones: hyperoxic (> 70 mmHg), periportal (60 – 70 mmHg), pericentral (35 – 60 mmHg), perivenous (25 – 35 mmHg), hypoxic (< 25 mmHg), and anoxic (<0.5 mmHg). In assessing which set of bioreactor operating conditions and type of HBOC replicated the in vivo-like oxygenation spectrum (25 – 70 mmHg) witnessed within the liver sinusoid, we calculated the percent of the ECS total volume that contained the three oxygenation zones essential for proper hepatic zonation, namely the periportal, pericentral, and perivenous zones. To calculate the volume of the ECS within any of the designated pO₂ zones we calculated volume integrals from each of the simulation results. Also, we integrated the entire ECS domain to determine the average pO₂ value. Calculating the ECS volume within the three desired pO₂ zones (25 – 70 mmHg) and the average ECS pO₂ allows us to assess how close we are to mimicking the in vivo oxygenation spectrum of the liver sinusoid.

4.3 Results

Numerical simulations of O₂ transport examined the effects of varying several operating parameters of the hollow fiber bioreactor. The flow rate (Q), concentration of
HBOC present in the circulating media (HbT), and inlet O₂ tension of the circulating media stream entering the hollow fiber (pO₂,in) were varied for each type of HBOC. The manipulation of each of these parameters produced trends that indicate strategies to possibly enhance oxygenation and provide the correct O₂ spectrum to hepatocytes cultured within a hollow fiber bioreactor. We observed that increasing flow rate and HBOC concentration both lead to enhanced O₂ delivery to the cultured hepatocytes. Additionally, each HBOC was simulated over a range of bioreactor inlet pO₂ values to determine the inlet pO₂ values that with maximize O₂ delivery. With the simulation results, we determined possible bioreactor operating conditions that increase the amount of O₂ delivered to the hepatocytes and exposed the hepatocytes to the in vivo-like O₂ tensions observed in the liver sinusoid (25 – 70 mmHg).

4.3.1 Varying Inlet Oxygen Tensions

The O₂ transport model was developed to allow input of any set of Adair (or Hill) parameters describing the OHEC of an HBOC. For the seven HBOCs selected, simulations were conducted over a range of inlet pO₂ values (0.25 – 155 mmHg) at an overall flow rate of 28.6 ml/min through the entire HF bioreactor (or ~33.6 μl/min through an individual hollow fiber) and HBOC concentrations of 0, 10, and 100% hHb (percent of the human in vivo hemoglobin concentration). Initially, simulations were conducted to determine O₂ delivery with no HBOC supplemented to the circulating media (0% hHb); these resulted were utilized as a basis to determine the amount of O₂ delivery enhancement (normalized OCR). The results of the simulations were separated into four normalized OCR plots to display the region of maximal O₂ delivery efficacy for each HBOC.
Figure 4.6 displays the normalized OCR of the three low $P_{50}$ (compared to the $P_{50}$ of bRBCs) HBOCs along with bRBCs at inlet $pO_2$ values ranging from 0.25 to 30 mmHg simulated at 10% hHb. It is apparent that each HBOC possess an inlet $pO_2$ where $O_2$ delivery is maximally enhanced compared to the case of no HBOC supplementation. Also apparent is the fact that each HBOC has varying degrees of efficacy in increasing the amount of $O_2$ delivered to cultured hepatocytes. The normalized OCR plots show a maximum in the normalized OCR, which approximately corresponds to the point of maximum slope on the OHEC. Generally, it was observed that as the $P_{50}$ of the HBOC increased, so did the inlet $pO_2$ corresponding to the maximum in the normalized OCR. The height of the predicted normalized OCR curves does not appear to correspond to $P_{50}$, $n$, or OHEC slope. Enhancement of $O_2$ delivery by the addition of an HBOC can only be fully quantified by the implementation of the newly developed $O_2$ transport model.

In Figure 4.6, we observed that for MP4 the normalized OCR curve does not appear to decline as the inlet $pO_2$ approaches zero. However, we know that mathematically the curve must go through the origin, meaning that this result is a mathematical anomaly that is probably due to inaccurate Adair parameters resulting from the difficulty of measuring the HBOC saturation ($S_{eq}$) measurements at low $pO_2$ values. The normalized OCR curve for XLbRBC1 displays a prominent peak and has a definite region of maximal $O_2$ delivery. However, Figure 4.6 indicates that XLbRBC1 enhancement of $O_2$ delivery is only slightly better than MP4 (at inlet $pO_2$ values above 5 mmHg) and bRBC has a higher normalized OCR (above 7 mmHg). Therefore, XLbRBC1 would most likely not be utilized in future experimental studies. The normalized OCR curve for XLbRBC2 appears almost flat as displayed in Figure 4.6.
However, a peak is present and the normalized OCR continues to decrease at inlet pO$_2$ values above 30 mmHg. With a normalized OCR that does not exceed a seven-fold enhancement in O$_2$ delivery, XLbRBC2 would probably not be an ideal HBOC for many systems. The normalized OCR curve for bRBC supplementation displays a well-defined peak, and within Figure 4.6 it can be observed that bRBCs would be the best HBOC for most of the intermediate inlet pO$_2$ values (8-26 mmHg).
Figure 4.6 Normalized OCR (OCR_{HBOC}/OCR_{No HBOC}) of low $P_{50}$ HBOCs at 10% hHb supplementation.
The three high $P_{50}$ HBOCs were also simulated at a flow rate of 28.6 ml/min and 10% hHb with inlet pO$_2$ values ranging from 1 to 155 mmHg (Figure 4.7). An inlet pO$_2$ of 155 mmHg was set as the maximum inlet pO$_2$ simulated, since this pO$_2$ corresponds approximately to atmospheric concentrations of O$_2$. At hyperbaric conditions (pO$_2 > 160$ mmHg) reactive O$_2$ radicals begin to form [199, 200], and can be harmful to cell cultures via lipid peroxidation and cytokine expression mechanisms [201]. Similarly to low $P_{50}$ HBOCs, maxima in the normalized OCR curves were observed for all high $P_{50}$ HBOCs. The normalized OCR curve for LEHb1 (Figure 4.7) has a well defined peak and would be the most advantageous of the high $P_{50}$ HBOCs from 20 to 60 mmHg for the case of cell cultures supplemented with 10% hHb. Above 60 mmHg, it was observed that LEHb2 has a higher normalized OCR than LEHb1 and PolyBvHb for the rest of the range studied (up to 155 mmHg). The maximum of the normalized OCR curve for PolyBvHb (which has the highest $P_{50}$) is toward the lower spectrum of inlet pO$_2$ values examined (~8 mmHg), which is not close to the $P_{50}$. Upon further inspection of the OHEC of PolyBvHb, we determined that the maximum slope in the PolyBvHb OHEC occurs at a low pO$_2$, resulting in the low pO$_2$ maximum of the normalized OCR curve. This indicates that $P_{50}$ may predict the region of maximum normalized OCR, but a better indicator of maximum O$_2$ offloading may be the OHEC slope.
Figure 4.7 Normalized OCR (OCR_{HBOC}/OCR_{No HBOC}) of high P_{50} HBOCs at 10% hHb supplementation.
Simulations conducted at low concentrations of HBOC (10% hHb) indicate that the amount of O₂ delivered to hepatocyte cultures can be increased by supplementation of the circulating media stream with HBOCs. However, we wanted to further enhance the O₂ delivery to the hepatocyte culture, so we increased the theoretical concentration of HBOC in the circulating media. The next sets of simulations were conducted under the same operating conditions, flow rate of 28.6 ml/min (≈33.6 μl/min through an individual hollow fiber) over the same range of inlet pO₂ values (0.25 – 30 mmHg for low P₅₀ and 1 – 155 mmHg for high P₅₀ HBOCs), except with a HBOC concentration of 100% hHb. Figure 4.8 displays the normalized OCR of low P₅₀ HBOCs and again shows similar maxima for these HBOCs. However, we observe that the normalized OCR has increased, and the peaks have slightly left-shifted compared to the 10% hHb case. We believe that this left-shift is due to an increased deviation from oxyHBOC equilibrium, with increased O₂ delivery the kinetics of O₂ offloading from HBOCs becomes increasingly important [202]. Additionally, the introduction of altered velocity and HBOC concentration profiles in the lumen decreases the concentration of HBOC near the membrane, and therefore will decrease the normalized OCR and affect the entire profile. For bRBCs, the maximum OCR has increased from ~18- to over 50-fold. This significant increase in O₂ delivery is promising in terms of utilizing HBOCs to improve the oxygenation of hepatic hollow fiber bioreactors.
Figure 4.8 Normalized OCR (OCR\textsubscript{HBOC}/OCR\textsubscript{No HBOC}) of low P\textsubscript{50} HBOCs at 100% hHb supplementation.
The high $P_{50}$ HBOCs simulated at 100% hHb (Figure 4.9) also exhibit similar maxima as in the 10% hHb case, with an increase in the normalized OCRs predicted. There was a significant increase in the amount of $O_2$ delivered with an increase in HBOC concentration; LEHb1’s maximum normalized OCR of ~6.1 (at 10% hHb) tripled to ~16.8 normalized OCR (at 100% hHb), and the other HBOCs increased by similar amounts. Again, like the low $P_{50}$ HBOCs, we observe a slight left-shift of the maxima in the normalized OCR curves displayed in Figure 4.9. The left-shift of the normalized OCR curves also changes the inlet $pO_2$ region where the HBOCs are most efficient at delivering $O_2$. The normalized OCR curve for LEHb1 indicates it would be most efficient from ~14 to 42 mmHg, than at intermediate inlet $pO_2$ values (~43 – 94 mmHg). LEHb2 becomes the best choice for a HBOC. At the highest inlet $pO_2$ values simulated (94 – 155 mmHg) PolyBvHb is the most efficient HBOC for the enhancement of delivery of $O_2$ to the cultured hepatocytes (highest normalized OCR). This elevated range of inlet $pO_2$ values (> 94 mmHg) is most likely to produce in vivo-like oxygenation (25 – 70 mmHg), and therefore we predict that PolyBvHb should be the most promising HBOC to create a fully functional BLAD.
Figure 4.9 Normalized OCR (OCR_{HBOC}/OCR_{No\ HBOC}) of high P_{50} HBOCs at 100% hHb supplementation.
4.3.2 Extracapillary Space Oxygen Profiles

In addition to the bioreactor inlet pO\textsubscript{2}, the overall bioreactor flow rate (Q) and total HBOC concentration (H\textsubscript{b7}) in the circulating media can be varied to determine the optimal bioreactor operating conditions. Several O\textsubscript{2} profiles (for each modified HBOC) were extracted from the O\textsubscript{2} transport model. The set of two-dimensional dissolved O\textsubscript{2} concentration profiles compare all of the simulated HBOCs at three different flow rates (Figure 4.10) and four different HBOC concentrations (Figure 4.11) to determine the effect of changing these parameters. The right hand column is the same within each of these figures and represents the best-case scenarios determined from our studies. First, we ran simulation at three different overall bioreactor flow rates of 1.77, 16.8, and 28.6 ml/min (2.08, 19.8, and 33.6 µl/min through an individual hollow fiber) at the higher inlet HBOC concentration of 100% hHb and an inlet pO\textsubscript{2} of 155 mmHg. The set of two-dimensional O\textsubscript{2} profiles in Figure 4.10 compare all of the simulated HBOCs in order of increasing P\textsubscript{50} (from top to bottom). From the colorimetric scale next to the O\textsubscript{2} profiles, we observe that with increasing flow rate (left to right) or increasing HBOC P\textsubscript{50}, the ECS is better oxygenated. At the lowest flow rate (1.77 ml/min) all of the HBOCs simulated contain an anoxic region in the ECS, which is defined as pO\textsubscript{2} values less than 0.5 mmHg as this is where the mitochondria of a cell will no longer function [203]. By increasing the overall bioreactor flow rate to 16.8 ml/min we observed that the anoxic region in the ECS for all of the HBOCs simulated is completely removed (Figure 4.10). Increasing the overall bioreactor flow rate from the minimum (1.77 ml/min) to the maximum (28.6 ml/min) increases the volume of the ECS that is maintained within the desired pO\textsubscript{2} range (25 – 70 mmHg) ~10- to 20-fold for the HBOCs examined. The O\textsubscript{2} profile for MP4
shows that ~45% of the ECS is anoxic and 1.1% of the ECS exhibits oxygenation within the desired pO$_2$ range (25 – 70 mmHg) at the lowest flow rate (1.77 ml/min). Due to the increase in flow rate (28.6 ml/min), the anoxic region is removed and the percent of the ECS volume in the desired oxygenation region is increased to 17%. For supplementation with bRBCs compared to the control, an anoxic region comprising 10% of the ECS volume was removed and 1.7% of the ECS volume exposed to \textit{in vivo}-like oxygenation (25 – 70 mmHg) increased to 30%. Finally, PolyBvHb shows the most improvement of the HBOCs examined in this study. Initially, the anoxic region comprised 13% of the ECS volume and 7.2% was within the desired pO$_2$ range. By increasing the overall bioreactor flow rate (from 1.77 to 28.6 ml/min), we removed the anoxic region and increased the ECS volume to 84% within the desired oxygenation spectrum (25 – 70 mmHg).
Figure 4.10 Oxygen concentration profiles at several flow rates for each of the HBOCs simulated at 100% hHb and $pO_{2,\text{in}} = 155$ mmHg (note: the fiber axes are not equal). The contour lines on each profile delineate hepatic oxygenation zones.
Next, the O\textsubscript{2} transport model was simulated at HBOC concentrations of 10, 25, 50, and 100\% hHb (percent of the human \textit{in vivo} hemoglobin concentration); all with an overall bioreactor flow rate of 28.6 ml/min and an inlet pO\textsubscript{2} of 155 mmHg. Figure 4.11 displays the predicted two-dimensional O\textsubscript{2} profiles for all HBOCs examined in order of increasing P\textsubscript{50} (from top to bottom). It was observed that as the concentration of HBOC increases (left to right) oxygenation of the hepatocytes within the hollow fiber bioreactor ECS space also increases. In the first column of Figure 4.11, it was observed that the anoxic region (pO\textsubscript{2} < 0.5 mmHg) present decreases in size with increasing P\textsubscript{50} for the low P\textsubscript{50} HBOCs (from MP4 to XLbRBC2). In fact, the anoxic region is completely removed in the bRBC supplemented case (and all of the high P\textsubscript{50} HBOCs). Additionally, the top row of Figure 4.11 shows that with an increase in HBOC concentration, the anoxic region decreases in size (from 10\% to 25\% hHb) and is completely removed at HBOC concentrations above 50\% hHb. These trends, which show a decrease in the size of the anoxic region of the ECS, are due to an increase in oxygenation of the hollow fiber, which also causes an increase in the volume of the ECS that exhibits \textit{in vivo}-like oxygenation (25 – 70 mmHg). For MP4, the percent of the representative ECS volume in the desired pO\textsubscript{2} spectrum increases from 8.7\% (at an HBOC concentration of 10\% hHb), to 10\% (at 25\% hHb), to 12\% (at 50\% hHb), and up to 17\% (at 100\% hHb). The increase in the percent ECS volume pO\textsubscript{2} within the desired oxygenation range is even more apparent for higher P\textsubscript{50} HBOCs. For bRBCs, it was observed increases in ECS pO\textsubscript{2} within the desired oxygenation range from 9.8\%, to 13\%, to 18\%, and up to 30\% (for the 10, 25, 50, and 100\% hHb cases, respectively). Finally, for PolyBvHb supplementation, it was observed increases in the percent of ECS volume within the desired oxygenation
range (25 – 70 mmHg) of 18%, to 35%, to 59%, and up to 84% (for HBOC concentrations of 10, 25, 50, and 100% hHb, respectively).
Figure 4.11 Oxygen concentration profiles at several HBOC concentrations for each of the HBOCs simulated at $Q = 28.6$ ml/min and $pO_2,\text{in} = 155$ mmHg (note: the fiber axes are not equal). The contour lines on each profile delineate defined hepatic oxygenation zones.
Overall, we observed that increasing the flow rate through the hollow fiber bioreactor (Figure 4.10) results in an increase in the amount of O₂ delivered to the hepatocytes cultured within the ECS and an improvement in the spectrum of O₂ tensions experienced by the hepatocytes. Also, we observed that as the P₅₀ of the HBOC increased (top to bottom) oxygenation continually improved throughout the ECS (Figure 4.10 & Figure 4.11). Figure 4.11 indicates that with increasing concentration of HBOC there is an increase in oxygenation of the hepatocyte culture in the ECS. Some of these oxygenation trends were previously observed in a similar hollow fiber bioreactor [137]. However, now the O₂ profiles can be interpreted to give a more accurate description of the oxygenation within the ECS environment. In addition to the colorimetric scale that describes the O₂ tensions throughout the bioreactor space, contour lines have been placed to separate the hepatic oxygenation zones described earlier and these can be utilized to calculate the volume of the ECS in each of the previously specified oxygenation zones.

4.3.3 Extracapillary Space Zonation

The volume of hepatocytes within the ECS that experience *in vivo*-like O₂ tensions can be calculated with volume integrals of the simulation results. The two-dimensional dissolved O₂ concentration profiles within the ECS (Figure 4.10 and Figure 4.11) were integrated to calculate the percent volume of the ECS space that would be within each hepatic oxygenation zone (defined pO₂ ranges). Figure 4.12 displays a breakdown of the ECS volume for all the HBOCs examined in this study at an inlet pO₂ of 155 mmHg, overall bioreactor flow rate of 26.8 ml/min, and HBOC concentration of 100% hHb. Generally, Figure 4.12 shows that with increasing P₅₀ of the HBOC (left to right), the hypoxic (pO₂ < 25 mmHg) volume of the ECS decreases and the percent of
ECS volume in the desired oxygenation range (25 – 70 mmHg) increases. We define tissue hypoxia as an inadequate supply of O₂ that results in compromising biological function [204]. Therefore, while hepatocytes may remain viable at pO₂ values below 25 mmHg, we consider this hypoxic because phenotypic hepatocyte functions are diminished [34].

For the control case of no HBOC supplementation of the circulating media stream (leftmost column) in Figure 4.12, the hypoxic region (which occupies ~91% of the ECS volume) is about half anoxic (pO₂ < 0.5 mmHg); with ~46% of the ECS volume being anoxic. Supplementation of the circulating media with any of the HBOCs studied completely removes the anoxic region within the ECS volume, thus the anoxic region is not included in Figure 4.12. We observed that for the cases examined in Figure 4.12, bRBCs did not maintain the trend of decreasing hypoxic ECS volume (and increasing volume within the desired pO₂ range) with increasing P₅₀. Nevertheless, this is an expected result because of the rapid decline in the normalized OCR curves for bRBC (Figure 4.6 and Figure 4.8). The normalized OCR curve for bRBC supplementation fell below the XLbRBC1 & 2 curves at high pO₂ values (~30 mmHg at 10% hHb and ~25 mmHg at 100% hHb). Again, this indicates that P₅₀ is not the best indicator of HBOC oxygenation efficacy. Finally, we observed that generally the percent volume of the ECS within the hyperoxic region (pO₂ > 70 mmHg) increases with increasing HBOC P₅₀. Hyperoxia can be as dangerous to cell viability and function as hypoxia [27], and we must try to minimize the ECS volume within both of these regions; i.e., maximize the ECS volume within the desired pO₂ spectrum (25 – 70 mmHg).
Figure 4.12 Zonation of the extra capillary space in each of the defined oxygenation zones for all HBOCs.
4.4 Discussion

The O₂ transport model developed in this article allows us to predict the O₂ concentration profile for a single hollow fiber within a hepatic hollow fiber bioreactor, and can be helpful in choosing an appropriately sized hollow fiber bioreactor and predicting optimal operating conditions in order to create a fully functional BLAD. Previously, we observed that supplementation of an HBOC (specifically bRBCs) increased the amount of O₂ delivered to hepatocytes cultured within a hollow fiber bioreactor [156]. However, we determined that bRBCs were not able to provide the desired hepatocyte oxygenation to cultured hepatocytes [137]. The O₂ binding/release kinetics of HBOCs influence the extent of O₂ delivery, as well as the O₂ concentration spectrum experienced by the hepatocytes in the ECS surrounding individual hollow fibers. Therefore, we investigated a wide range of HBOCs with P₅₀ values ranging from 5.35 to 54.2 mmHg (Table 4.1) to select the optimal HBOC and bioreactor operating conditions for our system. Additionally, we included a separately calculated velocity profile and HBOC concentration profile to the simulations to more accurately describe O₂ transport within our bioreactor. The results of our simulations validate our hypothesis that HBOC supplementation enhances O₂ delivery and that a high P₅₀ (decreased O₂ affinity) HBOC is most beneficial for the culture of hepatocytes in a hollow fiber bioreactor.

We investigated each of the HBOCs over a range of pO₂ conditions (0.25 – 155 mmHg) to determine a range of maximum normalized OCR. The normal mean arterial pO₂ in vivo ranges from 74 to 104 mmHg [27], and due to the additional diffusion barrier imposed by the hollow fiber membrane, we simulated inlet pO₂ values up to 155 mmHg
(pO₂ > 160 mmHg values can produce damaging reactive O₂ radicals [199, 200]). We observed that the HBOCs operate most effectively over a specific range of pO₂ values where they are most efficient in delivering O₂ (compared to the case of no HBOC supplementation). Low P₅₀ HBOCs (below the P₅₀ of normal bRBCs) were most efficient at bioreactor inlet pO₂ values < 30 mmHg (Figure 4.6 and Figure 4.8). For both the 10 and 100% hHb HBOC supplemented cases, we observed that as the P₅₀ of the HBOC increased so did the approximate location of the optimal inlet pO₂ where maximal relative O₂ offloading occurred (XLbRBC2 exhibited a very flat peak in the normalized OCR plots which may not be able to be accurately interpreted). Therefore, we infer that these low P₅₀ HBOCs may be specifically useful to culture cell types which require culturing in a low O₂ environment. Embryonic stem cells from both the peripheral and central nervous systems show enhanced neuronal proliferation and differentiation in a low oxygen environment [174, 175]. The use of low P₅₀ HBOCs for cell culture is beneficial compared to no HBOC supplementation (more than 10-fold for most cases); however, the low P₅₀ HBOCs studied are not optimal for the hepatic hollow fiber bioreactor application examined.

High P₅₀ HBOCs (above the P₅₀ of normal bRBCs) were simulated over the full span of inlet pO₂ values (1 – 155 mmHg) to determine their inlet pO₂ range of maximum efficacy (Figure 4.7 and Figure 4.9). Each of the high P₅₀ HBOCs show marked improvement in O₂ delivery to the ECS for the high inlet pO₂ values simulated. We observed that the normalized OCR peak for PolyBvHb is not at the highest inlet pO₂ value despite PolyBvHb having the highest P₅₀ of the HBOCs studied. This is due to PolyBvHb’s OHEC shape whose region of maximal slope is at a low pO₂, as explained
earlier. However, the right-shifted OHEC corresponding to PolyBvHb becomes the best HBOC choice at 100% hHb with inlet pO₂s > 94 mmHg. This elevated range of inlet pO₂ values (95-155 mmHg) has been determined to be very important in devoting in vivo-like oxygenation in hepatocyte cultures. Therefore, we infer that PolyBvHb should be the most effective HBOC in our hepatic hollow fiber bioreactor. We hypothesize that further increases in the P₅₀ (further right shifting of the OHEC) of an HBOC could continue to be beneficial for hepatocyte culture within a hollow fiber bioreactor. Ideally, the most optimal HBOC for our hepatic hollow fiber bioreactor system would have a further right-shifted region of maximum slope within the OHEC. However, all current methods for chemically right-shifting the OHEC of a HBOC result in a flattening of the OHEC (observable in Figure 4.5). Utilizing high P₅₀ HBOCs, we have found bioreactor operating conditions that meet our initial design criteria (significant enhancement in O₂ delivery to the hepatocytes within the hollow fiber bioreactor ECS and attainment of in vivo-like O₂ tensions in the ECS).

Initially, the P₅₀ of a HBOC was utilized to indicate the inlet pO₂ range where the HBOC should be most effective (in terms of enhancing O₂ delivery compared to a control of no HBOC supplementation), and this preliminary indicator appeared to be an accurate measure of O₂ transport efficiency for the normal bRBCs studied [137]. However, with a broader range of HBOCs, the assumption that P₅₀ indicates the inlet pO₂ of a HBOC’s maximum efficacy breaks down, and we have observed that the maximum slope of the OHEC is a more accurate predictor. By differentiating the OHEC for each HBOC with respect to pO₂, we are able to calculate the maximum slope of the OHEC (Matlab code provided in the Appendix). Next, from O₂ transport simulations we were able to
determine the approximate placement and height of the maximum normalized OCR (which determines the range of inlet pO2 values where there is significant enhancement in O2 delivery compared to a control case of no HBOC supplementation) at 10 and 100% hHb supplementation for the HBOCs studied (Figure 4.6 – Figure 4.9). Table 4.3 indicates that the maximum value observed for the derivative of the OHEC corresponds to the peak of the normalized OCR (nOCR) plots better than the P50 of the HBOC for almost all cases. We believe that HBOCs with a high Hill coefficient (i.e. bRBCs), show deviation from the predicted inlet pO2 values of maximum O2 transport efficacy due to a more sigmoidal OHEC shape.

<table>
<thead>
<tr>
<th>HBOC</th>
<th>HBOC P50</th>
<th>(d^2S_{eq}/dpO2^2 = 0)</th>
<th>Max nOCR at 10% hHb</th>
<th>Max nOCR at 100% hHb</th>
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</tr>
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</tr>
<tr>
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<td>8</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

Overall, we observed that the shape of the OHEC for the HBOC generally determines the inlet pO2 that corresponds to maximal O2 delivery (compared to the case of no HBOC supplementation) to the hepatocytes cultured in the ECS. HBOCs exhibiting left-shifted OHECs (compared to normal bRBCs) resulted in better
oxygenation of the ECS at low pO₂ values and visa versa. This result may be applicable when initially choosing an HBOC for a cell culture application with a known pO₂ environment. Also, increasing either or both the flow rate of media through the bioreactor or the total HBOC concentration yields an increase in the oxygenation of the ECS (Figure 4.10 and Figure 4.11). We previously observed similar results for bRBC supplementation of a similar hepatic hollow fiber bioreactor system [137].

In addition to quantifying the enhancement in O₂ delivered to hepatocytes cultures with HBOC supplementation compared to the case of no HBOC supplementation, we investigated the range of O₂ tensions experienced by hepatocytes within the ECS surrounding individual hollow fibers. As noted above, the range of pO₂ that hepatocytes experience influences their phenotype and viability, and must be included in the hepatic hollow fiber bioreactor design criteria. The integration of the two-dimensional O₂ concentration profiles for each of the HBOCs demonstrates that hepatocytes exposed to high P₅₀ HBOCs experience a more in vivo-like oxygen spectrum (Figure 4.12). Several promising HBOCs and bioreactor operating conditions (concentration of HBOC, inlet pO₂, and flow rate) were chosen, and the percent volume of ECS found in each of the oxygenation zones is presented along with the HBOC and bioreactor operating conditions (Table 4.4). Table 4.4 indicates that there are several acceptable sets of operating conditions for operating the bioreactor to obtain the desired criteria pO₂ spectrum experienced in the liver in vivo. Table 4.4 is arranged in order of increasing (top to bottom) volume of the ECS within the defined in vivo pO₂ spectrum (25-70 mmHg), ranging from ~54-84% of the total ECS volume. Additionally, the average pO₂ within the ECS (pO₂,Avg) can be calculated from the simulations by integrating the ECS domain.
The observed *in vivo* pO\textsubscript{2} for a healthy liver was then utilized as a design criteria, ~41 – 47 mmHg [119-121]. Hence, we conclude that at a HBOC concentration of 100% hHb, an inlet pO\textsubscript{2} of 155 mmHg and an overall bioreactor flow rate of 28.6 ml/min we can supplement the circulating media feed stream with either LEHb2 or PolyBvHb to create what should be a fully functional BLAD.

**TABLE 4.4**

PREDICTED VOLUME OF ECS IN EACH HEPATIC OXYGENATION ZONE, AND AVERAGE ECS PO\textsubscript{2} FOR SEVERAL PROMISING BIOREACTOR OPERATING CONDITIONS

<table>
<thead>
<tr>
<th>HBOC</th>
<th>H\textsubscript{b}T</th>
<th>pO\textsubscript{2,in}</th>
<th>Q</th>
<th>Periportal</th>
<th>Pericentral</th>
<th>Perivenous</th>
<th>pO\textsubscript{2,Avg}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEHb1</td>
<td>100</td>
<td>155</td>
<td>28.6</td>
<td>4.9%</td>
<td>27.1%</td>
<td>22.1%</td>
<td>33.1</td>
</tr>
<tr>
<td>PolyBvHb</td>
<td>50</td>
<td>155</td>
<td>28.6</td>
<td>6.5%</td>
<td>31.4%</td>
<td>21.4%</td>
<td>36.8</td>
</tr>
<tr>
<td>PolyBvHb</td>
<td>100</td>
<td>155</td>
<td>16.8</td>
<td>7.7%</td>
<td>38.9%</td>
<td>21.9%</td>
<td>38.9</td>
</tr>
<tr>
<td>LEHb2</td>
<td>100</td>
<td>155</td>
<td>28.6</td>
<td>7.6%</td>
<td>40.4%</td>
<td>23.9%</td>
<td>41.1</td>
</tr>
<tr>
<td>PolyBvHb</td>
<td>100</td>
<td>135</td>
<td>28.6</td>
<td>5.5%</td>
<td>41.1%</td>
<td>26.7%</td>
<td>37.1</td>
</tr>
<tr>
<td>PolyBvHb</td>
<td>100</td>
<td>155</td>
<td>28.6</td>
<td>13.8%</td>
<td>55.5%</td>
<td>16.3%</td>
<td>49.8</td>
</tr>
</tbody>
</table>

4.5 Conclusions

The need for a fully functional BLAD to assist in the recovery of ALF patients is self-evident. Hollow fiber bioreactors are currently the most promising bioreactor candidate to create a fully functional BLAD, and we believe that by supplementing the circulating media stream with a HBOC we can enhance O\textsubscript{2} delivery, while providing the correct pO\textsubscript{2} spectrum (25 – 70 mmHg) to cultured hepatocytes. Hepatocytes experiencing *in vivo*-like O\textsubscript{2} tensions should elicit zonal phenotypic differentiation, and
should perform the full range of liver functions required to sustain an ALF patient. In our oxygen transport simulations, we found that the shape of the OHEC of each of the HBOCs studied greatly affects their range of potential applications (high $P_{50}$ HBOCs are more efficient at delivering $O_2$ at high bioreactor inlet $pO_2$ values and visa versa). Simulations similar to what was performed in this study could be useful in evaluating low $P_{50}$ HBOCs for culture of various cell types requiring a low $pO_2$ environment. Finally, the work in this chapter demonstrates that by carefully choosing an appropriate (high $P_{50}$) HBOC and bioreactor operating conditions, we can enhance oxygen delivery to cultured hepatocytes, recreate the three main hepatic oxygenation zones, and replicate the average $pO_2$ present in vivo in the liver sinusoid.
This chapter, in part or in full, is a reprint of the material as it appears in:


The dissertation author was the first author on this paper with the dissertation committee chair as the primary investigator.
CHAPTER 5:

ENHANCED OXYGEN DELIVERY TO PRIMARY HEPATOCYTES WITHIN A HOLLOW FIBER BIOREACTOR FACILITATED VIA HEMOGLOBIN-BASED OXYGEN CARRIERS

5.1 Introduction

Primary rat hepatocytes have been shown to maintain differentiated function, over short periods of time, better than hepatoma cell lines in BLAD experiments [111]. Therefore, the C3A cells cultured in Chapters 3 were replaced with primary rat hepatocytes for the hollow fiber bioreactor studies presented in this chapter. Experimentally, oxygen provision to primary rat hepatocytes, cultured in the same manner as the Chapter 3 experimental studies, was measured. It was observed that supplementation with an oxygen carrier (bovine red blood cells at ~2% human hematocrit) did not significantly improve oxygenation compared to the absence of an oxygen carrier for all experimental bioreactor operating conditions. Therefore, an oxygen transport model of an individual hollow fiber within the bioreactor was developed and simulated (up to ~20% human hematocrit) to more fully examine the effect of oxygen carrier supplementation on oxygenation within the bioreactor. The modeling analysis, supported via the experimental results, was utilized to predict optimal bioreactor operating conditions for the delivery of in vivo-like oxygen gradients to cultured hepatocytes in clinically-relevant settings.
In this study, bovine red blood cells (bRBCs) were employed as an oxygen carrier within the experimental portion of this work, due to similar physical properties they share with human RBCs. Primary hepatocytes were cultured since they retain differentiated function. However, primary hepatocytes lack the proliferation potential of a hepatocyte cell line, which may affect cell density and distribution within the bioreactor. Additionally, primary hepatocytes have different oxygenation consumption demands compared to the cell line. Due to these changes in the experimental procedure, a new set of modeling parameters was derived from the experimental systems. Simulations were conducted with the updated oxygen transport model of an individual hollow fiber to facilitate the development of future hollow fiber bioreactors. Oxygen transport simulations, supported via data generated from the experimental system, were able to predict optimized bioreactor operating parameters for the given BLAD that provides the correct spectrum of oxygenation to cultured hepatocytes.

5.2 Materials and Methods

5.2.1 Hollow Fiber Bioreactor System

The CellMax® Quad™ (Spectrum Laboratories, Rancho Dominguez, CA) hollow fiber bioreactor system was utilized in this study, and consisted of a 250 ml media reservoir bottle (containing between 125 to 250 ml of culture media), ~2 meters of silicone tubing, a peristaltic pump, and inline dissolved oxygen probes (DO-166 Lazar Research Laboratories, Los Angeles, CA) attached to the bioreactor entrance and exit (see Figure 5.1). Two separate hollow fiber bioreactor systems were run during the
study. The experimental system was supplemented with bRBCs in the circulating media stream, while the control system lacked bRBCs in the circulating media stream. A small vortexer (12-812, Fisher Scientific, Hampton, NH) was implemented as a means of periodically, gently agitating the reservoir bottles to prevent sedimentation of bRBCs within the media bottle. A peristaltic pump circulated the media from the media bottle through the bioreactor at three different flow rates, in order to obtain hepatocyte oxygen consumption data at differing media flow rates. Finally, the entire bioreactor was maintained within a Heraeus incubator (Kendro Laboratory Products, Hanau, Germany) at 37°C and 5% CO₂.
Figure 5.1 Schematic of the experimental hepatic hollow fiber bioreactor, along with a cross-sectional view of a single representative hollow fiber. Solid rectangles within the enlarged fiber represent the modeled space.
The hollow fiber bioreactors (400-012, Spectrum Labs.) used in this study consisted of a 12 ml extra capillary space (ECS, the volume outside the hollow fibers, where the hepatocytes were cultured) volume, and hollow fibers with an outer surface area of 1500 cm$^2$ and a 95% particle size cut off of 0.3 μm. The small particle size cut off of the hollow fiber membrane within the bioreactor prevents bRBCs from entering the ECS.

The oxygen probes attached to the inlet and exit ports of the bioreactor provided a simple method of measuring the oxygen lost from the circulating media stream and consequently transported to the hepatocytes within the ECS of the bioreactor (hereafter referred to as the global bioreactor oxygen consumption rate, OCR). Prior to use within the system, the oxygen probes were calibrated with a two point calibration protocol consisting of first exposing the probes to a saturated sodium sulfite solution (zero initialization point), and subsequently to an oxygen saturated distilled water solution (high initialization point). After calibration, the probes were sterilized in 70% ethanol. The ethanol was then washed from the probes using sterile culture media, and the probes were finally inserted into the bioreactor system. After the achievement of steady state (~1 hour), the flow rate was varied and the system allowed to re-establish equilibrium. The dissolved oxygen concentration values were recorded, and this process was repeated for the other experimental system.

5.2.2 Hepatocyte Isolation and Culture Media

Primary rat hepatocytes were utilized within this study. Hepatocytes were isolated at Wayne State University using the method of Seglen as modified by Dunn [205, 206]. Rats were anesthetized, and a two stage collagenase perfusion of the liver
was performed via the portal vein. Dissociated hepatocytes were collected in 50 ml centrifuge tubes, and washed 3 times in Hanks balanced buffer solution. Cell counts were performed with a hemacytometer on each of the centrifuge tubes and viability was assessed via the exclusion of the Trypan blue stain. Finally, approximately 150 million viable cells were inoculated into the ECS of each of the hollow fiber bioreactors. The cells consequently occupied about an eighth of the ECS volume.

The complete cell culture medium utilized in the experimental study was composed of 90% Dulbecco’s modified Eagle’s medium (D6429, Sigma, St. Louis, MO), 10% fetal bovine serum (F6178, Sigma), 1000 Units/ml of penicillin and streptomycin, 0.5 Units/ml of insulin, 100 μg/ml of glucagon, 100 μg/ml of epidermal growth factor, and 750 μg/ml of hydrocortisone (Sigma). The medium supplying the experimental system was additionally supplemented with sterile washed bRBCs (943, Quad Five, Ryegate, MT) at about 100 million cells/ml, while the control system was not supplemented with an oxygen carrier. Finally, the medium inoculated into the ECS along with the hepatocytes did not contain an oxygen carrier, but was additionally supplemented with collagen extracted from rat tails at a concentration of 1 mg/ml.

Throughout the 10 day study, the media reservoir bottle was replaced daily with fresh complete medium. After removing the media from the system, aliquots were taken for analysis of various biomarkers which were intended to be indicative of the overall health of the cell culture. Briefly, glucose consumption was measured via a simple glucose strip test (Home diagnostics Inc., Fort Lauderdale, FL) and urea production was quantified using a kit based on the diacetylmonoxime method (0580, Stanbio Laboratory,
Boerne, TX). The results of these assays are not presented within this chapter, but were used to indicate that the cultures were behaving as expected.

5.3 Oxygen Transport Model

A single axisymmetric hollow fiber with a representative extra capillary space (ECS) was simulated in the oxygen transport model, which was developed in Chapter 2 and elsewhere [137, 206]. Briefly, the two-dimensional modeled space is presented at the bottom of Figure 5.1; where the upper rectangle represents the lumen containing flowing media, the middle rectangle represents the hollow fiber membrane, and the lower rectangle represents the ECS containing cultured hepatocytes. The hollow fiber bioreactor system in the experimental study was the basis for the dimensions used within the oxygen transport simulations. The geometry of an individual hollow fiber bioreactor contained within the bioreactor, along with measured flow rates, dissolved oxygen concentrations, and bRBC concentrations were used as input parameters in each of the oxygen transport simulations.

5.3.1 Model Components

The oxygen transport model described in Chapter 2, with a few modifications, was used for the oxygen transport simulations in this chapter. The first alteration to the oxygen transport model deals with the diffusivity of oxygen in the ECS. The diffusivity of oxygen in the ECS, equation 5.1, will be affected by the amount of cells present, since the diffusivity of oxygen though a hepatocyte is different than that of water. Therefore, to determine the effective diffusivity of oxygen through the ECS, we introduced a new
The parameter, the fractional volume of the ECS space that is filled with hepatocytes ($\varepsilon$), which was implemented in conjunction with both the diffusivity of oxygen in water ($D_{H_2O}$) and through hepatocytes ($D_{Hep}$) as shown in Equation 5.1.

$$D_{ECS} = D_{H_2O}(1 - \varepsilon) + D_{Hep} \cdot \varepsilon \quad \text{..........................................................(5.1)}$$

Within the lumen, for the control case of no media stream bRBC supplementation, the reaction term ($R$) in equation 2.3 is not present. However, for the experimental case (with media stream bRBC supplementation), the reaction term described oxygen binding/released to/from the bRBCs (discussed later). As the membrane within the hollow fiber device did not consume or produce oxygen within the control or experimental systems, the reaction term ($R$) was neglected for oxygen transport through the membrane. Within the ECS, the consumption of oxygen by hepatocytes has been shown to be most accurately represented by Michaelis-Menten kinetics [38, 140], equation 5.2. In addition to the oxygen diffusivity, equation 5.2 indicates that the consumption of oxygen is also influenced by the volume fraction of hepatocytes within the ECS ($\varepsilon$).

$$R = \frac{V_{Max} \cdot pO_2}{K_M + pO_2} \cdot \varepsilon \quad \text{..........................................................(5.2)}$$

where $V_{Max}$ and $K_M$ are the Michaelis-Menten parameters. With these equations, the control case of no bRBCs present in the system can now be solved numerically.

5.3.2 Model Parameters

The set of parameters, their values, and literature sources is presented in Table 5.1. In addition to these parameters, simulations were conducted over a range of flow
rates ($V_{max}$ values) and pO$_2$ values to match the experimentally measured data. The diffusivity of oxygen within water and through hepatocytes was reported in the literature [127, 140]. The diffusivity of oxygen within the ECS was found by utilizing literature values of the oxygen diffusivity through hepatocytes [148], as well as the oxygen diffusivity through water (determined via equation 5.1). The $V_{Max}$ value of 5.87 μM/s is within the range of values reported from several literature sources: 10 μM/s [127], 5 μM/s [11], and 4.45, 7.4 and 4.8 μM/s [38], utilizing a per unit volume oxygen consumption rate. This value was derived from a previously determined zero-order kinetics oxygen consumption rate of 48 amol/(cell-s) for rat hepatocytes in a three-dimensional gel [111]. The conversion assumes that ~150 million cells were inoculated in each of the ECS of the two hollow fiber bioreactors. This cell number yields about 12.5 million hepatocytes per cm$^3$ which is close to the 10 million per cm$^3$ cell density reported by Nyberg [111]. The $K_M$ of 3 mmHg is the median value that has been reported for hepatocytes, which ranges between 0.5 and 5.6 mmHg [127, 140]. Additionally, this $K_m$ value was previously used within modeling studies in Chapters 3 and 4, and was reported by the authors to agree with experimentally collected data [137, 138]. The void volume within the ECS was calculated by estimating two parameters, namely: the total volume of hepatocytes that were inoculated into the hollow fiber bioreactor, and the volume of the ECS. Given an average cell diameter of ~25 μm [207], it was determined that the inoculated hepatocytes filled between an eighth to a tenth of the total ECS volume. This consequently necessitated the inclusion of the fractional hepatocyte volume factor ($\varepsilon$) in the oxygen transport model, in order to account for the voids in the ECS. The concentration of circulating bRBCs was kept constant throughout the experiments at approximately 2% of
the normal human in vivo hematocrit, measured via a hemacytometer. The OHEC of bRBCs was measured with a Hemox Analyzer (TCS Scientific Corp., New Hope, PA), and the Adair parameters were regressed via a nonlinear six parameter model as described elsewhere [191].

TABLE 5.1
PARAMETERS OF THE HOLLOW FIBER BIOREACTOR UTILIZED IN OXYGEN TRANSPORT SIMULATIONS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusivity of oxygen in water, $D_{H_{2}O}$</td>
<td>$3.0 \times 10^{-5}$</td>
<td>cm$^2$/s</td>
<td>[11, 140, 148]</td>
</tr>
<tr>
<td>Diffusivity of oxygen in liver tissue, $D_{Hep}$</td>
<td>$2.0 \times 10^{-5}$</td>
<td>cm$^2$/s</td>
<td>[148]</td>
</tr>
<tr>
<td>Diffusivity of RBCs in water, $D_{RBC}$</td>
<td>$6.8 \times 10^{-10}$</td>
<td>cm$^2$/s</td>
<td>[160]</td>
</tr>
<tr>
<td>Maximum rate of oxygen consumption, $V_{Max}$</td>
<td>5.87</td>
<td>μM/s</td>
<td>[111]</td>
</tr>
<tr>
<td>Michaelis constant, $K_{M}$</td>
<td>3</td>
<td>mmHg</td>
<td>[127, 140]</td>
</tr>
<tr>
<td>Fractional hepatocyte volume in ECS, $\varepsilon$</td>
<td>0.1025</td>
<td></td>
<td>Measured</td>
</tr>
<tr>
<td>Total concentration of hemoglobin, $Hb_T$</td>
<td>0.003</td>
<td>g/cm$^3$</td>
<td>Measured</td>
</tr>
<tr>
<td>Reverse reaction rate constant, $k^-$</td>
<td>44</td>
<td>1/s</td>
<td>[161, 162]</td>
</tr>
<tr>
<td>Adair constants for bovine RBCs, $a_1$</td>
<td>$4.458 \times 10^{-2}$</td>
<td>mmHg$^{-1}$</td>
<td>Measured</td>
</tr>
<tr>
<td>$a_2$</td>
<td>$1.027 \times 10^{-4}$</td>
<td>mmHg$^{-2}$</td>
<td>Measured</td>
</tr>
<tr>
<td>$a_3$</td>
<td>$1.089 \times 10^{-5}$</td>
<td>mmHg$^{-3}$</td>
<td>Measured</td>
</tr>
<tr>
<td>$a_4$</td>
<td>$3.079 \times 10^{-6}$</td>
<td>mmHg$^{-4}$</td>
<td>Measured</td>
</tr>
</tbody>
</table>

5.4 Results and Discussion

As previously mentioned, hepatocytes were inoculated into the ECS of the two hollow fiber bioreactor systems and cultured for three weeks. For these experiments, the hepatocyte oxygen consumption parameters ($V_{Max}$ and $K_M$) were estimated. These parameters were subsequently used in oxygen transport simulations. Using the oxygen transport model, the most ideal bioreactor operating conditions for maintaining tissue-like
hepatocyte densities were determined. The predicted operating conditions are expected to be useful for the development of future BLADs as they are based on a more practical set of clinical requirements, such as providing an in vivo-like oxygen spectrum to cultured hepatocytes.

5.4.1 Experimental Data

Utilizing the number of cells within each hollow fiber bioreactor, the oxygen consumption rate was calculated to be very close to that previously reported for primary rat hepatocytes within a three-dimensional bioreactor. The range of oxygen consumption rates measured spanned a range of 65 to 87 amol/cell/s, and was within the range of values previously reported, 48 – 89 amol/cell/s [111, 208, 209]. The seeding densities of three-dimensional gels within previous experiments were also very similar (5 – 10 x 10⁶ cells/cm³) to that employed in these studies (~12.5 x 10⁶ cells/cm³).

The oxygen transport model employed in our study showed good agreement with measured experimental data, Figure 5.2. The experimental results showed only a marginal increase in oxygenation to cultured hepatocytes with media supplementation of bRBCs. This was likely due to the fact that under the experimental culture conditions, the hepatocytes were not oxygen limited, which was a result of the relatively low density of inoculated hepatocytes. Only at the lowest flow rate was a significant increase in oxygenation between the control case and the bRBC supplemented case observed. The model presented in this work was able to capture the observed phenomena, providing additional evidence for the validity of the model.
Figure 5.2 Comparison of experimentally measured oxygen consumption rates across the entire hollow fiber bioreactor along with values calculated by the oxygen transport model under identical conditions. □ - Plain media, ■ - RBC supplemented media, and ■ - Oxygen transport simulations for both cases. Oxygen consumption rates were measured for the following sets of Q [ml/min] and inlet pO₂ [mmHg]: A – 4.72/79, B – 8.35/85, C – 12.18/84, D – 4.72/77, E – 8.35/76, and F – 12.18/71.
5.4.2 Extracapillary Space Zonation

Oxygen transport simulations were used to calculate the oxygen concentration profiles for each set of model parameters, one of the resulting two-dimensional oxygen concentration distributions (for case A from Figure 5.2) is presented as Figure 5.3. Figure 5.3 shows the oxygen distribution within a single representative hollow fiber element. Contour lines have been added to delineate each of the hepatic oxygenation zones. The upper rectangle represents the flowing lumen, which contains bRBCs in the specific cases noted, the middle rectangle represents the semipermeable hollow fiber membrane that excludes bRBCs and limits radial flow of the media, and the lower rectangle represents the ECS which contains a continuum of hepatocytes. The two-dimensional cross-sections provide information on the distribution of oxygen concentration within the hepatocyte containing ECS. It is clear that these bioreactor operating conditions result in favorable oxygenation to cultured hepatocytes as the oxygen distribution is shown to be close to the desired goal of replicating all three oxygenation zones in the ECS. The resulting simulation data was cylindrically integrated over appropriate ranges of oxygen tension to provide the percent of the ECS volume within each of the hepatic oxygenation zones as: 18% periportal, 47% pericentral, and 20% perivenous. For this particular case, 85% of the ECS is within the desired oxygen spectrum, with 13% experiencing hyperoxic oxygenation and 2% experiencing hypoxic oxygenation. Additionally, the entire ECS was integrated to yield an average ECS pO₂ of ~50 mmHg. Similar well oxygenated values were found for the other experimental cases shown in Figure 5.2.
Figure 5.3 Oxygen concentration profile predicted by the oxygen transport model at one of the experimental conditions; \( u_{\text{max}} = 0.18 \, \text{cm/s} \), \( pO_2,\text{in} = 79 \, \text{mmHg} \), and no RBC supplementation – case A from Figure 5.2.

Figure 5.2 and Figure 5.3 indicate that the control case of no RBC supplementation at the lowest flow rate studied provided an almost ideal oxygenation environment to the cultured hepatocytes. However, as previously mentioned, only \(~10\%\) of the ECS volume was filled with hepatocytes during the experimental study. Utilizing this low hepatocyte density, an enormous number (~250) of hollow fiber bioreactors operating in parallel would be required in order to reach our desired minimum of 20\% of the \( in \, \text{vivo} \) hepatocyte mass necessary to sustain an ALF patient (10 – 40\% of the liver mass has been reported to be essential for maintaining proper liver function [37, 40, 113, 114]). For this reason, the model was also utilized to predict operating conditions with more feasible cell numbers, flow rates, and bRBC concentrations. Ideally, the hollow fiber bioreactor would be operated with hepatocytes at tissue-like densities in the ECS volume. This would provide enough cells to cover the surface of all of the hollow fibers within the bioreactor. However, the increased hepatocyte density within the hollow fiber
bioreactor would likely lead to the formation of significant hypoxic zones in the ECS volume, and even anoxic zones in some cases. This problem can be alleviated by increasing the oxygen carrier concentration within the circulating media stream. Utilizing the oxygen transport model, operating conditions that incorporate an increased concentration of RBCs can be shown to provide in vivo-like oxygenation to the ECS of the hollow fibers. These operating conditions will be discussed in the next section.

5.5 Simulated Hollow Fiber Bioreactor Operating Conditions

Comparison of experimentally measured oxygenation data to oxygen transport simulations provided supporting evidence for the validity of the oxygen transport model. Therefore, bioreactor operating conditions (presented in Table 5.2) likely to be more relevant for ALF patient support within a clinical setting were explored using the model. First, the hepatocyte cell mass within the hollow fiber bioreactor was increased to fill the entire ECS ($\varepsilon = 1$). Filling the entire ECS volume may not be experimentally attainable; however, an ideally operating bioreactor would have a tissue-like density of hepatocytes. This reduces the number of hollow fiber bioreactors needed to sustain a patient to $\sim 25$, which could be further reduced by utilizing bioreactors with larger ECS volumes. The flow rate through each of the bioreactors was calculated using a flow rate similar to that utilized for hemodialysis ($\sim 300$ ml/min) split up into each of the bioreactors, yielding an individual bioreactor flow rate of 12.18 ml/min. Additionally, two lower flow rates (4.72 and 8.35 ml/min) were examined, as it is desirable to remove as little blood from the patient as possible and because an increased flow rate can lead to Starling flow, which reduces hepatocyte metabolism due to increased shear forces on the cells [70, 210].
Generally, venous blood is fed into the BLAD, due in large measure to the accessibility of veins and the low venous blood pressure compared to arterial pressure. However, the average mixed venous dissolved oxygen tension is ~40 mmHg [123], which is too low to replicate all three oxygenation zones needed for proper zonation to develop. Therefore, the ALF patient’s venous blood would have to be oxygenated prior to entering the hollow fiber bioreactor. The inlet oxygen tension was examined over a range of values, to determine oxygen tensions that replicate all three oxygenation zones. It was determined that the average mixed arterial dissolved oxygen tension, 90 mmHg, was best suited as the inlet oxygen concentration entering the bioreactor. Finally, the RBC concentration within the hollow fiber bioreactor system was varied over a range of values. It was determined that the full human \textit{in vivo} hematocrit was not necessary to provide the desired oxygenation spectrum to cultured hepatocytes within the hollow fiber bioreactor. Increased RBC concentrations would be expected to lead to fouling of the membrane via RBC hemolysis; therefore, for future clinically-relevant BLAD designs, the concentration of human RBCs that pass through the bioreactor should be minimized by partial plasmapheresis.
TABLE 5.2

CLINICALLY-RELEVANT PARAMETERS OF THE HOLLOW FIBER BIOREACTOR EMPLOYED IN OXYGEN TRANSPORT SIMULATIONS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECS hepatocyte fraction, $\varepsilon$</td>
<td>1</td>
<td></td>
<td>Full ECS</td>
</tr>
<tr>
<td>RBC concentration, $Hb_T$</td>
<td>0.015</td>
<td>g/cm$^3$</td>
<td>10% [human RBC]</td>
</tr>
<tr>
<td>Inlet oxygen concentration, $pO_2_{in}$</td>
<td>90</td>
<td>mmHg</td>
<td>~Arterial pO$_2$ [123]</td>
</tr>
<tr>
<td>Adair constants, $a_1$</td>
<td>1.53x10$^{-2}$</td>
<td>mmHg$^{-1}$</td>
<td>[211]</td>
</tr>
<tr>
<td>$A_2$</td>
<td>1.10x10$^{-3}$</td>
<td>mmHg$^{-2}$</td>
<td></td>
</tr>
<tr>
<td>$A_3$</td>
<td>1.24x10$^{-7}$</td>
<td>mmHg$^{-3}$</td>
<td></td>
</tr>
<tr>
<td>$A_4$</td>
<td>1.81x10$^{-6}$</td>
<td>mmHg$^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

The Adair constants defining the oxygen-hemoglobin equilibrium curve (OHEC) for bovine hemoglobin were utilized in the initial modeling study presented above. However, in clinical usage this type of device would be operating with human RBCs present in the lumen, and thus the model was updated with Adair parameters (presented in Table 5.2) that describe the equilibrium binding of oxygen to hemoglobin contained within human RBCs. As the OHEC utilized for bovine and human RBCs are very similar (Figure 5.4), the model is expected to be equally valid with human RBCs as the oxygen carrier within the lumen.
Figure 5.4 Oxygen-hemoglobin equilibrium binding curves for bovine and human red blood cells predicted from the Adair constants presented in Table 5.1 and Table 5.2.
These new simulation parameters provided insight into possible clinical applications of this type of BLAD. The oxygenation zones within the hollow fiber bioreactor were calculated for the control case of no RBCs (complete plasmapheresis) and 10% of the normal human RBC concentration (partial plasmapheresis). Figure 5.5 indicates the fraction of the ECS volume that is within each of the specified hepatic oxygenation zones: periportal (60 – 70 mmHg), pericentral (35 – 60 mmHg), and perivenous (25 – 35 mmHg), under these conditions. In addition to the three desired hepatic oxygenation zones, hyperoxic (pO2 > 70 mmHg) and hypoxic (pO2 < 25 mmHg) zones were also observed. Furthermore, a white line is included to display the portion of the hypoxic region that is anoxic (<0.5 mmHg). For this set of results, it is observed that with no RBCs in the circulating media, there are severe hypoxic and anoxic regions within the hollow fiber bioreactor ECS. The addition of RBCs completely removes the anoxic region, and reduces the hypoxic region in each of the cases examined. Additionally, a near ideal oxygenation environment, as previously described, can be provided to hepatocytes within the hollow fiber bioreactor ECS for case L in Figure 5.5.

In addition to quantifying ECS zonation, the average ECS pO2 was calculated for each of the cases examined in Figure 5.5. For the control cases of G, H and I, the average pO2 values were calculated to be 5, 8, and 12 mmHg, respectively. In each of the control cases (those with complete plasmapheresis), the outlet oxygen concentration was reduced to almost 0 mmHg. However, with the addition of 10% of the normal human in vivo RBC concentration to the circulating media feed stream (partial plasmapheresis) a dramatic increase in the amount of oxygen delivered to the hepatocytes is observed. The average pO2 values within the hollow fiber ECS for the experimental systems (with
RBCs present) are 25, 34, and 41 mmHg for cases J, K, and L respectively. The zonation of the hollow fiber presented in Figure 5.5 indicates that case L approaches ideal oxygenation of the culture hepatocytes. It is fairly obvious that the volume of the periportal zone is smaller compared to the volume of either the pericentral and perivenous zones, but this is an expected result. Oxygen is consumed more rapidly by periportal hepatocytes located in a high pO$_2$ environment, due to the nature of the governing Michaelis-Menten kinetics.
Figure 5.5 Hepatic zonation of the ECS in each of the defined oxygenation zones for the predicted bioreactor operating conditions at an inlet dissolved oxygen tension of 90 mmHg at several flow rates: G&J – 4.72 [ml/min], H&K – 8.35 [ml/min], and I&L – 12.18 [ml/min], with either plain media circulating (G, H, & I) or circulating RBC supplemented media at 10% of the human in vivo RBC concentration (J, K, & L).
The axisymmetric cross-sections of a few representative oxygen concentration profiles are presented in Figure 5.6. Figure 5.6 contains three different simulations: cases I and L from Figure 5.5 (as cases a and b, respectively), and a case with a higher RBC concentration. Cases a and b can be directly compared to show a significant increase in the dissolved oxygen concentration profile within the ECS. It is observed that the outlet pO₂ is essentially 0 mmHg for a, and is raised to ~39 mmHg for b by the inclusion of RBCs within the BLAD feed stream. The volume of ECS within the desired oxygenation region (25-70 mmHg) increased from 21% to 95% by the addition of RBCs. Therefore, supplementation of RBCs to the circulating media stream has a significant impact on both the amount of oxygen, and range of oxygen tensions, available to the hepatocytes within the hollow fiber.

Next, the axisymmetric cross-sectional oxygen concentration profile of b was compared to c within Figure 5.6. In c the amount of RBCs present in the circulating media was increased to 20% of the average human in vivo hematocrit, and the inlet dissolved oxygen concentration was decreased to 80 mmHg. By increasing the concentration of RBCs, the oxygen carrying capacity of the solution entering the bioreactor is increased and thus the requirements of a high inlet oxygen partial pressure can be relaxed. The bioreactor operating conditions for case b was chosen such that b and c have almost identical overall oxygen consumption rates (OCRs). The inlet and outlet concentrations of dissolved oxygen are considerably different, from 90 to 39 mmHg in case b and from 80 to 48 mmHg in case c; however, it is anticipated that maintaining a similar OCR is more important. A similar average ECS pO₂ is observed between b and c, 40 versus 45 mmHg respectively, and both of these values are close to
the design criteria. The oxygen concentration profiles observed are significantly different under the bioreactor operating condition for cases b and c in Figure 5.6. In b, it was calculated that 95% of the ECS is within the desired range of oxygen tensions (25 – 70 mmHg), and in c almost all of the ECS (99.9%) is within the range of oxygen tensions. However, a better distribution of the three hepatic zones is found for case b. The pericentral zone in case c is significantly larger compared to case b, (81% versus 48% of the ECS volume), but the periportal (6% versus 7%) and perivenous (13% versus 40%) are smaller in case c compared to case b. The more even distribution of the oxygen profile, seen in case b, should provide a better distribution of the hepatic phenotypes.
Figure 5.6 Oxygen concentration profiles predicted by the oxygen transport model at three predicted experimental conditions; $u_{max} = 0.465$ cm/s and (a) $pO_2,_{in} = 90$ mmHg with no RBC supplementation – case I from Figure 5.5, (b) $pO_2,_{in} = 90$ mmHg with 10% supplementation of the human in vivo RBC concentration – case L from Figure 5, and (c) $pO_2,_{in} = 80$ mmHg with 20% supplementation of the human in vivo RBC concentration.
A standard one-at-a-time (OAT) sensitivity analysis was performed on each of the parameters examined as has been previously described in Chapter 3, and in the literature [137]. For the ranges of parameters examined, none of the parameters elicited convergence issues. Further simulations were conducted with higher RBC concentrations, and resulted in either over-oxygenation of the hepatocytes or, at lower inlet dissolved oxygen tensions, the hepatocytes only experienced a small portion of the desired oxygen concentration range. Therefore, within this chapter, the two most promising possible bioreactor operating conditions were examined. These conditions should allow the hepatocytes to experience an in vivo-like oxygenation spectrum (Figure 5.6). Utilizing the developed oxygen transport model, a set of optimal bioreactor operating conditions could be determined for any experimental setup.

The bioreactor outlet pO$_2$ calculated from the optimal simulation (case b) is 39 mmHg which approximates the in vivo mixed venous pO$_2$ and thus, when the patient’s blood is returned, a significant portion of the oxygen in the blood stream has not been removed. However, since current BLADs remove a patient’s venous blood, the feed stream to the hollow fiber bioreactor needs to be oxygenated to ~90 mmHg, for which several methods are available to accomplish this task. One promising method utilizes silicone tubing that is contained in a controlled, elevated oxygen environment [212, 213]. This work and previous work has indicated that bioreactor inlet pO$_2$ values of up to 95 mmHg can be attained with a reasonable length (~2 m) of silicone tubing at atmospheric oxygen concentrations. In the future, an oxygen transport analysis of silicone tubing could be employed to calculate the length of silicone tubing and ambient oxygen concentration required to produce the desired blood oxygen level.
5.6 Conclusions

In this study, the oxygen concentration spectrum within a hollow fiber bioreactor housing primary hepatocytes supplemented with RBCs as an oxygen carrier was examined. An oxygen transport model was developed and validated by experimentally measured data, and was subsequently utilized to predict the best bioreactor operating conditions for a viable BLAD. Previously, the use of hemoglobin-based oxygen carriers that could initially culture hepatocytes in an *in vivo*-like oxygen environment was investigated. In the work described here, the model developed in Chapter 2 was updated and its use has been extended to examine clinically-relevant settings of BLAD implementation, which is expected to aid in the development of commercially viable BLADs. The parameters examined in the study identified bioreactor operating conditions where hepatocytes would experience a biomimetic oxygenation environment. It is believed that a hepatic hollow fiber bioreactor operating in the manner explained could sustain an ALF patient until native liver regeneration can take place, or a suitable donor organ can be found.
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CHAPTER 6:
OXYGENATION WITHIN A HEPATIC HOLLOW FIBER BIOREACTOR: CLOSED CELL SPACE, OXYGEN CARRIER SUPPLEMENTATION, AND FLOW THROUGH THE CELL SPACE

6.1 Introduction

Lack of proper oxygenation to hepatocytes cultured within a hollow fiber bioreactor remains an important problem in the development of a viable BLAD. The oxygen demands of hepatocytes have not been completely fulfilled within the experimental systems utilized in preceding chapters (this is primarily due to the low concentrations of HBOC utilized for these studies). Traditionally, hepatic hollow fiber bioreactors were operated with the ECS port valves closed [85]. Thus, restricting media flow primarily to the lumen. This resulted in the development of insignificantly small Starling flows through the hollow fiber membrane and ECS, where diffusion is the dominant mechanism of mass transport to the cultured hepatocytes [148]. In this study, oxygen transport to housed hepatocytes was increased by allowing a fraction of the inlet media stream to permeate through the hollow fiber membrane, thereby flowing through the ECS, and exiting via the two ECS ports (permeate flow). We hypothesize that inclusion of convective transport to hepatocyte cultures will increase oxygen availability, leading to retention of differentiated functionality. Additionally, a bRBC supplemented
hollow fiber bioreactor system was operated and compared with the control and convection enhanced systems.

In the experimental study conducted in this chapter, all three experimental hollow fiber bioreactor systems were concurrently operated. Currently, most hepatic hollow fiber bioreactor systems operate with a stagnant cell space (closed ECS ports) to reduce shear forces on cultured cells to a minimum [1], and the first experimental system was operated in this manner as a control system. Also, an experimental hollow fiber bioreactor system, with closed ECS ports and an oxygen carrier (bRBCs) supplemented to the circulating media feed stream, was operated. This second experimental system was operated similar to the bRBC supplemented systems described in Chapters 3 and 5 of this dissertation. Finally, a third experimental hollow fiber bioreactor system was operated with the ECS port valves open to allow a fraction of the total inlet media flow to permeate through the hollow fiber membrane and flow through the ECS. This last experimentally operated system was an attempt to increase oxygen transport to the cultured hepatocytes via convection enhanced mass transfer. For all three hollow fiber bioreactor systems, metabolic, synthetic, and detoxification markers were tracked throughout the entire experiment and inline dissolved oxygen concentration measurements were taken at select time points. This study examines oxygen transport to hepatocytes within the hollow fiber bioreactor via bRBC supplementation, convection through the ECS, and compares the methods of enhancing oxygen transport.
6.2 Materials and Methods

6.2.1 Hollow Fiber Bioreactor System Operation

Three experimental hollow fiber bioreactor systems were inoculated and maintained in a similar manner to the cultures described in Chapter 3 and 5. Briefly, approximately three million C3A hepatoma cells were inoculated into the ECS of each hollow fiber bioreactor system (400-012, Spectrum Laboratories). All three experimental bioreactors were cultured concurrently within a Heraeus incubator (Kendro Laboratory Products) at 37°C and 5% CO₂. For the first week of cell culture, after inoculation, the ECS port valves remained closed in all three systems and no HBOC was supplemented to the media. Thus, all three hollow fiber bioreactor systems were operated in the same manner as the control system. This allowed time for the hepatocytes to acclimate to the bioreactor environment, adhere to the outside of the hollow fibers, and proliferate until the cultures achieved confluency. After a week of control operation, bRBCs (as a HBOC) were added to the media bottle of the bRBC supplemented system and the ECS port valves were opened in the convection enhanced system. Cell filters were placed after the ECS port valves in the convection system to maintain a separate and self-contained cell space, keeping dislodged hepatocytes from being flushed out of the hollow fiber bioreactor ECS and into the media bottle. A schematic of the hollow fiber bioreactor system is provided in Figure 6.1.

Figure 6.1 represents the setup of the experimental hollow fiber bioreactor systems in this chapter. Where complete media (with or without HBOCs) is pumped from the media reservoir (containing ~250 ml of media), through a length of silicone tubing (~2 m), to facilitate gas transfer with the incubator environment. Circulating media flows
through a turbine flow meter, dissolved oxygen probe, and into the hollow fiber bioreactor. Within the bioreactor, the media flows through hollow fibers, exchanging nutrients and waste with the cultured hepatocytes, collects and exits the bioreactor. In the convection enhanced system, some of the media flows through the membrane, leaks out the ECS ports, through another inline turbine flow meter and recombines with the bioreactor exit flow, Figure 6.1. Finally, the complete exiting media flows through another dissolved oxygen probe and returns to the media reservoir bottle.
Figure 6.1 Schematic of the convection enhanced hollow fiber bioreactor system utilized in the experimental study, along with a magnified view of a single hollow fiber simulated within the oxygen transport model.
The internal dimensions of the hollow fiber bioreactor’s utilized in these experimental trails have been measured, calculated, or are provided by the manufacturer. The single representative fiber presented in Figure 6.1 had a hollow fiber length (L) of ~10 cm, inner radius (r_L) of 165 µm, and average membrane thickness (t_m) of 50 µm. From the calculated number of fibers and total ECS volume, the radius of the representative ECS (r_ECS) was calculated to be 278 µm. This indicates that only approximately three layers of hepatocytes, with an average diameter of 20 – 25 µm [207], would be present around each fiber in the representative ECS. The tight hollow fiber spacing of this particular hollow fiber module is a major reason why it was selected for this study. In vivo hepatocyte plates within the liver sinusoids are only one or two cell layers thick [28].

As described in Chapter 5, to prevent sedimentation of bRBCs in the media reservoir within the bRBC system, a vortexer (Fischer Scientific) was set to shake the media bottle hourly in the incubator. Additionally, the hollow fiber bioreactor systems were equipped with inline turbine flow meters and inline dissolved oxygen concentration probes only when measurements were to be taken. Flow rates and oxygen concentrations were measured at select time points, and then the instruments were removed from the bioreactor system. In the convection system, when the ECS port valves were opened, the total flow rate entering the hollow fiber bioreactor and permeate flow rate exiting via the ECS (as seen in Figure 6.1) were recorded simultaneously, allowing fractional permeate flow through the ECS to be calculated.
6.2.2 Cell Line and Media Make-Up

The hepatocytes cultured in this study were the C3A hepatoma cell line (CRL-10741, ATCC, Manassas, VA), an immortalized human hybridoma derived from HepG2 cells. C3A cells were chosen because they exhibit most functional characteristics of human hepatocytes and maintain a rapid preconfluent doubling time (~1 day) with contact inhibition [214, 215]. Additionally, in Chapter 5, primary hepatocytes did not maintain high enough viability or cell density throughout the experimental studies, and this issue can be corrected with the usage of an immortalized cell line, such as the C3A hepatocyte.

The circulating culture media utilized in all hollow fiber bioreactor systems within this study was formulated similarly to the culture media in Chapter 3. Complete media was composed of 90% Dulbecco’s Modified Eagle’s Medium, 10% fetal bovine serum, and 200 U/ml of Penicillin and 0.2 mg/ml of Streptomycin (Sigma). For the case of the bRBC supplemented system, sterile bRBCs packed in 0.85% saline solution (Quad 5) were washed twice with complete media and added to the media reservoir bottle at ~10% of the human in vivo RBC concentration. Fresh media make-up and daily media changes were performed at the same time every morning for all three experimental systems. Daily media samples, taken directly from changed media bottles, were immediately frozen and stored for analysis. In the case of the bRBC supplemented system, media samples were spun down (4°C and 4000 rpm for 15 minutes) and only the supernatant was utilized to measure concentrations. Separating the bRBCs from the media in the bRBC supplemented system’s daily sample also gave an indication of bRBC concentration via fractional volume, supporting the bRBC counts.
6.2.3 Flow Rates and Membrane Permeability

Determining specific parameters and dimensions for the experimental setup was very important in calculating oxygen consumption by hepatocytes. Flow rates within the experimental systems were determined via inline turbine flow meters (DFS-2W, DigiFlow Systems, Lucas, OH) connected to a KrosFlo® pressure and flow meter (Spectrum Laboratories). In addition to flow rates through closed ECS cartridges, the flow rates with the ECS ports open were also measured. Inline turbine flow meters were placed at the inlet of the hollow fiber bioreactor (to measure total flow) and where the ECS ports were combined (to measure permeate flow), as is seen in Figure 6.1. Flow rates for closed and open ECS port systems were taken several times for each system, from which averaged flow rates and fractional permeate flow rates were calculated.

6.2.4 Inline Dissolved Oxygen Probes

Increasing the transport of oxygen to the hepatocytes cultured within the hollow fiber bioreactors is a primary aim of this study. In order to get quantitative measurements of the oxygen consumed by hepatocytes, inline dissolved oxygen probes (Lazar Research Labs) were inserted into the hollow fiber bioreactor systems, as previously described in Chapters 3 and 5. Briefly, inline dissolved oxygen concentration probes were calibrated by a two point calibration method, and then placed into the system immediately before and after the hollow fiber bioreactor. For the convection system, the outlet oxygen probe was placed after the permeate and outlet flows recombined, as shown in Figure 6.1. Once the oxygen probes were set in place and dissolved oxygen readings reached steady state (~1 hour), data points were recorded for various inlet and exit pO₂ values. This
procedure was repeated at several flow rates for each experimental hollow fiber bioreactor systems.

6.2.5 Cell Density

After the experimental study was concluded, the hollow fiber bioreactors (lumen and ECS) were filled with AccuMax (Innovative Cell Technologies, San Diego, CA) to loosen adherent hepatocytes, and dissociate them into a single cell suspension. Accumax is a replacement for trypsin (which had proved ineffective in previous trials) that has protease, collagenolytic, and DNase activity. Cell counts of the removed ECS media were performed with a hemacytometer, and viability determined via the Trypan Blue exclusion method. Initial cell counts were lower than expected, so a second soak with Accumax solution was performed. After a day in the AccuMax solution, the remainder of hepatocytes were removed, and another set of cell counts performed. These, and further cell counts, indicated significant proliferation of the hepatocytes within the ECS of all systems and a reasonably high viability, which supported selection of C3A hepatocytes for the experimental trial.

6.2.6 Key Hepatocyte Functional Markers

Maintaining hepatocyte functionality within a possible BLAD candidate is vital. Therefore, four different hepatocyte functional markers were tracked to examine impact of increased oxygenation on the function of hepatic hollow fiber bioreactors. Hepatocytes are multifunctional cells where tracking metabolic (glucose consumption and lactate production), synthetic (albumin production), and detoxification functions (ammonia removal) are standard markers of proper differentiated function. The
metabolic, synthetic, and detoxification profiles of the hepatocytes within each of the experimental bioreactors were determined via assaying daily media samples for all three experimental systems.

As mentioned in Chapter 3, and in the literature, glucose is the primary metabolite of C3A hepatocytes [111]. Previous studies indicated that bRBCs consume insignificant amounts of glucose compared to hepatocyte glucose consumption levels [156], and therefore this small amount of glucose consumption was not considered. Glucose concentration in daily media samples was quantified via a colorimetric assay (Quantichrom DIGL-200, BioAssay Systems, Hayward, CA). A small aliquot from a daily media sample was diluted 50:50 with water, 5 µl of the diluted sample was added to 500 µl of reagent, mixed, and placed in a boiling water bath for 8 minutes. Then, samples were placed in a cool water bath for 4 minutes, 200µl of room temperature sample was moved into a 96-well plate in duplicate, and the optical density was read at 630 nm on a plate reader (Bio-tek, Winooski, VT). The absorbance was converted to concentration via a calibration curve created from a set of standard glucose solutions (0 – 300 mg/dl) for each set of samples.

Glucose is primarily metabolized by hepatocytes via the citric acid (Kreb’s) cycle, where it can be fully broken down via glycolysis into two pyruvate molecules, and then into carbon dioxide and water via the oxidative phosphorylation pathway. However, in low oxygen environments, pyruvate can be converted into lactate, which is a less oxygen intensive (and lower energy) dead-end reaction [28]. Therefore, increased lactate production is a marker of hypoxia within the hepatocytes culture. Lactate concentration
levels alone do not provide enough information; instead a ratio of lactate production to glucose consumption is a better measure of cellular metabolism.

The lactate concentration within the daily media samples was determined via a lactate assay kit (A-108S, Biomedical Research Service Center, University of Buffalo, NY). A small aliquot from a daily media sample was diluted 50-fold with water, 20 µl of the diluted sample was combined with 50 µl of lactate assay solution, mixed, and incubated in a humidified 37°C environment for an hour. After incubation, the reaction was quenched by the addition of 50 µl of 3% (0.5 M) acetic acid and optical density was read at 492 nm on a plate reader. The measured absorbance was converted into lactate concentration via standard lactate concentration curve (0 – 250 µM) freshly generated for each set of samples. The ratio of glucose consumed to lactate produced is a standard metric of indirect hepatocyte oxygenation [156, 216]. Under hypoxic conditions, a single mole of glucose is broken down into two moles of lactate; a lower ratio of lactate production to glucose consumption indicates better oxygenation of the hepatocyte culture.

Synthesis of albumin is a standard marker of cell health and functionality within a hepatocyte culture, and is an important function for hepatocytes to maintain in vitro. C3A hepatocytes are a favored cell line for BLAD usage due to their maintenance of albumin synthesis [38]. Albumin is particularly important due to its role in regulation and transport activities within the liver. Additionally, previous studies have indicated that albumin synthesis is an indicator of hepatocyte cell culture confluence/maturation [215]. Production of albumin by hepatocytes is a highly oxygen intensive synthetic pathway, where 75 moles of glucose and 450 moles of oxygen are consumed for each
mole of albumin synthesized [217]. Albumin concentration of daily media samples was quantified via the BCG albumin assay kit (DIAG-250, BioAssay Systems). Five µl of an undiluted media sample was added to 200 µl of working albumin reagent in a 96-well plate. The plate was tapped lightly to mix, incubated for five minutes at room temperature, and the optical density was read at 620 nm on a plate reader. The measured absorbance was converted to albumin concentration via an eight point calibration curve (0 – 5 mg/dl) of bovine serum albumin.

Retaining detoxification capability is another necessary function of a viable BLAD. In vivo, ammonia removal is primarily facilitated by the urea cycle, which only occurs in the liver [217], and this is one of the only ways the body can get rid of ammonia. Build-up of ammonia within the blood can lead to, among other things, hepatic encephalopathy, a serious brain condition which can cause a decreased level of consciousness including coma and, ultimately, death [218]. Additionally, retention of a functioning urea cycle indicates a healthy and viable culture. The concentration of ammonia within the daily media samples was determined via an ammonia assay kit (200-02, Diagnostic Chemicals Ltd, Oxford, CT). A small aliquot from a daily media sample was diluted 50:50 with water, 20 µl of diluted sample was added to 300 µl of ammonia reagent, mixed, and incubated for one minute at room temperature. The optical density was read at 340 nm in a plate reader. Then, 2 µl of glutamate dehydrogenase (GLDH) was added into each well and incubated for six minutes, and the optical density at 340 nm was read again. The measured absorbance, with the initial absorbance and provided standard absorbance, allowed calculation of the ammonia concentration (0.17 – 0.80
µg/ml) within the media sample. The maintenance of all these important functions is necessary for the proper function of a BLAD.

6.3 Results and Discussion

The three different experimental hollow fiber bioreactor systems housing C3A hepatocytes were maintained for a period of over three weeks in this experimental study. Samples taken during the daily media changes were frozen, and these were later thawed and assayed to track the metabolic, synthetic, and detoxification functions of the hepatocyte cultures. Additionally, dissolved oxygen measurements were recorded at several flow rates for all three of the experimental hollow fiber bioreactor systems. The dissolved oxygen measurements yield a quantitative measure of oxygen utilization by the hepatocytes, and the functional markers provide information on the retention of hepatocyte differentiated function.

Cell density within the three hollow fiber bioreactors was determined via cell count and viability analysis, as previously described. After the experiment was terminated the bioreactors were determined to house 0.8 – 1.9 x 10⁹ hepatocytes per bioreactor ECS. This indicated approximately six doublings of the initially inoculated hepatocyte cell density. Since hepatoma cell line populations have been shown to double in 20 ± 3 hours during the early phase of cell growth [111], we can assume that the cartridges reached confluency after the initial week of bioreactor operation.

The total flow rates for the hollow fiber bioreactor system was measured and ranged between 5 – 23 ml/min for all pump settings utilized in these experimental trials. The fractional permeate flow rate in the convection system was determined to be up to
~10% of the measured inlet flow rate. Previous values found in the literature have measured fractional permeate flows of 20% and 30% in similar convection systems [127, 219], which indirectly indicated that high cell density will obstruct flow through the membrane. These previous studies were conducted on primary hepatocytes which do not proliferate, usually leading to a reduced cell density within the ECS of the hollow fiber bioreactor. Therefore, the lower values for fractional permeate flow determined for this experiment, compared to the literature, was expected.

6.3.1 Oxygen Consumption Rates

Oxygen provision within the hollow fiber bioreactor and hepatocyte health was measured via several different methods in this experimental study. During the first week of hepatocyte culture, all three systems operated like the control system until confluency was achieved. After a second week of operation, the effects of the experimental operating conditions (bRBC supplementation and enhanced convection) were assumed to be appreciable within the hollow fiber bioreactor systems. Only then were the inline dissolved oxygen probes inserted into each of the experimental systems, as described above. Dissolved oxygen measurements were taken on four separate days for each of the experimental hollow fiber bioreactor systems. From the recorded inlet and outlet dissolved oxygen concentration measurements, the total change in oxygen concentration across the bioreactors were calculated. Utilizing the difference in oxygen concentrations, along with the flow rates, volume of the ECS, and hepatocyte volume fraction, the oxygen consumption rate (OCR) for the bioreactor was calculated via equation 5.2.
Figure 6.2 presents observed OCRs for all three experimental bioreactor systems at four different measured flow rates. The overall media flow rates of media through the hollow fiber bioreactor systems utilized to measure oxygen consumption were: $Q_1 = 5.18$, $Q_2 = 11.1$, $Q_3 = 17.0$ and $Q_4 = 23.0$ ml/min. In Figure 6.2, the control case has the lowest measured oxygen consumption rate, and the bRBC supplemented and convection cases improve oxygen transport to the cultured hepatocytes for all measured flow rates. This effect is even more pronounced at higher flow rates for each bioreactor system. The increase in oxygen transport is significantly greater at the two highest flow rates ($Q_3$ and $Q_4$) and the convection enhanced system had significantly more oxygen transport compared to the bRBC supplemented system at the highest flow rate ($Q_4$). The range of values in the experimental measurements reported in Figure 6.2 compare well to previously reported literature values [38, 111]. As expected, increasing the flow rate through the bioreactor increases the OCR for all of the experimental hollow fiber bioreactor systems. Additionally, comparing the bRBC supplemented system to the convection system; it was observed that the measured OCR for the bRBC system is higher compared to the convection system at lower flow rates ($Q_{1-3}$). At the highest flow rate ($Q_4$), the convection system was found to have transported more oxygen to hepatocytes within the hollow fiber bioreactor system, Figure 6.2. This is hypothesized to happen because at higher overall flow rates, there is proportionally more permeate flow through the ECS and the effect of convective mass transport becomes more pronounced.
Figure 6.2 Oxygen consumption rate measured for each of the three experimentally operated hollow fiber bioreactor systems, at four different flow rates.
6.3.2 Key Hepatocyte Functional Markers

After the conclusion of the experiment, the media samples collected during daily media changes were assayed for key metabolic, synthetic, and detoxification functions. These key functional markers offer more evidence on oxygen availability to the hepatocyte cultures, providing an indication of cell health and proper functionality. The first assays to be performed were the metabolic assays, which measured the production of lactate and the consumption of glucose. As previously discussed, glucose is the primary metabolite in C3A hepatocyte culture, and lactate is generally produced in anaerobic environments. Therefore, the ratio of lactate produced to glucose consumed was calculated to demonstrate improvement of bioreactor oxygenation.

As discussed above, the ratio of lactate production to glucose consumption gives an indication of how well the citric acid cycle is performing [156]. Under well oxygenated conditions glucose can be converted into water and carbon dioxide; however, under hypoxic conditions glucose dead ends within the citric acid cycle producing two molecules of lactic acid. An average of the ratio of lactate produced to glucose consumed is presented as Figure 6.3. It is observed that during the beginning of the experiment, the ratios are similar for all systems and that in the latter days of hepatocyte culture, the control case has the highest ratio (indicating lower oxygen provision). The similarity in the average ratios for the first week is expected since all three bioreactors were operated in the same manner (ECS port valves closed and no oxygen carrier present). Next, a lag period was observed for the bioreactor systems during some of the second week, while the hepatocyte cultures adjust to altered bioreactor operating conditions. During the second week, average ratios begin to diverge: control system ~0.96, bRBC supplemented
system ~0.72, and convection system ~0.67 moles of lactate produced to moles of glucose consumed. For the final week of the experiment, diverging ratios were calculated: 1.5, 1.1, and 0.84 for the three systems, respectively. The overall lactate production to glucose consumption ratios for the entire experiment were 0.87 ± 0.6 for the control case, 0.67 ± 0.4 for the bRBC supplemented case, and 0.65 ± 0.3 for the convection case. In Figure 6.3, both bRBC supplemented and convection enhanced systems have significantly smaller overall ratios than the control; indicating that both the bRBC supplemented and convection enhanced systems were performing better than the control system. Additionally, the ratios seem to indicate that the convection enhanced system is slightly better oxygenated than the bRBC supplemented system, Figure 6.3. However, no significant difference between the bRBC supplemented system and the convection system was found for these data.
Figure 6.3 Averaged values of the ratio of lactate produced to glucose consumed for each of the three experimental hollow fiber bioreactor systems over the course of the entire experiment. Significant differences between the control case compared to the bRBC supplemented and convection enhanced cases are indicted by asterisks over the time point * (p < 0.025) and ** (p < 0.005).
The synthesis of albumin is a highly oxygen intensive process, making it another metric of increased ECS oxygenation. Additionally, albumin synthesis is an important function to maintain in designing a BLAD and an indicator of hepatocyte culture confluency/maturation. In previous studies, hollow fiber membranes with a smaller pore size were experimented with, and the ECS media had to be flushed for albumin concentrations to be assayed [220]. In this study, albumin was able to permeate through the membrane, escaping the ECS, and daily media change samples were able to be assayed, in triplicate, to determine albumin concentrations. The accumulated albumin production for all three experimental hollow fiber bioreactor systems is presented in Figure 6.4. An initial lag phase in lactate production to glucose consumption ratio is easily observed during the first week of this experiment, Figure 6.4. This was an indication that the hepatocyte cultures achieved confluency, as has been previously reported [220]. Mature hepatocytes in this experiment exhibited an average albumin production rate in each system of 7.6, 12.3, and 27.8 µg/day/10^6 cells for the control, bRBC supplemented, and convection enhanced systems, respectively. These reported albumin synthesis values compare well to previously published literature values, 5 – 30 µg/day/10^6 cells [221, 222]. More albumin molecules are produced per cell in the convection enhanced system; however, the total albumin production displayed in Figure 6.4 was utilized as the best measure of total BLAD function. After the first week of culture, the bRBC supplemented and the convection enhanced systems produced significantly more albumin compared to the control system, as shown in Figure 6.4. Increased albumin production was due to improved oxygen transport as well as higher energy availability from the more efficient glucose usage that was observed in the
previous section. For every mole of albumin synthesized six moles of glucose are consumed, all metabolic markers are tied in to oxygen provision and each other. Again, albumin synthesis data presented in Figure 6.4 does not show significant differences between the bRBC supplemented case and convection enhanced case for any of the data points throughout the experiment.
Figure 6.4 Averaged values of accumulated albumin produced by each of the three experimental hollow fiber bioreactor systems over the course of the entire experiment. Error bars were determined from multiple assays of each sample.
As previously mentioned, proper functioning of the urea cycle (chemical conversion of ammonia into urea) is an important detoxification function to recapitulate within a BLAD. Therefore, ammonia removal from the circulating media stream for all three hollow fiber bioreactor systems was monitored throughout the experimental study. Ammonia levels within daily media samples was monitored via colorimetric assay, in triplicate, where initial ammonia concentrations were subtracted from final ammonia concentrations to calculate daily ammonia removal. Figure 6.5 presents the accumulated ammonia removal from each of the experimental hollow fiber bioreactor systems. After the first week of bioreactor operation, bRBC supplemented and convection enhanced systems had higher ammonia removal rates, and thus were functioning better than the control case, Figure 6.5. Increased functionality over the control case is an important measure of BLAD efficacy. On a per cell basis the control system was found to remove from 0.51 – 1.8 μg/(hr·10^6 cells) with an average of 0.88 μg/(hr·10^6 cells), the bRBC supplemented system removed 1.1 – 2.6 μg/(hr·10^6 cells) with an average of 1.6 μg/(hr·10^6 cells), and the convection enhanced system removed 1.6 – 3.0 μg/(hr·10^6 cells) of ammonia with an average removal of 1.8 μg/(hr·10^6 cells). The per cell ammonia removal rates compare well with previously reported values from 0.68 – 2.2 μg/(hr·10^6 cells) for similar BLAD systems [223-225]. Additionally, the percentage of total ammonia removal was calculated for all daily media samples. On average, the control system removed 21% of ammonia available within the circulating media, the bRBC supplemented system removed 41%, and the convection system removed 37%. These values are all in relative agreement with previously reported values of percent ammonia removal by hepatoma cells [223]. The removal of ammonia is statistically
different between the bRBC supplemented and convection enhanced cases versus the control case, after the first week of the experimental hepatocyte culture, Figure 6.5. Again, no significant difference between the bRBC supplemented and convection enhanced cases was determined for this detoxification study.
Figure 6.5 Averaged values of total ammonia removal in each of the three experimental hollow fiber bioreactor systems over the course of the entire experiment. Error bars were determined from multiple assays of each sample.
Assay results for the final days of steady state hepatocyte culture provide the most informative data. Therefore, a table was made with the metabolic, synthetic, and detoxification activities in the final few days of culture of the three experimental hepatic hollow fiber bioreactor systems, Table 6.1. For the glucose and lactate data, the final four days of operation were averaged to yield the presented data in Table 6.1. The glucose production at the conclusion of the experimental study compares well to previously published glucose consumption rates within a hollow fiber bioreactor, reported as 0.04 – 0.8 µg/(10^6 cell·hr) by Nyberg et al. [226]. The calculated rates of lactate production by hepatocytes also indicated good agreement between literature values and the reported data [227, 228].

TABLE 6.1

KEY METABOLIC MARKERS OF METABOLISM, SYNTHESIS, AND DETOXIFICATION, FOR THE LAST FEW DAYS OF THE EXPERIMENTAL STUDY. ALL UNITS ARE IN µg/(10^6 cell·hr)

<table>
<thead>
<tr>
<th>Experimental System</th>
<th>Glucose Consumption</th>
<th>Lactate Production</th>
<th>Lactate Glucose</th>
<th>Albumin Synthesis</th>
<th>Ammonia Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.363 ± 0.047</td>
<td>0.265 ± 0.036</td>
<td>1.46 ± 0.28</td>
<td>0.427 ± 0.35</td>
<td>1.10 ± 0.087</td>
</tr>
<tr>
<td>bRBC</td>
<td>0.783 ± 0.081</td>
<td>0.379 ± 0.027</td>
<td>0.967 ± 0.12</td>
<td>0.657 ± 0.17</td>
<td>2.50 ± 0.26</td>
</tr>
<tr>
<td>Convection</td>
<td>0.426 ± 0.11</td>
<td>0.196 ± 0.022</td>
<td>0.917 ± 0.26</td>
<td>0.809 ± 0.48</td>
<td>3.47 ± 0.25</td>
</tr>
</tbody>
</table>

6.4 Conclusions

Oxygen provision is limiting in hepatic hollow fiber bioreactors, as has been seen in the literature and demonstrated in the experimental studies in this dissertation. In order
to remedy this problem, two methods of increasing oxygen delivery to hepatocytes were investigated and compared. First, supplementation of bRBCs to the circulating media feed stream can increase the oxygen carrying capacity of the media, allowing more oxygen to be available to the cultured hepatocytes; demonstrated in our previous work [137, 138, 156]. Second, an altered flow path method to increase oxygen transport was explored. This approach introduced permeate flow through the ECS to allow for enhanced convective oxygen mass transport to the cultured hepatocytes [127, 219]. In this study, convection and oxygen carrier supplementation were shown to be able to increase oxygen transport to hepatocytes cultured within a hollow fiber bioreactor, compared to a control system with no oxygen carrier supplementation or convective flow through the ECS. Additionally, both methods increased key metabolic, synthetic, and detoxification functions of the hepatocytes, compared to the control case. For the fractional permeate flow rate and oxygen carrier (bRBC) concentration utilized in the two altered experimental systems within this study, no significant differences was observed. The implementation of both oxygen carrier supplementation and a convection flow path should further increase oxygen availability to hepatocyte cultures.
This chapter, in part or in full, will be submitted for publication in 2007 as the following manuscript:


The dissertation author was the first author on this paper with the dissertation committee chair as the primary investigator.
CHAPTER 7:

CONCLUSIONS AND RECOMMENDATIONS

7.1 Summary/Lessons Learned

Developing an *in vitro* replica of the liver sinusoid that closely emulates *in vivo* hepatocyte differentiated function is a key step in advancing treatment of liver diseases via a bioartificial liver assist device (BLAD). Provision of oxygen within the proper gradient experienced in the liver sinusoid microenvironments, as well as the cell-cell interaction fostered by hepatic hollow fiber bioreactors, is crucial in reproducing a successful multifunctional hepatocyte culture. Increasing and targeting oxygen transport to hepatocytes within a hollow fiber bioreactor leading to recapitulation of many of their metabolic, synthetic, and detoxification functions is a central goal of the original work presented in this dissertation.

Hepatic hollow fiber bioreactor systems were designed and operated to assess methods of improving oxygen transport and the impact of increased oxygenation on hepatocyte differentiated function. Each of the experimental and oxygen transport simulation studies have indicated that oxygen provision to hepatocytes cultured within a hollow fiber bioreactor can be improved by the addition of an oxygen carrier or the inclusion of convection through the hollow fiber membrane. Agreement between the experimental data collected and the developed oxygen transport model predictions was observed for all experimental studies performed. The correlation of experimental and
theoretical work is promising for prediction of optimal oxygen provision, future scale-up, and production of a fully functional hepatic hollow fiber bioreactor. The main criterion examined in these studies is proper oxygenation of the hepatocyte culture microenvironment; attaining an average ECS pO$_2$ of approximately 44 mmHg with an oxygen concentration gradient from 25 – 70 mmHg. Exposing hepatocytes to physiological oxygenation should allow for maximum viability and differentiated (zonal) function [34].

Preliminary work on the addition of an oxygen carrier to the circulating media stream of a hepatic hollow fiber work was performed in our lab, indicating via indirect measure an improvement in oxygenation [156]. In Chapter 3, inline dissolved oxygen probes were added to the experimental hollow fiber bioreactor systems for a quantitative measure of oxygen transport to hepatocytes. The oxygen transport model, supported by dissolved oxygen concentration measurements, illustrated the oxygen concentration profile throughout the bioreactor ECS. The control system (no oxygen carrier supplementation) measurements indicated that a lack of oxygen may be limiting further development of this type of BLAD. However, in the experimental hollow fiber bioreactor system (bRBCs supplemented to the circulating media stream as an oxygen carrier) a significantly improvement in oxygen transport was observed. This initial study supported the hypothesis that the addition of an oxygen carrier can improve oxygenation to hepatic hollow fiber bioreactors.

Probing further into improvement of oxygen provision, via oxygen carrier supplementation, a wide range of parameters was simulated in the oxygen transport model developed in Chapter 2. Many hollow fiber bioreactor operating conditions were
varied within the model in Chapter 4, including: inlet oxygen concentration, bioreactor flow rate, oxygen carrier concentration, and characteristics of the oxygen carrier supplemented. Hemoglobin-based oxygen carriers of varying size, production method, and oxygen affinity were examined with the oxygen transport model parameters attained in the experimental study presented in Chapter 3 of this dissertation. It was determined that high oxygen affinity oxygen carriers (low $P_{50}$) were more appropriate for low oxygen concentration environments, such as neural stem cell cultures. And, that low oxygen affinity oxygen carriers (high $P_{50}$) were best for high oxygen concentration requirements, such as the hepatocyte experiments presented within this dissertation. The oxygen transport model predicted several bioreactor operating conditions leading to proper oxygenation of hepatocytes cultured within a hollow fiber bioreactor supplemented with altered hemoglobin-based oxygen carriers. However, the implementation of hemoglobin-based oxygen carriers with a hepatic hollow fiber bioreactor is better suited for the production of a research in vitro liver model than a BLAD.

The experiments in Chapter 3 and 4 examined the culture of C3A hepatoma cells within hollow fiber bioreactor systems. However, some groups have found that in short-term cultures, primary hepatocytes retain more differentiated function than cell lines [111, 113]. Therefore, an experimental study was designed in Chapter 5 to examine the culture of primary rat hepatocytes within the hollow fiber bioreactor systems. The experimental systems operated similarly to the previous C3A studies, with a control case and an oxygen carrier (bRBC) supplemented case. Additionally, the oxygen transport model was altered to represent the dimensions of the hollow fiber bioreactor system, oxygen consumption kinetics of primary hepatocytes, and the inclusion of a hepatocyte
ECS volume fraction. As in the previous studies, oxygen transport simulations accurately predicted the oxygen distribution within the hollow fiber bioreactor for both experimental systems. However, due to a lower cell density (primary hepatocytes do not usually proliferate *in vitro*) the bioreactor was found to be well oxygenated for both experimental systems. Therefore, a set of more clinically relevant operating conditions were entered into the newly verified oxygen transport model, including: human RBC oxygen binding properties, a higher hepatocyte density in the ECS, and increased oxygen carrier concentrations. With the new biophysical parameters, oxygen transport simulations predicted optimal bioreactor operating conditions for primary hepatocyte culture within a hollow fiber bioreactor.

The lack of proliferation of the primary hepatocytes was problematic for the hollow fiber bioreactor system. Consequently, the next experiment was conducted with C3A hepatocytes cultured in the bioreactor ECS. The proliferation potential of a cell line increases hepatocyte density and also improves the cell distribution within the hollow fiber bioreactor, which can influence cell-cell interaction. Chapters 3 and 5 demonstrated that supplementation of an oxygen carrier improves oxygen delivery, so an additional approach to increasing oxygen availability to the hepatocytes was examined. To enhance oxygen transport to hepatocytes, the ECS port valves were opened during operation to produce additional convection of oxygen through the membrane and ECS of hollow fiber bioreactors. The experimental hepatic hollow fiber bioreactor systems operated in this study verified the improvement of oxygenation in the oxygen carrier (bRBC) supplemented case and demonstrated increased oxygen transport in the convection enhanced case, compared to a control case of no oxygen carrier and a stagnant ECS.
Both methods significantly improved oxygen transport to hepatocytes and differentiated function of the hepatocytes by a similar magnitude. With a combination of the two methods, oxygen carrier supplementation and a fraction of the media flowing through the ECS, hepatocytes should be cultured in an ideal oxygen environment.

Viability and hepatocyte function are highly dependent on oxygenation and the knowledge gained from these studies should be helpful in the design of future BLADs. This project is significant in that it provides an assessment of the importance of proper oxygenation within a hollow fiber bioreactor and possible methods to reproduce in vivo-like oxygen environments for cultured hepatocytes. The construction of an oxygen transport model that is verified by experimental measurements is vital to designing future BLADs. Hopefully, the understanding gained within these studies fosters further work in this area and eventually leads to production of a biomimetic hepatic hollow fiber bioreactor. Thus, producing a commercial, fully functional BLAD, that could benefit the treatment of thousands of liver failure patients.

7.2 Proposed Future Studies

7.2.1 Further Convection Studies

The addition of convection to the hollow fiber bioreactor system, studied in Chapter 6, is a very promising method for improving oxygen delivery. However, more experimental and simulation work needs to be done in this area to fully examine possibilities for a BLAD. First, the oxygen transport model could be updated to include flow through the ECS via several additional assumptions, including: homogeneous flow
through the membrane, even distribution of flow through each fiber, and the flow rates through each ECS port. Additionally, a convection experiment performed on a hollow fiber bioreactor system operated with the ECS ports open and bRBCs supplemented to the media, would provide additional information on the oxygen provision to the cultured hepatocytes. This appears to be the next logical step in the progression of this project.

7.2.2 Addition of Pulsatile Flow to Oxygen Transport Model

The use of a peristaltic pump to move the media through the hollow fiber bioreactor may cause pulsatile flow to be present through the hollow fibers. Pulsatile flow has been shown to increase the oxygen delivery via mixing within the lumen [229]. If this type of flow is observed in the lumen it could be modeled, providing a more accurate picture of the oxygen concentration profile. The equations for pulse flow through a cylindrical tube have been solved previously [230, 231]. Experimentally, the frequency, period, and amplitude could be experimentally determined for the hollow fiber bioreactor utilized. The inclusion of pulsatile flow may be important in elucidating the full oxygen concentration profile within a hollow fiber bioreactor.

7.2.3 Collection of pO2 Data through Non-Invasive Oximetry

To better understand the oxygenation provided to the hepatic HF bioreactor, more in depth experimental work needs to be accomplished. BLAD oxygenation data (for the case were bRBCs are present) can be obtained with a functional magnetic resonance imaging (MRI) technique using blood oxygen level dependent (BOLD) contrast. This technique can ascertain the amount of deoxyhemoglobin present along a flowing vessel by the way it alters the proton signal of surrounding water molecules. BOLD works on
the principle that when the normally diamagnetic HbO₂ gives up its oxygen, the resulting deoxy-Hb is paramagnetic, and this difference is “felt” by the adjacent water molecules [232]. With this technique, the fractional Hb saturation profile through a fiber can be measured and may be able to improve the modeling of oxygen transport within the hollow fiber bioreactor.

Many new techniques to measure oxygenation are emerging, especially using MRI [233-236]. Overhauser enhanced MRI has been developed to visualize tissue oxygen concentration within tumors by a double resonance technique that couples MRI with electron paramagnetic resonance imaging [237]. Also, oxygen transport and metabolism can be assessed in small tissue regions (or possibly in a hollow fiber bioreactor) with positron emission tomography using tracer ¹⁵O-oxygen [238]. With any one of these methods the oxygen distribution in the bioreactor could be mapped and better understood, therefore improving the model.

7.2.4 Additional Experimental Metrics of Hepatocyte Function

Maintaining a culture of hepatocytes within a hollow fiber bioreactor over a period of several weeks has allowed the measurement of key hepatocyte functions and oxygen consumption. Previously, enhancement of oxygen provision and metabolic functions (i.e., glucose, lactate, albumin, ammonia) were observed within this type of BLAD, Chapters 3 and 6. Additional compounds that could be monitored for the hepatic hollow fiber bioreactor experiments are glutamine, transferrin, or total protein synthesis.

In order to determine the different amounts of periportal and perivenous hepatocyte phenotypes within the BLAD a glutamine assay (GLN2, Sigma-Aldrich, St. Louis, MO) could be performed on all samples. Glutamine is one of the many
compounds that is both metabolized and synthesized in the liver to maintain homeostasis [239]. Glutamine, an important metabolic fuel for dividing cells and a nontoxic shuttle of ammonia between tissues, is present in plasma at 0.6 mM [240, 241]. Studies indicate that periportal hepatocytes generate glutamine [239, 240], while glutamine synthetase is highly localized to perivenous hepatocytes [242]. The C3A hepatoma cells have been shown to take up glutamine faster than normal human cells [243]; however, significantly different levels of glutamine in the experimental systems should indicate that the hepatocytes are exhibiting different phenotypes.

Transferrin is a large protein (~80 kDa) that is involved in iron binding and transport within plasma. The C3A synthesis of transferring could be measured in a similar manner as albumin was in Chapter 3, with an ELISA assay kit (Bethyl Laboratories, Cat. #E80-129). Lastly, BioRad (Hercules, CA) has a general colorimetric assay that measures total protein production. Based on the Bradford dye-binding procedure, Coomassie Brilliant Blue G-250, binds to mainly basic and aromatic amino acids residues, allowing quantification of the protein production by the hepatocytes.

Biotransformation functions of the cultured hepatocytes must be preserved to create a viable BLAD. The efficacy of these functions could be examined by the addition of a known quantity of a compound to the system’s circulating media stream and measurement of its concentration decrease over time. Phase I biotransformation can be examined by the addition of lidocaine and phase II biotransformation via the metabolism of 4-methylumbelliferone. For the phase I biotransformation (cytochrome P450 activity) study, lidocaine concentration can be assessed by a method described in the literature [244]. Phase II biotransformation could be examined via 4-methylumbelliferone (4-MU),
which undergoes both glucuronidation and sulfation. Medium containing 60 μM of 4-MU would replace the standard media for a media change and samples would be taken to follow the removal of the 4-MU from the system [209].

7.2.5 Bioreactor Sectioning and MicroArray for Cell Hypoxia Pathways

After the conclusion of a future experimental study, the cultured hepatocytes could be immobilized via cryofreezing the hollow fiber bioreactor, or by the addition of formaldehyde to the circulating media steam. Next, the bioreactor could be sectioned into radial slices allowing separate examinations at various lengths down the bioreactor. These sections could provide information of cell distribution and hepatocyte DNA could be extracted for further study. Microarrays are available to examine the hypoxia signaling pathways (SuperArray Bioscience Corporation, Frederick, MD, Cat. #OHS-032), which would provide evidence of hypoxia (or the lack thereof) in the bioreactor ECS. This microarray would also allow verification of the predictions made in the oxygen transport model. A sectioned hollow fiber bioreactor could provide valuable information on the morphology and density of hepatocytes. One of the previously discussed disadvantages of a hollow fiber bioreactor is uneven cell distribution and if the proliferation of C3A hepatocytes induce a more homogeneous cell density this would support the use of C3A hepatocytes within hollow fiber bioreactors.

7.2.6 Hepatocyte – Nonparenchymal Cell Co-Culture

Cell–cell contact is very important to the culture of healthy, viable, and properly differentiated hepatocytes. This is one of the main advantages of a hollow fiber bioreactor to culture hepatocytes. However, many studies have shown that co-culturing
nonparenchymal cells (sinusoidal endothelial cells, Kupffer cells, Ito cells, and/or liver associated lymphocytes) with hepatocytes increases function and viability [70, 245, 246]. These co-culture experiments were performed on flat plate bioreactors, where most preliminary hepatocyte culture experiments have been conducted. Future experimental trials could incorporate nonparenchymal cells with the inoculated hepatocytes in a hollow fiber bioreactor system. This could determine if co-culturing provides similar advantages in a hollow fiber bioreactor system as it does in flat plate systems. Additionally, the different types of cells inoculated, ratio of cell type populations, and method of inoculation could all be explored.
A.1 Derivation of Oxygen-Hemoglobin Equilibrium Curve Equations

A.1.1 Hill Equation

The Hill equation was derived before the number of binding sites on a hemoglobin tetramer was definitively known [145]. Therefore, the model is based on hemoglobin having a variable number of oxygen binding sites ($n$).

\[
Hb + nO_2 \rightleftharpoons [Hb(O_2)_n] \tag{A.1}
\]

From this rate law the reaction kinetics can be found.

\[
R_{Hill} = k^+ [Hb][O_2]^n - k^- [Hb(O_2)_n] \tag{A.2}
\]

The total concentration of hemoglobin is defined as the sum of the deoxygenated and oxygenated hemoglobin.

\[
Hb_T = [Hb] + [Hb(O_2)_n] \tag{A.3}
\]

Next, the fractional saturation of hemoglobin binding sites is defined for both oxygenated and deoxygenated hemoglobin.

\[
S = \frac{[Hb(O_2)_n]}{Hb_T} = 1 - \frac{[Hb]}{Hb_T} \tag{A.4}
\]

Defining $pO_2$ as the dissolved oxygen concentration we can now simplify the reaction rate by substituting in the fractional saturation of hemoglobin.

\[
R_{Hill} = k^+ Hb_T (1 - S) pO_2^n - k^- Hb_T S \tag{A.5}
\]
At equilibrium the reaction rate is zero \( (R_{\text{Hill}} = 0) \). Therefore, the fractional saturation of hemoglobin in terms the concentration of oxygen can be determined.

\[
k^+ pO_2^n = S(k^- - k^+ pO_2^n) \tag{A.6}
\]

\[
S = \frac{pO_2^n}{k^- / k^+ + pO_2^n} \tag{A.7}
\]

\( P_{50} \) is defined as the fractional saturation of hemoglobin when half of the binding sites contain bound oxygen molecules.

\[
S = \frac{1}{2} = \frac{P_{50}^n}{k^- / k^+ + P_{50}^n} \quad \text{and therefore} \quad P_{50}^n = \frac{k^-}{k^+} \tag{A.8}
\]

Substituting gives the final form of the Hill equation.

\[
S = \frac{pO_2^n}{P_{50}^n + pO_2^n} \tag{A.9}
\]

Within the oxygen transport model developed in Chapter 2, oxygen bound to hemoglobin is considered in equilibrium when implementing the Hill equation, as equation 2.11. Therefore, in equation A.9 the fractional saturation of oxygen found is the equilibrium value, \( S = S_{\text{eq}} \).

A.1.2 Adair Equation

Adair recognized that the hemoglobin protein contained four oxygen binding sites, giving hemoglobin four different intermediate conformations/compounds [146].

\[
Hb(O_2)_i \xrightleftharpoons{\kappa^-}{\kappa^+} O_{2i} \xrightarrow{\nu} Hb(O_2) \tag{A.10}
\]

Where \( i \) represents each of the four oxygen binding sites, i.e., \( i = 1 \rightarrow 4 \). From each of the four rate laws the reaction of oxygen binding/release can be found.
\[ R_i = k_i[Hb(O_2)]_i \prod[O_2] - k_{-i}[Hb(O_2)]_i = 0 \] \hspace{1cm} (A.11)

Where brackets indicate a concentration of the molecule and we are assuming equilibrium, i.e., \( R_i = 0 \). With the reaction rate we can solve for each of the intermediate hemoglobin compounds, where \( K_i = k_i/k_{-i} \).

\[ [Hb(O_2)]_i = K_i[Hb(O_2)]_i \prod[O_2] \] \hspace{1cm} (A.12)

Now the set of equations above can be plugged into the general formula for the fractional saturation of hemoglobin, below. Where the number of hemoglobin binding sites occupied with oxygen is in the numerator and the number of total hemoglobin oxygen binding sites.

\[
S = \frac{[Hb(O_2)] + 2[Hb(O_2)]_2 + 3[Hb(O_2)]_3 + 4[Hb(O_2)]_4}{4[Hb] + 4[Hb(O_2)] + 4[Hb(O_2)]_2 + 4[Hb(O_2)]_3 + 4[Hb(O_2)]_4} \] \hspace{1cm} (A.13)

Which can be further expanded.

\[
S = \frac{K_1[Hb]O_2 + 2K_1K_2[Hb]O_2^2 + 3K_1K_2K_3[Hb]O_2^3 + 4K_1K_2K_3K_4[Hb]O_2^4}{4[Hb] + 4K_1[Hb]O_2 + 4K_1K_2[Hb]O_2^2 + 4K_1K_2K_3[Hb]O_2^3 + 4K_1K_2K_3K_4[Hb]O_2^4} \] \hspace{1cm} (A.14)

To simplify we divide through by the concentration of hemoglobin ([Hb]). Again, the concentration of oxygen is considered the dissolved oxygen, i.e., \([O_2] = pO_2\).

Additionally, the reaction rate constants were renamed, i.e., \( a_i = \prod K_i \).

\[
S = \frac{a_1pO_2 + 2a_2pO_2^2 + 3a_3pO_2^3 + 4a_4pO_2^4}{4(1 + a_1pO_2 + a_2pO_2^2 + a_3pO_2^3 + a_4pO_2^4)} \] \hspace{1cm} (A.15)

Within the oxygen transport model developed in Chapter 2, the oxygen bound to hemoglobin is in equilibrium when using the Adair equation. Therefore, in equation A.15 the fractional saturation of oxygen found is the equilibrium value, \( S = S_{eq} \). This
value of the fractional saturation of hemoglobin oxygen binding site is what is presented in equation 2.12 and substituted into equation 2.10.

A.2 Development of Oxygen Transport Model

A.2.1 Oxygen Transport

The mass transport of oxygen in the oxygen simulations is calculated by the standard convection-diffusion equation (equation 2.3). The full convection-diffusion equation in cylindrical coordinates can be written as equation A.16. In equation A.16, D is the diffusivity of oxygen, C is the concentration of oxygen, r is the radius, \( \theta \) is the azimuth, and \( z \) is the axial direction.

\[
D \left( \frac{1}{r} \frac{d}{dr} \left( r \frac{dC}{dr} \right) \right) + D \left( \frac{1}{r^2} \frac{d^2C}{d\theta^2} \right) + D \left( \frac{d^2C}{dz^2} \right) = R + \frac{dC}{dt} + U_r \frac{dC}{dr} + U_\theta \frac{dC}{r d\theta} + U_z \frac{dC}{dz} \quad (A.16)
\]

Each of the terms within equation A.16 represent a mode and direction of oxygen transport or is a reaction term. The left side of equation A.16 contains the diffusive flux terms, from left to right: radial, azimuth, and axial. On the right side of equation A.16 there is the reaction term \( R \), which used for oxygen consumption by hepatocytes equation 2.4 or oxygen released by an oxygen carrier equation 2.10, followed by the time dependent term \( dC/dt \), which is not present in any of models in this dissertation since all simulations operate at steady state, and the convection terms. The convection terms on the right side of equation A.16 are, from left to right: radial, azimuth, and axial convection. The oxygen transport model we are constructing is axially symmetric, meaning that there can be no dependence on azimuth position and both the convection
and diffusion terms can be considered zero. With the elimination of the azimuth terms and the time dependent term equation A.16 becomes equation A.17.

\[
D \left( \frac{1}{r} \frac{d}{dr} \left( r \frac{dC}{dr} \right) \right) + D \left( \frac{d^2C}{dz^2} \right) = R + U_r \frac{dC}{dr} + U_z \frac{dC}{dz} \tag{A.17}
\]

With all of the terms within the convection diffusion equation defined we can simplify the equation by eliminating negligible terms. The mass transfer Peclet number \((Pe)\) is a dimensionless quantity that is defined as proportional to the convective mass transport divided by the diffusive mass transport; an example of the Peclet number in the z-direction is given in equation A.18. Small Peclet numbers \((Pe \ll 1)\) indicate that diffusion is the main mode of mass transport and the convective term within equation A.17 can be removed with insignificant changes to the solution determined. Conversely, at large Peclet numbers \((Pe \gg 1)\) convection dominates mass transport and the diffusive term within equation A.17 can be eliminated. Equation A.18 can be calculated for both the radial direction \((x = r)\) or the axial direction \((x = z)\).

\[
Pe_x = \frac{U_x \cdot x}{D} \tag{A.18}
\]

In the oxygen transport model developed in Chapter 2 we have calculated the Peclet number within each of the domains. In the lumen domain, axial convection and radial diffusion dominate, thus eliminating axial diffusion and radial convection. Also as noted in Chapter 2, if no oxygen carrier is present the reaction term within equation A.17 can be removed. In the membrane and ECS there is no fluid flow, which eliminates the remaining convection terms. In the membrane domain, the reaction term within equation A.17 is zero, since no oxygen is consumed or produced, giving equation A.27 in the analytical models. In the ECS domain, the reaction term of equation A.17 is utilized to
account for hepatocyte consumption of oxygen in the analytical oxygen transport model (equation A.28) and the numerical model (described in Chapter 2).

A.2.2 Variable Reaction Rate Kinetics

In Chapter 2, variable reaction rate kinetics are quickly derived to indicate how oxygen binding/release with hemoglobin with the oxygen carriers is accounted for within the oxygen transport model. An additional step in the derivation of the intermediate compound hypothesis is provided in this section. From equation 2.7 the rate law in equation 2.8 is derived, as discussed in Chapter 2. These equations define the equilibrium saturation of hemoglobin binding sites with oxygen, and at equilibrium the reaction rate of equation 2.8 is zero \(R_{oxyHBOC} = 0\), thus giving equation A.19.

\[
0 = k^+ \cdot pO_2 (1 - S) \cdot Hb_{r} - k^- \cdot S \cdot Hb_{r} \tag{A.19}
\]

Equation A.19 is rearranged by solving for \(k^+\) and eliminating the \(Hb_r\) term, thus giving equation 2.9. As noted in the previous section, the implementation of the oxygen binding curve in the Adair equation is for an equilibrium value, thus \(S\) in equation A.19 is \(S_{eq}\) in equation 2.9. Now the association rate constant \((k^+)\) is defined (equation 2.9), it can be substituted into equation 2.8, giving equation 2.10, as noted in Chapter 2.

A.2.3 Velocity and Oxygen Carrier Profiles within Lumen

The fluid flow through the lumen is found to be Poiseuille flow by calculating the Reynolds number, which is well within the laminar flow regime for all flow rates examined throughout this work, as described in Chapter 2. However, the addition of an oxygen carrier to the flowing lumen causes a disruption in the flow pattern resulting in a more blunted flow profile and aggregation of the oxygen carrier toward the centerline of
the lumen, Figure 3.2 in Chapter 3, as previously determined [134]. The characteristic length \( (L_C) \) for the oxygen carrier concentration and velocity profiles to develop was calculated for each of the simulations conducted according to equation 2.2. When the characteristic length of induction for the profiles is less than the total length of the hollow fiber \( (L_C < L) \), the profiles must be determined and applied to the oxygen transport simulation. For a few of the cases examined with the oxygen transport model simulations, with large oxygen carriers at high concentrations, there is a significant change in the radial velocity and oxygen carrier concentration profiles. Therefore, these profiles within the lumen were calculated for each of the situations according to the following method.

The oxygen carrier concentration and velocity profiles within the lumen are determined by an iterative calculation where the oxygen carriers are considered rigid flowing particles. The assumption of rigid particles is reasonable in large diameter tubes, like the hollow fiber considered here, because the oxygen carriers examined in this dissertation should not significantly deform. Initially, the oxygen carrier volume fraction at the wall of the lumen \( (C_w) \) was guessed. From the guessed oxygen carrier concentration, the wall shear stress \( (\tau_w) \) was calculated according to the experimentally determined formula equation A.20, which should be valid for any radial position.

\[
\tau = 2.27 \cdot C^3 \exp(2.34 \cdot C) \quad \text{..........................................................(A.20)}
\]

Next, with the determined wall shear stress from equation A.20 the radial shear stress profile within the lumen was calculated according to equation A.21, where, 1.56 is an experimentally determined constant [136].

\[
\tau(r) = \frac{\tau_w}{r^{1.56}} \quad \text{..........................................................(A.21)}
\]
With the known radial shear stress profile determined from equation A.21 the oxygen carrier concentration profile, $C(r)$, can be reverse mapped according to equation A.20. In the oxygen carrier profile calculated above the oxygen carrier concentration blows up near the centerline of the lumen (as $r \to 0$). However, due to particle volume exclusion there is a maximum volume fraction of oxygen carrier ($C_m = 0.62$), and therefore the profile flattens at shear stresses above calculated maximum shear stress, which is found from equation A.22, as seen in Figure A.1.

\[ C(r) = C_m : \tau(r) > \tau_m \]

(A.22)

Figure A.1 Two graphs of the calculated oxygen carrier concentration profiles, where the concentration blows up as the radius approaches zero (left) and where the concentration is capped due to particle volume exclusion (right).
Now, with the radial oxygen carrier concentration profile calculated, the viscosity profile ($\mu$) in the lumen was calculated according equation A.23.

$$\mu(r) = \frac{\exp(-2.32 \cdot C(r))}{\left(1 - \frac{C(r)}{C_m}\right)^3} \quad \text{(A.23)}$$

Next, the velocity profile within the lumen was calculated by integrating the velocity equation presented in equation A.24.

$$\frac{du}{dr} = -\frac{r}{2} \exp(-2.32 \cdot C(r)) \left(1 - \frac{C(r)}{C_m}\right)^3 \quad \text{(A.24)}$$

Finally, with the oxygen carrier concentration profile and the velocity profile known we can determine the flow averaged concentration. The flow averaged concentration ($\overline{C}$) is the ratio of the integrals of the radial oxygen carrier concentration profile to the velocity profile (equation A.25).

$$\overline{C} = \frac{\int u(r) \cdot C(r) \cdot r \, dr}{\int u(r) \cdot r \, dr} \quad \text{(A.25)}$$

If the calculated flow averaged oxygen carrier concentration matches the known oxygen carrier concentration the correct profiles have been determined. If the flow averaged oxygen carrier concentration is not accurate a new starting wall concentration must be guessed. This procedure is iterated until the calculated oxygen carrier concentration is the same as the experimental or modeling concentration. Then, the velocity and oxygen carrier concentration profiles are correctly calculated. The Matlab codes for these calculations are presented in Appendix A.3.1. The calculated fully developed flow profiles are presented along with the assumed inlet flow profiles in Chapter 3 as Figure 3.2.
A.2.4 Evolution of Profiles within Lumen

For the oxygen carrier simulations where an altered velocity and oxygen carrier concentration profiles will develop within the length of the bioreactor, the method to determine these new profiles is presented in Appendix A.2.3. The inlet profiles for the velocity and oxygen carrier concentration as still considered to be Poiseuille flow and homogeneous oxygen carrier concentration, as discussed in Chapter 2 and presented in Chapter 3 as Figure 3.2. Therefore, a method to calculate the evolution of the inlet profile to the altered profiles was determined. This was accomplished by an exponential evolution from the assumed inlet velocity and oxygen carrier concentration profiles to the calculated fully developed profiles. The Matlab code for these calculations is presented in Appendix A.3.2. The calculated oxygen carrier concentration and velocity profiles are presented in Chapter 4 as Figure 4.1 and Figure 4.2 respectively.

A.3 Willaert Oxygen Transport Models

A previously developed analytical oxygen transport model by Willaert et al. [147], is derived in this section for comparison to the oxygen transport model developed in Chapter 2 of this dissertation. In this section, an attempt was made to keep the variable symbols the same as in the originally cited model to allow easy reference to the source material. If not noted, the symbols are the same as the more complete oxygen transport model described earlier in this text. The analytical solutions in this section were calculated using Matlab (provided later in Appendix A.4).
Several additional assumptions are required in the analytical models compared to the numerical oxygen transport model developed in Chapter 2. The first major difference was the assumption in the analytical model that the majority of resistance to oxygen mass transport is present in the hollow fiber membrane and ECS. The second assumption maintains that the lumen contains a homogeneous radial oxygen concentration profile (e.g., \( pO_2 \) is the same at the lumen midline as the inner hollow fiber wall). In the third assumption, oxygen consumption by the hepatocytes was simplified to either of the two asymptotic limits of Michaelis-Menten kinetics, i.e., zero- and first-order kinetics. An oxygen mass balance on a single fiber and the appropriate boundary conditions are presented below for each of the three regions examined (lumen, membrane, and ECS).

Since no radial oxygen concentration profile is considered in the lumen, only axial convection of the dissolved oxygen is calculated in the Willaert model, in equation A.26. \( U_z \) is the average axial velocity through the fiber lumen, \( D_i \) is the diffusivity of dissolved oxygen, and \( C_i \) is the concentration of dissolved oxygen in the \( i^{th} \) domain, where the lumen is represented by domain 1, the membrane is represented by domain 2, and the ECS is represented by domain 3. The boundary condition for this equation is a defined concentration at the inlet of the individual hollow fiber (\( C_0 \)). The calculated dissolved oxygen concentration of the lumen (\( C_l \)) is the dissolved oxygen concentration on the inner surface of the membrane. In equation A.26, \( z \) is the axial length, \( R \) is the inner radius of the hollow fiber, and \( n \) is the number of fibers. In equations A.26 – 28, the asterisks designate that these are all dimensional quantities.

\[
\frac{dC^*_1}{dz^*} = \frac{2nD_2}{U_z R} \cdot \frac{dC^*_2}{dr^*} \bigg|_{r^*=R}^{z^* = 0} ; C^*_1 = C_0 \quad \text{...............................................(A.26)}
\]
Fluid flow through the membrane was not considered; hence mass transport of oxygen through the membrane was via diffusion only, equation A.27. Additionally, the reaction term within the convection-diffusion equation is not present, since there is no oxygen consumption or production in the hollow fiber membrane. The boundary conditions for the equation presented indicate continuity in dissolved oxygen concentration and flux.

\[
\frac{D_2}{r^*} \frac{d}{dr^*} \left( r^* \frac{dC_2^*}{dr^*} \right) = 0, \quad r^* = R : C_2^* = \gamma C_1^* \\
\]

\[
\begin{align*}
    & r^* = \beta R : D_2 \frac{dC_2^*}{dr^*} = D_3 \frac{dC_3^*}{dr^*} \text{ and } C_2^* = \gamma C_3^* \end{align*}
\]

Within the ECS, transport of dissolved oxygen is only accomplished by radial diffusion, and the reaction term \((V)\) represents the hepatocyte oxygen consumption rate (Note: the variable \(V\) in equation A.28 is an \(R\) in the previous section, equations 2.3 and 2.4). Equation A.28 provides the simplified convection-diffusion equation with boundary conditions of continuity between the membrane and ECS and no flux out of the modeled cylinder.

\[
\frac{D_3}{r^*} \frac{d}{dr^*} \left( r^* \frac{dC_3^*}{dr^*} \right) = V, \quad r^* = \delta R : \frac{dC_3^*}{dr^*} = 0 \\
\]

\[
\begin{align*}
    & r^* = \beta R : D_3 \frac{dC_3^*}{dr^*} = D_2 \frac{dC_2^*}{dr^*} \text{ and } C_3^* = C_2^* \frac{1}{\gamma} \end{align*}
\]

The partition coefficient is considered to be unity for the hollow fiber membrane, i.e., \(\gamma=1\). Additionally, the equations are solved for a single fiber \((n = 1)\), further simplifying the equations. In the analytical oxygen transport model, \(R\) is the inner radius of the fiber, \(\beta R\) is the outer radius of the fiber, and \(\delta R\) is the radius of the theoretical ECS per fiber. With this set of starting equations for each of the domains the oxygen
consumption profile can be solved. The kinetics of oxygen consumption leads to different solutions. In the next sections, the addition of zero- and first-order hepatocyte oxygen consumption kinetics in equation A.28 is explored.

A.3.1 Zero-Order Reaction Kinetics

Zero-order kinetics is defined as a reaction rate that is independent of reactant concentration. This is a simplification of the MM kinetics reaction rate law (equation 2.4) when the dissolved oxygen concentration is much greater than the $K_M$. For this limiting case of zero-order kinetics, the reaction rate term in equation A.28 ($V$ and $R$ in equation 2.4) is approximated by the Michaelis-Menten maximum reaction rate ($V_{Max}$), equation 2.13. Equations A.27 and A.28 were non-dimensionalized and solved to yield the radial oxygen concentration profile in the membrane (equation A.29) and in the ECS (equation A.30); asterisks were removed because the variables are now dimensionless.

$$C_2 = \frac{\phi_0^2 \beta^2}{2} \xi \left(1 - \left(\frac{\delta}{\beta}\right)^2\right) \ln r + \gamma C_1 \hspace{1cm} \text{.................................................................(A.29)}$$

$$C_3 = \frac{\phi_0^2 \beta^2}{4} \left[\left(\frac{r}{\beta}\right)^2 - 1 + 2 \left(\frac{\delta}{\beta}\right)^2 \ln \frac{\beta}{r} + 2 \frac{\xi}{\gamma} \left(1 - \left(\frac{\delta}{\beta}\right)^2\right) \ln \beta\right] + C_1 \hspace{1cm} \text{...........................................................(A.30)}$$

Where $\xi$ is the ratio of $D_3$ to $\gamma D_2$ and $\phi_0$ is the zero-order Thiele modulus, defined as $\phi_0^2 = R^2 V_{Max} / \left(D_3 C_0\right)$. Next, differentiating equation A.29 and substituting into equation A.26 and integrating yields the axial dissolved oxygen concentration profile in the lumen, equation A.31.

$$C_4 = \frac{n \phi_0^2 \beta^2 \xi L}{Pe_z R} \left[1 - \left(\frac{\delta}{\beta}\right)^2\right] z + 1 \hspace{1cm} \text{.................................................................(A.31)}$$
Where $L$ is the axial length of the bioreactor and $Pe_z$ is the axial Peclet number, defined as $Pe_z = U_z R / D_2$, as found from equation A.18. Now, the axial oxygen concentration profile in the lumen was be calculated, and from the concentration of dissolved oxygen at the wall, a radial dissolved oxygen concentration profile in the membrane and ECS can be calculated for any specified axial length, via equation A.29 and A.30 respectively.

The analytical model was used to plot the radial dissolved oxygen concentration profiles (Figure A.2) at three zero-order Thiele moduli ($\phi_0 = 0.5, 1.0, 1.5$), calculated at three dimensionless axial positions ($z = 0, 0.5, 1$). Figure A.2 is a reproduction of a figure from the original Willaert paper, which verifies the correct replication of the analytical model. Utilizing zero-order reaction kinetics in the original complete oxygen transport model over predicts the amount of oxygen consumed by the cells, finding that the dissolved oxygen concentration decreases to zero within the first centimeter of the bioreactor. Therefore, this analytical solution cannot be utilized to correctly model oxygen transport within the hollow fiber bioreactor system.
A.3.2 First-Order Reaction Kinetics

First-order reaction kinetics is defined as a reaction rate that linearly depends on the concentration of one reactant. This is another asymptotic simplification of the MM kinetic rate law, valid when the concentration of oxygen in the ECS is much greater than $K_M$. For the limiting case of first-order kinetics, the reaction rate law in equation A.29 ($V$, and $R$ in equation 2.4) is approximated by the first order Michaelis-Menten reaction rate, equation 2.14. Equations A.27 and A.28 were non-dimensionalized and solved to yield
the radial dissolved oxygen concentration profile in the membrane (equation A.32) and in the ECS (equation A.33); asterisks were removed because the variables are now dimensionless.

\[ C_2 = \gamma C_1 \left( \frac{\phi_i \beta \xi^2}{\gamma - \phi_i \beta \xi^2} \ln r + 1 \right) \quad \text{with} \quad \Psi = \frac{K_i(\phi_i \delta)I_1(\phi_i \beta) - I_1(\phi_i \delta)K_0(\phi_i \beta)}{K_1(\phi_i \delta)I_0(\phi_i \beta) + I_1(\phi_i \delta)K_0(\phi_i \beta)} \ldots (A.32) \]

\( I_i \) and \( K_i \) are the \( i^{th} \) order modified Bessel functions of the first and second kind, respectively. Also, presented in equations A.32 and A.33 is the first-order Thiele modulus \( \phi_1 \), defined as \( \phi_1^2 = R^2 V_{\text{max}} / (D_3 K_m) \). A mistake was made in the originally cited literature source for equation A.33 [147], which has been fixed here. The modified Bessel function of the first kind in the numerator \( (I_0) \) was mistakenly defined with a subscripted 1 \( (I_1) \) in the cited article.

\[ C_3 = \gamma C_1 \left[ \frac{1}{\gamma - \phi_i \beta \xi^2} \ln \beta \right] \quad \Psi = \frac{K_i(\phi_i \delta)I_1(\phi_i \beta) - I_1(\phi_i \delta)K_0(\phi_i \beta)}{K_1(\phi_i \delta)I_0(\phi_i \beta) + I_1(\phi_i \delta)K_0(\phi_i \beta)} \ldots (A.33) \]

Differentiating equation A.31 and substituting into equation A.26 and integrating yields the axial dissolved oxygen concentration profile in the lumen, equation A.34.

\[ C_4 = \exp \left[ \left( \frac{2nL}{Pe} \right) \frac{\phi_i \beta \xi^2}{1 - \phi_i \beta \xi^2} \right] \ldots (A.34) \]

The analytical oxygen transport model with first-order oxygen consumption kinetics was solved for a single fiber \( (n = 1) \). Figure A.3 shows the radial dissolved oxygen concentration profiles at four first-order Thiele moduli \( (\phi_i = 0.5, 1, 5, \text{and} \ 10) \), calculated at three dimensionless axial positions \( (z = 0, 0.5, 1) \). The first-order oxygen consumption kinetics model constructed, Figure A.3, compares well to the same figure within the cited literature data.
Figure A.3 Radial O$_2$ concentration profiles in the membrane and ECS of the hollow fiber for first-order kinetics at various axial positions $z = 0$ (—), 0.5 (•••), and 1 (- - -), with the first-order Thiele modulus as a variable parameter.

The parameters within this model were altered to mimic the hollow fiber bioreactor system in the numerical oxygen transport model developed in Chapter 2. However, the implementation of first-order kinetics in the oxygen transport model under predicts oxygen consumption by hepatocytes, leading to large hyperoxic regions within the bioreactor ECS. The application of an analytical solution with first-order oxygen consumption kinetics was found to be inferior to the numerical model at some of the simulated dissolved oxygen concentrations.
A.4 Matlab Codes

A.4.1 Velocity and Oxygen Carrier Concentration Profiles

% Determination of concentration and velocity profiles
% For "rigid" bRBCs in tube flow
clear

step=0.01;
step=0.01;
r=[0:step:1]; % normalized tube radii
d=-0.56; % experimentally determined constant
Cw=0.345; % vol. fraction - wall concentration of bRBC
Cm=0.62; % vol. fraction - maximum concentration of bRBC
G=1; % constant
toomuch=1;

toomuch=1;

% Start of Loop
while toomuch < 500;
    toomuch=toomuch+1
end

% Shear stress at the wall and maximum concentration
Sw=2.27*Cw^3*exp(2.34*Cw);
Sm=2.27*Cm^3*exp(2.34*Cm);

% Shear profile
count=1;
while count < (1/step+2);
    S(count)=Sw/r(count)^(1-d);
    count=count+1;
end

% Reverse map Concentration
x=linspace(Cw,Cm);
y=x;
count=1;
while count < 101;
    y(count)=2.27*x(count)^3*exp(2.34*x(count));
    C=interp1(y,x,S,'spline');
    count=count+1;
end

% Max concentration section
rm=(Sw/Sm)^(1/(1-d));
count=1;
while count < (1/step+2);
    if r(count)<rm C(count)=Cm;
    else C(count)=C(count);
    end
    count=count+1;
end

% Viscosity profile
M=exp(-2.32.*C.)/(1-C./Cm).^3;
%Velocity profile
R=linspace(0,1,101);
u=ode45(@DuDr,[1 0],0,[],R,C);
%plot(R,deval(u,R))

%Numerator calculation at Numerator.m (below)
Top=quad(@numerator,0,1,[],[],u,C);

%Denominator calculation at Denominator.m (below)
Bottom=quad(@denominator,0,1,[],[],u);

%Average flow concentration
AvgConc=Top/Bottom;

%Difference between known and calculated concentration
Error=0.45-AvgConc
if Error<-0.001
    Cw=Cw-0.0001;
elseif Error>0.001
    Cw=Cw+0.00005;
else tooomuch=500;  %End Loop
end
plot(R,deval(u,R))
%plot(r,C)
Velocity=deval(u,R);
%plot(R,Velocity)

Numerator.m

function top=numerator(r,u,C)
R=linspace(0,1,101);
vel=deval(u,r);
Cnow=interp1(R,C,r);
top=vel.*Cnow.*r;

Denominator.m

function top=denominator(r,u)
R=linspace(0,1,101);
vel=deval(u,r);
%Cnow=interp1(R,C,r);
top=vel.*r;

A.4.2 Velocity and Oxygen Carrier Concentration Profile Evolution

%Evolution of profiles from profile.m (A.3.1)
clear;
points=20;
zpoints=50;
r=linspace(0,0.0165,points); %radius from Femlab
z=linspace(0,12,zpoints); %axial distance from Femlab
umax=13.7; % maximum velocity at the centerline (Poiseuille flow)
Rc=0.00347; % Normalized maximum packing radius
Lc=2.56; % Characteristic length found by profiles.m
develop=(z/Lc).^3; % Normalized length of lumen to Lc (change function)

% Inlet profiles
c_const_old=0.15;
for i=1:rpoints;
    c_const(i)=c_const_old;
end
u_const=umax*(1-(r/0.0165).^2);

% Fully developed flow and concentration profiles
for count=1:1:
    if r(count)<Rc;
    c_profile(count)=0.211;
    elseif r(count)>Rc;
    c_profile(count)=3.384204e6*r(count)^4-174846.5*r(count)^3+3562.926*r(count)^2-37.84596*r(count)+0.3069423;
    end
end

% Profiles depending on r and z
HbT=ones(zpoints,rpoints);
for i=1:zpoints;
    if develop(i)<1;
    HbT(i,:)=c_const-develop(i)*(c_const-c_profile);
    elseif develop(i)>1;
    HbT(i,:)=c_profile;
    end
end
HbT2=HbT./0.15;
HbT3=HbT2.*100;
mesh(r,z,HbT3,'EdgeColor','black')
xlabel('Radius [cm]')
ylabel('Length [cm]')
ylabel('O2 Carrier Concentration [% hRBC]')
velocity=ones(zpoints,rpoints);
for i=1:zpoints;
    if develop(i)<1;
    velocity(i,:)=u_const-develop(i)*(u_const-u_profile);
    elseif develop(i)>1;
velocity(i,:)=u_profile;
end
figure, mesh(r,z,velocity,'EdgeColor','black')
end

A.4.3 Willaert – Zero-Order Kinetics

syms %r z %C1 C2 C3
%Dimensional Variables
R=0.165/1000; %m Radius of lumen
betaR=0.215/1000; %m Radius of membrane
deltaR=0.29/1000; %m Radius of cell space (calculated)
D2=3e-9; %m^2/s Diffusivity in membrane
D3=2e-9; %m^2/s Diffusivity in the cell space
Co0=4.175e-7; %mol/l Inlet concentration for 1st order reaction
%Co1=2; %mol/l Inlet concentration for 0th order reaction
%Vmax_over_Km=0.01; %s-1
Vmax=1e-6; %mol/l-s Maximum reaction rate
%Km=Vmax/Vmax_over_Km; %mol/l
Uz=0.00655; %m/s Flow rate (laminar flow)
%Uz=Um/2; %Average flow rate if
L=12/100; %m Length of HF cartridge
n=1; %Number of axially parallel fibers - *For a single fiber*
gamma=1; %Membrane partition coefficient

%Non-dimensional Variable
%r=R/R; %radius of lumen
beta=betaR/R; %radius of membrane
delta=deltaR/R; %radius of cell space
xi=D3/gamma/D2;
Theta0=R^2*Vmax/D3/Co0; %Thiele modulus
%Theta1=R^2*Vmax/D3/Co1; %Thiele modulus
Pe=Uz*R/D2; %Radial Peclet number

z=0.01:0.001:1;
%test1=n*Theta0*beta^2*xi*L/Pe/R
%test2=(1-(delta/beta)^2)
%slope=test1*test2
%test11=n*Vmax*beta^2*L/Co0/Uz/gamma
%Theta0=(0.5)^2; %set for Figure
%Zero Order Kinetics
Z=1; %Length for Radial solutions
C1=n*Theta0*beta^2*xi*L/Pe/R*(1-(delta/beta)^2)*z+1;
r2=1:0.01:beta;
C2=Theta0*beta^2*xi/2*(1-(delta/beta)^2)*log(r2)+gamma*C1(Z);
r3=beta:0.01:delta;
C3=Theta0*beta^2/4*((r3/beta).^2-1+2*(delta/beta)^2)*log((beta./r3)+2*xi/gamma*(1-
(delta/beta)^2)*log(beta))+C1(Z);
hold on
A.4.4 Willaert – First-Order Kinetics

syms % r z %C1 C2 C3
% Dimensional Variables
R=0.5/1000; %m Radius of lumen
betaR=0.6/1000; %m Radius of membrane
deltaR=0.82/1000; %m Radius of cell space (calculated)
D2=0.5e-10; %m^2/s Diffusivity in membrane
D3=1.0e-10; %m^2/s Diffusivity in the cells space
Co0=0.03; %mol/l Inlet concentration for 1st order reaction
Co1=2; %mol/l Inlet concentration for 0th order reaction
Vmax_over_Km=0.01; %s
Vmax=0.001; %mol/l-s Maximum reaction rate
Km=Vmax/Vmax_over_Km; %mol/l
Uz=0.3; %m/s Flow rate (laminar flow)
%Uz=Um/2; %Average flow rate if
L=26/100; %m Length of HF cartridge
n=110; %Number of axially parallel fibers
gamma=1; %Membrane partition coefficient

% Non-dimensional Variable
% r=R/R; %radius of lumen
beta=betaR/R; %radius of membrane
delta=deltaR/R; %radius of cell space
xi=D3/gamma/D2;
Theta0=R^2*Vmax/D3/Co0; %Thiele modulus
Theta1=R^2*Vmax/D3/Co1; %Thiele modulus
Pe=Uz*R/D2; %Radial Peclet number
z=0.0:0.01:1;

% First Order Kinetics
Theta1=10;
Z=1; %Length for Radial solutions
psi=(besselk(1,Theta1*delta)*besseli(1,Theta1*beta)-
besseli(1,Theta1*delta)*besselk(1,Theta1*beta))/(besselk(1,Theta1*delta )*besseli(0,Theta1*beta)+besselk(1,Theta1*delta)*besselk(0,Theta1*beta) );

C1_1=exp((2*n*L/Pe/R)*(Theta1*beta*xi*psi)/(1-
Theta1*beta*xi*psi))'*(Theta1*beta*xi*psi)/(1-
Theta1*beta*xi*psi))'*z);
C2_1=gamma*C1_1(2)*(Theta1*beta*xi*psi)/(gamma-
Theta1*beta*xi*psi)*log(beta))'*log(r2)+1);
A=(besselk(1,Theta1*delta)*besseli(0,Theta1*r3)+besselk(1,Theta1* delta)*besselk(0,Theta1*r3))/(besselk(1,Theta1*delta)*besseli(0,Theta1* beta)+besseli(1,Theta1*delta)*besselk(0,Theta1*beta));
\[ C_{3_1} = \gamma C_{1_1}(Z)/(\gamma - \Theta_1 \beta \xi \psi \log(\beta)) * A; \]

\[ \text{plot}(r2,C_{2_1},'--r'),\text{title('First Order')} \]
\[ \text{plot}(r3,C_{3_1},'--r') \]

**A.4.5 Differentiating the Adair Equation**

```matlab
syms P
A1=0.0437; %2.6e-3;
A2=0.0013; %1.7e-3;
A3=1.67e-5; %1.9e-11;
A4=1.02e-7; %1.4e-6;


dfdP=simple(diff(f));
%pretty(dfdP)
ddfdP=simple(diff(dfdP));
%pretty(dfdP)

x=5:0.05:11;
fplot=double(subs(f,P,x));
dfplot=double(subs(dfdP,P,x));
ddfplot=double(subs(ddfdP,P,x));

subplot(1,3,1),plot(x,fplot,'-b'), title('Adair equation')
subplot(1,3,2), plot(x,dfplot,'--r'),title('Derivative of Adair equation')
subplot(1,3,3),plot(x,ddfplot,'-g'),title('Double derivative')
xlabel('pO_2 (mmHg)')

Exp_f=subs(f,P,x);
Exp_dfdP=subs(dfdP,P,x);
Exp_ddfdP=subs(ddfdP,P,x);
```


