IMPROVED OXYGEN DELIVERY TO HEPATIC HOLLOW FIBER BIOREACTORS

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Abstract

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Oxygen provision to hepatocytes (and other types of cells) maintained within the extracapillary space of a hollow fiber (HF) bioreactor is believed to be transport limited because of the low solubility of oxygen in aqueous media and the high cellular oxygen demand. This problem limits hepatocyte functional capabilities. Alleviating this problem is of significant biomedical interest, since hepatic HF bioreactors can serve as both an in vitro model for studying liver biotransformation, detoxification, and metabolic functions, and as a device used to sustain patients suffering from acute liver failure.

One means of improving the oxygen carrying capacity of a HF bioreactor media stream is via supplementation of the circulating media stream with bovine red blood cells (bRBCs). This dissertation describes the initiation and development of a project aimed at employing bRBC
supplementation to improve the oxygen environment within the HF bioreactor hepatocyte space.

The work presented is organized into three sections. The first presents mathematical models depicting oxygen transport within the bioreactor system. One of these models was also utilized to demonstrate the potential benefit of bRBC supplementation. The remaining sections present the results of experiments aimed at evaluating bRBC supplementation within a C3A cell containing HF bioreactor and at examining the ability to engineer bRBC properties. Briefly, it was found that a C3A cell containing HF bioreactor maintained with media bRBC supplementation exhibited signs of an improved C3A cell space oxygen environment in comparison to a control (a C3A cell containing HF bioreactor not maintained with bRBC supplementation). Additionally, from the research conducted on engineering the properties of bRBCs, the ability to improve the cell’s osmotic stability and to alter its oxygen binding/dissociation properties was demonstrated. This could allow for the creation of a tunable, novel oxygen carrier well-suited for use within a HF bioreactor system. Lastly, the results obtained from this work have led to the design of a rigorous set of experiments which utilize bRBC supplementation to explore the impact (on function, etc) of oxygen provision on hepatocytes maintained within a HF bioreactor.
To my mother, Janel Hedges, for her constant encouragement, love, and support, and whose strength has always been a source of inspiration.
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CHAPTER 1

INTRODUCTION

1.1 Motivation

Hollow fiber (HF) bioreactors, first described by Knazek et al (1), are finding increasing utilization in providing three-dimensional environments for cell cultures (2). These devices, which are intended to be biomimetic, are composed of bundles of fibers enclosed in a cylindrical manifold (see Figure 1.1). Culturing media is passed through these fibers in order to deliver nutrients to cells, which are typically maintained outside of these fibers within the extracellular space (ECS). Simultaneously, toxins are removed from the cellular environment via dilution into the circulating media stream. HF bioreactors additionally provide a large membrane surface area to volume ratio that permits the attainment of high solute mass transfer coefficients (3), while also providing ample surface area for cellular attachment. However, it is a commonly held belief that oxygen transport within HF bioreactors is severely limited (4-9), which consequently hampers cell culture. This is likely a result of large diffusion distances, large cell densities at the fiber membrane, and poor aqueous oxygen solubility: ~0.22 mM at 37°C and 1 atm (10).
This oxygen limited transport problem is believed to be especially harmful to the maintenance of hepatocytes (the major parenchymal cell of the liver) within such a HF bioreactor because of their characteristically high oxygen demand (5, 11-14). This obstacle is of significant interest as a hepatocyte containing HF bioreactor represents a device that has a distinct possibility of finding use as both an in vitro liver model, and perhaps most importantly as a bioartificial liver assist device (BLAD, described further below) (6, 15-17). The development of such a device that could sustain patients suffering from certain types of end stage liver disease to the point where either native liver recovery can take place, or to the point where the patient has been stabilized and a liver can be found for transplantation, would be of immeasurable benefit to the medical community.

Oxygen limited transport in hepatic HF bioreactors is thus a major obstacle in the development of efficient and effective HF-based BLADs that
must be overcome in order to further the development of a practical BLAD (4, 5, 7, 8). In considering this challenge, it must be realized that hepatocytes are highly oxygen dependent, as evidenced by the fact that the human liver consumes 20-30% of the body's basal oxygen supply \textit{in vivo} (5, 11-14). Additionally, hepatocyte viability and several critical hepatocyte activities, such as their attachment to a matrix (hepatocytes are adherent cells), and much of their differentiated function, are highly dependent on adequate oxygen provision (5, 11-14).

Further complicating this oxygen transport picture is that delivery of sufficient oxygen to hepatocytes must be balanced with the fact that hyperoxic conditions (pO$_2$>160 mmHg) may cause cellular injury via formation of reactive oxygen species (14, 18). Within the liver's hepatic sinusoid, blood enters the periportal region via the hepatic artery and portal vein at a pO$_2$ of ~65 - 70 mmHg. Blood then leaves the sinusoid in the perivenous region via the central vein at a pO$_2$ of ~25 - 35 mmHg (see Figure 1.2). This oxygen gradient along the sinusoid capillary is believed to be at least partially responsible for the localization of cellular function along the sinusoid (referred to as functional zonation, see Figure 1.2) (14). There is some evidence within the literature that provision of oxygen to hepatocytes within a BLAD at levels similar to that seen \textit{in vivo} leads to a better functioning device. For example, one study found that oxygen gradient formation can help produce a heterogeneous distribution of protein levels in bioreactor cultures similar to that seen \textit{in vivo} (14).
Figure 1.2. This figure shows a schematic of a single liver sinusoid and a list of metabolic and synthetic activities that occur predominantly in either the periportal or perivenous regions (functional zonation).

There is thus considerable interest in exploring the impact of the local oxygen environment on hepatocytes maintained within a HF bioreactor. In order to ascertain the optimum hepatocyte space oxygen levels for maintaining hepatocytes within a HF bioreactor, the impact of local oxygen tensions (pO$_2$) within the hepatocyte space, and changes in the percent of the cells experiencing periportal, pericentral, or perivenous oxygen tensions on hepatocyte viability and function should be determined. However, accomplishing this aim requires that the oxygen carrying capacity of the aqueous culturing media stream circulating within HF bioreactors be improved in order to present increased oxygen levels to the maintained hepatocytes. This must be accomplished in a manner that does not preclude the provision of in vivo-like oxygen gradients over the bioreactor length (as this is expected
to yield optimum cellular function), and does not require that the hepatocytes be subjected to a hyperoxic environment. Bovine red blood cell (bRBC) supplementation of the circulating media presents one simplistic and novel means of accomplishing this aim, and consequently has been the focus of the work presented within this dissertation.

1.2 Acute Liver Failure: Primary Driving Force for the Creation of a BLAD

End-stage liver disease is estimated to claim 26,000 lives and to cost 9 billion dollars annually within the U.S. alone (19). The only effective treatment for end-stage liver disease remains orthotopic liver transplantation, OLT (20). Consequently, as of 2003, there were approximately 17,700 patients on the liver transplant waiting list within the U.S. alone. There were roughly 5,600 transplants performed in 2003, but over 1,800 people on the transplant list died that year waiting for an organ to become available (21). Unfortunately, OLT is problematic in that it requires immediately available organs, sufficient patient stability prior to the procedure, and costs between $70,000 to upwards of $240,000 per transplant (15, 22, 23). One form of end-stage liver disease generally treated by OLT is acute liver failure (ALF), which accounts for 6% of all liver disease related deaths (24), and is characterized by its sudden and rapid onset and high mortality rate (15, 22, 23, 25). However, unlike most instances of chronic liver failure, the liver often retains some capacity for regeneration after ALF, with spontaneous recovery occurring in approximately 25% of cases (23, 26). The physician must consider this along with the fact that OLT is typically most successful when carried out as early as possible
after diagnosis of ALF. This is a difficult task, and hence there is an obvious need for a liver assist device (LAD) that could be used as a bridge to either OLT or native liver regeneration.

1.3 Liver Assist Devices

In general, there are three categories of LADs; artificial, biological, and bioartificial. Hemodialysis (27), charcoal hemoperfusion (28), and plasma exchange (29) represent common examples of artificial LADs. These devices attempt to carry out the detoxification functions of the liver via artificial means. While some improvements in the neurological state of patients have been observed in limited clinical trials, in general these treatments have resulted in little improvement in the survival rate of ALF patients (6, 30, 31). Biological devices, which attempt to provide global liver support by replicating at least some portion of each of the major functions of the liver, include extracorporeal heterologous or homologous liver perfusion and cross circulation systems (25, 32). While showing some encouraging results in limited studies (33), these devices are severely limited by organ availability, inconsistent function, risk of infection, and limited life span (34).

The lack of effectiveness of purely artificial devices combined with the successes seen from the use of OLT and purely biological devices has largely pushed LAD research towards the BLAD (15, 16). This system utilizes an artificial device to house hepatocytes in an attempt to provide global liver support. The hepatic HF bioreactor is perhaps the most prominent of the proposed bioartificial devices (17). These devices, which are composed of a
large number of fibers enclosed in a cartridge, have been found to be clinically useful for hemodialysis due to the large surface area to volume ratio that results in high solute mass transfer coefficients (3). This large surface area to volume ratio should also be advantageous in the development of a BLAD. Additionally, the fibers within the HF device allow for cell attachment, and the bioreactor provides a three-dimensional structure similar to that seen in vivo. The approaches taken in constructing these devices are varied, and thus the following discussion of current hollow fiber BLAD devices is not intended to be comprehensive. More detailed information can be found elsewhere (3, 6, 15, 16).

Within BLADs, hepatocytes have been placed in either the inner-fiber space of the HF bioreactor (lumen) or in the space outside of these fibers (extracapillary space, ECS). The device created by Nyberg et al., which utilizes primary porcine hepatocytes (PPHs, \( \sim 6 \times 10^7 \)) suspended in a collagen solution, is an example of the former case where cells were inoculated into the lumen space (35, 36). As the collagen suspension gels, it contracts, thus providing a gap in the lumen through which culture medium can be perfused. However, the majority of BLADs currently in clinical trials utilize hepatocytes placed in the ECS. This configuration is believed to be advantageous in that turbulent flow should be reduced, thus minimizing blood clotting as the patient’s blood is passed through the lumen as opposed to the ECS (37). Devices that place hepatocytes in the ECS include the Arbios Systems Inc. HepatAssist® device (38-41), the Vitagen ELAD™ (26, 42, 43), and Gerlach’s
The HepatAssist® device utilizes collagen-coated dextran microcarriers to provide a large surface area for cell attachment of PPHs ($5 \times 10^9$). The Vitagen device makes use of the C3A hepatoma cell line by attaching $\sim 2 \times 10^{11}$ cells to cellulose acetate membranes with 70 kDa molecular weight cutoff pores. Finally, PPHs ($\sim 2.5 \times 10^9$) are attached to Matrigel™ coated fibers in the BELS device. This device is unique in that it is composed of four separate bundles of fibers and allows for a coculture of hepatocytes with sinusoidal endothelial cells in an attempt to improve their in vitro function (3, 46). The different bundles within this device are individually intended for plasma inflow, plasma outflow, oxygen delivery and carbon dioxide removal, and one containing the sinusoidal endothelial cells.

Unlike most other organs, the liver has several main functions, including synthetic, metabolic, biotransformation, and detoxification activities (3, 47, 48). The complexity of the liver and the broad set of functions it performs makes it unclear as to what specific liver functions are vital for patient survival, and consequently it has been difficult to establish a specific set of criteria that a potential BLAD must fulfill (15). However, one general requirement includes support of a minimum cell mass of at least 20-40% of the in vivo cell mass (a minimum of $10^{10}$ cells, as determined by liver biopsies from ALF patients) for at least several days (49, 50). Additionally, the device must be able to maintain normal hepatocyte metabolic functions, be easily accessible and maintainable in a hospital setting, and maintain sterility throughout its usage (50). Some basic detoxification and biotransformation
criteria for such a device have been set forth based on the accepted pathways of pathogenesis of hepatic coma in acute liver failure, including ammonia accumulation and endogenous benzodiazepine accumulation (49). Consequently, elimination of ammonia (via a functioning urea cycle and/or the production of glutamine) and biotransforming abilities (phase I cytochrome P450 activity and phase II conjugation reactions) must be conserved to at least some degree (49).

Given the uncertainty in the liver functions that must be replicated by a BLAD, it is desirable that a prospective device provide as much global support as possible. This should be considered in the design and engineering of the device, and in the selection of the type of hepatocyte implemented since performance will vary with the type of hepatocyte. A complete review of different types of hepatocytes has been provided by Stange and Mitzner (51). Primary human hepatocytes (PHHs), primary xenogenic hepatocytes (PXHs), hepatocyte cell lines (HCL) (6, 49), and stem cells (24, 52-54) are being considered for bioreactor implementation. PHHs, which would seem an ideal choice for liver support, are limited in their availability (6, 49) and tend to rapidly lose differentiated function in vitro (15, 55, 56). Improvements in bioreactor design and support matrix have increased the in vitro life span of primary hepatocytes (15), but maintenance of differentiated function over a prolonged period is still problematic (46). Primary porcine hepatocytes (PPHs) are the most commonly used PXH due to their availability, functional characteristics, and size (3, 57). However, the
The use of these cells presents the risk of immunological responses occurring in patients, escape of immunological molecules, and transmission of the opportunistic porcine endogenous retrovirus (49, 58, 59). The use of membranes with appropriate pore sizes reduces these risks, but they must still be considered. Storage issues are also of concern for primary cells, since they tend to lose differentiated function quickly (similar to PPHs) and thus cannot be kept indefinitely in culture (49). The most likely storage method, cryopreservation, is hampered by reduced viability levels upon thawing (60, 61).

The ability of hepatocyte cell lines to proliferate, which permits longer treatment periods and simplified storage, has made them an attractive option. However, the majority of cell lines that have been studied do not retain the same level of differentiated function that is witnessed initially in primary hepatocytes (51, 62). There are two general types of cell lines that are currently being studied in BLADs; tumor-derived and immortalized hepatocytes. The C3A hepatoma cell line is probably the most studied of the tumor-derived HCLs and is utilized in the Vitagen ELAD that is currently undergoing clinical trials (16, 49). This cell line is a clonal derivative of the Hep G2 line (a hepatoblastoma based cell line), and has been shown to produce high levels of albumin and to display strong nitrogen metabolizing activity (49, 63). Unfortunately, cytochrome P450 IA1 activity has been shown to be reduced in this cell line compared to primary hepatocytes (49, 51), though there are reports of inducible cytochrome P450 activity (34).
There is also concern regarding the potential for tumor cell propagation (62, 64), however; the use of immunoisolative membranes with appropriately sized pores and cell filters minimizes this risk (6).

Immortalization of hepatocytes typically occurs through either coculture with cells from another species (i.e. rat epithelial cells) or transfection with a replication defective recombinant retrovirus carrying a temperature sensitive variant of the simian virus 20 large tumor (SV40T) antigen gene (6, 49). Coculture with xenogenic cells can represent potential immunological disadvantages as previously described for primary xenogenic cells; however, several cell lines (such as HH01, HH02, HH08 and HH25) have been produced in this manner and exhibit maintenance of some level of differentiated function (65). The OUMS-29 (66) and NKNT-3 (67) cell lines have both been produced using the SV40T gene, and have been shown to express a variety of differentiated hepatocyte functions (49, 66). There have, however, been reports of the development of hepatocellular carcinoma in hepatocytes expressing SV40T within experimental animals (68), although tumorigenic features were not witnessed in initial work with the OUMS-29 line transplanted into athymic nude mice (69). Finally, efforts are also being made to produce immortalized human hepatocyte cell lines without using any oncogenes or carcinogens. One such cell line, HHY41, was produced from regular hepatocytes in culture within a collagen gel sandwich (70). This cell line synthesizes albumin and other proteins at levels comparable to those
found in vivo (71). Unfortunately, much of the cytochrome P450 activity is sustained at relatively low levels (70).

Stem cells, though only recently considered, represent a promising source of proliferating cells that could be directed to express the hepatocyte phenotype. The use of stem cells would also be beneficial in answering safety concerns with regards to immunological, and tumorigenic concerns as the cells could be drawn directly from the patient (24). Stem cells from the liver itself, from other adult populations, from fetal liver tissue, or from embryonic stem cells that undergo ex vivo differentiation are being considered (24). However, differentiation of embryonic stem cells towards exhibiting hepatocyte phenotype has thus far only been reported in murine cells (61, 72). Research examining the use of stem cells to produce mature hepatocytes for use in a BLAD is currently in its infancy, and much work remains before this becomes a viable option.

1.4 Oxygenation of HF-Based BLADs

Significant work remains for HF-based BLADs to progress to the commercial stage (16, 46). Optimization of such factors as cell loading, attachment matrices, hormone gradients, and mass transfer is still progressing. As previously mentioned in the motivation section, proper oxygen transport to the hepatocytes maintained within such a device is believed to be hampering its advancement (4, 5, 7, 8). This concern warrants specific, individual attention given that hepatocyte attachment, viability, and
much of its differentiated functions are dependent on proper oxygen provision (11-14).

Most current BLADs rely on either the patient’s venous blood ($pO_2 \sim 40$ mmHg) or an inline oxygenator to oxygenate either plasma (separated from the venous blood) or culturing media (when not attached to a patient) in an attempt to deliver sufficient oxygen to hepatocytes (17). However, analyses of various devices have often shown that providing appropriate oxygenation remains a limiting factor in the efficient and effective functioning of these devices (8, 36, 73). Venous blood is poorly oxygenated, and thus does not represent an effective means of providing adequate oxygenation. Inline oxygenators can be used to increase the dissolved oxygen within bioreactor inlet streams (culturing media or plasma). However, oxygen is sparingly aqueous soluble, and thus delivering media/plasma to the inlet of a HF device oxygenated to a physiological level (~70 mmHg) is expected to result in a significant anoxic region within the ECS. In an attempt to solve this problem, many groups have oxygenated bioreactor inlet streams to supraphysiological levels (~225 mmHg), which ignores physiological oxygen tensions and gradients within the device, and likely results in a portion of the cells being exposed to hyperoxic conditions that are toxic to the cells (14).

The addition of tetrameric hemoglobin (Hb) to the oxygenated circulating media represents one potential mechanism for improving oxygen delivery within a HF bioreactor without necessarily resorting to the use of supraphysiological oxygen tensions. In vivo, Hb serves to increase the
concentration of oxygen within human arterial blood by about 65 fold (from 130 µM to 8630 µM) (74). Hemoglobin’s oxygen binding capacity is provided by the heme group, a non-polypeptide prosthetic group present on each of the four globin chains making up the Hb tetramer. Each of these heme groups is capable of binding one molecule of oxygen. The oxygen binding properties of Hb are described by its oxygen half saturation point ($P_{50}$), and its cooperativity coefficient ($n$).

One previous study showed that the addition of several oxygen carriers, including PEG modified bovine Hb, buffer stabilized bovine Hb, and chemically stabilized bovine Hb to a HF bioreactor containing Hybridoma 3C11 cells resulted in improved antibody production (75). However, it was also found that the addition of normal bovine Hb to the HF system did not result in an improvement in antibody production. The oxidation of iron in the 2+ valence state within normal bovine Hb to produce methemoglobin (methemoglobin, metHb, is a form of Hb incapable of binding oxygen as the iron is in the 3+ valence state) was suggested as a likely reason for the lack of improvement in antibody production. Additionally, interaction of the heme group with many cell types can potentially provide a toxic environment to the respective cells (76-78), as was witnessed by Shia et al. when normal bovine Hb was added to a static culture of Hybridoma 3C11 cells at a concentration greater than 1% (w/v) (75). Further complicating the matter is the fact that Hb, which consists of two $\alpha$ and two $\beta$ polypeptide chains possessing a total molecular weight of about 68 KDa, can disassociate into pairs of $\alpha\beta$ dimers,
making it more difficult to isolate free Hb and its dimers from a cell culture (75, 76). However, many of the difficulties associated with the use of tetrameric bovine Hb could be alleviated by utilizing intact bovine RBCs (bRBCs) that contain Hb. Bovine RBCs are readily available and provide the added benefit of possessing an enzymatic reduction system to control the formation of metHb (77). As bRBCs are approximately 7 µm in diameter, their passage through the membrane of a HF device is prohibited, thus minimizing toxicity concerns that could result in harm to cultured hepatocytes. The large size of bRBCs is also important in that it allows HF membranes with larger pore sizes (70 kDa to 0.3 µm) to be utilized in a BLAD, facilitating increased solute transport across the membrane.

1.5 Cross-Linking BRBCs

Of additional interest is the fact that the rheological and O₂ binding properties of bRBCs can be engineered as desired via cross-linking (fixing) proteins both on the surface of the bRBC and within the bRBC using the 5-carbon dialdehyde cross-linker glutaraldehyde (Figure 1.3). Dialdehydes, in particular glutaraldehyde, have been used to cross-link cells for many purposes, including imaging via electron microscopy studies and rheological evaluation (79, 80). Cross-linking cells typically maintains the size, morphology, and internal structure of the cells in their pre-treated state (80), and, at least when glutaraldehyde is used as the cross-linker, the activity of many different cellular proteins (including Hb) is preserved (81). Glutaraldehyde is a nonspecific amino acid cross-linker, reacting with the
amino groups of lysine and valine amino acid residues, the sulfhydryl groups of cysteine residues, imidazole rings of histidine residues, and phenolic rings of tyrosine residues (82, 83). Hemoglobin polymers of various sizes have been synthesized using glutaraldehyde as a cross-linking agent (84). This process also alters the oxygen binding properties of the Hb polymer, and Hb polymers of varying sizes and oxygen binding properties have been investigated as potential artificial blood substitutes (85-89). When reacted with bRBCs, glutaraldehyde cross-links proteins on the surface of the cell. This imparts mechanical stability to the bRBC (90), reduces its deformability (91), and reduces diffusion of macromolecules across the cell membrane (92). Glutaraldehyde is also able to diffuse into the bRBC where it cross-links Hb molecules, thus altering its oxygen binding/dissociation properties. In previous studies conducted on human RBCs, the oxygen affinity of the hRBC was found to be increased ($P_{50}$ decreased) and its cooperativity diminished after glutaraldehyde cross-linking (90-92). The polymerization of intracellular Hb molecules combined with the increased stability and reduced deformability of the bRBC is expected to reduce shear-induced hemolysis in a HF media feed stream, thereby lessening the amount of free Hb in the circulating media. Lastly, of added benefit, glutaraldehyde treatment sterilizes the bRBC solution, thus inactivating pathogens and viruses that may be present in the bRBC suspension (92).
1.6 Objective and Scope of Research

The ultimate goal of the research presented within this dissertation is to utilize bRBCs (both normal and engineered) supplemented within the circulating media stream of a HF bioreactor to improve the oxygen carrying capacity of the respective media stream, and to consequently improve the oxygenation of hepatocytes maintained within the HF bioreactor. Utilizing bRBC supplementation of the circulating media feed stream, a rigorous study on the impact of differing oxygen concentrations throughout the ECS of a bioreactor on the function of hepatocytes (C3A cells) maintained within the device has been initiated. The effects of providing increased oxygen levels to the ECS were gauged by examining the oxygen lost from the circulating media within the bioreactor (which is related to hepatocyte oxygen consumption), the metabolic state of the hepatocytes (glucose consumption and lactate production), and their synthetic ability (synthesis of albumin). Additionally, a Krogh-based model was developed and employed to further explore the impact of bRBC supplementation on the hepatocyte space oxygen concentration within a HF bioreactor. Lastly, studies were initiated that were aimed at examining the potential of engineering bRBCs with properties desirable for oxygen delivery to cells maintained within a HF bioreactor.
1.7 Outline of Dissertation

The work presented herein provides evidence that supports the hypothesis that bRBCs can be supplemented to the circulating culturing media stream of a HF bioreactor in order to improve its oxygen delivering capacity. Studies (both experimental and modeling) have been undertaken that examine the ability of bRBCs to improve the oxygenation of hepatocytes maintained within a HF bioreactor. The impact of bRBCs on the metabolic state, synthetic abilities, and oxygen usage (judged by oxygen lost from a circulating media stream within the bioreactor) of the hepatocytes was also investigated. The influence of oxygenation on hepatocyte function is anticipated to be a strong predictor of the performance of these cells when maintained under similar oxygenation conditions within a HF bioreactor attached to an anhepatic animal model. Therefore, the results presented here, and the results of future studies discussed at the end of this dissertation, are expected to be of benefit not only to the future design of BLADs, but also to the development of a system to investigate how oxygen delivery impacts the phenotype of cells cultured within a 3-D environment.

The second chapter of the dissertation is devoted to presenting mathematical models that describe the oxygen transport processes involved within the HF bioreactor system. These models have been kept relatively simple as they are intended to provide a quick *a priori* means of examining the transport processes occurring within the bioreactor. The bioreactor essentially has two oxygen transport stages that are of interest. First, the
media is brought from a reservoir bottle to the entrance of the bioreactor using silicone tubing. During this period, oxygen travels from the ambient atmosphere of the incubator through the silicone tubing and dissolves into the flowing media stream, thereby oxygenating the media stream. A model describing this process was created to provide a means of predicting how much tubing would be required at a given oxygen concentration within the incubator ambient atmosphere in order to oxygenate a media stream from the average oxygen concentration in the reservoir to the desired oxygen concentration at the inlet of the bioreactor. Next, the media traverses the bioreactor, where it releases oxygen into the extracellular space of the device. A model based on the Krogh Tissue Cylinder was formulated to describe the release of oxygen from the media stream to the hepatocytes in the ECS based on the oxygen consumption rate of the hepatocytes and other factors characteristic of the system.

The experiments conducted within a hepatic HF bioreactor with bRBC supplementation are described within the third chapter of this dissertation. These experiments utilized C3A hepatoma cells as the type of hepatocytes maintained. Throughout the initial study, the metabolic state (judged by glucose consumption and lactate production) of the cells and their synthetic abilities (albumin production) were gauged. A subsequent experiment was performed in order to obtain a measure of oxygen lost from the circulating media within the C3A cell containing bioreactor (directly related to C3A cell oxygen consumption).
The fourth chapter of this dissertation examines the impact of glutaraldehyde cross-linking of bRBCs on several properties of the bRBCs. As previously discussed, the oxygen binding/dissociation properties and osmotic strength of the bRBCs can be altered, potentially creating a novel oxygen carrier that could be used preferentially over normal bRBCs in many HF bioreactor cell culture situations. Consequently, the impact of this cross-linking reaction on several properties of the bRBCs was examined. The Krogh-based model described in the second chapter was also utilized to provide a priori predictions as to the impact that these engineered bRBCs (based on their measured oxygen binding/dissociation properties) would have on the oxygen transport carrying capacity of a HF bioreactor media stream that feeds a hepatic HF bioreactor.

Lastly, the fifth chapter is provided as a concluding chapter. This chapter summarizes the main conclusions drawn from the work presented within the dissertation and outlines the expected future progression of this project.
CHAPTER 2

MATHEMATICAL MODELING OF OXYGEN TRANSPORT WITHIN A HF BIOREACTOR SYSTEM

2.1 Introduction

2.1.1 Background

The main goal of this project is to examine the impact of the local hepatocyte-space oxygen environment on the ability of the hepatocytes to perform metabolic, biotransformation, and detoxification functions. This dissertation describes the beginning of a series of experiments aimed at achieving this goal and outlines the expected future progression of the project toward this goal. However, this process can be simplified, thus saving both time and money, by examining oxygen distribution within the system under a given set of operating conditions (namely the oxygen content of the circulating media at the inlet to the bioreactor, which is a function of both the dissolved oxygen concentration within the media and the bRBC supplementation concentration of the circulating media, and the flow rate of the circulating media) prior to laboratory experimentation via mathematical modeling. The development of a mathematical model of oxygen transport within the hepatic HF bioreactor system would give:
• The ability to determine the length of silicone tubing required at a specific ambient incubator pO$_2$ in order to oxygenate the circulating media stream from a given pO$_2$ (at the media reservoir) to a desired pO$_2$ (at the entrance to the bioreactor).

• The ability to examine the impact of different oxygen carriers and operating conditions (such as the bioreactor inlet pO$_2$) on the oxygen levels provided to cells maintained within the ECS of the bioreactor.

• The ability to estimate the amount of ECS exposed to each of the oxygenation zones that hepatocytes experience in vivo, along with the amount of cells exposed to non-physiological pO$_2$ values.

• The ability to estimate oxygen consumption parameters ($V_{max}$, which is the maximum rate of oxygen consumption, and $K_m$, which is referred to as the Michaelis-Menten parameter and describes the pressure at which the actual consumption rate is half of $V_{max}$) for the hepatocytes.

2.1.2 Research Overview

The development of relatively simplistic mathematical models describing oxygen transport within this HF bioreactor system was carried out and is illustrated within this chapter because of the potential benefits described above. This entailed constructing a model for each of the two oxygen transport situations of interest within the HF bioreactor system. The first of these models presented within this chapter describes the oxygenation of the circulating media as it travels within the silicone tubing on its way from the media reservoir to the HF bioreactor. The second model presented was created to depict the loss of oxygen from the media to the hepatocytes within the HF bioreactor.
2.2 Theoretical Background – Oxygenation of the Circulating Media Stream

The entire experimental HF bioreactor system is displayed in Figure 2.1. The first process of interest that occurs is the oxygenation of the media stream as it passes through a silicone tube of known radius and length on its way to the inlet of the HF bioreactor. In modeling this oxygenation process, a tube of known length, $L$, placed within a controlled atmosphere and containing flowing media supplemented with bovine red blood cells was considered. The geometry for this situation is shown in Figure 2.2. Oxygen from the ambient incubator air passes through the silicone tubing and dissolves into the flowing media. The oxygen then diffuses radially within the continuous plasma. During this process, oxygen is internalized by the red blood cells (which are assumed to be uniformly dispersed throughout the silicone tubing), where it binds to hemoglobin (assumed to be instantaneous as oxyHb is in equilibrium with dissolved oxygen). Lastly, both dissolved and bound oxygen are transported in the axial direction by convection. Detailed descriptions of this model can be found in the literature (93-95).
Figure 2.1. This figure shows a schematic of the HF bioreactor system. The media stream is oxygenated within the silicone tubing as it is carried from the reservoir to the bioreactor, and then oxygen is lost from the media stream within the bioreactor due to hepatocyte consumption.

Figure 2.2. This figure shows the silicone tubing geometry utilized within the mathematical model describing the oxygenation of the circulating media stream. Media supplemented with bovine RBCs enters the tube of known radius, $R$, at an inlet $\text{pO}_2$ of $P_i$, and exits the tube after traveling a distance, $L$, at an exit $\text{pO}_2$ of $P_{\text{out}}$. The tube is placed within a controlled atmosphere with a constant bulk $\text{pO}_2$, $P_w$, which is the value at the wall of the tube.
In creating a basic model of this oxygenation process, several general assumptions can be made to simplify this problem, including:

- The bRBC suspension is considered to be a continuous hemoglobin solution. This is reasonable given that the bRBCs are expected to be uniformly dispersed since the diameter of the silicone tube is much greater than approximately 200 µm (74). In tubes with a larger diameter, a plasma layer does not develop as the bRBCs do not preferentially migrate to the centerline. Additionally, the resistance of the bRBC membrane to oxygen transfer is considered negligible, thus allowing for the suspension to be viewed as a hemoglobin solution.

- Oxygen is considered to chemically react with hemoglobin instantaneously and reversibly. This is expected to be valid in larger diameter tubes as the major resistance to transport is expected to be the diffusion of oxygen through the media.

- The hemoglobin contained within the bRBCs does not escape its enclosure.

- The silicone tubing presents relatively little oxygen mass transfer resistance in comparison to the diffusion of oxygen through the media (94-96). Consequently, the pO2 in the bulk air of the incubator is considered to be that at the outside edge of the flowing media.

- The oxygenation process is angularly symmetric, and thus there is no dependence on the angular coordinate.

- Convective transport in the radial direction is considered negligible as there is no radial velocity. Axial diffusive transport is also negligible. The justification of the later assumption is shown via the Peclet number, which is a ratio of convection to diffusion, and is as shown:

\[
Pe = \frac{\pi R^2 U_{ave} \frac{P_i}{H}}{\pi R^2 D \frac{P_i}{L \times H}} \gg 1
\]  

(2.1)

where \(U_{ave}\) is the average media velocity in the tube, \(H\) is the Henry’s law constant, \(D\) represents the diffusivity of oxygen within the media, and the remaining variables are as earlier defined. Using the characteristic parameters of the HF system given below in Table 2.1 (Section 2.3), the Peclet number is found to be much greater than 1, and thus axial diffusion can be ignored.
A conservation of mass balance for oxygen flowing within a cylindrical tube can be written as:

\[
D \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC}{dr} \right) + \frac{1}{r^2} \frac{d^2 C}{d\theta^2} + \frac{d^2 C}{dz^2} \right] + K = \frac{dC}{dt} + U_r \frac{dC}{dr} + U_\theta \frac{dC}{r d\theta} + U_z \frac{dC}{dz} \tag{2.2}
\]

where \( r \) is the radial coordinate within the tube, \( z \) is the axial component within the tube, \( \Theta \) is the angular coordinate within the tube, \( C \) is the dissolved oxygen concentration, \( t \) is time, \( K \) represents the oxygen reaction term (in this case oxygen loss as it is picked up by the bRBCs), \( U_r, U_\theta, \) and \( U_z \) represent the bRBC suspension velocities in the radial, angular, and axial directions respectively, and the remaining variables are as earlier specified. The bracketed terms on the left of the equation describe the transport of oxygen by diffusion, \( K \) represents a generation/consumption term, the first term on the right side of the equation represents an accumulation term, and the remaining terms on the right side of the equation describe the transport of oxygen by convection. Considering the assumptions described above and how they regulate the oxygen transport scenario within Figure 2.2, equation 2.2 can be reduced to:

\[
D \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC}{dr} \right) \right] + K = U_z \frac{dC}{dz} \tag{2.3}
\]

Similarly, a conservation of mass balance equation can be written for the transport of oxygen bound to hemoglobin within a cylindrical tube.
\[
D \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC'}{dr} \right) + \frac{1}{r^2} \frac{d^2C'}{d\theta^2} + \frac{d^2C'}{dz^2} \right] + G = \frac{dC'}{dt} + \frac{U_r}{r} \frac{dC'}{d\theta} + U_z \frac{dC'}{dz} \]

(2.4)

where \( C' \) represents the concentration of oxygen bound to hemoglobin, \( G \) represents the release of oxygen from the bound state (and thus the production of dissolved oxygen), and the remaining variables are as earlier defined. Considering the above described assumptions, and that the hemoglobin cannot diffuse, equation 2.4 can be reduced to:

\[
G = U_z \frac{dC'}{dz} \]

(2.5)

Equations 2.3 and 2.5 are linked through the generation/consumption terms (\( G \) and \( K \)) as they are simply the negatives of each other. The combination of these two equations leads to:

\[
D \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC}{dr} \right) \right] - U_z \frac{dC}{dz} = U_z \frac{dC}{dz} \]

(2.6)

The concentration of hemoglobin bound oxygen and dissolved oxygen can also be linked through an oxygen dissociation curve. This curve plots the fractional saturation of hemoglobin bound to oxygen against the concentration of dissolved oxygen within a given suspension. The relationship between bound and dissolved oxygen can be expressed as:

\[
\frac{dC'}{dz} - \frac{dC}{dz} \left( \frac{dC'}{dC} \right) = m \frac{dC}{dz} \]

(2.7)
After combining this with the use of Henry’s law ($pO_2 = HC$), equation 2.6 can be rewritten as:

$$D \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dpO_2}{dr} \right) \right] = U_z (1 + m) \frac{dpO_2}{dz} \quad (2.8)$$

The \( m \) term, which is dimensionless, can be related to the \( pO_2 \) of the bRBC suspension using Henry’s law:

$$m = \frac{dC'}{dC} = HC'_{sat} \frac{dY}{dpO_2} \quad (2.9)$$

where \( C'_{sat} \) represents the concentration of oxygen bound to hemoglobin when the hemoglobin within the suspension is fully saturated with oxygen, \( Y \) represents the fractional saturation of hemoglobin bound to oxygen, and the remaining variables are as earlier specified. An equation for hemoglobin saturation can be written utilizing Hill’s equation:

$$Y = \frac{(pO_2)^n}{(pO_2)^n + P_{50}^n} \quad (2.10)$$

where \( n \) represents the cooperativity of the hemoglobin solution and \( P_{50} \) the \( pO_2 \) where half of the hemoglobin oxygen binding sites are saturated. The \( m \) term can now be expressed as:

$$m = nP_{50}^nHC'_{sat} \frac{(pO_2)^{n-1}}{((pO_2)^n + P_{50}^n)^2} \quad (2.11)$$
The entrance length of tubing required for the velocity profile to become fully-developed laminar flow (Poiseuille flow) is less than ~3 cm (97), and thus the velocity profile can be represented as:

\[ U_z = 2U_{ave} \left( 1 - \left( \frac{r}{R} \right)^2 \right) \]  

(2.12)

The initial condition for this situation is known given the inlet pO\textsubscript{2} value, and the boundary conditions are known because the pO\textsubscript{2} at the tube wall is specified and due to the existence of axial symmetry. These conditions are respectively:

\[ P(z=0) = P_i \]  

(2.13)

\[ P(r=R) = P_w \]  

(2.14)

\[ \left. \frac{dP}{dr} \right|_{r=0} = 0 \]  

(2.15)

Equation 2.8 can now be solved numerically. This was accomplished using the partial differential equation (PDE) solver command \texttt{pdepe} that is part of the Matlab program (The MathWorks, Inc., Natick, MA, version 6.5). The code for this Matlab program can be found as Appendix A.1. The \texttt{pdepe} command can be utilized to solve initial-boundary value problems for systems of parabolic and elliptic PDEs. The PDEs must be in the one space variable \( x \), and time \( t \). A second-order spatial discretization based on specified spacing in both the \( x \) and \( t \) directions is used to convert the PDEs to ODEs. The \texttt{ode15s} solver is utilized for the time integration. This method is
described further in the literature (98). In order to apply this solver to the problem described here, the radial component of the cylinder is considered as the one space variable, $x$, and the axial element of equation 2.8 is represented by the time component that is expected by this PDE solver.

The Matlab program provided the oxygen partial pressure as a function of both the radius and the axial distance into the silicone tubing. However, ultimately the average $pO_2$ value for any given distance into the tube is of interest as this is the value that will be measured by an oxygen probe incorporated into the system. This value can be determined from:

$$P_{\text{ave}} = \frac{\int_0^R 2\pi r P(r,z)dr}{\pi R^2} \quad (2.16)$$

where the integral is determined using Simpson’s 1/3 rule.

Lastly, an estimate of the length of tubing required before the oxygen concentration becomes fully-developed ($L_D$) is provided for by (74, 99):

$$L_D > 0.05^*Re^*Sc^*D_c \quad (2.17)$$

where $Re$ is the Reynolds number, $Sc$ is the Schmidt number, and $D_c$ is the tube diameter. According to this equation, the length of tubing required before the oxygen concentration becomes fully developed is greater than the length of silicone tubing. Consequently, the boundary layer for oxygen transport into the tube will still be forming at the end of the silicone tubing. Within the boundary layer, the oxygen concentration would be expected to
change rapidly. In order to accurately account for this, the radial step-change must be kept small (thus the pO$_2$ profile is found for a large number of radial points). In order to ensure that the boundary layer was adequately considered, the model was solved three times for several random cases, each with a different sized radial step-change. The radial step-change was considered small enough when the average pO$_2$ calculated for the exit of the tubing did not change significantly with a further reduction in the size of the radial step-change.

2.3 Analysis of the Media Stream Oxygenation Model

The media stream oxygenation model was compared to data generated from an experiment designed to examine the oxygenation of a media stream flowing through silicone tubing. Briefly, this experiment was conducted by flowing Dulbecco’s Modified Eagles Medium, which had had nitrogen bubbled through it in order to reduce the dissolved oxygen concentration, at two different flow rates and through varied lengths of silicone tubing. Flow through dissolved oxygen probes (Lazar Research Laboratories, Los Angeles, Ca, model DO-166FT) were attached at the beginning of the silicone tubing and at a known length of the silicone tubing from the inlet probe. The probes were calibrated using water saturated with air and zeroed with a saturated sodium sulfite solution prior to the experiment. At each combination of tube length and media flow rate, the inlet and outlet pressures were recorded over a period of at least 15 minutes to ensure
stability of the readings. The characteristics of this experimental system are given within Table 2.1.

The described mathematical model was run using the property values given in Table 2.1 and compared to the experimentally obtained oxygenation results. Since the circulating media was not supplemented with bRBCs in the experiment, $C_{sat}$ was set to zero within the model. Figure 2.3 shows the model predicted percent increase in media dissolved oxygen concentration for each combination of tube length, media flow rate, and initial media $pO_2$. As can be seen, the model tends to predict the outlet $pO_2$ about 5-15 % higher than the experimentally obtained results. This is likely an indication that the silicone tubing does offer some resistance to oxygen mass transport. However, the simple model offered here was found to provide a reasonable estimate as to the amount of oxygen picked up by a media stream circulating at a known flow rate within a specified length of silicone tubing.

TABLE 2.1

CHARACTERISTICS OF A MEDIA STREAM OXYGENATION EXPERIMENT

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion coefficient, $D$</td>
<td>$2.3 \times 10^{-5}$</td>
<td>cm$^2$/sec</td>
</tr>
<tr>
<td>Average velocity, $U_{ave}$</td>
<td>3.3, 6.6</td>
<td>cm/sec</td>
</tr>
<tr>
<td>Silicone tubing radius, $R$</td>
<td>0.159</td>
<td>cm</td>
</tr>
<tr>
<td>Initial oxygen partial pressure within the tubing, $P_i$</td>
<td>~50-90</td>
<td>mmHg</td>
</tr>
<tr>
<td>Tubing wall oxygen partial pressure, $P_w$</td>
<td>~155</td>
<td>mmHg</td>
</tr>
<tr>
<td>Tube length, $L$</td>
<td>~50-350</td>
<td>cm</td>
</tr>
</tbody>
</table>
Figure 2.3. This figure shows a comparison of the silicone tubing media oxygenation model with experimentally collected media stream oxygenation data at three different tube lengths \((L)\) and two different media velocities \((U_{ave})\). Each bar shows the percent increase in the average \(pO_2\) within the tube from the initial oxygen probe \((P_{in})\) to the downstream oxygen probe for each respective situation. For the experimentally collected data, the percent increase shown was calculated from the average of measurements taken over at least 15 minutes (to ensure steady state readings). The model was then run using the experimentally found average initial \(pO_2\) \((P_{in})\) for each case. Legend: ■ is the experimental data and □ is the model predicted data.
The HF bioreactor system utilized within the experimental studies described in chapter 3 contains about 2 meters of silicone tubing. The system is as described in Table 2.1, with the exception that the diffusivity of oxygen within media at 37°C is around $3 \times 10^{-5}$ cm$^2$/sec, the concentration of saturated hemoglobin is expected to vary from 0-4400 µM (0 – $2.5 \times 10^9$ bRBCs/mL), the average media velocity can vary, and the bioreactor inlet and wall pO$_2$ values can vary. The oxygenation of the media stream within a given hepatocyte experiment can thus be analyzed via the description of the system provided by these parameters.

Ultimately, the pO$_2$ of the circulating media at the entrance to the bioreactor should ideally be ~70 mmHg, and ~25-30 mmHg at the exit of the bioreactor. Consequently, after leaving the bioreactor, the media stream must be oxygenated back to ~70 mmHg within the silicone tubing before it returns to the bioreactor. The model can be utilized to investigate this requirement, as shown in Figure 2.4. Within this figure, the media stream pO$_2$ is predicted over the 2 meters of silicone tubing for the situation of ~$5 \times 10^8$ bRBCs/mL (~10% of the human in vivo concentration) media supplementation, a $P_w$ of 155 mmHg, and varied media flow rates as given within the figure. As can be seen from the figure, at the slowest flow rate, it is predicted that the media pO$_2$ can be brought back to the desired level under the specified conditions. However, for the remaining flow rates (which are more likely to be utilized within an experiment), the media pO$_2$ falls short of the required value at the bioreactor entrance. Consequently, it becomes
apparent that either the ambient pO$_2$ within the incubator needs to be increased or the length of the silicone tubing needs to be increased in order to re-oxygenate the media stream to ~70 mmHg.

This mathematical model can thus be utilized to investigate the mechanism of oxygenation of the media stream within the HF bioreactor system used in these studies. Unfortunately, as experimentation is still in its early stages, the results of this model have not yet been utilized to alter the

![Figure 2.4](image)

Figure 2.4. This figure shows the media stream pO$_2$ after traveling a given length of silicone tubing as predicted by the model for differing media flow rates. The remaining parameters describing the situation are given in the text. Legend: $U_{ave} = 3$ cm/min; $U_{ave} = 5$ cm/min; $U_{ave} = 6.5$ cm/min; $U_{ave} = 8$ cm/min.
conditions under which a media stream is oxygenated. The example above (shown in Figure 2.4) describes a desired situation, but one that has not yet been encountered within these experimental studies. Regardless though, this example was included to demonstrate the future worth of this model in analyzing employed HF bioreactor systems, and in demonstrating the potential benefits that utilizing a model such as this can have on experimental design. Most practically, the model will be utilized in the future to assist in determining the required pO$_2$ within the ambient incubator atmosphere in order to oxygenate the circulating media as desired. However, the model could also be utilized to investigate the design of the tubing system in order to optimize oxygen transfer. This would involve examining the impact of tube radius, length, and media flow rate on the oxygenation process.

2.4 Theoretical Background – Oxygen Transfer to the Hepatocytes in the ECS

The second oxygen transfer process of interest that occurs within the HF bioreactor system is the loss of oxygen from the circulating media to hepatocytes maintained within the ECS of the bioreactor cartridge. Describing this process mathematically is of considerable interest, and consequently several approaches can be found within the literature (7, 8, 10, 100, 101). Most of these approaches are based on the model created by Krogh and Erhlang (102). This original model was created to describe the transfer of oxygen to muscle tissue from the capillary that feeds the tissue. The transport phenomena that occurs within this process includes the diffusion of dissolved oxygen from within the capillary to the capillary wall.
Simultaneously, oxygen is released from the flowing red blood cells, which are relegated to the inner-capillary volume because of their size, as the dissolved oxygen concentration decreases (based on the assumed equilibrium between dissolved oxygen and oxyHb). At the capillary wall, oxygen diffuses radially through the wall where it reaches the tissue space. Once in the tissue space, oxygen continues to diffuse radially and is consumed by the tissue. This consumption of oxygen thus provides the driving force for oxygen transport within the system.

At a basic level, the Krogh model carries with it several assumptions that greatly simplify the situation (74). Some of the main assumptions include:

- The tissue space is considered a continuous phase rather than as being comprised of discrete cells so that the diffusion of $O_2$ through this space can be described by a simple effective diffusivity.
- Blood flowing through the capillary is considered to be in plug flow.
- The particulate nature of blood is ignored, as is any mass transfer resistance provided by the RBC membrane. Hb within the RBC is carried along at the same velocity as the RBC, and thus there is no diffusive transport of Hb.
- The capillary membrane is ignored due to its relatively minimal thickness.
- Tissue oxygen consumption occurs by a zero-order process.
- The physical situation is composed of numerous capillaries, all assumed to be evenly spaced and parallel to each other. Consequently, a cylinder of tissue of specific radius is assumed to be fed by only one capillary, and thus there is no oxygen transport outside of the far edge (also called the Krogh radius, $R_t$) of the tissue.
Within the capillary, radial convective transport is considered negligible as there is no radial velocity. Axial diffusive transport is also negligible as the Peclet number (Eqn. 2.1) is found to be ~250 for an average capillary. Within the tissue space, transport is only by radial diffusion as there is no flow in either the axial or radial directions. Axial diffusion is considered negligible as the ratio of oxygen transport by radial diffusion to axial diffusion is much greater than unity:

\[
\frac{C_o \cdot D'}{\frac{2\pi R_c L}{R_t - R_c}} = \frac{2R_c L^2}{(R_t - R_c)(R_t^2 - R_c^2)} \sim 2000
\]

where \(D'\) represents the diffusivity of oxygen within the tissue space and \(R_c\) the radius of the capillary.

The majority of mass transfer resistance arises within the tissue space as a result of the dense growth of cells causing a relatively small diffusion coefficient in comparison to that found in the capillary. Consequently, the concentration of dissolved oxygen within the capillary can be radially averaged.

The above listed assumptions are inherent to the fundamental Krogh model. The derivation of this model begins with a conservation of mass balance for dissolved oxygen within the capillary, for oxygenated Hb within the capillary, and for dissolved oxygen within the tissue space:

\[
D \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC}{dr} \right) + \frac{1}{r^2} \frac{d^2 C}{d\theta^2} + \frac{d^2 C}{dz^2} \right] + K = \frac{dC}{dt} + U_r \frac{dC}{dr} + \frac{U_b}{r} \frac{dC}{d\theta} + U_z \frac{dC}{dz}
\]

\[
D' \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC'}{dr} \right) + \frac{1}{r^2} \frac{d^2 C'}{d\theta^2} + \frac{d^2 C'}{dz^2} \right] + G = \frac{dC'}{dt} + U_r \frac{dC'}{dr} + \frac{U_b}{r} \frac{dC'}{d\theta} + U_z \frac{dC'}{dz}
\]

\[
D' \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC'}{dr} \right) + \frac{1}{r^2} \frac{d^2 C'}{d\theta^2} + \frac{d^2 C'}{dz^2} \right] - \Gamma = \frac{dC'}{dt} + U_r \frac{dC'}{dr} + \frac{U_b}{r} \frac{dC'}{d\theta} + U_z \frac{dC'}{dz}
\]

where \(\Gamma\) represents the metabolic consumption of oxygen by the tissue, \(C'\) the dissolved oxygen concentration within the tissue, and the remaining symbols...
are as defined earlier in section 2.2. Given these equations and the
previously mentioned assumptions, it is straightforward to solve the system
and obtain a set of coupled equations that describe the radially averaged
capillary $\text{pO}_2$ and the tissue $\text{pO}_2$ (74).

The equations describing oxygen transport in the lumen are again
linked via the $m$ term as previously explained. One simplistic means of
handling the $m$ term is to assume it is a constant, $M$, and is calculated from
the average capillary $\text{pO}_2$ over the length of the capillary. This requires an
initial estimate of the capillary $\text{pO}_2$ at the end of the capillary in order to find
the average capillary $\text{pO}_2$. Using the calculated $M$ value, the solution can be
obtained via equations 2.22 and 2.23. The final calculated capillary $\text{pO}_2$ is
then compared to the estimated value, and the process is repeated until the
two converge within a specified error tolerance. Equations 2.22 and 2.23 are:

\[
\langle \text{pO}_2(z) \rangle = \langle \text{pO}_2 \rangle_{in} - \frac{\Gamma H^t}{(1+M)U_{avg}} \left[ \left( \frac{r_1}{r_c} \right)^2 - 1 \right] z \tag{2.22}
\]

\[
\text{pO}_2^t(r,z) = \langle \text{pO}_2(z) \rangle - \frac{r_c^2 \Gamma H^t}{4D^t} \left[ 1 - \left( \frac{r}{r_c} \right)^2 \right] - \frac{r_c^2 \Gamma H^t}{2D^t} \ln \left( \frac{r}{r_c} \right) \tag{2.23}
\]

where $\langle \text{pO}_2(z) \rangle$ represents the radially averaged capillary $\text{pO}_2$, $H^t$ is the
Henry’s constant in the tissue space, and $\text{pO}_2^t$ the tissue $\text{pO}_2$.

The HF bioreactors utilized in the experiments described in this
dissertation (originally shown in Figure 1.1) can essentially be considered as
containing a grouping of evenly-spaced capillaries feeding surrounding tissue.
As a result of their even-spacing, a model of oxygen transport within the HF bioreactor need only consider an individual fiber and its surrounding membrane and ECS. Figure 2.5 shows a schematic of such an individual fiber that runs through a bioreactor and its surrounding membrane and ECS. Initially, the basic Krogh model described above, with the exception of how the $m$ term was handled, was employed to examine the HF bioreactor systems utilized. This $m$ term varies considerably with significant changes in dissolved oxygen concentration, and consequently using a single value for a fiber of the length seen within the HF bioreactor (12 cm in the bioreactors utilized here) that is feeding cells with a high oxygen demand can lead to significant error in the determination of $pO_2$. Instead, a localized $m$ value was used when calculating the $pO_2$ in the lumen at any given point. This value was calculated from the average of the three preceding calculated radially-average lumen $pO_2$ values. A marching method was then used to calculate the $pO_2$ values within the lumen beginning at the inlet and proceeding to the exit of the bioreactor. This model was created within Matlab (version 6.5).

![Figure 2.5](image)

Figure 2.5. This figure shows a schematic of an individual bioreactor fiber, membrane, and surrounding ECS.
Unfortunately, a few of the original assumptions applied to the Krogh tissue cylinder are at least somewhat problematic with a fiber of the dimensions (both lumen radius and wall thickness) seen within these HF bioreactors. Within a capillary (which has a diameter of about 10 µm or less), there is significant formation of a marginal zone, which means that the flowing red blood cells migrate to the centerline of the capillary \(^{103}\). Additionally, there is a relatively small distance that oxygen must diffuse to reach the capillary wall. These two phenomena help to justify the assumptions of plug flow and the radial averaging of the capillary \(pO_2\). However, within a fiber with the diameter of that seen in the HF bioreactors used in these studies (>~200 µm), little marginal zone formation is expected \(^{74}\). In these fibers, the flow can be better represented as Poiseuille flow (equation 2.12) as the Reynolds number places the flow within the laminar region \((Re<100)\) and entrance effects are negligible. The radial dependence of the dissolved oxygen concentration within the fiber must thus be considered. Also of interest is the assumption that the capillary wall imposes little mass transfer resistance. Regrettably, the larger thickness of the fiber membrane (>15 µm) in comparison to the human capillary (~0.5 µm) results in a significant additional oxygen diffusion distance that must be considered. Additionally, the hepatocytes maintained within the ECS consume oxygen more in agreement with Michaelis-Menten kinetics rather than the constant consumption imposed by zero order kinetics \((104, 105)\). Michaelis-Menten kinetics (described by equation 2.24) claim that oxygen consumption is based on the available
dissolved oxygen concentration, the maximum oxygen consumption rate ($V_{\text{max}}$), and a Michaelis-Menten parameter ($K_m$) that is the pO$_2$ that corresponds to an actual oxygen consumption rate that is half of the maximal value. Consequently, as the dissolved oxygen concentration decreases, the oxygen consumption rate of the hepatocytes will also decrease. At these lower dissolved oxygen concentrations, the hepatocytes will reduce or even shut off many of their functions in order to direct the limited available oxygen towards essential survival functions (11-13). Lastly, it should also be mentioned that the assumption regarding the treatment of the hepatocyte space as a continuous phase likely introduces some inaccuracy into the model. This is largely because of the adherent nature of the hepatocytes and that oxygen consumption will not be uniform within each individual hepatocyte. This assumption could potentially be relaxed through the inclusion of concentric rings of hepatocytes within the ECS, each with differing oxygen consumption parameters, or by multiplying the oxygen consumption rate for the hepatocytes by a radially decreasing function. However, in the interest of maintaining simplicity, this assumption was preserved within the mathematical model presented in this dissertation.

$$\text{O}_2 \text{ consumption rate} = \frac{V_{\text{max}} \times \text{pO}_2}{K_m + \text{pO}_2} \quad (2.24)$$

The additional transport barrier imposed by the membrane requires the addition of a third shell balance:
The three differential equations describing oxygen transport within the fiber, membrane, and ECS are respectively:

\[
D^m \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dC^m}{dr} \right) + \frac{1}{r^2} \frac{d^2C^m}{d\theta^2} + \frac{d^2C^m}{dz^2} \right] = \frac{dC^m}{dt} + U_r \frac{dC^m}{dr} + U_\theta \frac{dC^m}{d\theta} + U_z \frac{dC^m}{dz} \tag{2.25}
\]

The boundary conditions required to solve this system are as follows:

\[
D^m \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dpO_2^m}{dr} \right) \right] = 2U_{avg} \left( 1 - \left( \frac{r}{R_o} \right)^2 \right)(1 + m) \frac{dpO_2}{dz} \tag{2.26}
\]

\[
\left[ \frac{d}{dr} \left( r \frac{dpO_2^m}{dr} \right) \right] = 0 \tag{2.27}
\]

\[
D^i \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dpO_2^i}{dr} \right) \right] = \frac{H^i V_{max} pO_2^i}{K_m + pO_2^i} \tag{2.28}
\]

where \( pO_2^m \) represents the pO\(_2\) within the membrane, \( m \) is as defined in equation 2.11, and all other variable are as earlier defined. These equations indicate that within the fiber, transport is by diffusion only in the radial direction and convection only within the axial direction (no radial velocity and \( Pe >> 1 \)). Within the membrane, there is no flow in either the radial or axial direction, and thus transport only occurs in the radial direction by diffusion (equation 2.18 >> 1). Lastly, within the hepatocyte space, there is again no flow in either the radial direction or axial direction, and transport is thus only in the radial direction by diffusion (equation 2.18 >> 1).
For $0 \leq z \leq L$ and $0 \leq r \leq R_c$:

**BC1**: $p_O^2 (z = 0) = p_O^2 \text{(inlet)}$  \hspace{1cm} (2.29)

**BC2**: \[
\left. \frac{dp_O^2}{dr} \right|_{r=0} = 0 \hspace{1cm} (2.30)
\]

**BC3**: $p_O^2 (r = R_c) = p_O^m (r = R_c)$ and $D \left. \frac{dp_O^2}{dr} \right|_{r=R_c} = D^m \left. \frac{dp_O^m}{dr} \right|_{r=R_c}$  \hspace{1cm} (2.31)

For $0 \leq z \leq L$ and $R_m \leq r \leq R_t$:

**BC4**: $p_O^l (r = R_m) = p_O^m (r = R_m)$ and $D^l \left. \frac{dp_O^l}{dr} \right|_{r=R_m} = D^m \left. \frac{dp_O^m}{dr} \right|_{r=R_m}$  \hspace{1cm} (2.32)

**BC5**: \[
\left. \frac{dp_O^l}{dr} \right|_{r=R_t} = 0 \hspace{1cm} (2.33)
\]

These differential equations (equations 2.26-2.28) were solved using Matlab (version 6.5, see Appendix A.2). It is important to recognize that the fiber and ECS equations are linked through the continuity boundary conditions at the inner and outer edges of the fiber membrane. Also, the bioreactor was axially discretized into small intervals in order to allow for a marching solution approach. The lumen equation at each axial point was first solved using the *pdepe* command in Matlab. However, the third boundary condition, which is for the wall of the lumen and necessary in order to solve the lumen equation, requires knowledge of the pressure on the membrane side at the lumen wall. In order to accomplish this at the inlet of the bioreactor (the first axial point, $z=0$, at which the equations are solved), zero-order oxygen consumption kinetics (constant oxygen consumption) within the
ECS was assumed (oxygen consumption rate = $V_{\text{max}}$). This allowed for the ECS equation to be solved analytically, which then allowed for the membrane equation to also be solved analytically using the continuity boundary condition at the outer edge of the membrane wall. The continuity boundary condition can then be used at the inner edge of the membrane with the analytical solution to the membrane equation in order to provide the required final boundary condition for the lumen equation. The analytical solution to the membrane equation was used to provide the pO$_2$ distribution within the membrane at the inlet of the bioreactor. Lastly, the differential equation describing oxygen transport within the ECS was re-solved using Michaelis-Menten kinetics rather than using the zero-order oxygen consumption assumption. This differential equation was numerically solved using the $bvp4c$ Matlab command.

The third boundary condition was found in a different manner for the remainder of the bioreactor. For the $z_i$ axial point, the numerically found derivative at the ECS side of the membrane/ECS wall for the $z_{i-1}$ axial point was utilized to solve the membrane equation and also the lumen wall boundary condition, which was needed to solve the lumen equation. The axial step-change was kept small in order to account for rapid pO$_2$ changes. This procedure allowed for Michaelis-Menten kinetics to be employed both in the ECS, and in determining the driving force for oxygen transfer from the lumen into the membrane and ECS for the remainder of the bioreactor. A copy of this Matlab code can be found in Appendix A.2.
The assumption of zero order oxygen consumption kinetics within the ECS in order to determine the driving force for oxygen transfer from the lumen at the inlet of the bioreactor is expected to be valid as long as the $pO_2$ within the ECS at the inlet remains significantly greater than the $K_m$ value for the hepatocytes. In the case where the $pO_2$ drops near the $K_m$ (which could occur when using bioreactors with thick membranes, a large ECS volume surrounding each fiber, and cells with a high $V_{max}$), the assumption of zero order oxygen consumption kinetics at the inlet will result in a significantly inflated oxygen transfer driving force at the bioreactor inlet. This would consequently introduce considerable error into the solution of the differential equations governing oxygen transfer within this system.

In order to solve this problem, these equations have been solved using FEMLAB (COMSOL, Inc., Burlington, MA, version 3.1), which allows for the three equations to be easily solved simultaneously. Consequently, the assumption of zero-order oxygen consumption kinetics at the bioreactor is not required. This work has been conducted by Jesse Sullivan, a fellow graduate student within the Palmer research group, whose research has been focused on the modeling of oxygen transport within the HF bioreactor. However, within this dissertation, the Matlab solution (Appendix A.2) discussed here is utilized to investigate oxygen transport within a bioreactor. Those cases that present problems for this solution approach are indicated as they are encountered (sections 3.2.6 and 3.3.5).
2.5 Illustrating the HF Bioreactor Oxygenation Problem via Modeling

The model described above can be utilized to investigate the oxygen environment of hepatocytes maintained within a representative HF bioreactor. For these simulations, the bioreactor described in section 3.2.1 of this dissertation is employed. Briefly, this bioreactor has a length of 12 cm, a lumen radius of 90 µm, a wall thickness of 15 µm, and a Krogh radius of 160 µm. Unless otherwise specified, the diffusivity of oxygen within the lumen and within the cellulosic membrane is $3 \times 10^{-5}$ cm$^2$/sec, and that within the ECS is $2 \times 10^{-5}$ cm$^2$/sec. The diffusivity of this cellulosic membrane has not been reported in the literature. In estimating the oxygen diffusivity of the membrane, it is assumed to be fully hydrated, and consequently the diffusivity is taken to be that of oxygen in water ($\delta$).

The predicted impact of bRBC supplementation of culturing media feeding a hepatic bioreactor is shown in Figure 2.6. In this case, the flow rate through the system was designated as 30 ml/min (and thus the velocity through each fiber was $\sim 0.7$ cm/sec), the bioreactor inlet $pO_2$ as 70 mmHg, the $V_{max}$ as 15 µM/s, and the $K_m$ as 5 mmHg (these oxygen consumption parameters have been previously presented as approximately representative for C3A cells maintained within a HF bioreactor ($\delta$)). Bovine red blood cell supplementation was taken at 10% of the human in vivo RBC concentration, and it is assumed that the void volume within the ECS is negligible. The figure shows the $pO_2$ at the lumen centerline, at the edge of the lumen, at the far edge of the membrane, and at the far edge of the ECS (the Krogh radius).
As can be seen, solely relying on the dissolved oxygen within the circulating media is expected to be problematic as the limited oxygen supply is quickly consumed within the bioreactor. In this case, it would be expected that less than a third of the bioreactor would be experiencing adequate oxygenation. However, supplementation of the circulating media stream is shown to significantly aid in oxygen delivery to the cell culture. Under these hypothetical conditions, it would be expected that no part of the bioreactor ECS would experience anoxic conditions.

As each of the operating parameters describing the bioreactor impacts the oxygen delivery situation, it is important to utilize the model to compare the predicted benefits of bRBC supplementation to the impact that other user-controlled parameters could potentially have on oxygenation. Essentially, the user-controlled parameters of this system include bRBC supplementation concentration, media flow rate, the pO$_2$ of the media at the bioreactor inlet, the oxygen consumption characteristics of the hepatocytes ($V_{max}$ and $K_m$), and the oxygen binding/dissociation characteristics of the supplemented bRBCs. The impact of the oxygen binding/dissociation characteristics of the supplemented bRBCs is discussed in chapter 4, and thus will not be considered within this section of the dissertation. An easy means of comparing the impact of each of these parameters on oxygen delivery is to normalize the predicted global bioreactor oxygen consumption rate (rate of oxygen loss to the hepatocytes within the bioreactor) in each case to a base
Figure 2.6. This figure shows the model predicted pO$_2$ profiles at the lumen centerline (r=0), lumen edge (r=R$_c$), membrane edge (r=R$_m$) and ECS edge (r=R$_t$) for a representative bioreactor (characterized within the text). The pO$_2$ profiles are shown for the case of no bRBC supplementation (blue), and bRBC supplementation at 10% of the human in vivo RBC concentration (red).

case, which was taken to be the situation presented above with no bRBC supplementation, a media flow rate of 30 ml/min, a $V_{\text{max}}$ of 15 µM/s, a $K_m$ of 5 mmHg, and a bioreactor inlet pO$_2$ of 70 mmHg. The predicted global bioreactor oxygen consumption rate was calculated based on the predicted oxygen loss rate from the circulating media between the inlet and exit of the bioreactor.
The impact of bRBC supplementation concentration, media flow rate, and the bioreactor inlet pO$_2$ on the predicted global bioreactor oxygen consumption rate is shown in Figures 2.7, 2.8, and 2.9 respectively. As can be seen from the figures, oxygen delivery to a hepatic HF bioreactor can be improved in a variety of manners. Figure 2.7 shows that a relatively minimal bRBC supplementation concentration is predicted to significantly improve

![Graph showing the impact of bRBC concentration on global bioreactor oxygen consumption rate.

Figure 2.7. This figure shows how oxygen provision to a representative bioreactor is predicted to improve with increasing bRBC supplementation of the circulating media stream. The global bioreactor oxygen consumption rate is presented as a ratio with respect to a base case (no bRBC supplementation). The characteristics of the bioreactor are presented within the text.
oxygen delivery to the maintained hepatocytes. However, to significantly improve oxygen delivery via oxygenating the circulating media stream (increasing the media stream dissolved oxygen concentration at the inlet of the bioreactor), the model predicts that the inlet $pO_2$ must be raised to an excess of 200 mmHg (Figure 2.8). This also complicates the formation of \textit{in vivo}-like oxygen gradients along the length of the bioreactor (as shown in Figure 2.10). The impact of increasing the average media velocity through

![Figure 2.8](image)  
Figure 2.8. This figure shows the impact that varying the bioreactor inlet dissolved oxygen concentration within the circulating media stream has on the model predicted global bioreactor oxygen consumption rate (shown as a ratio in comparison to the base case described within the text).
the bioreactor is shown in Figure 2.9, and as expected, oxygen delivery can be increased with increasing media velocity. However, the model predicts that the average media velocity must be approximately quadrupled in order to achieve the same improvement in oxygen delivery as attained through a minimal bRBC supplementation concentration (<5% of the human in vivo concentration). It is again also easier to maintain in vivo-like oxygen gradients utilizing bRBC supplementation rather than using an increased

Figure 2.9. This figure shows the impact that the average media velocity within the lumen of a given fiber has on the model predicted global bioreactor oxygen consumption rate (shown as a ratio with respect to the base case described within the text).
media velocity. In other words, it is easier to control the system for oxygen delivery within a physiological range through bRBC supplementation of the circulating media. This is a result of the manner in which oxygen is released from bRBCs according to the oxygen dissociation curve. Consequently, significant amounts of oxygen will be held within the bRBCs and then released within the bioreactor when the lumen pO$_2$ is in a physiological range.

Figure 2.10. This figure shows the impact on the model predicted ECS pO$_2$ distribution of increasing the pO$_2$ (to 200 mmHg at the bioreactor inlet, shown as the area between the blue lines) within the circulating media as compared to supplementing the circulating media with bRBCs (at a concentration of 10% of the human in vivo RBC concentration, shown as the area between the red lines). As can be seen, simply increasing the pO$_2$ at the inlet of the bioreactor results in a significant portion of the bioreactor exposed to either hyperoxic (z<~4 cm) or hypoxic conditions (z>~6 cm).
Lastly, the oxygen consumption characteristics of the maintained hepatocytes can impact the amount of oxygen that must be delivered to the bioreactor. This is important as differing types of hepatocytes do not necessarily have the same oxygen requirements. For instance, hepatic cell lines, such as the C3A cell line, are expected to have reduced metabolic demands in comparison to primary hepatocytes (6). Figure 2.11 shows the predicted ECS oxygen profiles for different $V_{\text{max}}$ values. As can be seen, as $V_{\text{max}}$ increases, the circulating media stream cannot supply sufficient oxygen without further improvement of its oxygen carrying capacity (increasing the bRBC supplementation concentration). Consequently, primary hepatocyte cultures could potentially benefit even more from media stream bRBC supplementation.

Figure 2.12 indicates that the $K_{\text{m}}$ value has a relatively minimal impact in comparison to the $V_{\text{max}}$ value. The figure shows the impact that this term has on the pO$_2$ profile within the ECS space of a bioreactor maintained under the same conditions as those described for the bRBC supplementation case outlined within Figure 2.6. Within the Michaelis-Menten equation, the $K_{\text{m}}$ value serves to reduce the oxygen consumption rate as the pO$_2$ drops. Consequently, as $K_{\text{m}}$ increases, the actual hepatocyte oxygen consumption rate will be predicted to drop quicker with a decreasing ECS pO$_2$. However, practical $K_{\text{m}}$ values typically range from about 0.5-5.6 mmHg for hepatocytes (8), and therefore this term is expected to have a minimal impact on the ECS pO$_2$ profile as long as the pO$_2$ is greater than ~15-20 mmHg.
Figure 2.11. This figure shows the model predicted impact that the maximum hepatocyte oxygen consumption rate ($V_{\text{max}}$) has on the oxygen profile within the ECS for a bioreactor operating under the same conditions as those of the bRBC supplementation case shown in Figure 2.6. As can be seen, increasing the $V_{\text{max}}$ value results in an increased oxygen delivery demand. The $V_{\text{max}}$ values shown in this figure include 5 µM/s (red), 15 µM/s (blue), 25 µM/s (green) and 50 µM/s (black).
Figure 2.12. This figure shows the model predicted impact of the $K_m$ value on the ECS pO$_2$ profile for a bioreactor maintained under the same conditions as those specified for the bRBC supplementation case presented in Figure 2.6. As can be seen, this term has relatively minimal impact until the ECS pO$_2$ drops below ~15-20 mmHg. Also, as $K_m$ increases, the ECS pO$_2$ profile decreases at a slower rate. The $K_m$ values shown in this figure include 0.5 mmHg (red), 3 mmHg (blue), and 5 mmHg (green), and each set of curves provides the boundaries for the ECS pO$_2$ profile.
2.6 Concluding Remarks

As described in the introduction to this chapter, there is obvious benefit to examining specific processes occurring within a system, such as the transfer of oxygen within the hepatic HF bioreactor, via a modeling approach. The accurate modeling of oxygen transport within the hepatic HF bioreactor would allow for future experimental studies to be more focused on utilizing operating conditions likely to lead to the most ideal ECS oxygen levels.

Therefore, as this project was developing within the laboratory, time was also devoted to modeling the transport of oxygen within the system. Eventually, a second graduate student began to work on the modeling aspect of this project, and accordingly only the initially developed models are presented within this dissertation.

The first model presented within this chapter described the transfer of oxygen from the ambient air within an incubator into a media stream flowing within a silicone tube. This model can be utilized to estimate the ability of the HF system to oxygenate the flowing media stream after it has left the bioreactor in an oxygen-depleted state. Consequently, an estimate of the length of silicone tubing required and the pO$_2$ of the ambient incubator air necessary in order to adequately re-oxygenate the circulating media stream can be made. Lastly, the model was compared to experimentally collected media stream oxygenation data to show that the model as presented here slightly over-predicts the media stream pO$_2$ after a given distance traveled through a silicone tube. This is most likely a result of the assumption that the
tube wall, which is made of silicone, provides negligible resistance to oxygen mass transfer. However, given the desire to keep the initial models simplistic and the relatively small error witnessed (5-15% over-prediction of the media stream pO$_2$ at the exit of the tubing), this model is believed to be useful as a tool in analyzing the hepatic HF bioreactors employed within this project.

The second oxygen transport process of interest within this system is the loss of oxygen from the circulating media to the hepatocytes maintained within the ECS of the bioreactor. The second model presented within this chapter describes this situation through three linked differential equations and is based on the famous Krogh Tissue Cylinder model. Initially employed in a very basic manner that is more applicable in describing oxygen transport from an in vivo capillary to surrounding tissue, the model was expanded to account for such important phenomena as the resistance posed by the lumen wall, to provide for a more realistic media velocity profile (which impacts the transport of oxygen), and to incorporate a more realistic description of the oxygen consumption rate of the maintained hepatocytes. The development of the model to this point is described within this chapter. However, the model as presented here will over-predict the driving force for oxygen transfer from the lumen to the ECS when a significant portion of the ECS at the bioreactor inlet is subjected to low dissolved oxygen concentrations (where the pO$_2$ is approaching the $K_m$ value). This is mainly expected to occur with the use of a bioreactor that has a large ECS volume surrounding each individual fiber along with hepatocytes that exhibit a substantial $V_{max}$. This problem has
since been alleviated within a similar model of the system created within the FEMLAB program by a fellow graduate student whose work is focused on the modeling of oxygen transport within this system.

Lastly, the model as presented within this dissertation was used to demonstrate the problem of oxygen delivery to hepatocytes maintained within a representative HF bioreactor. A comparison was made between the predicted pO$_2$ profiles for a bioreactor being fed with bRBC supplemented media and media lacking bRBC supplementation. Figure 2.6 clearly shows that the model predicts that the bRBC supplemented media is better suited for oxygen delivery to maintained hepatocytes. The model was then utilized to perform a basic investigation into the other main user-controlled parameters of the HF system that could potentially impact oxygen transport. Figures 2.7, 2.8, and 2.9 show the impact of bRBC supplementation, the media stream dissolved oxygen concentration at the inlet of the bioreactor, and the media stream velocity within the bioreactor on oxygen delivery. As can be seen, increasing the bRBC supplementation concentration, the initial media stream dissolved oxygen concentration, and the media stream velocity can all improve the amount of oxygen delivered to the maintained hepatocytes. However, significant increases in the media stream dissolved oxygen concentration and velocity are required in order to match the oxygen carrying capacity of a media stream supplemented with bRBCs. Additional problems, such as maintaining in vivo-like oxygen gradients also makes bRBC supplementation an attractive option for improving oxygen delivery to
maintained hepatocytes. The final figures presented within this chapter show the predicted impact of the hepatocyte oxygen consumption characteristics on the pO$_2$ profiles within the ECS. As expected, an increase in the hepatocyte $V_{max}$ results in an increased demand on the oxygen delivery capability of the circulating media stream. This is important to consider given that different types of hepatocytes have differing oxygen consumption characteristics, with primary cells expected to require significantly more oxygen than most hepatocyte cell lines. The $K_m$ value, the other oxygen consumption characteristic, was shown to have minimal impact on the ECS pO$_2$ profiles. This parameter impacts the profiles as the pO$_2$ drops below ~15-20 mmHg, and according to the Michaelis-Menten equation, serves to show that hepatocyte oxygen consumption is reduced at low oxygen tensions.
CHAPTER 3

THE IMPACT OF CIRCULATING MEDIA BRBC SUPPLEMENTATION ON C3A HEPATOMA CELLS MAINTAINED WITHIN A HF BIOREACTOR

3.1 Introduction

3.1.1 Background

As described in the introductory chapter of this dissertation, the selection of hepatocyte cell type utilized within a hepatic HF bioreactor is of significant importance. Differentiated function varies significantly with cell type and must be considered in conjunction with the liver functions required to sustain life. Additionally, the ease of maintaining the hepatocytes in cell culture, the length for which they can be maintained, and their nutritional requirements vary among the different cell types (51). One type of cell that has received significant attention is the hepatocyte cell line, especially the C3A hepatoma cell line. This type of cell line presents an interesting option for use within a hepatic HF bioreactor given that it generally has reduced nutritional requirements in comparison to primary cells (including oxygen dependence), and can easily be maintained in culture for an extended period of time due to its ability to proliferate (6). However, these cells typically
exhibit reduced levels of many hepatocyte differentiated functions in comparison to that exhibited by primary cells (51, 62).

The C3A hepatoma cell line, which is a clonal derivative of the Hep G2 hepatoblastoma cell line, has been subjected to fairly extensive analysis as it has been the cell type of choice within many hepatic bioreactors, including the ELAD (Extracorporeal Liver Assist Device) currently undergoing clinical trials (16, 49). Consequently, this cell line was also utilized within the HF bioreactor oxygenation studies presented in this chapter. One of the main advantages offered in using this cell line is that the cells will proliferate for an extended period of time, thus allowing for extended studies. There are reports within the literature of the maintenance of this cell line within culture for upwards of 8 months (34). Additionally, these cells are commercially readily available and do not require a complex harvesting process. As a result of their extensive use, C3A cells have been fairly well characterized. For instance, it is known that they produce high levels of albumin and exhibit a robust nitrogen metabolizing activity (106, 107). Albumin is an important hepatocyte product as it is utilized within the blood for pH and osmotic pressure regulation, and it serves to bind and deliver various toxins to the hepatocytes for biotransformation or detoxification (74). Nitrogen metabolism is important since the hepatocytes must serve to prevent the accumulation of ammonia within the blood. This is accomplished through either the conversion of ammonia into glutamine or glutamate, or through the use of ammonia within the urea cycle (49).
3.1.2 Research Overview

As mentioned above, C3A hepatoma cells have seen extensive use within HF bioreactor cell culture, including use within the ELAD which is currently undergoing clinical trials (3, 26). Even though these cells likely have a reduced oxygen demand in comparison to primary cells, one modeling analysis of the ELAD has shown that when fed with a patient’s venous blood (as would likely be the case when attached to a patient), it is still likely functioning with significant regions of non-physiological oxygen levels (8). Consequently, this cell line was employed within the HF bioreactor oxygenation studies described in this chapter.

These oxygenation studies relied on the supplementation of the circulating culturing media with bovine red blood cells in order to improve oxygen delivery to the maintained C3A cells. An initial experiment was conducted that was aimed at indirectly assessing the ability of media stream bRBC supplementation to improve oxygen delivery to the C3A cells in comparison to a media stream lacking bRBC supplementation. This experiment relied on the measurement of hepatocyte metabolic (glucose utilization and lactate production) and synthetic (albumin production) functions in order to gauge the oxygen environment present within each of the bioreactor’s ECS. The results from this study have been published within the literature (108).

After the conclusion of the first experiment described above, oxygen probes were acquired for measuring the amount of oxygen lost from the
circulating media to the hepatocytes within the bioreactor (referred to as the global bioreactor oxygen consumption, GBOC). This prompted a second experiment utilizing C3A cells within the ECS of a HF bioreactor. This experiment was conducted similarly to the initial experiment described in sections 3.2.1-3.2.5, with the exception of the differences detailed in section 3.2.6. While the metabolic functions (glucose consumption and lactate production) were again followed in order to gauge the health of the cells, this experiment was conducted solely in order to determine the GBOC rate for a bioreactor containing C3A cells. Unfortunately, the bioreactor utilized within this study possessed a small number of fibers (~80), and thus the cell space surrounding each individual fiber was large ($R_m = 0.0215$ cm and $R_t = 0.0348$ cm). Consequently, this bioreactor presented an example of a situation in which the Krogh-based model described in section 2.4 did not accurately model oxygen transport within the system. Therefore, only the experimentally found data is presented within this dissertation. A rigorous modeling study from these results has been undertaken and published by a collaborating graduate student, and the C3A cell oxygen consumption characteristics ($V_{max}$ and $K_m$) found from this published study are presented within this chapter (109).
3.2 Materials and Methods

3.2.1 The Hollow Fiber Bioreactor System

The hollow fiber bioreactor system employed in the studies described in this chapter was the commercially available CellMax® system (Spectrum Laboratories, Rancho Dominguez, CA). A schematic of this system was shown earlier as Figure 1.1. A picture of the actual bioreactor system is shown here in Figure 3.1. Within the system, culturing media was pumped from the reservoir bottle into the hollow fiber cartridge via about 2 feet of silicone tubing (reduced from the ~2 m of tubing that is contained within the system as purchased) possessing a radius of ~0.159 cm. This silicone tube permits gas transfer between the incubator’s ambient atmosphere and the circulating media, and thus the circulating media was oxygenated as it traveled to the bioreactor from the reservoir. A mathematical model describing this process was presented earlier within section 2.2. The culturing media then entered the lumen of the bioreactor where it delivered nutrients and oxygen to the C3A cells maintained within the ECS of the bioreactor and removed various released toxins and metabolites via dilution into the circulating media. Mass transport between the lumen and the ECS occurred via diffusion. A mathematical model describing the transport of oxygen from the lumen into the ECS was also presented earlier in section 2.4.

The HF bioreactors selected for these studies were purchased from Spectrum labs. However, the cartridges selected for each experiment were not necessarily identical. Within this first study, the cartridge selected,
Figure 3.1. This figure shows a digital picture taken of the HF bioreactor system utilized within the studies described in this chapter.

catalog #410-011, was composed of a cellulosic membrane containing pores with a 95% 50 kDa molecular weight cutoff. This cartridge contained ~2800 fibers, a lumen volume of ~8.5 mL, and an ECS volume of ~15 mL. The small molecular weight cutoff of this bioreactor was considered beneficial in that it prevented free hemoglobin (Hb that had been released from the bRBCs due to hemolysis of the cells within the media stream) from crossing the fiber walls and entering the cell space. This is beneficial as free hemoglobin can be toxic to cell cultures (75-78). However, this small pore size within the membrane of these fibers also prevented transport of albumin (with a
molecular weight of ~68 kDa) and other high molecular weight proteins from the ECS, where they were synthesized by the hepatocytes, through the fiber walls and into the flowing media. This complicated the determination of the C3A cell production rate of albumin, and required the periodic flushing of the ECS in order to obtain media aliquots for assay of albumin.

During the duration of all experiments, the HF bioreactor system was maintained at 37°C, 21% O₂, and 5% CO₂ within a Heraeus (Kendro Laboratory Products, Hanau, Germany) incubator. The CO₂ concentration within the incubator was verified periodically with the use of a Fyrite (Bacharach, New Kensington, PA) instrument. These conditions were intended to be biomimetic, and to thus provide an ideal environment for cell culture.

3.2.2 Cell Inoculation and Maintenance

The C3A cells used in these studies were purchased frozen from the ATCC (Manassas, VA, Cat. #CRL-10741). A picture of these cells plated on a petri dish can be seen as Figure 3.2. Upon arrival, the cells were immediately thawed in a 37°C water bath, counted using a hemacytometer, and analyzed for determination of the percent of the cells remaining viable using a trypan blue stain that specifically dyes the cell membranes of dead cells. The cell count and viability for the first experiment was ~7.4×10⁶ cells and ~93% respectively. The cells were then inoculated into two separate HF bioreactors via the ECS ports (~3.7×10⁶ cells per cartridge). The culturing media utilized within this study was composed of 90% Eagle’s Minimum
Essential Medium (MEM; ATCC, Cat. #30-2003), 10% fetal bovine serum (FBS; ATCC, Cat. #30-2020), and 0.2% Normocin (an antibiotic/antimycotic agent; Invivogen, San Diego, CA, Cat. #ant-nr-o). In the bioreactor system designated as the experimental system, sterile, washed bRBCs (Quad Five, Ryegate, MT, Cat. #943) were added to the media at ~5×10^8 cells per mL (~10% of the human \textit{in vivo} RBC concentration). The other system was not supplemented with bRBCs and was designated the control system. A third HF bioreactor system was employed that utilized the same media formulation as the experimental system (including bRBC supplementation), but lacked an actual HF bioreactor. Therefore, this system was simply media circulating through silicone tubing. This third system was provided in order to gauge the extent of metabolic functions (bRBCs maintain anaerobic glycolytic and pentose phosphate pathways) occurring within the bRBCs in order to account for these functions in the experimental system. Lastly, media flow in each system was maintained at ~33 mL per minute.

Figure 3.2. A brightfield image of C3A cells plated on a collagen-coated petri dish (20X). The image dimensions are 0.25 mm × 0.25 mm.
Throughout the study, the reservoir (which was initially 125 mL and later increased to 250 mL) was replaced with fresh complete media every 24-48 hours (fresh bRBCs were also replaced at the same time within the experimental system). At the same time that the media reservoirs were replaced, an aliquot was also taken from both the spent media being removed from each system and the fresh media being placed into each system. These aliquots were utilized to determine hepatocyte glucose consumption and lactate production. Additionally, since albumin is too large to diffuse from the ECS into the flowing media, the ECS space was flushed with complete media every 2-4 days (lacking bRBCs in both systems) to provide a sample for the determination of albumin production by the hepatocytes. As hepatocytes are adherent cells (requiring a surface to attach to in order for them to remain viable), only dead cells should have been flushed from the system when the ECS was flushed.

3.2.3 Glucose, Lactate, and Albumin Assays

The aliquots collected from the media reservoirs were stored at -20\(^\circ\)C until they were analyzed for glucose consumption, lactate production, and albumin synthesis. Glucose consumption was determined via a commercially available colorimetric assay kit (Diagnostic Chemicals Limited, Oxford, CT, Cat. #220-32). The concentration of glucose within the sample was determined via the production of Quinoneimine (Q) dye according to the following reaction scheme:
\[
\beta-D\text{-}glucose + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} D\text{-}gluconic Acid + H_2O_2
\]

\[
H_2O_2 + \text{hydroxybenzoate} + 4\text{-}aminoantipyrine \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O
\] (3.1)

Therefore, the concentration of the Quinoneimine dye was followed via UV spectroscopy as it was produced, and through comparison to a known standard (5 mM glucose), it was possible to quantify the concentration of glucose in each sample. The final amount of glucose consumed was determined by analyzing the glucose concentration within an aliquot prior to the reservoir being placed into the HF bioreactor system, and within an aliquot taken from the same reservoir after it was removed from the HF bioreactor system. Lastly, each analysis was carried out in triplicate on a plate reader (Bio-tek, Winooski, VT).

Lactate concentration was also determined using a commercially available colorimetric assay kit (Biomedical Research Service Center, SUNY at Buffalo, Buffalo, NY, Cat. #A-108). In this assay, lactic acid was utilized to protonate NAD\(^+\) with lactate dehydrogenase acting as a catalyst. This in turn ultimately resulted in the reduction of a tetrazolium salt to formazan via a coupled reaction. The formazan caused the sample solution to turn a bright red color, making it easy to follow via spectroscopy. Similar to the glucose assay, aliquots from the reservoir prior to being placed into the system and after being removed from the system were analyzed against a set of standards (25 \(\mu\)M – 400 \(\mu\)M) in order to determine the amount of lactate.
produced by the hepatocytes. Each sample was run in duplicate for this assay, and the assay was again carried out on the same plate reader as previously mentioned.

Lastly, albumin synthesis was followed with the aid of a commercially available enzyme linked immunosorbent sandwich assay kit (Bethyl Laboratories, Montgomery, TX, Cat. #E80-129). Briefly, this assay is based on the binding of albumin to the surface of a well cell within an immunoplate. Each well was first coated with a capture antibody (goat anti-human albumin-affinity purified antibody). A blocking solution (mixture of Tris buffer and bovine serum albumin in a sodium chloride solution) was then added to block available sites still remaining on the surface of the well cell. The sample and standards were next added. The albumin present within the sample or standard then binded to the capture antibody while all other components of the aliquots were washed away. Next, a detection antibody (goat anti-human albumin-HRP conjugate) was added. The detection antibody, which binded to the captured albumin, had first been conjugated with an enzyme that reacted with the addition of a detection substrate (TMB). This antibody-substrate reaction resulted in the formation of a yellow color that could be followed by spectroscopy. The reaction was stopped with the addition of sulfuric acid, and the albumin present in the sample was quantified by comparison with known standards. In the analysis presented here, each aliquot taken from the ECS was analyzed in duplicate on the plate reader. Again, aliquots were taken prior to entering the HF system and after being removed from the
system in order to determine the amount of albumin produced by the C3A cells.

3.2.4 BRBC Oxygen Binding/Dissociation Properties

The oxygen binding/dissociation properties ($P_{50}$ and $n$) of the bRBCs were determined after each shipment of washed bRBCs was received. These properties were analyzed using a Hemox™-Analyzer (TCS Scientific Corporation, New Hope, PA). In order to analyze the bRBCs, a suspension was first created by mixing 5 mL of Hemox Buffer (TCS Scientific Corporation), 20 µL of Hemox A additive (TCS Scientific Corporation), 10 µL of an antifoaming agent (TCS Scientific Corporation), and 50 µL of the original bRBC suspension into a holding cell that was maintained at 37°C within the instrument. The instrument then oxygenated the suspension to a pO$_{2}$ of ~150 mmHg using a compressed air stream, and then subsequently de-oxygenated the suspension via the addition of nitrogen gas. A Clark oxygen electrode was employed by the instrument in order to measure the oxygen tension within the sample suspension. During the de-oxygenation process, the instrument followed the change in concentration of deoxyHb via dual wavelength spectrophotometry. This measurement technique takes advantage of the fact that the absorption spectrum of oxyHb and deoxyHb differ at most wavelengths between 500 and 600 nm, but do crossover at a few wavelengths (referred to as isosbestic points). The absorbance of the sample suspension will thus remain essentially constant throughout the deoxygenation process at the isosbestic points. However, at a wavelength of
558 nm, deoxyHb exhibits its maximum absorbance while the absorbance of oxyHb is at a local minimum. The measured absorbance at this wavelength will thus change drastically during the deoxygenation process as the deoxyHb concentration increases. The Hemox thus measures this absorbance change against the isosbestic point found at a wavelength of 568 nm in order to provide raw deoxyHb absorbance data as a function of \( pO_2 \). Utilizing this raw data in conjunction with the Adair equation, a complete oxygen binding/dissociation curve was generated. In order to accomplish this though, the fractional oxygen saturation of the bRBCs at a given oxygen tension within the suspension (\( Y \)) needs to be determined using the raw absorbance values. This would ideally be accomplished according to:

\[
Y = \frac{\text{Abs}(pO_2 = i) - \text{Abs}(pO_2 = 0)}{\text{Abs}(pO_2 = \infty) - \text{Abs}(pO_2 = 0)}
\]

where \( \text{Abs} \) refers to the absorbance at the specified \( pO_2 \) value and \( i \) simply denotes each individual measured absorbance value. Utilizing this equation (equation 3.2), it must be assumed that the Hemox can both fully oxygenate and fully de-oxygenate each suspension. However, this is not the case, and consequently absorbance values at these two points must be determined. This was accomplished by combining equation 3.2 with the Adair equation, and considering the maximum and minimum absorbance values as two additional parameters, \( A_0 \) and \( A_\infty \):
\[
Y = \frac{\text{Abs}(pO_2 = i) - A_0}{A_\infty - A_0} = \frac{A_1 pO_2 + 2 A_2 pO_2^2 + 3 A_3 pO_2^3 + 4 A_4 pO_2^4}{4(1 + A_1 pO_2 + A_2 pO_2^2 + A_3 pO_2^3 + A_4 pO_2^4)}
\]  

(3.3)

where \(A_1, A_2, A_3, \) and \(A_4\) are empirical Adair parameters. Initial guesses for each of the 6 parameters \((A_0, A_\infty, A_1, A_2, A_3, \) and \(A_4\)) were then fed into a Matlab code (version 6.5, see Appendix A.3) that carried out a 6-parameter nonlinear regression on the raw data in order to determine the Adair parameters. To check the accuracy of the generated parameters, these fitted parameters were utilized within the Adair equation to produce a graph of absorbance values with respect to oxygen partial pressure. These Adair calculated absorbance values were then compared to the raw absorbance values to determine the accuracy of the non-linear regression. If the accuracy of the generated Adair parameters did not agree within a desired tolerance level to the raw absorbance value, new initial guesses were utilized within the Matlab code in order to generate refined Adair parameters. Lastly, these Adair parameters were utilized to generate a complete oxygen binding/dissociation curve, from which the \(P_{50}\) and cooperativity of the bRBCs were determined.

3.2.5 Determination of BRBC MetHb Concentration

As discussed in the introduction of this dissertation, the formation of metHb from Hb is troublesome as metHb, which is an oxidized form of Hb, is incapable of binding oxygen. Consequently, it was important throughout these studies to monitor the metHb concentration within the circulating media in order to ensure that it did not climb to problematic levels. In order to
measure the metHb concentration, the Hb had to be freed from the interior of the bRBCs. This was accomplished by centrifuging (500×g for 15 minutes) a reservoir media aliquot immediately after each reservoir bottle was removed from the HF system in order to pellet out the bRBCs. The supernatant was removed and cold distilled water (the oxidation rate of Hb to metHb is reduced at cooler temperatures) was added to the pellet in order to lyse the cells. After being allowed to sit for at least 24 hours at 4°C (to ensure complete lysis of the bRBCs), the solution was centrifuged again (500×g for 15 minutes) in order to remove cell debris. The supernatant, which contained free Hb released from the lysed cells, was filtered (0.2 µm) to further ensure the removal of all cell debris.

A colorimetric cyanomethemoglobin procedure \((110)\) was then utilized to determine the concentration of metHb within the supernatant. This procedure relied on the conversion of metHb to cyanometHb, which unlike Hb and metHb, does not absorb at 630 nm. Consequently the concentration of metHb within each aliquot could be determined. This procedure was carried out in duplicate for each aliquot.

3.2.6 Measurement of the GBOC

As described in the introduction section of this chapter, determining the amount of oxygen released from the circulating media stream to the hepatocytes inside of a HF bioreactor is of interest. However, the oxygen probes required to accomplish this task were not acquired till after the experiment described in sections 3.2.1-3.2.5 was completed. Consequently,
a second experiment was conducted with C3A cells maintained within a HF bioreactor in order to obtain this information. The culturing procedure was the same as that described above with the exception of the differences described in this section.

After the first experiment, the bioreactor utilized (Spectrum Labs, Cat. #400-011) was switched to one that contained fiber walls with pores large enough to permit the transport of albumin but small enough to prevent the bRBCs from entering the ECS. The bioreactor selected for this study (Spectrum Labs, Cat. #400-014) contained ~80 hydrophilic polyethylene fibers with pores exhibiting a 0.3 µm 95% molecular weight cutoff. The lumen volume within this bioreactor was ~0.8 mL, the ECS volume was ~2.3 mL, and the fiber radius was ~165 µm.

In this study, rather than using 2 HF bioreactors, only 1 was utilized. C3A cells were inoculated into this cartridge; ~1.06×10^6 cells with a viability of ~73%. The cells were then allowed to proliferate within the bioreactor for upwards of a month. After calibration (using air saturated water) and zeroing (using sodium sulfite saturated water), sterilized (using 70% ethanol) oxygen probes (Lazar Labs, Los Angeles, CA, Model #DO-166FT) were incorporated into the system at the inlet and exit of the bioreactor. These probes measured the dissolved oxygen concentration within the media stream as it entered and exited the bioreactor. Utilizing Henry’s law, the dissolved oxygen concentration was converted to oxygen tension. The amount of oxygen bound to hemoglobin at the inlet and exit could then be determined from the
oxygen tension at each respective point utilizing the Hill equation (equation 2.10). Consequently, it was possible to determine the total oxygen lost to the C3A cells within the bioreactor.

The culturing media utilized within this experiment was the same as that described for the control culture within section 3.2.2. However, the media flow rate was maintained at ~8.35 mL per minute through the system. Once the oxygen probes were observed to reach a steady state, the inlet and exit dissolved oxygen concentrations were recorded. Typically, ~1 hour was given for the oxygen probes to reach a steady state. The flow rate was then varied to ~4.75 and then later to ~16.8 mL per minute. At each of these flow rates, the dissolved oxygen concentration at the inlet and exit of the bioreactor was recorded. Sterile, washed bRBCs were then supplemented to the circulating media at a concentration of ~2.5×10^8 cells/mL (~5% of the human in vivo RBC concentration). The inlet and exit bioreactor dissolved oxygen concentrations were then recorded for the same three flow rates.

Lastly, throughout this study, glucose and lactate concentrations were followed as a measure of the health of the maintained C3A cells.

3.3 Results and Discussions

3.3.1 BRBC Oxygen Binding/Dissociation Properties and MetHb Formation

The oxygen binding/dissociation properties of the washed bRBCs were consistently found to be within reason. The values for $P_{50}$ ranged from about 26-27 mmHg, and the values for $n$ ranged from about 2.4-2.9.
Typically, the metHb concentration within a human being is less than 1%. Throughout this experiment, the metHb level of bRBCs after being employed within the HF bioreactor system was found to not exceed about 3%. Consequently, it was concluded that the formation of metHb was not of significant concern through this study as long as the washed bRBCs placed into the system were relatively fresh (no more than 1-2 weeks old), and the bRBCs within the system were replaced after a few days.

3.3.2 Metabolic Assays

Figure 3.3 shows the bRBC and C3A cell consumption of glucose and production of lactic acid over the course of the first 16 days of this study. As can be seen, the bRBCs by themselves consumed little glucose and produced little lactic acid throughout the study. The average bRBC glucose consumption rate throughout the study was found to be $0.1 \pm 0.3$ mg/hr, while the average lactic acid production rate was measured as $0.2 \pm 0.1$ mg/hr. These values were subtracted from the measured C3A cell glucose consumption and lactic acid production values in order to correct for the approximate metabolic activity of the bRBCs.
Figure 3.3. This figure shows glucose consumption (A) and lactic acid production (B) during the experiment. The experimental system was corrected for the observed average bRBC glucose consumption and lactate production rates. Values are averages of triplicate sample measurements for glucose consumption and duplicate measurements for lactic acid production, and error bars indicate standard errors. A significant difference from the control system is indicated by an asterisk over the respective time point *(P<0.025), and **(P<0.005). Legend: Control system (C3A cells and no bRBC supplementation); Experimental system (C3A cells and bRBC supplementation); and bRBC control system (bRBC supplementation but no C3A cells)

After the inoculation of the C3A cells, relatively little glucose was consumed by the C3A cells within either the control or experimental systems until the 9th day of the study, at which point the glucose consumption rate began to increase rapidly (to a maximum of ~3 mg/hr by day 16). Throughout the 16 days, no significant difference was detected in the glucose consumption rate between the control and experimental systems. Similar to what was observed for glucose consumption, little C3A cell lactic acid production was detected until approximately the 12th day. However, it was consistently found from the 6th day on (with the exception of the 12th day) that the experimental system produced significantly less lactic acid.
Most likely, the lack of glucose consumption and lactic acid production at the beginning of the study reflected a lag (or adjustment) phase of the cultured C3A cells as they became acclimated to their new environment. However, a 9-day lag phase seems like an abnormally lengthy lag period. This might be a result of the relatively small number of cells inoculated into each HF bioreactor (the seeding density into each bioreactor was \( \sim 2.5 \times 10^5 \) cells/mL). It is conceivable that this low seeding density might have impaired cell-to-cell communication during the early stages of cell culture, and consequently slowed down the proliferation and function of the C3A cells during this period. After the 9\(^{th}\) day though, glucose utilization began to increase significantly. It is interesting to note that in conjunction with the increase in glucose utilization, the C3A cells also began to synthesize albumin, which is a highly energetic process, in significantly larger quantities than before (shown beginning with the day 8-12 measurement period of Figure 3.5). It is also of interest that lactic acid production was found to be consistently lower in the experimental system than in the control system, even though glucose consumption remained relatively similar between the two. This would seem to indicate the presence of a more aerobic environment within the experimental system. This point is further discussed below.

The argument that the metabolic characteristics of the two systems indicates that a more aerobic environment was present within the experimental system is better demonstrated by considering the ratio of lactic acid production to glucose consumption. This can be explained by
considering the main metabolic pathways available to the C3A cells. Under both aerobic and anaerobic conditions, 1 mole of glucose (C$_6$H$_{12}$O$_6$) will be converted to 2 moles of pyruvate via glycolysis, which results in the production of 2 moles of ATP (adenosine triphosphate). Pyruvate is then utilized within the citric acid cycle, which employs the oxidative phosphorylation pathway, when an aerobic environment is present. Ultimately, this results in the additional production of 36 moles of ATP. Under anaerobic conditions, pyruvate is converted to lactic acid (C$_3$H$_6$O$_3$), which is essentially a metabolic dead end. The overall reaction scheme for both aerobic and anaerobic glucose metabolism is displayed as equations 3.4 and 3.5. Given the increased ATP production, aerobic metabolism is considerably more efficient than anaerobic metabolism.

Aerobic metabolism:

$$C_6H_{12}O_6 + 38\text{ADP} + 38\ P_i + 6O_2 \rightarrow 6\text{CO}_2 + 44\text{H}_2\text{O} + 38\text{ATP}$$  \hspace{1cm} (3.4)

Anaerobic metabolism:

$$C_6H_{12}O_6 + 2\text{ADP} + 2\ P_i \rightarrow 2C_3H_5O_3 + 2H^+ + 2\text{H}_2\text{O} + 2\text{ATP}$$  \hspace{1cm} (3.5)

where P$_i$ is inorganic phosphate, CO$_2$ is carbon dioxide, H$_2$O is water, H$^+$ is a hydrogen proton, and the remaining chemical formulas are defined above.

Considering the differences between aerobic and anaerobic glucose metabolism, it is clear that the ratio of lactic acid produced to glucose consumed is an indicator of the level of oxygenation seen within the C3A cell space. Under completely anaerobic conditions, this ratio should approach 2,
whereas aerobic conditions should result in a smaller ratio. Figure 3.4 shows that this ratio was generally found to be smaller for the experimental system, thus supporting the hypothesis that the C3A cells maintained within this system were presented with a more aerobic environment. A significant difference can be seen for days 6-16, with the exception of day 12-13. While the ratio was fairly consistent for the control data (the average ratio value was found to be 1.4 ± 0.2), there was some variability in the ratio for the experimental system (0.8 ± 0.6). The variability seen in the experimental system was likely a reflection of the bRBCs settling out within the reservoir bottle with time. Attempts were made to prevent this by manual shaking of the reservoir bottle, but bRBC settling remained a problem throughout the study (the reservoir bottle could only be manually shaken so often as continual opening of the incubator is not ideal). The increase in the lactic acid production to glucose consumption ratio on day 12-13 was likely a reflection of this problem.

3.3.3 Albumin Synthesis

Conservation of albumin synthesis is important in a hepatic HF bioreactor culture. Besides its previously described regulation and transport uses within the blood stream, albumin synthesis can also be utilized as an indicator of an in vitro hepatocyte culture having reached confluency/maturation (111). In vivo, fetal hepatocytes synthesize low levels of albumin, however, upon reaching a mature state, albumin secretion increases dramatically (111). In this study, albumin accumulated in the ECS
Figure 3.4. This figure shows the ratio of lactic acid produced to glucose consumed. The values are each an average of ratios calculated from at least 2 measurements of both lactic acid production and glucose consumption, and standard errors are indicated by the error bars. A 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 since it is larger than the HF membrane pore molecular weight cutoff.

Consequently, periodic flushing of the ECS was required. As C3A cells (and all hepatocyte types) are adherent cells, this should not have impacted viable cells, but rather flushed out dead cells from the bioreactor.

C3A hepatoma albumin production over the course of this study is displayed in Figure 3.5. Throughout the study, albumin was produced to a
greater extent within the experimental system, and the difference between the
two systems was found to be significant for days 8-16. This supports the
hypothesis that a more aerobic environment was provided to the C3A cells
maintained within the experimental system. However, there would seem to
be two likely reasons for the increased albumin synthesis. The first is simply
that the improved aerobic environment of the experimental system directly
resulted in increased albumin synthesis. Albumin production is a highly
energy intensive process. Assuming this energy is provided for by glucose
metabolism, and complete glucose oxidation occurs (requiring aerobic
metabolism), 75 µmol of glucose and 450 µmol of oxygen are required to
synthesis 1 µmol of albumin in vivo (112). As a result of this high-energy
demand, albumin synthesis would be expected to be greater within more
aerobic systems. It is also possible that increased proliferation might have
occurred within the experimental system as a result of the improved oxygenation. This would have consequently resulted in an increase in
albumin concentration within this system (more functioning cells leads to
more albumin production). However, as it is difficult to measure cell number
within a HF bioreactor for an adherent cell line, and since the experimental
system was not maintained through the establishment of confluency (which
would have allowed for a rough estimation of the final cell number and thus
proliferation rate), it is difficult to draw conclusions regarding C3A cell
proliferation in this study. Regardless though, it seems that the increased
albumin synthesis witnessed within the experimental system supports the
hypothesis that bRBC supplementation improves the oxygenation of the maintained C3A cells.

3.3.4 The Establishment of Confluency Within the Control System

While the experimental system was only maintained for about 16 days, the control system was allowed to proliferate to the point of confluency. The establishment of confluency within the system was determined on the basis of

![Image](image.png)

Figure 3.5. This figure shows albumin production over the course of the study. Values shown are each an average of triplicate sample measurements, and standard errors are shown by error bars. A significant different from the control culture is indicated by an asterisk over the respective time point *(P<0.005). Legend: blue is the control system and red is the experimental system.
a significant increase in albumin production (see Figure 3.6), which occurred around day 33 of the study. At this point, the hepatocytes within the system were synthesizing $104 \pm 7.4$ nmol/day of albumin.

As previously mentioned, it is difficult to determine the number of C3A cells within the bioreactor at a given time point as these cells are adherent cells. However, it has previously been reported that upon culture maturation within the ELAD system, C3A cells synthesize approximately $3.3 \times 10^{-9}$ µmol of

![Figure 3.6](image-url)  

Figure 3.6. This figure shows C3A cell albumin production within the control culture over the entire period of time that the control system was maintained. The values shown are each averages of triplicate sample measurements, and standard errors are indicated by error bars.
albumin per cell per day (34). Therefore, if any possible differences in C3A cell albumin synthesis rates based on oxygenation levels is ignored, it can be estimated based on this synthesis rate that the control system contained approximately 0.3 grams or \(3.15 \times 10^7\) cells upon reaching confluency. These results thus indicate that the culture was maintained through confluency, and provides further evidence that these C3A cells behave similarly to normal hepatocytes in their progression from a fetal state to a mature state.

3.3.5 Experimentally Measured GBOC Rate

As previously mentioned, the amount of oxygen lost to the C3A cells within a bioreactor (GBOC) was experimentally examined. This experiment was conducted on a single hepatic HF cartridge. At first, the bioreactor was fed with media not supplemented with bRBCs at three different media flow rates. Following this, bRBCs were then added to the circulating media, and again the media flow was varied between the same three different rates. At each media flow rate, the rate of oxygen loss from the circulating media within the bioreactor was calculated. In the situations where the media was not supplemented with bRBCs, this was simply calculated using Henry’s law and the inlet and exit measured pO\(_2\) values. However, with the addition of bRBCs, the amount of oxygen bound to Hb had to also be calculated. This was accomplished using Hill’s equation (equation 2.10) in conjunction with the inlet and exit experimentally measured pO\(_2\) values.
The impact of bRBC supplementation on the GBOC rate is shown in Figure 3.7. As can be seen, at each media flow rate, bRBC supplementation increased the amount of oxygen delivered and released within the bioreactor. Also as expected, increasing the flow rate resulted in an increase in the GBOC rate when no bRBCs were present. However, it is difficult to determine a trend in the GBOC rate when bRBCs were present in regards to a dependence on media flow rate given that the fastest flow rate resulted in the least amount of oxygen lost within the bioreactor. This could be a result of error in the inlet pO$_2$ measurement as this value was observed to be reduced (~90 mmHg) as compared to that observed for the slower media flow rates (~95 and 97 mmHg). Regardless though, the data shows that bRBC supplementation in this bioreactor increased the GBOC rate between ~1.4 and 2.8 fold.

Lastly, a rigorous modeling analysis of this hepatic HF bioreactor system has been completed by a collaborating graduate student (109). Utilizing the experimentally found bioreactor inlet and exit pO$_2$ values within the oxygen transport model, a $V_{\text{max}}$ of 25 µM/s and a $K_m$ of 3 mmHg was determined for the C3A cells.

3.4 Concluding Remarks

The results produced from this study were encouraging in their support of the hypothesis that bRBC supplementation of a circulating media feed stream can improve oxygen delivery and release to a hepatic HF bioreactor. This chapter presented both direct and indirect evidence of a more aerobic
Figure 3.7. This figure compares the global bioreactor oxygen consumption rate within a bioreactor when the circulating media was not supplemented with bRBCs (blue) and when it was supplemented with bRBCs at $5 \times 10^8$ cells/mL (red) for three different media flow rates. The GBOC rate was found to be increased with bRBC supplementation as expected.

environment within the experimental system. The indirect evidence was provided through the reduction in the lactic acid production to glucose consumption ratios (Figure 3.4), and an improvement in albumin synthesis (Figure 3.5) for the experimental system. The improvement in albumin synthesis is likely explained either through an increase in C3A cell number (the system would have thus witnessed improved proliferation) or through an improvement in the metabolic efficiency of the C3A cells (albumin synthesis is
a highly energetic process, and consequently an improvement in energy production would facilitate synthesis). It is expected that improved oxygenation of the C3A culture should positively impact both of these phenomena (proliferation and metabolic efficiency). The reduction in the lactic acid production to glucose consumption ratio is indicative of an improved metabolic efficiency, which would occur as a result of more available oxygen.

In order to collect direct evidence of bRBC supplementation improving oxygen delivery, a C3A cell-containing HF bioreactor was operated with oxygen probes at the inlet and exit of the device. Consequently, the oxygen lost from the circulating media stream to the C3A cells within the bioreactor could be calculated (GBOC rate). As can be seen from Figure 3.7, an increase in oxygen offloading from the media stream to the bioreactor was witnessed when the media stream was supplemented with bRBCs.
4.1 Introduction

4.1.1 Background

The previous chapter of this dissertation described experiments aimed at examining the impact of an improved oxygen carrying capacity of a HF bioreactor circulating media stream on C3A cells maintained within the bioreactor. The oxygen carrying capacity of that media stream was improved via supplementation with bovine red blood cells (bRBCs). Bovine red blood cells were selected as an oxygen carrier because of their strong oxygen binding capacity, their availability, and their ease of use within the HF bioreactor system.

In certain circumstances, however, it might be desirable to modify some of the properties of normal bRBCs to create novel oxygen carriers that are more effective in delivering oxygen to a HF bioreactor cell culture. One such property is the oxygen binding/dissociation characteristics of the bRBC. The majority of oxygen release from these cells occurs when the suspension
\( pO_2 \) is near the \( P_{50} \) of the bRBCs, which is \(~26-27\ \text{mmHg}\) for normal bRBCs. Altering the \( P_{50} \) of bRBCs would thus change the oxygen delivery profile of a bRBC supplemented circulating media stream. This could potentially provide a means of targeting oxygen delivery to a HF bioreactor cell culture within a specified media \( pO_2 \) range. Consequently, engineering the oxygen binding/dissociation properties of bRBCs could provide an interesting means of adjusting the oxygen environment within the hepatocyte space of a hepatic HF bioreactor, thus facilitating a rigorous study on the effects of varied oxygen provision on these cells. Additionally, certain cell cultures, such as many stem cell cultures, are ideally maintained at fairly low \( pO_2 \) (<10 mmHg) values \((113, 114)\). Therefore, increasing the oxygen affinity of bRBCs (decreasing their \( P_{50} \)) could potentially allow for oxygen delivery specifically tailored for these types of cell cultures.

The modification of bRBC rheological properties could also be of interest in creating an oxygen carrier specifically suited for HF bioreactor oxygen transport. Bovine RBCs contain Hb, which is known to be toxic to many cell cultures when in direct contact with the culture \((75, 78)\). Unfortunately, within a circulating media stream, some degree of hemolysis (rupturing of the bRBC and subsequent release of Hb into the circulating media stream) is to be expected. Imparting further osmotic stability to the bRBC could be a simplistic solution to reducing the extent of hemolysis that occurs within a bRBC supplemented circulating media stream.
As described in the introductory chapter to this dissertation, many properties of the bRBC, including those mentioned above, can be engineered via a cross-linking (fixing) procedure performed using the dialdehyde cross-linker glutaraldehyde. Glutaraldehyde serves to cross-link proteins on the surface of the bRBC, which imparts additional mechanical stability to the bRBC (90) and reduces its deformability (91). This cross-linker is also capable of diffusing into the bRBC where it can cross-link Hb molecules, resulting in a change in the oxygen binding/dissociation properties of the bRBC.

4.1.2 Research Overview

As described above, the ability to engineer the properties of bRBCs could lead to a novel oxygen carrier with properties specifically tailored for targeted oxygen delivery within HF bioreactor systems. Consequently, the impact of glutaraldehyde cross-linking on select properties of the bRBC is described in this chapter. Specifically, the osmotic stability, electrophoretic mobility, and oxygen binding/dissociation properties were measured for the engineered bRBCs and compared to normal bRBCs. The cross-linking reaction was carried out at different glutaraldehyde to Hb (Hb was contained within the bRBC) molar ratios and for different reaction durations.

The ability of these engineered bRBCs to improve oxygen delivery to a cell culture maintained within a HF bioreactor operated under different bioreactor inlet pO\textsubscript{2} values is also of interest in order to determine the most ideal oxygen carrier for each bioreactor inlet pO\textsubscript{2}. To this aim, the Krogh-
based model described in the second chapter of this dissertation was utilized to predict oxygen delivery to a HF bioreactor maintaining C3A cells.

4.2 Materials and Methods

4.2.1 Glutaraldehyde Cross-Linking of BRBCs

For the cross-linking reactions, sterile washed bRBCs were purchased from Quad Five (Ryegate, MT, Cat. #943) and a 25% aqueous glutaraldehyde solution was obtained from Sigma (St. Louis, MO, Cat. #G-5882). Prior to use, glutaraldehyde was stored at -20°C and bRBCs were stored at 4°C. All steps involved in the handling of the bRBCs and in the cross-linking reaction were carried out at 4°C in order to minimize the oxidation of Hb to metHb.

In preparation for the cross-linking reaction, the sterile bRBCs were washed three times with sterile (via triple filtration through a 0.2 µm filter) phosphate buffered saline (PBS, pH=7.4) in order to remove any free Hb from the suspension along with any remaining plasma proteins. After addition of the PBS, the bRBCs were pelleted from the suspension (500×g, 15 minutes) and the supernatant removed. Fresh PBS was added to re-suspend the bRBCs.

After the bRBC washing procedure, an aliquot was removed and the bRBCs were pelleted from the aliquot suspension (500×g, 15 minutes). The cells were then subjected to hypotonic lysis via the addition of cold distilled water for a period of at least 24 hours (to ensure complete bRBC lysis). The solution was then centrifuged (500×g, 15 minutes) to pellet cell debris. The
supernatant was removed and filtered through a 0.1 µm filter to ensure removal of all cell debris. Utilizing the modified cyanomethemoglobin procedure described in section 3.2.5 of this dissertation, the concentration of Hb within the solution, and thus within the original bRBC suspension, was determined. The original bRBC suspension was then diluted to a final Hb concentration of 3.5 mM using fresh PBS.

The cross-linking reaction was carried out on the washed bRBCs at 4 different molar ratios of glutaraldehyde to Hb (again, the Hb is contained within the bRBCs); specifically 1:1, 2:1, 5:1, and 10:1. Additionally, the impact of the cross-linking procedure was examined after 1, 2, 6, and 12 hours of reaction duration, with the 12-hour reactions carried out in duplicate while the remaining reaction durations were carried out once. To initiate the cross-linking reaction, 12 mL of bRBC suspension (3.5 mM Hb) was transferred to a 50 mL centrifuge tube wrapped in aluminum foil (glutaraldehyde is light sensitive), and an appropriate amount of glutaraldehyde (according to the desired reaction molar ratio) was added to the suspension. Lastly, the centrifuge tube was placed on a 3-D rotator (to promote mixing) for the duration of the cross-linking reaction.

As mentioned above, the impact of the cross-linking procedure was examined as a function of reaction duration. This was accomplished by removing 4 mL of reacted bRBC suspension from each of the 50 mL reaction vessels after each of the shorter reaction periods (1, 2, and 6 hours). The removed reacted suspension was transferred to a 15 mL centrifuge tube and
sodium borohydride (NaBH₄) was added at a 2:1 molar ratio with respect to the initial glutaraldehyde concentration in order to quench the reaction via the reduction of free unreacted aldehyde groups into alcohols (88). This step, the addition of NaBH₄ also helped to stabilize the imine bonds that form between amino groups and glutaraldehyde through the reduction of imine bonds into stable amine bonds (88). This is advantageous as it prevents the future dissociation of glutaraldehyde from cross-linked proteins. If glutaraldehyde were to dissociate from a cross-linked bRBC while flowing within a HF bioreactor circulating media stream, it would be able to cross-link proteins within the circulating culturing media and within the maintained cell culture. Each 15 mL tube was placed on the 3-D rotator for 30 minutes, after which the reaction suspension was spun down at 500×g for 15 minutes, the supernatant removed, and fresh sterile PBS was added in order to re-suspend the cross-linked bRBC pellet. This final washing procedure was repeated 3 times in order to ensure the removal of all remaining free glutaraldehyde and NaBH₄. After the last PBS wash, fresh PBS was added to the cross-linked bRBC pellet in order to bring the final suspension Hb concentration to ~1 mM. Lastly, the 12 hour duration reaction suspension was also quenched with NaBH₄, washed with fresh PBS, and stored in PBS at a final Hb suspension concentration of ~1 mM.

4.2.2 Oxygen Binding/Dissociation Properties of Normal/Cross-Linked BRBCs

The oxygen binding/dissociation properties (P₅₀ and n) of both normal and engineered bRBCs were determined using a Hemox™-Analyzer. The
procedure followed for this determination was outlined in section 3.2.4 of this dissertation. As before, Adair constants were found through the regression of instrument provided raw absorbance data within a 6-parameter, non-linear modified form of the Adair equation (equation 3.3). These constants were then utilized to construct a complete oxygen binding/dissociation curve. The \( P_{50} \) and \( n \) values for each bRBC suspension were determined from this curve (115). Lastly, duplicate measurements were performed on each bRBC suspension.

4.2.3 Electrophoretic Mobility Determination for Normal/Cross-Linked BRBCs

The electrophoretic mobility of each bRBC suspension was measured using a Zetapals instrument (Brookhaven Instrument Corp., Holtsville, NY). Prior to analysis, the instrument’s noble metal electrode was preconditioned using PBS. PBS was placed into the instrument’s holding cell, and the instrument was programmed to make 10 runs with 30 cycles for each run. The PBS solution was then removed from the holding cell and an aliquot of a dilute bRBC suspension prepared for this analysis was added to the cell. The dilute bRBC suspension utilized within this analysis was prepared by adding one drop of either normal or engineered bRBC suspension to 15 mL of PBS solution. The dilute bRBC suspension sample was then analyzed by the instrument (10 runs, 20 cycles). All electrophoretic mobility measurements were conducted under the following conditions: pH of 7.4, conductance of 45500-52000 \( \mu \)S, viscosity of 0.89 cP, dielectric constant of 78.54, sample count rate of 275-850 kcps, reference count rate of 1340-1830 kcps,
wavelength of 656 nm, voltage of 4 volts, and an electric field of 4.29-5.32 V/cm. Lastly, each bRBC suspension was analyzed in duplicate.

4.2.4 Normal/Cross-Linked BRBC Osmotic Stability Determination

The osmotic stability study was initiated by centrifuging each bRBC suspension (500×g for 15 minutes) in order to pellet the bRBCs and remove the PBS supernatant. To the bRBC pellet, cold distilled water was added at 10 times the volume of packed bRBCs to produce a hypotonic suspension. The suspension was then mixed and left at 4ºC for at least 24 hours prior to analysis. To determine the extent of osmotic bRBC lysis, an aliquot was taken from each hypotonic sample and centrifuged (500×g for 15 minutes) to pellet intact bRBCs and cell debris. The supernatant was removed and filtered through a 0.1 µm filter to further ensure the removal of all cell debris. The concentration of Hb within the supernatant was then determined using the cyanomethemoglobin method described in section 3.2.5 of this dissertation. This allowed the extent of osmotic bRBC lysis to be determined by comparison of the Hb concentration within the supernatant to that in the initial isotonic bRBC suspension. Lastly, each analysis was carried out in duplicate.

4.2.5 Krogh-Based Model Prediction of Oxygen Delivery Within a HF Bioreactor

As previously mentioned, the ability of these novel engineered bRBC oxygen carriers to deliver oxygen effectively within a HF bioreactor is of ultimate interest. One means of evaluating these modified bRBCs to this end
is through a simple modeling study of their efficacy as oxygen delivery vehicles. This study was conducted utilizing the Krogh-based model presented in section 2.4 of this dissertation.

For this modeling analysis, the HF bioreactor was assumed to contain C3A cells and to have a negligible cell space void volume. Consequently, the oxygen consumption characteristics used within the model are those given in the literature for C3A cells (8, 116). The model was also operated using a range of bioreactor inlet pO$_2$ values in order to evaluate the ability to create cross-linked bRBCs suitable for targeted oxygen delivery. The remaining parameters utilized within the model are characteristic of the HF cartridge described in section 3.2.1 of this dissertation. Table 4.1 lists the parameters utilized within the model.

The oxygen binding/dissociation properties utilized within the model were those found for the normal and engineered bRBCs according to the procedure outlined in section 4.2.2. Bovine RBC supplementation was considered at 0% and 10% (~5×10$^8$ cells/mL) of the human in vivo RBC concentration. The bRBC supplementation concentration was kept relatively low as this would ideally be the situation within an actual HF bioreactor experiment. Additionally, Figure 2.7 in chapter 2 of this dissertation shows that this Krogh-based model predicts that a significant increase in oxygen delivery (in comparison to the situation where the media is not supplemented with an oxygen carrier) can be achieved with a minimal bRBC
supplementation concentration, thereby further justifying focusing on a small bRBC supplementation concentration.

4.3 Results and Discussions

4.3.1 Oxygen Binding/Dissociation Properties of Normal/Cross-Linked BRBCs

Within the literature, there is a variety of reports on the impact of the glutaraldehyde reaction on the oxygen binding/dissociation properties of human RBCs (hRBCs), but little mention of work done examining the effects of the reaction on these properties for bRBCs. These literature findings indicate that when the cross-linking reaction is carried out within an aerobic environment, the oxygen affinity of the resulting cross-linked hRBCs is
increased and their cooperativity is decreased (90-92). In the work presented here, glutaraldehyde cross-linking of bRBCs was also found to increase their oxygen affinity. This can be seen in Figure 4.1, which shows typical oxygen binding/dissociation curves measured for the normal and cross-linked (at a 1:1, 2:1, 5:1, and 10:1 glutaraldehyde to Hb molar ratio) bRBCs utilized within this current study. As can be seen, glutaraldehyde cross-linking of the bRBC caused a left-shift in the oxygen binding/dissociation curve, which indicates an increase in oxygen affinity of the oxygen carrier. The actual oxygen binding/dissociation properties, $P_{50}$ and $n$, for the bRBCs subjected to the cross-linking reaction for 12 hours are shown in Figure 4.2. The $P_{50}$ of normal bRBCs (the control) was found to be approximately 26 mmHg. As expected, the $P_{50}$ of the bRBCs decreased with an increased glutaraldehyde concentration within the reaction. At a reaction molar ratio of 10:1 glutaraldehyde to Hb, the $P_{50}$ of the engineered bRBCs decreased to as low as approximately 3 mmHg. The $P_{50}$ of each of the engineered bRBCs was found to be significantly different ($P<0.01$) from normal bRBCs. Additionally, the figure shows that a significant difference ($P<0.025$) between the $P_{50}$ values of each of the cross-linked bRBC suspensions was found (for example, between the $P_{50}$ value of the bRBC suspension produced from the 5:1 molar ratio reaction and that produced from the 2:1 molar ratio reaction).

The other oxygen binding/dissociation property measured, the cooperativity coefficient, was found to be ~2.6 for normal bRBCs within these studies. In agreement with the general literature findings previously
Figure 4.1. This figure shows oxygen binding/dissociation curves for normal bRBCs (orange) and bRBCs cross-linked at a 1:1 (green), 2:1 (red), 5:1 (blue), and 10:1 (purple) glutaraldehyde to Hb molar ratio.

As mentioned, the cross-linking reaction resulted in a reduction of bRBC cooperativity. For instance, the 10:1 molar ratio reaction (12 hour duration) yielded a cooperativity of only ~1.1. All of the cross-linking reactions produced engineered bRBCs with cooperativities significantly different (P<0.0005) from normal bRBCs. The cooperativity of each of the cross-linked bRBC suspensions was also found to be significantly different from the cooperativity of the other cross-linked bRBC suspensions (P<0.025).
Figure 4.2. This figure shows the oxygen binding/dissociation properties, $P_{50}$ (red) and $n$ (blue), of normal and cross-linked (for a duration of 12 hours) bRBCs. The results shown are an average of at least duplicate measurements performed on aliquots from duplicate cross-linking reactions, and standard errors are indicated by the error bars shown on the figure. The oxygen binding/dissociation properties of each of the cross-linked bRBCs were found to be significantly different from normal bRBCs and from the bRBCs cross-linked at different glutaraldehyde to Hb ratios (confidence levels are given in the text).
Unfortunately, it is difficult to precisely compare this work with that previously reported within the literature (90-92). As previously mentioned, these literature studies were mainly focused on hRBCs. However, further complicating the situation, the cross-linking reactions and oxygen binding/dissociation analyses were conducted at different temperatures within these literature studies than within this work. The cross-linking reaction was carried out at a lower temperature within this study in order to minimize the formation of metHb. The presence of elevated levels of metHb, up to 20%, was detected within one of the literature studies (92). In the current studies, the metHb concentration was found to be less than 5% for the 1:1 and 2:1 molar ratio cross-linking reaction suspensions according to the cyanomethemoglobin method described in section 3.2.5 (the other suspensions would not undergo significant hypotonic lysis and thus the cyanomethemoglobin method could not be conducted). Also, the oxygen binding/dissociation properties of the cross-linked bRBCs were measured under physiological temperature conditions (37ºC) since it is ultimately intended that they be utilized as novel oxygen carriers within a HF bioreactor cell culture maintained at this temperature. It is known that the oxygen binding/dissociation curve is left-shifted at the lower temperatures under which the oxygen binding/dissociation curve was measured within the literature studies (74).

Lastly, the duration of the cross-linking reaction was found to have little impact on the oxygen binding/dissociation properties of the resulting
engineered bRBCs within this study. For instance, the $P_{50}$ for bRBCs cross-linked for 1 hour at a glutaraldehyde to Hb molar ratio of 1:1 was measured to be 24.3 ± 0.4 mmHg, while this value was measured to be 23.7 ± 2.0 mmHg when the reaction was allowed to proceed for 12 hours. As the glutaraldehyde to Hb molar ratio increased, a larger gap in the $P_{50}$ values was seen with time, though this change remained relatively small. On the other hand, essentially no dependence on the reaction duration was witnessed in the resulting cooperativity of the engineered bRBCs. These results thus suggest that glutaraldehyde is quickly internalized into the bRBC where it cross-links the internal proteins. This is in agreement with previous work conducted on hRBCs (80).

4.3.2 Electrophoretic Mobility for Normal/Cross-Linked BRBCs

The electrophoretic mobility of a cell is an indication of the overall charge that exists at the surface of the cell (117). Figure 4.3 shows the electrophoretic mobilities measured for both normal bRBCs and the cross-linked (for a duration of 12 hours) bRBCs created within this current work. Normal bRBCs were found to exhibit a mobility of approximately -1.05 µ-V/cm-s, which is very similar to that previously reported, -1.08 µ-V/cm-s (118). Interestingly, all of the electrophoretic mobilities obtained for both normal and engineered bRBCs were found to be between -1.0 and -1.15 µ-V/cm-s. Additionally, the duration of the cross-linking reaction was not found to impact the electrophoretic mobility of the resulting engineered bRBCs.
Figure 4.3. This figure shows the electrophoretic mobility of both normal and cross-linked (for a duration of 12 hours) bRBCs. The results shown are an average of duplicate measurements performed on aliquots from duplicate cross-linked bRBC suspensions, and the error bars shown within the figure indicate standard errors.

It has previously been reported that at a pH greater than 6.0, glutaraldehyde treatment unexpectedly increased the negative mobility of hRBCs by 10%, to about -1.20 $\mu$-V/cm-s (80). Within the current studies presented here, a slight negative increase (~7 to 9%) in the mobility of bRBCs cross-linked at a molar ratio of 1:1 and 2:1 glutaraldehyde to Hb was witnessed. However, a negative increase in the mobility of the bRBCs cross-linked at larger glutaraldehyde to Hb molar ratios (5:1 and 10:1) was not
observed. It should also be considered that the error in the electrophoretic mobility measurement ranged from ~0.1 to 0.18 µ-V/cm-s, and consequently a 10% change in mobility could easily be absorbed within the error of the measurement. Therefore, the results presented here seem to indicate that the glutaraldehyde cross-linking reaction does not alter the electrophoretic mobility of bRBCs.

4.3.3 Normal and Cross-Linked BRBC Osmotic Stability

It has previously been noted that one of the effects of the glutaraldehyde cross-linking reaction on hRBCs is to impart additional osmotic stability to the cell as a result of the cross-linking of membrane bound proteins on the surface of the cell (91, 119, 120). The results of the osmotic stability test carried out here on normal bRBCs and bRBCs subjected to the cross-linking reaction for 12 hours are shown in Figure 4.4. Normal bRBCs completely lysed when placed in a hypotonic solution (100% lysis), thus providing a control for comparison with cross-linked bRBC hemolysis. The 1:1 glutaraldehyde to Hb molar ratio cross-linking reaction resulted in little added osmotic stability to the bRBCs. However, the 2:1 molar ratio reaction provided a significant amount of additional osmotic stability, and essentially no osmotically induced bRBC lysis occurred for the 5:1 and 10:1 molar ratio cross-linking reactions. Additionally, the osmotic lysis study showed that there was little difference in the results regardless of whether the cross-linking reaction was allowed to proceed for 1 hour or for 12 hours.
Figure 4.4. This figure shows the extent of hypoosmotic lysis undergone by normal and cross-linked (for a duration of 12 hours) bRBCs as a result of being placed in cold distilled water for at least 24 hours. The results shown are an average of duplicate measurements performed on aliquots from duplicate cross-linking reactions, and the error bars show standard errors. The percent lysis of all of the cross-linked bRBC suspensions, with the exception of the 5:1 and 10:1 molar ratio reaction suspensions, was significantly different (P<0.025) from the other suspensions.
The osmotic stability results obtained from the studies presented within this dissertation are in agreement with trends previously observed regarding the use of glutaraldehyde to cross-link RBCs (91, 119, 120). Bovine RBCs were found to become more resistant to hypoosmotic stress and better able to withstand severe hemolytic conditions with increased cross-linking reaction glutaraldehyde concentration. The engineering of bRBCs such that shear induced hemolysis is significantly reduced would be beneficial within a HF bioreactor setting in that it would help to prevent free Hb from being released into the circulating media stream where it could diffuse through the HF membrane and interact directly with the cells maintained in the ECS. This is of importance to the biotechnology industry since free Hb is known to be cytotoxic to many cell types (76-78).

4.3.4 Krogh-Based Model Prediction of Oxygen Delivery Within a HF Bioreactor

The Krogh-based model described earlier (section 2.4) was utilized in order to predict the ability of the studied normal and engineered bRBCs to deliver and release oxygen to a hepatic HF bioreactor. This model was run over a bioreactor inlet pO$_2$ range of 10-70 mmHg for the case of no circulating media bRBC supplementation and circulating media bRBC supplementation (at a concentration of 10% of the human $in$ vivo RBC concentration). For the situations where bRBC supplementation was considered, the oxygen binding/dissociation properties experimentally found for the normal and engineered bRBCs were employed. The Krogh-based model was used to
provide lumen, membrane, and cell space pO$_2$ profiles for each situation modeled. Figure 4.5 shows representative lumen, membrane, and ECS pO$_2$ profiles for a bioreactor inlet pO$_2$ of 70 mmHg. The oxygen tension profiles within this figure were generated assuming no bRBC supplementation and bRBC supplementation (10% of the human $in$ vivo RBC concentration) using normal bRBCs and bRBCs cross-linked at a glutaraldehyde to Hb molar ratio of 2:1 and 5:1. As can be seen, the addition of bRBCs (both normal and engineered) helped to improve the local hepatocyte space oxygen environment throughout the majority of the bioreactor.

Utilizing the pO$_2$ profiles predicted from the Krogh-based model, the global bioreactor oxygen consumption (GBOC) rate was determined at each of the inlet oxygen tensions in combination with the differing oxygen binding/dissociation properties of the normal and engineered bRBCs. This was done so that a direct comparison could be made at each bioreactor inlet oxygen tension as to the efficacy of the use of normal and engineered bRBCs in delivering and releasing oxygen to a hepatic HF bioreactor. In each case, the predicted GBOC rate was normalized using the GBOC rate that was predicted at each bioreactor inlet oxygen tension when the circulating media was not supplemented with bRBCs. Figure 4.6 shows the GBOC rate ratios for the considered scenarios. As can be seen, at all studied inlet pO$_2$ values, bRBC supplementation was observed to increase the amount of oxygen delivered to the maintained hepatocytes. As expected, for the cases assuming larger bioreactor inlet pO$_2$ values (30-70 mmHg), normal bRBCs
Figure 4.5. This figure shows the Krogh-based model predicted pO\textsubscript{2} profiles within the lumen (A), membrane (B), and ECS (C) of a representative C3A cell containing HF bioreactor operating with an inlet pO\textsubscript{2} of 70 mmHg. The situations considered include the case of no bRBC supplementation of the circulating media (black), bRBC supplementation using normal bRBCs (blue), and bRBC supplementation using bRBCs cross-linked at a 2:1 (green) and 5:1 (red) glutaraldehyde to Hb molar ratio.

were predicted to increase oxygen delivery to the C3A cells more than the engineered bRBCs (though all showed an improvement over no bRBC supplementation at all considered bioreactor inlet pO\textsubscript{2} values). This is reasonable given the cooperative nature of the bRBC oxygen binding/dissociation curve (see Figure 4.1). This curve predicts that the maximum improvement in oxygen delivery and release will occur when the suspension pO\textsubscript{2} is near the P\textsubscript{50} of the employed bRBCs. Consequently, for larger bioreactor inlet pO\textsubscript{2} values, normal bRBCs (which possess a larger P\textsubscript{50}
value in comparison to the engineered bRBCs considered) should improve oxygen delivery to a greater extent.

On the other hand, for the situations considered where the bioreactor inlet $pO_2$ is assumed to be relatively low (10 and 15 mmHg), the cross-linked bRBCs would be expected to be better suited for oxygen delivery than normal bRBCs given their left-shifted oxygen binding/dissociation curves. This was

Figure 4.6. Krogh-based model prediction of the rate of oxygen lost to C3A cells (global bioreactor oxygen consumption rate) maintained within a representative HF bioreactor at different bioreactor inlet oxygen tensions utilizing either normal (red) or engineered bRBCs (cross-linked a glutaraldehyde to Hb molar ratio of 1:1 (green), 2:1 (orange), 5:1 (blue), and 10:1 (purple)). The data is presented after normalization to the rate of oxygen lost at each respective bioreactor inlet oxygen tension when the circulating media is not supplemented with bRBCs.
indeed the prediction made by the model (see Figure 4.6). This result is of interest as it indicates that bRBCs could be engineered through cross-linking as novel oxygen carriers capable of providing improved oxygen delivery at reduced oxygen tensions. As previously mentioned, this could be of benefit to culturing specific cell types, such as stem cells, that prefer reduced oxygen tensions (113, 114).

4.4 Concluding Remarks

There are several Hb based oxygen carriers, both acellular (86-89) and cellular (121-124), that could potentially function as oxygen delivery vehicles for HF bioreactor cell cultures. However, bRBCs offer a novel, simplistic, and potentially superior means of providing improved oxygenation to HF bioreactor cell cultures. One example of the use of bRBCs to improve oxygen delivery to a HF bioreactor culture was provided in chapter 3 of this dissertation, where oxygen delivery to a C3A culture was found to be improved with bRBC supplementation of the circulating culturing media. Stemming from the positive results obtained from the experiments described in chapter 3, it became of interest to examine the potential of engineering many of the properties of bRBCs, including their osmotic stability and oxygen binding/dissociation properties. As explained in the introductory section of this chapter, improving the osmotic stability of bRBCs could lead to reduced hemolysis within the circulating media stream, and changing the oxygen binding/dissociation properties of bRBCs could allow for the creation of novel
oxygen carriers that could be designed to target oxygen delivery to certain pO$_2$ ranges. This chapter thus described work done examining the impact of glutaraldehyde cross-linking of the bRBC (and the internal Hb) on several key bRBC properties, including the oxygen binding/dissociation properties, electrophoretic mobility, and osmotic stability.

The results of this study were presented within this chapter. Briefly though, the cross-linking reaction was observed to improve bRBC osmotic stability, have no impact on bRBC electrophoretic mobility, and to decrease both the $P_{50}$ and cooperativity of bRBCs.

Following the work done examining the changes in bRBC properties that resulted from glutaraldehyde cross-linking, a Krogh-based model (see section 2.4) was employed to predict the efficacy of the engineered bRBCs (based on their measured oxygen binding/dissociation properties) to deliver oxygen to a C3A culture maintained within a representative HF bioreactor. It was found that all of the engineered bRBCs were predicted to significantly improve oxygen delivery over the situation where the circulating media stream lacks an oxygen carrier. At the lower bioreactor inlet pO$_2$ values examined, the engineered bRBCs were seen to be better suited to oxygen delivery in comparison to normal bRBCs. This is reasonable given the decreased $P_{50}$ values found for the engineered bRBCs. As the bioreactor inlet pO$_2$ was increased though, the normal bRBCs were seen to improve oxygen delivery to a greater extent than the cross-linked bRBCs. This would imply that engineered bRBCs, which carry with them a desirable improved osmotic
stability, are best suited for situations in which oxygen delivery at low pO\textsubscript{2} values is desired. There is, however, work within the literature suggesting that the oxygen binding/dissociation properties of hRBCs are impacted less when the cross-linking reaction is carried out under anaerobic conditions \((90)\). This suggests the possibility of also creating hemolysis resistant bRBCs capable of delivering oxygen to essentially the same extent as normal bRBCs at elevated pO\textsubscript{2} ranges.

The results presented within this chapter are thus exciting as they indicate the ability to engineer bRBCs such that they are well suited for targeted oxygen delivery within a HF bioreactor system.
CHAPTER 5

CONCLUSIONS AND PROPOSED FUTURE STUDIES

5.1 Conclusions

As discussed in the introductory chapter to this dissertation, the provision of proper oxygen levels to hepatocytes maintained within a HF bioreactor is widely considered to be a significant problem facing the development of an effective bioartificial liver assist device (4, 5, 7, 8). One potential means of improving the local oxygen environment within the cell space of a HF bioreactor is through the supplementation of the culturing media circulating through the system with bovine red blood cells. This dissertation described the initiation and development of a research project aimed at examining the ability of bRBCs to improve oxygen delivery to C3A hepatoma cells maintained within a HF bioreactor, and the resulting impact on the metabolic functions and synthetic abilities of the C3A cells. Additionally, the potential to engineer bRBCs (via glutaraldehyde cross-linking of the proteins on the surface of the cell and inside the cell) in order to develop novel oxygen carriers well suited for oxygen delivery within a HF bioreactor system was explored.

The work presented within this dissertation was grouped into three distinct chapters. Within the second chapter, mathematical models were
presented that described oxygen transport within the HF bioreactor system. The ability to describe a system such as the HF bioreactor system through mathematical modeling is advantageous for a variety of reasons. Utilizing accurate models, oxygen transport throughout the system can be explored, which could lead to more appropriate experimental design with regards to oxygen transport to the maintained hepatocytes. Consequently, time, effort, and money could be saved through focused model-driven experimentation.

The first model presented was meant to portray the oxygenation of the circulating media as it travels from the media reservoir into the HF bioreactor through silicone tubing. In order to simplify the model, any resistance to oxygen mass transport offered by the silicone tubing wall was ignored. This resulted in a slight over-prediction of the amount of oxygen transferred to the flowing media within a given silicone tubing length in comparison to experimental findings. However, this model is expected to be useful in providing reasonable estimates of the requirements (silicone tubing length and oxygen concentration within the incubator ambient atmosphere) placed on the system in order to achieve the desired media stream oxygenation prior to entry into the HF bioreactor.

The second oxygen transfer process for which a mathematical model was presented is the loss of oxygen from the circulating media to the cells maintained within the HF bioreactor. This model was developed based on the original Krogh Tissue Cylinder model. Improvements to this basic model that were integrated here include accounting for the oxygen mass transfer
resistance offered by the lumen wall and the inclusion of Poiseuille flow within the lumen. Unfortunately, due to a required assumption at the bioreactor inlet, the model will not accurately predict \( pO_2 \) distributions for all situations (primarily when the ECS volume surrounding a given fiber is large in conjunction with the use of cells possessing a large \( V_{max} \)), but is expected to be useful in most instances. For a given set of operating conditions, the model can be utilized to predict the \( pO_2 \) profile within the lumen, membrane, and ECS of a HF bioreactor. Within this chapter, this model was utilized to show that bRBC supplementation is expected to be a more effective means of improving oxygen delivery to a C3A cell culture maintained within a representative HF bioreactor in comparison to either increasing the media stream dissolved oxygen concentration to supraphysiological levels or increasing the media flow rate.

The third chapter of this dissertation presented experimental work involving examining the impact of bRBC supplementation of circulating media on C3A hepatocytes maintained within a HF bioreactor. Metabolic (glucose consumption and lactate production) and synthetic (albumin production) functions were measured for the C3A cells in both the experimental system (containing bRBC supplementation) and a control system (no bRBC supplementation). Over a 16-day study duration, the lactate production to glucose consumption ratio was consistently found to be lower for the experimental system while albumin synthesis was found to be improved for this system. Both of these situations indicate the presence of a more aerobic
environment within the experimental system, and thus provide indirect evidence that bRBC supplementation of the experimental HF bioreactor system improved the local C3A cell space oxygen environment.

A subsequent experiment was conducted using C3A cells within a HF bioreactor that also employed oxygen probes at the inlet and exit of the bioreactor. Consequently, the rate of oxygen delivery and removal from the bioreactor could be calculated (as the global bioreactor oxygen consumption, GBOC, rate). The GBOC rate was determined for a HF bioreactor at 3 different media flow rates under the operating conditions of no bRBC supplementation and bRBC supplementation. It was found that the GBOC rate was higher for the cases of bRBC supplementation, suggesting that bRBC supplementation improved oxygen delivery to the maintained C3A cells. The bioreactor employed for this study contained a relatively large cell space around each fiber, and in combination with the relatively high oxygen demand of C3A cells, presented a case where the assumption of zero order oxygen consumption kinetics for the C3A cells at the bioreactor inlet was invalid, thus preventing the modeling of this system with the developed model.

The fourth chapter of this dissertation described work performed with the aim of examining the ability to engineer bRBCs (via glutaraldehyde cross-linking) to create novel oxygen carriers suited for targeted oxygen delivery within a HF bioreactor cell culture. Briefly, glutaraldehyde cross-linking was found to decrease both the $P_{50}$ and cooperativity of bRBCs, to have no impact
on bRBC electrophoretic mobility, and to increase the osmotic stability of the bRBCs. Utilizing the experimentally found oxygen binding/dissociation properties of the engineered bRBCs within the Krogh-based model (see section 2.4) run on a representative HF bioreactor, the efficacy of the engineered bRBCs was examined. It was observed that as a result of their reduced $P_{50}$ values, engineered bRBCs as produced in these studies allow for oxygen delivery targeted at low pO$_2$ ranges. In conjunction with their improved osmotic stability, engineered bRBCs thus provide an oxygen carrier well suited for oxygen delivery at reduced pO$_2$ ranges within a HF bioreactor.

5.2 Proposed Future Studies

5.2.1 The Impact of Oxygenation on the Hepatic HF Bioreactor

The work presented within this dissertation has provided the groundwork for the planning of a rigorous study on oxygen provision within a hepatic HF bioreactor. As previously mentioned, it is generally believed that the local oxygen environment within the cell space of a HF bioreactor is insufficient for the proper maintenance of a significant number of hepatocytes. Consequently, bRBCs were utilized and will continue to be employed in future studies to improve the ability of the circulating media to deliver oxygen to the maintained hepatocyte culture.

Beyond simply increasing the amount of oxygen delivered to the maintained hepatocytes, it is of interest to determine the oxygen delivery conditions, which regulate the oxygen tension within the cell space (such as
the bioreactor inlet $pO_2$), that result in the most efficiently functioning cells. It is proposed that this be determined through a comprehensive study that employs HF bioreactors operating at differing bioreactor inlet $pO_2$ values (tentatively to be varied between 25-150 mmHg) and differing bRBC supplementation concentrations (0-50% of the human $in vivo$ RBC concentration). The bioreactor inlet $pO_2$ will be varied either by adjusting the silicone tubing length or by changing the oxygen concentration within the incubator ambient atmosphere. Throughout these studies, the bioreactor inlet and exit $pO_2$ values would be monitored using flow through dissolved oxygen probes, thereby allowing for the determination of the amount of oxygen lost from the media stream to the hepatocytes within the bioreactor. Through manipulation of the bioreactor inlet $pO_2$ and the bRBC supplementation concentration, it is expected that the cell space oxygen gradient can be considerably varied. Lastly, in order to alleviate the problem of bRBC settling witnessed in the studies presented in chapter 3 (and to thus create the intended oxygen delivery situation), a shaking apparatus has been implemented into the system for future studies using a Fisher Vortex Genie 2 (Fisher Scientific, Hampton, NH, Cat. #12-812) set to gently shake the media reservoir every few hours.

Similar to the work presented within chapter 3 of this dissertation, the measured global bioreactor oxygen consumption rate, metabolic functions, and synthetic functions of the maintained hepatocytes will be utilized to gauge the health and differentiated function capabilities of the maintained
hepatocytes. This in turn will help to elucidate the most appropriate oxygen delivery conditions for the hepatocytes. Another synthetic function that will be measured within these experiments is the production of transferrin, an 80 kDa protein involved in iron binding and transport within plasma. Additionally, a generalized assay that can be utilized to quantitate total hepatocyte protein synthesis will also be incorporated into the studies.

5.2.2 Oxygenation and Hepatocyte Biotransformation/Detoxification Function

Biotransformation and detoxification functions are vital to liver function and must be conserved within an effective BLAD. Consequently, it is also of interest to determine the impact of the local cell space oxygen environment on hepatocyte biotransformation and detoxification capabilities. In order to conduct these tests, the circulating media will be supplemented with an appropriate compound intended to act as a chemical stress for the maintained hepatocytes. Over the duration of the study, aliquots would then be collected from the media reservoir and analyzed in order to gauge the ability of the cells to appropriately manage the chemical stress.

The detoxification capabilities of the hepatocytes would be gauged through their ability to convert ammonia (presented to the system as ammonium chloride) into either urea or glutamine. The concentration of both ammonia and urea within the aliquots taken from the circulating media will be determined using commercially available colorimetric assay kits.

The capability of the hepatocytes to conduct standard biotransformation functions will be gauged via lidocaine, diazepam, and 4-
methylumbelliferone (4-MU) biotransformation. Lidocaine and diazepam biotransformation occurs via phase I (cytochrome P450) activity, and thus it is expected that the cells will be better able to handle these chemical stresses when the local cell space pO$_2$ environment is within the perivenous range. Lidocaine, which is used mainly as a local anesthetic, is predominantly metabolized to monoethylglycinexylidide and 3-hydroxylidocaine in the human liver. The concentration of lidocaine and these two metabolites will be followed via high performance liquid chromatography (HPLC) analysis (125). Diazepam biotransformation is of specific interest because it is believed that benzodiazepine-like substances are involved in the onset of hepatic encephalopathy (126). The biotransformation of this compound results in the formation of ternazepam, oxazepam, and nordiazepam (126). Diazepam and its metabolites will also be assayed for via a HPLC procedure (126).

A phase II biotransformation challenge will be presented to the maintained hepatocytes via the addition of 4-MU. In vivo, 4-MU undergoes both glucuronidation and sulfation. The presence of 4-MU and its biotransformation products will be measured via another HPLC procedure previously described within the literature (127). Lastly, given that 4-MU biotransformation is a phase II activity, it is expected that periportal oxygen conditions within the hepatocyte cell space will be most appropriate for this type of differentiated function.
5.2.3 Hepatocyte Type Employed within the HF Bioreactor

The experiments described within this dissertation employed C3A Hepatoma cells within the HF bioreactor. It is anticipated that these cells will also be utilized throughout the studies proposed above. However, the use of primary hepatocytes is also of interest, especially given their improved differentiated function and increased oxygen demand in comparison to C3A cells. Unfortunately, as described in the introductory chapter of this dissertation, primary cells do not proliferate within \textit{in vitro} culture, and typically their differentiated function declines after relatively short cell culture durations. It has been demonstrated, though, that culturing primary hepatocytes within a 3-dimensional collagen gel can improve the maintenance of differentiated function within these cells. Consequently, it is suggested that studies similar to those described above be conducted using primary rat hepatocytes within the HF bioreactor. Work within this laboratory has begun aimed at developing experience with primary rat hepatocyte HF bioreactor culture. The primary rat hepatocytes that have been utilized were obtained from Dr. Howard Matthew at Wayne State University (Detroit, MI). These hepatocytes were entrapped within collagen within the ECS of a HF bioreactor. Utilizing a commercially available colorimetric assay, the primary rat hepatocytes were observed to produce urea, and thus it was concluded that at least some of the cells remained viable and exhibited differentiated function after inoculation into the HF bioreactor cartridge. This work is currently in its infancy, and thus it is
suggested that the use of primary rat hepatocytes within oxygenation studies performed on HF bioreactors be further developed.

5.2.4 Selection of the Type (Normal or Cross-linked) of Bovine RBC Utilized

The creation of a novel, tunable oxygen carrier designed for use within a HF bioreactor system was described in the fourth chapter of this dissertation. This cross-linking procedure was found to improve the osmotic stability of bRBCs. This would be beneficial for a HF bioreactor system as hemolysis would be reduced, and thus the amount of free Hb within the circulating media would be of less concern. However, it was found that the cross-linking procedure also had a relatively significant impact on reducing both the $P_{50}$ and cooperativity of the bRBC. Consequently, these engineered bRBCs seem to be best suited for delivering oxygen at reduced oxygen tension ranges.

If it is later found that there is significant hemolysis occurring within the HF bioreactor circulating media stream, then the potential of creating, through glutaraldehyde cross-linking under an anaerobic environment, osmotically stable bRBCs with oxygen binding/dissociation properties more similar to normal bRBCs should be considered. This suggestion is based on literature work that demonstrated that when the cross-linking reaction is carried out under an atmosphere devoid of oxygen, there is a much reduced impact on the oxygen binding/dissociation properties of the RBC (90). It is thus proposed that work be carried out examining the impact on select bRBC properties of glutaraldehyde cross-linking under an anaerobic atmosphere.
An additional problem that could be faced with the inclusion of engineered bRBCs within a HF bioreactor system involves the necessity of a significant amount of cross-linked bRBCs over the duration of a HF bioreactor oxygenation experiment. The reaction as carried out within the studies described here produced only 12 mL of engineered bRBC. Therefore, work will have to be conducted examining the scale-up of this reaction. One suggestion is to consider the possibility of carrying this reaction out within a flowing HF bioreactor system. This would enable a bRBC suspension to be fed from a large reservoir (250 mL or greater) through the fibers of a bioreactor while a glutaraldehyde solution is flowed through the ECS of that bioreactor. Ideally then, the glutaraldehyde would diffuse into the fibers of the bioreactor and react with the flowing bRBC suspension.
APPENDIX

MATLAB CODES

A.1 Simulation of the Oxygenation of Media Flowing Through Silicone Tubing

function siliconeoxygenationmarch62006
clear
clc

%Required parameters
Uave = 2.63; %(cm/sec; Average velocity)
H = 0.74; %(mmHg/uM; Henry's constant)
Pi = 59.5; %(mmHg; initial pO2)
Pw = 155; %(mmHg; wall pO2)
R = 0.15875; %(cm, tube radius)
D = 2.4E-5; %(cm^2/sec; diffusion coefficient for O2)

%RBC supplementation characteristics
N = 2.6; %(cooperativity)
Csat = 0; %(uM; concentration of OxyHb when fully saturated)
P50 = 26; %(mmHg; pO2 at half saturation)

%Discretization information
rintervals = 1000; %the number of intervals in the r direction
zintervals = 2000; %the number of intervals in the axial direction
tlength = 200.7; %(cm; tubing length)
rstep=R/(rintervals-1); %step change in radial direction

UaveD=2*Uave/D; %this is a constant in the pdepe solver
NP50H = N*(P50^N)*H*Csat; %this is a constant in the pdepe solver

%Use pdepe solver to solve the PDE
m=1;
x = linspace(0,R,rintervals);
t = linspace(0,tlength,zintervals);
sol = pdepe(m,@pdex2pde,@pdex2ic,@pdex2bc,x,t,[],UaveD,R,NP50H,N,P50,Pi,Pw);
u=sol(:,:,1);

%Use Simpsons 1/3 rule to calculate the ave. O2 conc.

for z=1:zintervals
    %first we must find the values used to solve the numerator
    toptopofintegral(1)=0; %set first one equal to zero
    for r=2:rintervals-1
        toptopofintegral(r)=x(r)*u(z,r);
    end

    %next, we sum the even elements of the numerator
    even=topofintegral(2);
    for r=2:2:rintervals-2
        even=topofintegral(r)+even;
    end
    %now sum the odd elements
    odd=topofintegral(3);
    for r=3:2:rintervals-1
        odd=topofintegral(r)+odd;
    end

    %now Pave can be calculated
    Pave(z)=2*((rstep/3)*((u(z,rintervals)*x(rintervals)+4*even+2*odd))/(R^2));
end

%save the Pave values
Pavetranspose=Pave';
csvwrite('SitubepO2.txt',Pavetranspose);

%plot Pave as a function of distance into tube
figure(3)
plot(t,Pave)
title('Average lumen pO2 as a function of distance into the lumen')
xlabel('Z (cm)')
ylabel('pO2 (mmHg)')

%subfunctions for determining pO2
---------------------------------------
function  [c,f,s] = pdex2pde(x,t,u,DuDx,UaveD,R,NP50H,N,P50,Pi,Pw)
\( c = U_{aveD} \times (1-(x/R)^2) \times (1+(NP50H \times ((u^{(N-1)})/((u^N)+(P50^N))^2))) \);
\( f = DuDx; \)
\( s = 0; \)

%--------------------------------------------------------------
function u0 = pdex2ic(x,UaveD,R,NP50H,N,P50,Pi,Pw)
u0 = Pi;
%--------------------------------------------------------------
function [pl,ql,pr,qr] = pdex2bc(xl,ul,xr,ur,t,UaveD,R,NP50H,N,P50,Pi,Pw)
pl = 0;
ql = 1;
pr = ur-Pw;
qr = 0;

A.2 Final Krogh Model Describing Oxygen Transport from the Flowing Media to the Hepatocytes

function Kroghmodelfinal

%Required parameters
Uave=0.7; % (cm/sec; Average velocity)
Pin = 10; % (mmHg; initial pO2)
Pave(1)=10; % mmHg; average pO2 within lumen at inlet

%O2 consumption characteristics
Vmax=10; % (uM/s; max hep O2 consumption rate)
Km = 0.5; % (mmHg, Michaelis-Menten constant)

%Bioreactor characteristics
Rc = 0.0090; % (cm, fiber radius)
Rm = 0.0105; % (cm, membrane radius)
Rt = 0.0160; % (cm, tissue radius)
D = 3E-5; % (cm^2/sec; diffusion coefficient for O2 in RBC suspension)
Dm = 3E-5; % (cm^2/sec; diffusion coefficient for O2 in membrane)
Dt = 2E-5; % (cm^2/sec; diffusion coefficient for O2 in tissue space)
H=0.74; % (mmHg/uM; Henry's constant)
Ht=0.74; % (mmHg/uM, Henry's constant in the tissue space)
btl=12; % bioreactor total length (cm)

%RBC supplementation characteristics
N = 2.65; % (cooperativity)
Csat = 0; % (uM; concentration of OxyHb when fully saturated)
\[ P50 = 26.1 \; \text{mmHg; } \text{pO2 at half saturation} \]

%Discretization information
\[ R_{\text{mintervals}} = 100; \; \text{%intervals in the r direction within the membrane} \]
\[ R_{\text{tintervals}} = 100; \; \text{%intervals in the r direction within the ECS} \]
\[ r_{\text{intervals}} = 100; \; \text{the number of intervals in the r direction} \]
\[ z_{\text{intervals}} = 10; \; \text{the number of intervals in the axial direction for each axial} \]
\[ t_{\text{length}} = .001; \; \text{cm; tubing length} \]
\[ z_{\text{change}} = t_{\text{length}}/(z_{\text{intervals}} - 1); \]
\[ r_{\text{co}} = \text{linspace}(0,R_c,r_{\text{intervals}}); \; \text{vector containing the r values used} \]
\[ r_{\text{change}} = R_c/(r_{\text{intervals}} - 1); \; \text{%step change in radial direction in lumen} \]
\[ r_{\text{mchange}} = (R_m - R_c)/(R_{\text{mintervals}} - 1); \; \text{%step change in radial direction in} \]
\[ z_{\text{axialpoints}} = 12000; \]

%Make a vector for the inlet pO2
\text{for} \; \text{counter} = 1:r_{\text{intervals}}
\quad \text{Pi(counter)} = \text{Pin};
\text{end}

%constants used used within the PDE's
\[ N_{\text{P50H}} = N \times (P50^N) \times H \times C_{\text{sat}}; \]
\[ U_{\text{aveD}} = (2 \times U_{\text{ave}})/D; \]
\text{axialcounter} = 1; \; \text{%counter for global axial change} \]

%initial derivative based on analytical solution of Pt for determination of
%Plumen and Pmembrane
\text{DerivPt(axialcounter)} = ((0.5 \times H_t \times V_{\text{max}})/(D_t)) \times ((R_m - ((R_t^2)/(R_m))));

%Begin loop for determining the pressure distribution
\text{for} \; \text{axialcounter} = 1:z_{\text{axialpoints}}

%this section sets the derivative at the inside tissue edge for the current axial
%step
\text{if} \; (\text{axialcounter} > 1) \; \& \; (\text{axialcounter} < 6)
\quad \text{DerivPt(axialcounter)} = \text{Pecsderivative(2)}; \; \text{%derivative at inside tissue} \]
\text{edge}
\text{end}
\text{if} \; \text{axialcounter} > 5
\quad \text{if} \; \text{Pecs(2,1) < DerivPt(axialcounter-1)}
\quad \quad \text{DerivPt(axialcounter)} = \text{DerivPt(axialcounter-1)}; \; \text{%derivative at} \]
\text{inside tissue edge}
else
  \text{otherwise if it is larger than previous, use current derivative value}
  \text{DerivPt(axialcounter)=Pecsderivative(2); \text{derivative at inside}}
  \text{tissue edge}
end
end

BC= -(((Dt/D)*Rm*DerivPt(axialcounter))/Rc); \text{this is the BC used within the}
\text{lumen PDE}

\%First we find how pO2 within the fiber varies
options = odeset(’RelTol’, 1E-3); \text{this is in case we want to decrease the}
\text{error tolerance}

\%The following is used to solve the lumen PDE
m=1; \text{this value specifies the geometry -- 1 indicate cylindrical}
x = linspace(0,Rc,rintervals); \text{this sets the r values for the PDEPE solver}
t = linspace(0,tlength,zintervals); \text{this sets the z values for the PDEPE}
\text{solver}
sol =
pdepe(m,@pdex2pde,@pdex2ic,@pdex2bc,x,t,options,UaveD,Rc,NP50H,N,
P50,rco,Pi,BC);
u=sol(:,:,1);

\%for the next points, the lumen pressure distribution must be saved for the
\%pdepe solver
for counter=1:rintervals
  Pi(counter)=u(zintervals,counter);
end

\%the following steps are for finding the pressure distribution within the
\%membrane and tissue
for zcounter=1:zintervals \%determine the pressure distribution step-wise in
\%the axial direction
  r=Rc; \%initial r value at the inner edge of the membrane
  \%the following is to determine Pm
  for rmcounter=1:Rmintervals \%determine the P distribution step-wise in the
  \%radial direction for given axial point
    Pm(zcounter,rmcounter)=u(zcounter,rintervals)+(Dt/Dm)*Rm*DerivPt(axialcounter)*(log(r/Rc));
    \%in case the membrane pressure drops to less than 0, set remaining
%Pm to 0
if Pm(zcounter,rmcounter)<0
    Pm(zcounter,rmcounter)=0;
end % for if on line 104
r=r+rmchange;
end % for on line 100

%next, the P distribution within the ECS is determine as long as 
Pm(r=Rm)>0
if Pm(zcounter,Rmintervals)>0

    PmRm=Pm(zcounter,Rmintervals); %initial guess for the Pt(r=Rm)
    solinit=bvpinit(linspace(Rm,Rt,Rtintervals),[PmRm,-2000]);
    sol = bvp4c(@Pt4ode,@Pt4bc,solinit,options,PmRm,Ht,Vmax,Dt,Km);
    j = linspace(Rm,Rt,Rtintervals); %create a vector for solving the Pm at 
    %specified radial points
    Pecs = deval(sol,j);
    Pcsderivative(zcounter)=Pecs(2,1); %this is the derivate at Pt(r=Rt)

end %for on line 122

%for the next axial points create a matrix, Pt, that stores the pO2 within 
%the ECS
for rtcounter=1:Rtintervals
    Pt(zcounter,rtcounter)=Pecs(1,rtcounter);
    
    if Pt(zcounter,rtcounter)<0 %in case Pt<0, set Pt=0
        Pt(zcounter,rtcounter)=0;
        end %for if on line 125

    %in case the Pt doesnt decrease futhur into ECS, set next point =
    % previous point
    if rtcounter>1
        if Pt(zcounter,rtcounter)>Pt(zcounter,rtcounter-1)
            Pt(zcounter,rtcounter)=Pt(zcounter,rtcounter-1);
        end %for if on line 131
    end %for if on line 130
end %for on line 122
end %for if on line 112

%in case the Pm<=0 at r=Rm, then set all Pt=0
if Pm(zcounter,Rmintervals)<=0
    for rtcounter=1:Rtintervals
        Pt(zcounter,rtcounter)=0;
    end %for on line 140
end %for if on line 139
end %for if on line 95
%next, the code is used to determine Pave within the lumen using simpson’s 1/3 rule must first find the values used to solve the numerator

topofintegral(1)=0; %set first one equal to zero
for r=2:rintervals-1
    topofintegral(r)=x(r)*(u(zintervals,r));
end

%next, we sum the even elements of the numerator
even=topofintegral(2);
for r=2:2:rintervals-2
    even=topofintegral(r)+even;
end
%now sum the odd elements
odd=topofintegral(3);
for r=3:2:rintervals-1
    odd=topofintegral(r)+odd;
end

%now Pave can be calculated
Pave(axialcounter+1)=2*((rchange/3)*((u(zintervals,rintervals)*x(rintervals)+4*even+2*odd))/(Rc^2));

%save the Pm value at r=Rm, the Pt value at r=Rt, and the P value at r=0,Rc
Pmsaved(axialcounter)=Pm(1,Rmintervals);
Ptsaved(axialcounter)=Pt(1,Rtintervals);
Pcenterline(axialcounter)=u(1,1);
Pedge(axialcounter)=u(1,rintervals);
end

%save the P values for the exit point
Pmsaved(axialcounter+1)=Pm(zintervals,Rmintervals);
Ptsaved(axialcounter+1)=Pt(zintervals,Rtintervals);
Pcenterline(axialcounter+1)=u(zintervals,1);
Pedge(axialcounter+1)=u(zintervals,rintervals);

%now we transpose these vectors to make them into column vectors (easier for manipulation in excel)
PCL=Pcenterline’;
PRC=Pedge’;
Pmem=Pmsaved’;
Ptissue=Ptsaved’;
Paverage=Pave’;

%Calculate the global bioreactor Oxygen consumption rate
%This is the moles of oxygen lost within the bioreactor over time
Cin=(Pin/H)*(1/1000); % Cin in umol/cm^3
Cout=(Pave(axialcounter+1)/H)*(1/1000); % Cout in umol/cm^3
Qfiber = Uave*pi*(Rc^2); % Q in cm^3/sec
DissolvedO2=Qfiber*(Cin-Cout) % umol/sec
Sin=(Pin^N)/((Pin^N)+(P50^N)); % fraction of Hb as OxyHb at inlet
Sout=(Pave(axialcounter+1)^N)/((Pave(axialcounter+1)^N)+(P50^N)); % fraction of Hb as OxyHb at exit
BoundO2change=Qfiber*((Sin*Csat)-(Sout*Csat))*(1/1000) % umol/sec
GBOCrate=DissolvedO2+BoundO2change; % total oxygen lost
for GBOCcounter=2:axialcounter+1
    GBOCrate(GBOCcounter)=0;
end
GBOCrate=GBOCrate';
parameters=[PCL Paverage PRC Pmem Ptissue GBOCrate];
csvwrite('Pressuredistribution.txt',parameters)

% Subfunctions for determining P
%-------------------------------------
function  [c,f,s] = pdex2pde(x,t,u,DuDx,UaveD,Rc,NP50H,N,P50,rco,Pi,BC)
c = (UaveD*(1-(x/Rc)^2))*(1+((NP50H)*((u^(N-1))/((u^N)+(P50^N))^2)));
f = DuDx;
s = 0;

% Subfunctions for determining Pt
%-------------------------------------
function dydx=Pt4ode(x,y,PmRm,Ht,Vmax,Dt,Km)
dydx = [ y(2)
        (((Ht*Vmax/Dt)*y(1)/(Km+y(1)))-(1/x)*y(2))];
function res = Pt4bc(ya,yb,PmRm,Ht,Vmax,Dt,Km)
res = [ ya(1)-PmRm]
A.3 Bovine Red Blood Cell Adair Constant Determination Using Raw Hemox Generated Absorbance Data

%This file utilizes the raw Hemox absorbance data to determine the Adair constants via a 6 parameter nonlinear fitting. This file was written on September 1, 2005.

%This file requires the m files dissociation.m, adaircurve.m, and P50.m. Additionally, one must create a tab delimited text file in Microsoft Excel named Adairdata.txt, with column A as the raw pressure data, column B as the raw absorbance data, %column C as the absorbance data normalized to saturation data assuming that the %high and low absorbances measured %by the Hemox are a Y of 1 ans 0, and column D %as the Adair parameters %as estimated by the Hemox.

%This file then utilizes the Hemox adair parameters as a first guess and %solves the Adair equation for the normalized saturation data (4 parameter %fit). The revised Adair parameters are then utilized, along with initial %values of the absorbance at infinite pressure and no pressure (which comes %as the high and low Hemox measured absorbances) to fit the raw %absorbance data to a 6 parameter Adair equation.

%The output of this program is a text file called Adairparameters.txt. %This file gives 3 columns of data, which correspond to the initial guesses %for the parameters, the 6 parameter absorbance fitted data, and the %normalized saturation fitted data. The output is of the form: A1, A2, A3, A4, %A(infinity), A(0), the residual of the fit, the p50, and the saturation %values at the high and low Hemox measured pO2 values.

%It is important that one pays attention to the residual that is given to %ensure reasonable estimates of the Adair parameters and the p50 value. I %have found that you should look for a residual of at least less than 1E-3. %Also, please make sure you look at the graph comparing the raw %absorbance data to that predicted by the 6 parameter fit.

%There is also a version of this code that fits the raw absorbance data to %a 5 parameter equation (A(0) is assumed as the last absorbance measured %by the Hemox, and A(infinity) is kept as an unknown parameter).

%The last thing to note is that if your p50 is out of the range of 1 to 30, %then you will need to change these bounds below within the matrix, uy %%(code line 49).
clear

%load data
M = dlmread('Adairdata.txt','	');

p=M(:,1); %read pressure data into p
den=M(:,2); %read absorbance data into den
sat=M(:,3); %read normalized saturation data into sat
adairguess=M(:,4); %read Hemox Adair parameters into adairguess
uy=[1;30]; %is the bounds for the p50 value

L=length(den); %number of data points
absinf=den(1); %the absorbance at the highest pO2
abszero=den(L); %the absorbance at the lowest pO2

%-------------------------------------------------------------
%NON-LINEAR LEAST SQUARES FIT TO DISSOCIATION CURVE TO
%DETERMINE %OVERALL ADAIR CONSTANTS
%-------------------------------------------------------------

%Hemox Adair parameters
ai = [adairguess(1)
     adairguess(2)
     adairguess(3)
     adairguess(4)
     absinf
     abszero];

xdata=p; %place pressure data into xdata
ydata=sat; %place normalized saturation data into ydata

x0=ai; %starting guess

%Set the upper and lower bounds
UB = [Inf
      Inf
      Inf
      Inf
      2
      ai(6)];

LB = [0
      0
      0
      0
      0
      0]
% Set the options for the lsqcurvefit command
options = optimset('MaxFunEvals', 10000, 'MaxIter', 5000, 'TolFun', 1e-10, 'TolX', 1e-10);

% nonlinear least squares fit command
[k, resnorm, residual, exitflag, output] = lsqcurvefit(@adaircurve, x0, xdata, ydata, LB, UB, options);
k(5) = absinf; k(6) = abszero; % reset these back to original values
x0 = k; % place new guess data into X0

% find the P50 from saturation data
A1 = k(1); A2 = k(2); A3 = k(3); A4 = k(4);
Z = fzero(@P50, uy, options, A1, A3, A4);
fit = (k(1).*p + 2.*k(2).*p.^2 + 3.*k(3).*p.^3 + 4.*k(4).*p.^4)./(4.*(1 + k(1).*p + k(2).*p.^2 + k(3).*p.^3 + k(4).*p.^4));

% save the parameters for the normalized saturation estimation for output
saturationparameters = [k(1)
  k(2)
  k(3)
  k(4)
  0
  0
  resnorm
  Z
  fit(1)
  fit(L)];

% nonlinear least squares fit command using absorbance data
ydata = den; % switch ydata to raw absorbance data
[x, resnorm, residual, exitflag, output] = lsqcurvefit(@dissociation, x0, xdata, ydata, LB, UB, options);

fit = (x(1).*p + 2.*x(2).*p.^2 + 3.*x(3).*p.^3 + 4.*x(4).*p.^4)./(4.*(1 + x(1).*p + x(2).*p.^2 + x(3).*p.^3 + x(4).*p.^4));

% find the P50
A1 = x(1); A2 = x(2); A3 = x(3); A4 = x(4); A5 = x(5); A6 = x(6);
Z = fzero(@P50, uy, options, A1, A3, A4);

% code for finding the sum of squares difference between calculated % absorbance and raw data absorbance
counter=1;
sumofsquares=0;
pressure=p';

while counter<L+1
absorbance(counter)=((A1*pressure(counter)+2*A2*pressure(counter)^2+3*A3*pressure(counter)^3+4*A4*pressure(counter)^4)/(4*(1+A1*pressure(counter)+A2*pressure(counter)^2+A3*pressure(counter)^3+A4*pressure(counter)^4)))*(A5-A6)+A6;
sqd=(absorbance(counter)-ydata(counter))^2;
sumofsquares=sumofsquares+sqd;
counter=counter+1;
end

%save the required parameters to a file
parameters=[x(1)
x(2)
x(3)
x(4)
x(5)
x(6)
resnorm
Z
fit(1)
fit(L)];

initialA=[ai(1)
ai(2)
ai(3)
ai(4)
ai(5)
ai(6)
0
0
0
0];
overallparameters=[initialA parameters saturationparameters];

absorbancecolumn=absorbance';
csvwrite('Adairparameters.txt',overallparameters);
csvwrite('Adairabsorbance.txt',absorbancecolumn);

%plot the Adair predicted saturation curve
plot(p,fit,'o-')
hold off
legend('NLLS fit')
%Display data
output
disp('Adair constants')
disp('initial final')
format short e
[ai x]

%plot the adair absorbance data versus raw absorbance
figure(2)
plot(pressure,absorbance,'b-')
hold on
plot(p,ydata,'r--')
hold off

%exit flag command for problems with the fit
if exitflag==1
    disp('SOLUTION FOUND: Function converged to a solution x')
elseif exitflag==2
    disp('SOLUTION FOUND: Change in x was less than the specified
tolerance')
elseif exitflag==3
    disp('SOLUTION FOUND: Change in the residual was less than the
specified tolerance')
elseif exitflag==4
    disp('SOLUTION FOUND: Magnitude of search direction smaller than
the specified tolerance')
elseif exitflag <=0
    disp('Routine terminated prematurely')
end

%Subfunctions:
function F=dissociation(x,xdata) %calculates the nonlinear least squares
%regression of the raw absorbance data to a 6 parameter version of the Adair
%equation
%F is the oxy hemoglobin raw absorbance data
%x is the vector of overall adair coefficients and absorbances at P=0 and
%P=infinity, %xdata is the oxygen tension (pO2)
F=((x(1)*xdata+2*x(2)*xdata.^2+3*x(3)*xdata.^3+4*x(4)*xdata.^4)./(4*(1+x(1)*
xdata+x(2)*xdata.^2+x(3)*xdata.^3+x(4)*xdata.^4))).*(x(5)-x(6))+x(6);

function F=adaircurve(k,xdata) % provide a nonlinear least squares
%regression %analysis of the normalized saturation data to the traditional adair
%equation.
%F is the oxy hemoglobin saturation, x is the vector of overall adair
%coefficients
%xdata is the oxygen tension (pO2)
F=((k(1)*xdata+2*k(2)*xdata.^2+3*k(3)*xdata.^3+4*k(4)*xdata.^4)./(4*(1+k(1)*xdata+k(2)*xdata.^2+k(3)*xdata.^3+k(4)*xdata.^4)));

function F=P50(Z,A1,A3,A4) %calculates P50 of suspension
F=2+A1*Z-A3*Z^3-2*A4*Z^4;
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