Rapid Separation of Bacteria from Blood for Sepsis Diagnosis

Mahsa Alizadeh
Brigham Young University

Follow this and additional works at: https://scholarsarchive.byu.edu/etd
Part of the Chemical Engineering Commons

BYU ScholarsArchive Citation
Alizadeh, Mahsa, "Rapid Separation of Bacteria from Blood for Sepsis Diagnosis" (2018). All Theses and Dissertations. 7038.
https://scholarsarchive.byu.edu/etd/7038

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu, ellen_amatangelo@byu.edu.
Rapid Separation of Bacteria from Blood
for Sepsis Diagnosis

Mahsa Alizadeh

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

William G. Pitt, Chair
Bradley C. Bundy
Alonzo D. Cook

Department of Chemical Engineering
Brigham Young University

Copyright © 2018 Mahsa Alizadeh
All Rights Reserved
ABSTRACT

Rapid Separation of Bacteria from Blood for Sepsis Diagnosis

Mahsa Alizadeh
Department of Chemical Engineering, BYU
Master of Science

Sepsis is a severe blood infection caused by bacteria entering the blood stream. Sepsis caused by antibiotic resistant bacteria is very dangerous with a high mortality rate. The current clinical diagnostic methods for sepsis require culturing the blood sample prior to other steps of the diagnosis procedure. Culturing the blood samples is a time-consuming step which increases the time required for the diagnostic procedure. Considering the fact that the mortality rate of the sepsis increases as time passes, it is essential to find methods for sepsis diagnosis that do not require culturing the samples.

The first step of a new diagnostic method for antibiotic resistant sepsis, we have developed a new method for rapid separation of the bacteria from whole human blood based on centrifugal force. Density and size differences between blood cells and bacteria lead to different sedimentation velocities for each of these cells and microorganisms in a centrifugal field. Spinning blood inoculated with bacteria in our designed hollow disks at the specified speed for a designated period of time creates fairly well-separated layers of blood cells and plasma. Red and white cells have higher sedimentation velocities due to higher densities or larger sizes compared to bacteria, forming a region of dense cells close to the wall of the spinning disk. Bacteria sediment slower than red and white cells, moving to and remaining in plasma. By carefully slowing the spinning speed after separation, we are able to avoid remixing of the blood cell layer and bacteria, thus keeping the bacteria separated from rest of the blood cells.

This thesis involves the experimental methods for increasing the recovery of the bacteria from human blood by mechanical and chemical methods. It also explains the theory behind the separation technique used.

Keywords: sepsis, diagnosis, sedimentation, separation
ACKNOWLEDGEMENTS

I would like to thank Dr. Pitt for his mentorship, patience and kindness over the last three years. Thank you for teaching me how to design and conduct the experiments and to analyze the results. Thank you for giving me the opportunity to be in your research group.

I want to thank Daniel McClellan, Colin Bledsoe, Rae Blanco, Alex Hunter, Madison Wood, Tanner Ravsten, Clara Buchanan, Caroline Hickey, Alexandra Carter, Jacob Stepan, Cameron Beard, Evelyn Welling and Rebekah Torgesen who helped me conducting the experiments. I enjoyed working with you in the lab. I want to thank Ryan Wood for his help with analyzing the data related to the number of the red blood cells.

I would also like to thank the faculty who taught me in class and Dr. Cook and Bundy for serving on my committee.

I acknowledge my deep thanks to my husband, Mahmood Rahmati, for his love and support during graduate school. I wouldn't have been able to concentrate on my research if you were not patient.

I want to thank my father, Hashem, my mother, Pourandokht Baradaran Nateghi and my brother, Sina, for their everlasting love and support, even from continents away.

This work was supported by the National Institute of Health grant number R01AI116989.
TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................................................ vi
LIST OF FIGURES ................................................................................................................................................ vii

1 Introduction .................................................................................................................................................. 1

2 Background ................................................................................................................................................ 4

2.1 Infectious Diseases ............................................................................................................................. 4
2.2 Sepsis ................................................................................................................................................ 6
2.3 Etiology and Site of Infection ............................................................................................................. 7
2.4 Current Diagnosis Assays for Sepsis ............................................................................................. 10
2.5 Nucleic-Acid Based Technologies for Sepsis Diagnosis ................................................................. 12
2.6 Centrifugation and Sedimentation ................................................................................................. 13

3 Aims and Objectives .................................................................................................................................. 15

3.1 Aim and Primary Objective .............................................................................................................. 15
3.2 Overview ........................................................................................................................................... 15
3.3 Sub-objectives ..................................................................................................................................... 16

3.3.1 Determine the Influence of Anti-coagulant on Bacterial Recovery ....................................... 16
3.3.2 Influence of Disk Design on Bacterial Recovery by Sedimentation ...................................... 17
3.3.3 Determine the Influence of Sedimentation Process Parameters on Blood Cell Separation .......................................................................................................................... 17

4 Materials and Methods .......................................................................................................................... 18

4.1 Disk Design ......................................................................................................................................... 18
4.2 Disk Motors .......................................................................................................................................... 21
4.3 Disk Platform ....................................................................................................................................... 22
4.4 Control System for Chemical Aspects Experiments ...................................................................... 22
4.5 Control System for Mechanical Aspects Experiments .................................................................. 24
4.6 Preparing Bacteria ............................................................................................................................. 24
4.7 Spinning Blood .................................................................................................................................... 25
4.8 Collecting Plasma ............................................................................................................................... 26
4.9 Counting Bacteria in Recovered Plasma and Initial Blood ............................................................... 26
4.10 Red Cell Count .................................................................................................................................. 27
4.11 Bacteria Concentration Recovery ................................................................................................. 27
4.12 Total Bacteria Recovery .................................................................................................................... 28
4.13 Platelet Imaging ............................................................................................................................... 29
LIST OF TABLES

Table 2-1: Frequency and mortality rate of sepsis associated with site of infection .................. 9
Table 2-2: Species and organisms associated with sepsis and their frequencies ...................... 11
Table 5-1: Mean average results for disks with various number of baffles .............................. 33
Table 5-2: P-values for comparisons between disks with different number of baffles ............... 34
Table 5-3: Blood and PBS volume used for each size of the disk and disk measurements for each size of the disk .................................................................................................................................. 36
Table 5-4: Y-intercept for disks of various diameter .................................................................. 42
Table 5-5: Y-intercept for disks of various diameter ................................................................. 43
Table 5-6: Slopes for red cell recovery vs. characteristic length ................................................. 47
Table 5-7: P-values for red cell recovery vs. characteristic length ............................................. 48
Table 6-1: Average RBC recovery for experiments with various type of anticoagulants ........... 56
Table 6-2: P-values for statistical analysis comparing red cell recovery of three types of anticoagulants .................................................................................................................................................. 56
LIST OF FIGURES

Figure 4-1: Schematic design of the hollow disk................................................................. 20
Figure 4-2: Detailed design of the disk.................................................................................. 21
Figure 4-3: Design of the disk platform used to connect the disk to the motor............... 22
Figure 4-4: Plot of spinning speed of the disk versus spinning time used for experiments studying chemical aspects of spinning................................................................. 23
Figure 5-1: Bacteria total recovery versus characteristic length for a 10-cm-diameter disk......37
Figure 5-2: Bacteria total recovery versus characteristic length for a 12-cm-diameter disk.....38
Figure 5-3: Bacteria total recovery vs characteristic length for a 16-cm-diameter disk.........38
Figure 5-4: Integrated plot of bacteria concentration recovery vs. characteristic length for 10-cm-disk, 12-cm-disk and the 16-cm-disk................................................................. 39
Figure 5-5: Plot of bacteria concentration recovery versus characteristic length for 10-cm-diameter disk ................................................................................................................. 40
Figure 5-6: Plot of bacteria concentration recovery versus characteristic length for 12-cm-diameter disk ................................................................................................................. 40
Figure 5-7: Plot of bacteria concentration recovery versus characteristic length for 16-cm-diameter disk ................................................................................................................. 41
Figure 5-8: Plot of plasma volume fraction versus characteristic length.......................... 43
Figure 5-9: Red cell recovery versus characteristic length for the 10-cm disk.................. 45
Figure 5-10: Red cell recovery versus characteristic length for 12-cm disk.................... 45
Figure 5-11: Red cell recovery versus characteristic length for the 16-cm disk.............. 46
Figure 5-12: Schematic design of disk height and its influence on the plasma menisci...... 49
Figure 5-13: Plot of bacteria concentration recovery versus characteristic length comparing two various disk weir designs ................................................................. 50
Figure 6-1: Plot of volume of recovered plasma versus the gender of the donor and the type of anticoagulant used ................................................................................................. 53
Figure 6-2: Concentration of platelets in the recovered plasma as a function of anticoagulant and gender ............................................................................................................. 58
Figure 6-3: Concentration of platelets in the recovered plasma as a function of anticoagulant and ADP
1 INTRODUCTION

Bacteria in the blood stream of a human can cause a severe blood infection called sepsis. Sepsis caused by antibiotic resistant bacteria can be very dangerous and fatal and has a high mortality rate. Sepsis can be caused by both gram-positive and gram-negative bacteria\textsuperscript{1-4}. Gram-negative bacteria can acquire resistance to antibiotics by plasmid transfer easier than gram-positive species, which cause more concerns for clinicians and researchers\textsuperscript{1, 5-6}. The most troubling gram-negative bacteria that cause sepsis are Klebsiella pneumonia (\textit{K. pneumoniae}), Escherichia coli (\textit{E.coli}), and members of the genus Enterobacter\textsuperscript{6}. The carbapenem class of antibiotics effectively treats sepsis except in case of Carbapenem-resistant enterobacteria (CRE). Carbapenem-resistant bacteria pose greater challenges to the healthcare system since they are very difficult to treat with standard antibiotics\textsuperscript{7} and can only be treated with Colistin, a fairly toxic antibiotic\textsuperscript{8}. These infections become life-threatening even when the concentration of bacteria is as low as 10 colony-forming-units per milliliter (CFU/ml) of blood. Because blood is a suitable medium for bacteria, their number increases as the time passes, increasing the mortality rate of the infection. Current clinical methods used to diagnose the microorganism and its antimicrobial resistance involve culturing the blood and then performing additional experiments such as phenotypic assays, fluorescence in situ hybridization, mass spectrometry, and polymerase chain reaction (PCR) to identify whether resistance genes are present. PCR methods can identify the pathogen and its antimicrobial resistance quickly and precisely.
However, the efficiency of these methods is very low in blood since blood contains inhibitors that interfere with the PCR reactions\textsuperscript{3}. A rapid and effective diagnosis method, such as tests based on nucleic acids, can identify the pathogens in less than one hour when the bacteria has already been separated from blood. Blood components such as red and white cells must be separated from the bacteria so that they will not interfere with DNA-based detection tests.

The objective of my M.S. thesis was to develop and analyze rapid separation methods for bacteria from blood. Chapter 2 of this thesis includes the previous methods used to diagnose sepsis mentioned in the literature, while Chapter 3 contains the prime objective for this thesis and the sub-objectives including the steps to achieve the prime objective.

The method used to separate the bacteria from blood was sedimentation. A hollow disk was designed which has the capacity to hold a pre-specified volume of blood and spin at a specific speed for a designated time. As the disk stops, plasma rich in bacteria was collected for bacteria identification. The details on disk design are discussed mainly in Chapter 4, Materials and Methods.

The first sub-objective of this thesis was to select the appropriate spinning parameters (spinning speed and time) that will maximize bacteria recovery while reducing red cell recovery in the plasma. Chapter 5 provides a summary of the experiments designed and conducted to find the optimum spinning conditions.

The second sub-objective of this thesis was to select a proper anticoagulant to prevent blood from coagulation while conducting experiments. This and other chemical aspects are discussed extensively in Chapter 6.
Chapter 7 discusses the data of Chapters 5 and 6, and the next crucial steps for the identification of the bacteria that are not part of this thesis and suggestions to further improve this technique.
2 BACKGROUND

2.1 Infectious Diseases

An infection results when a pathogen invades and begins growing within a host. Healthcare-associated infections (HAIs) – infections patients can get while receiving medical treatment in a healthcare facility or after they have been discharged from the medical facility – are a major, yet often preventable, threat to life. The World Health Organization (WHO) estimates that the number of patients infected by HAIs worldwide exceeds hundreds of millions annually, causing a significant mortality and an enormous economic burden on health care systems. According to the Centers for Disease Control and prevention (CDC), medical costs of HAIs for US hospitals is between 36 to 45 billion dollars ($ US) annually. Six to 7 percent of patients that are hospitalized at any given time will unfortunately get at least 1 infection that can be classified as HAIs in developed countries. In developing countries, at least 10% of hospitalized patients will face the same problem. The current methods for the prevention and treatment of the infections are losing their effectiveness because the microorganisms are becoming more resistant to the available medicine. Antimicrobial resistance (AMR) is threatening global health; patients with antimicrobial- resistant infections have to pay higher costs of healthcare compared to patients who suffer non-resistant infections since the medications are much more expensive and the illness lasts longer than a non-resistant one.
Antimicrobial resistance is the result of the change in the genetic structure of the microorganisms, occurring as these antimicrobial drugs are prescribed to battle the infection. As the microorganisms acquire resistance to the antimicrobial drugs globally, we lose our once-effective treatments for the infections, and we are becoming unable to deal with common infections. As a result, these common infections cause long-lasting illnesses, disability and eventually death\textsuperscript{10}.

Without proper antimicrobials, we will not have any medications to prevent and treat infections. The high risk of infection might introduce new challenges to medical procedures such as organ transplant, cancer treatments and major surgeries such as caesarean section\textsuperscript{10}.

The development of resistance to antimicrobial drugs is a natural procedure that eventually happens over time. However, misapplication of the antimicrobials stimulates this natural phenomenon\textsuperscript{10}.

Drug-resistant bacteria cause infections that lead to severe conditions for the patient who may die. These patients demand more health-care resources compared to patients with non-resistant species of the same bacteria. Hospital-acquired bloodstream infections threaten newborns and intensive-care unit patients (patients with weaker immune systems). In some countries, carbapenem is ineffective for half of these patients with \textit{K. pneumoniae} due to resistance developed by that species\textsuperscript{10}.

\textit{E. coli} resistance to fluoroquinolone antibiotics, which are commonly used to treat urinary tract infections, is prevailing. In some countries, this antibiotic is ineffective for half of the cases prescribed\textsuperscript{10}.
2.2 Sepsis

Sepsis is defined as the systemic illness which is caused by the body's response to bacteria entering the body. This term differentiates an illness of microbial origin from an identical clinical syndrome that might appear in several non-microbial conditions, for example pancreatitis. Sepsis is defined as the association of several non-specific inflammatory responses with the signs, or suspicion, of a microbial cause. When this syndrome is accompanied by some extent of hypoperfusion (a condition of severe peripheral circulatory failure that is caused by improper circulatory control or as a result of the loss of circulating fluid) or at least one organ failure, the situation is referred to as “severe sepsis”. Finally, septic shock happens when severe sepsis is accompanied by very low blood pressure or need for vasopressors. Increasing severity of sepsis leads to increasing mortality rate, which rises from 25-30% for severe sepsis up to 40-70% for septic shock.\textsuperscript{11}

The signs and symptoms of sepsis are affected by many factors including virulence, the severity or harmfulness of a disease or poison, and bioburden defined as the number of live bacteria present. Other factors affecting the symptoms of sepsis include the portal of entry, host susceptibility (the resistance the patient's body shows towards an infection), and the temporal evolution of the condition. Symptoms are non-specific but can all be related to the overall picture of systemic illness.\textsuperscript{11}

Diagnosis and confirmation of the microbial origin of a disease is accomplished either visually by microscopy of pathogens in tissue samples or, more commonly, by culturing tissue samples, especially culturing the blood of the suspected patient. Culturing the samples of the patient helps to assure the microbial origin of the febrile condition and identify the antimicrobial agents to which the pathogen is susceptible.\textsuperscript{12} However, it takes at least 24 hours before the
results from cultures become available, and even then only 50% of the suspicious cases can be
classified as positively having a microbial origin. This low yield might be due to many factors
such as sampling error, antimicrobial regimen prior to sampling, or the presence of fastidious or
slow growing pathogens which need longer incubation time11, 13.

As explained previously, sepsis diagnosis usually involves culturing the blood samples,
which may take a long time. Therefore, antimicrobial treatment is usually started empirically
based on the patient's symptoms and the epidemiological data11-12. The results of cultures and
sensitivity testing (which is performed to test the susceptibility of the pathogen towards
antibiotics) are used only for confirmation of prior empirical diagnosis and to sometimes change
the recommended treatment. The inadequate treatment caused by delayed identification of the
pathogen may be eliminated by new molecular methods. These new methods assure the detection,
identification, quantification, and determination of the pathogen and its resistance genes from
patient's samples within a few hours, eliminating the step of sample culturing. These methods are
often automated and include a step of amplification of specific DNA sequences by polymerase
chain reaction (PCR). These new molecular methods, depending on the target DNA sequence
and the detection method used (e.g. DNA sequencing, DNA microarray, fluorescent probe), test
the sample for the presence of single pathogens, members of entire pathogenic species, or even
members of entire phylogenetic kingdoms such as the eubacteria by using 16S ribosomal RNA
probing11.

2.3 Etiology and Site of Infection

The inflammatory response of the body's immune system is affected by the microbial
etiology and source of sepsis. The most common site of infection among all studied septic cases
were respiratory tract infections, especially pneumonia. Moreover, these infections have the highest mortality rate among all the septic cases\textsuperscript{14}.

Most pneumonia cases occur in men and alcoholics, while the frequent site of sepsis for women is reported as genitourinary infections. Other common infection sites are reported as abdominal, skin, and soft tissue, device-related (catheter-related sepsis), central nervous system, and endocarditis\textsuperscript{14} (see Table 2-1). Motzkus \textit{et al} have reported that it is not easy to estimate the effect of infection site on the hospital mortality rate of septic patients due to misclassification among infections and diseases\textsuperscript{15}.

The type of microbial origin of severe sepsis directly influences the outcome of the infection. The number of septic cases associated with a gram-positive organism as a cause of infection have increased over time and are now as common as gram-negative infections, probably due to greater use of invasive procedures\textsuperscript{14}.

The predominant organisms associated with sepsis are reported as \textit{Staphylococcus aureus} (20.5\%), \textit{Pseudomonas} species (19.9\%), \textit{Enterobacteriaceae} (mainly \textit{E. coli}, 16.0\%), and fungi (19\%). \textit{Acinetobacter} was seen in 9\% of all infections, with higher infection rates in Asia (19.2\%) compared to North America (3.7\%)\textsuperscript{14} (see Table 2-2).

Antibiotic resistance has increased over time as a result of more common use of broad-spectrum antibiotics for sick patients who remain in the ICU for longer periods of time. Antibiotic resistance is problematic, increasing the needed length of stay in intensive care units for patients\textsuperscript{14}. The predominant organisms associated with sepsis are reported as \textit{Staphylococcus aureus} (20.5\%), \textit{Pseudomonas} species (19.9\%), \textit{Enterobacteriaceae} (mainly \textit{E. coli}, 16.0\%), and
fungi (19%). Acinetobacter was seen in 9% of all infections, with higher infection rates in Asia (19.2%) compared to North America (3.7%)\textsuperscript{14} (see Table 2-2).

Antibiotic resistance has increased over time as a result of more common use of broad-spectrum antibiotics for sick patients who remain in the ICU for longer periods of time. Antibiotic resistance is problematic, increasing the needed length of stay in intensive care units for patients\textsuperscript{14}.

<table>
<thead>
<tr>
<th>Site of infection</th>
<th>Frequency (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Respiratory</td>
<td>41.8</td>
<td>35.8</td>
</tr>
<tr>
<td>Bacteremia, site unspecified</td>
<td>21.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>10.3</td>
<td>18.0</td>
</tr>
<tr>
<td>Abdominal</td>
<td>8.6</td>
<td>8.1</td>
</tr>
<tr>
<td>Device- related</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Wound/ soft tissue</td>
<td>9.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Other/ unspecified</td>
<td>6.7</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*Data taken from reference\textsuperscript{14}.

The microbial diagnosis of sepsis directly from whole blood using the method of plating the blood samples has been limited for a long time by the low number— often 1 to
10 CFU/mL—of circulating live microorganisms during such infection. Reimer et al have reported that the number of bacteria in blood of a septic patient is often less than 1 CFU/mL. As the number of the living bacteria in blood increases, the risk of septic shock increases depending on the type of the bacteria and the age of the patient.

### 2.4 Current Diagnosis Assays for Sepsis

Traditional methods used for sepsis diagnosis involve a step of culturing the samples and analyzing the symptoms. A positive culture from the cultured sample which can be blood, urine, cerebrospinal fluid, or bronchial fluid is the most certain diagnosis method.

It is critical to diagnose sepsis early since proper treatment should be chosen in the first 6 hours to reduce the mortality rate of the infection. One-fourth of the septic patients are severely affected by inaccurate treatment due to misdiagnosis of the origin of the infection.

Another problem with culturing the blood samples is the time period needed for this method. Twenty-four to 48 hours are needed for accurate results by this method. Most of the time, if the sampling from the patient is done after the start of antimicrobial treatment, or if the number of the bacteria in the blood is very low, or in case of fastidious infections, the blood culture results are going to be negative.

Other high-tech methods for bacteria detection in the samples have been used. These automated culturing systems use solution pH or change in the CO$_2$ levels of the samples to detect the bacteria. The detection time varies between 11-31 hours and the error in diagnosis is between 2-3%. 
Table 2- 2: Species and organisms associated with sepsis and their frequencies

<table>
<thead>
<tr>
<th>Organism</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>20.5</td>
</tr>
<tr>
<td>MRSA</td>
<td>10.2</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>10.9</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>10.8</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>4.1</td>
</tr>
<tr>
<td>other</td>
<td>6.4</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>19.9</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>16.0</td>
</tr>
<tr>
<td>Klebsiella species</td>
<td>12.7</td>
</tr>
<tr>
<td>Acinetobacter species</td>
<td>8.8</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>7.0</td>
</tr>
<tr>
<td>other</td>
<td>17.0</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>4.5</td>
</tr>
<tr>
<td>Other bacteria</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Table 2-2: continued

<table>
<thead>
<tr>
<th>Organism</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Candida</td>
<td>17.0</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>1.4</td>
</tr>
<tr>
<td>Other</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Other organisms</strong></td>
<td>3.9</td>
</tr>
</tbody>
</table>

*Data taken from reference 14.*

### 2.5 Nucleic-Acid Based Technologies for Sepsis Diagnosis

Nucleic-acid based techniques that are commercially available for sepsis diagnosis give results within 6-12 hours. These methods use primers of DNA or RNA to amplify the genomic sequences of the pathogens. The sequences that are unique to the pathogen are later compared with the available signatures in the databases to identify the genus or species of the microorganism that might be present in the whole blood sample\(^{19}\).

The systems are expensive as a result of the high capital cost of the instruments. These equipment costs range from $75,000 to $200,000. If we consider the cost of the reagents and the operators, we can estimate that these techniques are currently very expensive\(^ {13}\).

Nucleic Acid based tests can lead to false positive results which might be due to contamination of the samples or the very low numbers of the pathogens in the sample. To reduce
this problem, it has been recommended to increase the volume of the sample. It is also recommended that these tests be combined with the clinical conditions of the patient to be more reliable\textsuperscript{13}.

Culturing the blood samples is the most common technique that is used for sepsis diagnosis. However, a 2015 study by Cohen et al shows that blood cultures in 60 to 70\% of severe septic patients are negative\textsuperscript{20}.

2.6 **Centrifugation and Sedimentation**

Centrifugal force is force which appears in a rotating system. Considering a blood sample containing bacteria which rotates around a specified point, centrifugal force, which is proportional to the mass of particles, will operate on all the cells (blood components) and the bacteria present. Sedimentation is the process of moving particles in a suspension as a result of an external force from an external field such as a gravitational field, centrifugal field, electric field, etc. During sedimentation, the centrifugal (or other) force causes particles to move with a velocity called sedimentation velocity which is proportional to the density difference between the particle and the surrounding fluid. Efficient separation according to density will occur if there is enough time for the suspended particles with different densities to travel unimpeded to various positions as a result of the centrifugation force.

There are several reports of devices such as centrifugal disks that are capable of separating plasma from microliter volumes of blood. Haeberle et al designed a disk which separates 2 \mu L of plasma from 5 \mu L of whole blood using a sedimentation technique with a spinning disk. Blood is injected to a chamber and the disk starts spinning. After spinning for a specific time, the suspended particles in a blood sample will form separate layers which will
remain separated even after slowing down the spinning speed, making the process a feasible separation method\textsuperscript{21}.

Amasia \textit{et al} have fabricated a similar centrifugal system on a spinning disk format which is capable of processing large volumes of whole blood. As blood enters the specified chamber in their device, the disk starts to spin, resulting in formation of the various layers of blood components. As the disk stops spinning, plasma which forms the upper layer travels into another chamber by siphoning\textsuperscript{22}.

Centrifugal systems have both advantages and disadvantages for bacteria isolation. Advantages of these techniques include simplicity in pressure control which eliminates the need to use a pump or a valve. One disadvantage is the presence of platelets in the collected plasma after spinning which will be presented in detail in Chapter 5. Furthermore, current disks reported in the literature have the limit of processing small volumes of whole blood which is not enough for our purpose of processing blood\textsuperscript{21-23}. 
AIMS AND OBJECTIVES

3.1 Aim and Primary Objective

My study was part of a larger study to develop processes to identify bacteria causing sepsis in blood infections, particularly bacteria with Carbapenem resistance. The principal aim of my research study was to develop a rapid method for efficiently separating bacteria from blood, which method can be done in no more than 10 minutes so that there would be sufficient time for lysing the bacteria and collecting its DNA for identification of the pathogen and identifying if an antibiotic resistance gene is present. The primary practical objective was to recover from blood the most bacteria possible to send them forward to the identification steps of this research. This study was limited to recovery of E. coli from 7 mL of human blood and did not include any research on lysing or genetic identification.

This main objective is divided into three sub-objectives which are determining the effect of anticoagulant on bacterial recovery, studying the effect of disk design on bacterial recovery and determining the influence of sedimentation process parameters on bacterial recovery and blood cell separation.

3.2 Overview

A literature study showed that microfluidic processes and filtration were inadequate to rapidly separate a few smaller bacteria from billions of larger blood cells in 7 ml of blood in less
than 10 minutes. High numbers of RBCs would interfere with subsequent processing of the bacteria. In fact, the most challenging aspect of separating bacteria from the blood components was to significantly reduce the number of the red cells while not reducing the number of bacteria. To achieve this objective, we carefully designed and built a hollow disk that had the capacity to process whole blood and to separate blood cells from plasma. The process relied on sedimentation. The blood containing bacteria was put in the center of the disk. As the disk started to spin, the blood quickly moved to the disk wall where there was a chamber to hold the specified volume of blood cells. As the disk continued to spin, all particles in the blood reached a steady state velocity called sedimentation velocity. This velocity depended on the size and density of particles. Therefore, by selecting the proper spinning speed and spinning time for the hollow disk, the sedimentation velocity of the red cells was different from the sedimentation velocity of bacteria so red cells would sediment in a separate layer than the bacteria. If the disk spinning speed was reduced slowly, we could prevent red cells from mixing with the bacteria layer, while not spinning all the bacteria into the red cell pack.

3.3 Sub-objectives

3.3.1 Determine the Influence of Anti-coagulant on Bacterial Recovery

Selecting a proper anticoagulant in order to maximize the bacterial recovery was an important first goal for our experiments. We studied the three types of anticoagulants commonly used in medical laboratories: ethylenediaminetetraacetic acid (EDTA), heparin and citrate.
3.3.2 Influence of Disk Design on Bacterial Recovery by Sedimentation

Disk design has a direct impact on the volume of the recovered plasma and the number of the red blood cells present in it. We performed experiments to verify how changing parameters (such as disk height, weir design, baffles, disk diameter) affected the efficiency of plasma volume recovery, bacterial recovery and RBC concentration in plasma. For example, the disk weir was designed to prevent the red cell pack from flowing down and mixing with the clarified plasma while enhancing flow of the plasma (which contains the bacteria) into a space separate from the blood cells. Furthermore, the volume of a trough was calculated to have the capacity to retain all the red cells. Baffles were added to the disk to reduce mixing and to promote the smooth flow of plasma after the spinning stopped. Disk ceiling height was studied to see whether it had any effect on the plasma volume recovered from the disk. All of these design parameters were studied.

3.3.3 Determine the Influence of Sedimentation Process Parameters on Blood Cell Separation

Red blood cells are present in extremely high numbers in the blood so it is essential to reduce the number of the red blood cells in the recovered plasma sample while keeping the recovery of bacteria high.
4 MATERIALS AND METHODS

4.1 Disk Design

The hollow disk is made from a photopolymerizable monomer (Vero ClearTM and Vero BlueTM Resins) using a 3D printer (Stratasys Object 30 Prime, Eden Prairie, MN, USA). The 12-cm-diameter disk is designed to process 8.5 ml of fluid consisting of 7 ml of whole blood and 1.5 ml of phosphate buffer solution (PBS), since the volume of the blood taken from patients is usually 7 ml or greater. As soon as the disk starts to spin, the fluid is flung to the chamber by the disk wall. This chamber is called "vestibule" and is the space bordered by the disk lid, disk back wall and bottom part of the chamber. This space is shown by the green and purple color in Figure 4-1 C. While spinning, various particles in the blood with different sizes and densities will have different sedimentation velocities. Particles with higher densities and larger sizes, will have higher sedimentation velocities, while particles with smaller densities and sizes will sediment much slower. Considering a specific period of time for spinning the disk, slow-sedimenting particles will travel a shorter distance in the disk than fast-sedimenting particles. As a result, slow sedimenting particles (which are bacteria and platelets) remain in the plasma while fast sedimenting particles (mainly red and white cells) collect at the back wall of the hollow disk. As the disk continues to spin, these cells become more concentrated at the back of the disk.

When the disk slows to a stop, these densely packed cells will slide down and become entrapped in the designated space in the bottom part of the vestibule. This section of the disk,
called the "trough", is designed to hold the cell pack as the disk stops spinning. (see purple area of Figure 4-1C, and Figure 4-2 part 6). Considering the average hematocrit level of the patients to be around 0.35 to 0.50, the trough volume is approximately half of the volume of the human blood used. As mentioned before, the upper surface of the vestibule is formed by a thin plastic lid (see Figure 4-2 part 8). By changing the height of the lid, we can change the volume of the vestibule. There is a round fillet at the corner connecting the back wall of the disk to its top lid (Figure 4-2 part 10). This round fillet is intended to reduce the entrapment of fluid in the meniscus formed in this corner and increase the volume of the recovered plasma. The lower surface of the disk inward from the vestibule, which is called the bowl (see Figure 4-2 part 1), is slanted steeply near the weir (Figure 4-2 part 9) to help the plasma drain fast and to increase the volume of the recovered plasma by pulling down the small plasma pools draining down along the baffles. Baffles are thin fins of plastic that are designed to stabilize the red cell pack while helping the plasma to drain by guiding small pools of plasma from over the edge of the weir and down to the disk bowl (Figure 4-2 part 2). These triangular plates of plastic extend from the back wall of the disk to the slope of the bowl. Each baffle has two windows. A triangular window in the upper portion of the vestibule helps the fluid to move freely (Figure 4-2 part 3) while a rectangular window in the trough (Figure 4-2 part 4) allows easier cleaning of the disk since the disks designed for our lab were not disposable. A standard 12-cm disk was designed with 16 baffles.

Near the outer edge of the hollow disk, the bowl turns upwards to the part called "weir" (Figure 4-2 part 5). The top of the trough connecting to the weir is curved toward the outer wall to prevent the cell pack from coming directly up and inward over the weir. The shape of the weir affects the volume of the recovered plasma since plasma will drain over the weir as the disk
stops spinning. The outward edge of the weir, or the edge of the weir towards the disk wall, extends slightly over the trough. This extension is intended to skim the layer of the bacteria in the plasma increasing the volume of the recovered plasma and bacteria concentration recovery. The top of the weir is horizontal (Figure 4-2 part 7) and the inward edge of the weir, or in other words, the edge of the weir towards the center of the disk has a square corner. A nearly vertical slope at the connection of the weir to the bowl of the disk helps plasma drainage (Figure 4-2 part 9).

Figure 4-1: Schematic design of the hollow disk: a) a 3-D view of the disk b) Close-up view of cross section c) Color shows the volume occupied by spinning blood; the purple section is the trough that retains blood cells after spinning stops.
4.2 Disk Motors

The motor used for our preliminary experiments was a CD player motor that was retrieved from an old boom box. The motor used for further experiments was the same OT-1140 spindle motor (Wagner Co). All the experiments to investigate the effect of chemical aspects of sedimentation and the experiments to study the mechanical aspects used the same types of motors.

Figure 4-2: Detailed design of the disk: 1) Bowl; 2) Baffle; 3) Baffle window; 4) Trough window; 5) Weir; 6) Trough rectangular window; 7) Weir top; 8) Disk lid; 9) Vertical slope of weir; 10) Round corner fillet.
4.3 Disk Platform

The original device used to spin the disk was a CD player motor. To secure the disk on the motor, a flat disk-shaped platform with a central shaft in the upper part was designed. A hollow axial tube was embedded in the bottom of the platform to connect to the shaft of the motor. The spindle of the motor was connected to this hollow tube by press fit. As the disk sits on the upper part of the platform, it was secured by a butterfly nut to avoid any displacement of the disk while spinning. Figure 4-3 shows the design for the platform.

![Figure 4-3: Design of the disk platform used to connect the disk to the motor; The motor spindle is inserted into the hollow cavity at the bottom. The hollow disk sits on the horizontal surface. The upper rod is threaded so a butterfly nut can secure the disk to the platform.](image)

4.4 Control System for Chemical Aspects Experiments

An Arduino Uno (ATmega 328P) micro controller was powered by a 12V power supply. The output of this micro controller connected to a L298N stepper motor driver (DROK
Electronics). This stepper motor was directly connected to the output control dial of a KPS 620M (KEPCO INC, Sanford, New York) power supply using electrical tape. A KEPCO power supply was directly attached to the OT-11400 CD spindle motor (Wagner Electronics Super Store, Ashfield, Australia) which was used to spin the disk. Using this setup, programmable changes in the Arduino were precisely translated as voltage output for the motors to control the rotational velocity of the disk. The plot of rotational velocity of the disk versus spinning time for all experiments performed to investigate the chemical aspects of spinning (Chapter 6) is shown below in Figure 4-4. A similar speed profile was used in the experiments examining mechanical aspects of the process of spinning blood (see Chapter 5), but with different maximum velocities and hold times at that velocity.

![Figure 4-4: Plot of spinning speed of the disk versus spinning time used for experiments studying chemical aspects of spinning.](image-url)
4.5 Control System for Mechanical Aspects Experiments

For a few of the experiments reported in Chapter 5, the Labview software was used to precisely control the spinning process of the hollow disk powered by the Maxon motor (model 301039). Labview programming was done by an undergraduate student, Daniel McClellan.

4.6 Preparing Bacteria

Bacteria used for all the experiments were *E. coli* (strain BL21 or Star DE3), which were streaked from a frozen stock onto a nutrient agar plate. This plate was then incubated for 24 hours at 37°C. The next day, the plate was carefully examined by eye and, using a sterile loop, a single colony of bacteria was transferred to a 250-ml baffled flask containing 50 mL of nutrient broth (Difco, Becton Dickinson, Franklin Lakes, New Jersey). The flask was incubated at 37°C with shaking at approximately 200 rpm. After 24 hours, the bacteria suspension was centrifuged at 8000xg for 10 minutes to separate the bacteria from nutrient broth. The pellet at the bottom of the centrifuge tube was washed and resuspended in PBS. After repeating this process twice more, the bacteria, now free of nutrient broth, were diluted to the target concentration in PBS estimated by optical density at 660 nm in a spectrometer (Agilent UV/vis spectrophotometer, model Cary 60). The typical target concentration of the bacteria was approximately $10^6$ CFU/ml in the blood used in the spinning disk. This high concentration was used so that plate counting could be employed to quantitate bacterial recovery.

The PBS solution used in all experiments contained 8 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of Na$_2$HPO$_4$ and 0.24 g/L of KH$_2$PO$_4$ dissolved in 1 liter of distilled water. It was sterilized by autoclaving.
4.7 Spinning Blood

Human blood was collected by venipuncture from healthy volunteers by a qualified phlebotomist into vacutainer tubes. The three main types of vacutainer tubes used for the experiments contained EDTA (BD #366643 10 mL Becton Dickinson, Franklin Lakes, New Jersey), Heparin (BD #367874 10 mL) or Citrate (BD # 363083, 4 mL). The blood collection process followed the protocol approved for student collection by Brigham Young University and was collected by a trained student in the Medical Technology Program at BYU.

The tubes were then refrigerated prior to use for the experiments on the same day. The refrigeration time did not exceed six hours. To prepare for spinning, the blood tubes were taken out of the refrigerator 15 to 20 minutes prior to spinning in order to raise the temperature of the blood to room temperature. In order to resuspend the gravity-sedimented cells, the tubes were gently inverted several times by hand to regain the uniform color of the blood. Eight milliliters of blood were pipetted into a sterile plastic tube (Fisherbrand, Culture Test Tube 17x100mm) and were mixed with 100µL of diluted E. coli pipetted into the blood in the sterile tube, producing a concentration of around $10^6$ CFU/mL. The exact concentration of the live bacteria in the blood prior to spinning was subsequently measured exactly by serial dilution and plate counting. In some experiments, 115µL of various concentrations (0.5 to 1.1 g/L) of adenosine 5’-diphosphate (ADP) in PBS were also added to the blood prior to spinning. The tube was inverted for about 10 seconds by hand to assure good mixing of the bacteria in the blood and/or ADP. For 12-cm disk experiments, 7 ml of the blood was pipetted into the bowl of the disk. Then 1.5 ml of PBS was added to the disk bowl prior to spinning the disk. The volume of the blood and PBS was adjusted for 10-cm and 16-cm disks proportional to disk diameters.
4.8 Collecting Plasma

Approximately 15 seconds after the disk came to a complete stop, the recovered plasma was collected by a pipette into pre-weighed sterile plastic tubes. The weight of the tubes was re-measured and, considering the density of plasma as 1.024 g/L, the volume of the recovered plasma was calculated, and was designated $V_{\text{plasma}}$.

4.9 Counting Bacteria in Recovered Plasma and Initial Blood

The number of the bacteria in the whole blood samples prior to spinning and in the recovered plasma samples after spinning must be accurately measured to provide information about the overall bacteria recovery of the process. Serial dilution of the samples and bacterial plating were the techniques used to estimate an accurate number of bacteria. First, 100 µL were pipetted from the tube containing the blood or plasma sample of interest (either blood or recovered plasma) into the tube containing 900 µL sterile PBS. The tube was vortexed and 100 µL were pipetted from this tube to another tube of 900 µL sterile PBS. This process was repeated at least 3 more times, depending on the estimated number of the bacteria in the sample prior to spinning. Then samples of 50 µL were pipetted from the tubes and were plated on nutrient agar plates by the single drop technique. The plates were incubated for 24 hours at 37°C. The colonies of bacteria on the plates were counted the next day. To back calculate the exact number of the bacteria in the sample, a plate section that had 45 or more colonies of bacteria was selected. The number of the colonies in the blood was calculated as:

$$\text{Number of the bacteria in the initial tube containing blood or plasma} = N_{B \text{ tube}} = \frac{\text{Number of colonies on the plate}}{\text{Volume of the sample plated} \times \text{dilution factor}}$$
Dilution factor is defined as:

\[
Dilution Factor = \left( \frac{Volume \ of \ sample}{Volume \ of \ sample + Volume \ of \ dilutent} \right)^n
\]

where \( n \) is the number of serial dilutions performed.

4.10 Red Cell Count

One important aspect of this procedure was to reduce the number of the red blood cells in the recovered plasma since they would interfere with the subsequent steps of the process. The first method used to quantitate the number of the red blood cells in the whole blood or plasma was to count the number of the red cells in the dilute solution using a hemocytometer. The number of the red cells in the solution was estimated considering the volume of a diluted blood or recovered plasma sample put on the hemocytometer, and the dilution factor of the solution. Since this method was time consuming, another method used to estimate red cell counts used UV/vis spectrometer absorbance data at 280, 421, 600 and 700 nm. The concentration of the red cells in the target solution, either whole blood or plasma was estimated from calibration curves of diluted blood and diluted washed RBCs, using the absorption of protein at 280 nm, hemoglobin at 421 nm and the cellular scattering of cells and platelets at 600 and 700 nm. The absorbance values of these diluted blood and diluted washed RBCs were correlated with the cell calibration data for diluted blood and recovered plasma.

4.11 Bacteria Concentration Recovery

As the disk spins, the red and white blood cells sediment through the plasma towards the disk wall. Much of the slower-sedimenting bacteria remain in the plasma. "Bacteria concentration recovery" refers to the concentration of the bacteria in the collected plasma,
(\(C_{b,\text{plasma}}\)) divided by the concentration of bacteria in the original spiked blood (\(C_{b,\text{blood}}\), before dilution by PBS). It was calculated as:

\[
Bacteria\ Concentration\ Recovery = C_{c,\text{bact}}^* = \frac{C_{b,\text{plasma}}}{C_{b,\text{blood}}}
\]

Experimentally, \(C_{c,\text{bact}}^*\) is calculated easily from experiments as:

\[
C_{c,\text{bact}}^* = \frac{\text{Number of bacteria colonies on the plasma plate}}{\text{Dilution factor of the plasma plate} \times \text{Dilution factor of the whole blood plate before spinning} \times \text{Number of the bacteria colonies on the whole blood plate}}
\]

In experiments, bacteria concentration recovery sometimes exceeded 1.0, indicating that the bacteria migrated inward into the plasma layer while the blood cells migrated toward the back of the vestibule.

**4.12 Total Bacteria Recovery**

To quantitate and optimize the entire spinning process, we measured the total amount of bacteria recovered by combining the bacteria concentration recovery and plasma volume recovery. Bacteria total recovery was defined as:

\[
Bacteria\ Total\ Recovery = C_{T,\text{bact}}^* = \frac{C_{b,\text{plasma}} \times V_{\text{plasma}}}{C_{b,\text{blood}} \times V_{\text{blood}}}
\]

This dimensionless parameter provided information about the bacteria recovery as well as the efficiency of the spinning process in concentrating bacteria in the plasma layer. If a change in the system, such as change in the spinning speed affected the total bacteria recovery, we
attributed this change to either a change in the volume of the recovered plasma or to concentrating bacteria more efficiently:

Red cell recovery was a parameter defined as the concentration of the RBCs in the recovered plasma divided by the concentration of the RBCs in the whole blood:

\[
Red \ Cell \ Recovery = C_{RBC}^* = \frac{\text{Concentration of RBCs in the recovered Plasma}}{\text{Concentration of RBCs in whole blood}}
\]

4.13 Platelet Imaging

Platelets are small cell fragments that play an important role in the blood coagulation cascade and clotting. Since their sedimentation velocity is near to bacteria sedimentation velocity, the recovered plasma contained high numbers of platelets. To count the number of platelets, dilute samples from the recovered plasma were prepared, and portions of these dilute samples were placed on microscope slides. Using the microscope (Zeiss), digital images from these slides were taken which were later processed by the software Image J to visually distinguish between the red cells and platelets. The number of the platelets in plasma were estimated by considering the dilution factor and the average number of the platelets recognized by the software.

4.14 Statistical Analysis and Methods

The statistical analysis of the data was done using JMP software from SAS (Statistical Analysis System). Data were first analyzed using student-t-tests and ANOVA (Analysis of Variances). Then other tests were used as post discussion such as a t-test (2-sided \( \alpha=0.05 \)) or Tukey's HSD when comparing analyzing more than two sub-sets of data.
5 MECHANICAL ASPECTS

5.1 Introduction

In this chapter, the effects of various mechanical aspects of disk design—such as disk diameter, height, weir, baffles and spinning speed and time of spinning—on the key parameters for bacterial recovery were studied. These key dependent parameters were the volume of the recovered plasma that flowed over the weir and was collected ($V_P$), the concentration of bacteria in the collected plasma ($C_{b,plasma}$) and the concentration of other blood components such as red blood cells, white blood cells and platelets.

If a suspension of particles with various sizes and densities is placed in a centrifugal field, the particles will start to move due to the forces that act on them. The velocity of the particles will quickly reach a steady value called "sedimentation velocity". Sedimentation velocity of the particles in a fluid medium can be calculated by balancing the various forces acting on the particles as they move in the medium.

The superposition of centrifugal force and the gravity force on the mass and buoyancy of the particles results in a net force $F_c$ on a spherical particle in our horizontal spinning device.

$$F_c = \frac{\pi}{6} D_p^3 (\rho_p - \rho_f) \sqrt{R^2 \omega^4 + g^2}$$

where $R$ is the rotational radius of the particle, $D_p$ is the particle diameter, $\omega$ is the rotational angular velocity, $\rho_p$ is the particle density, $\rho_f$ is the fluid density, and $g$ is the gravitational
constant. In less than a second, the particles reach a steady state sedimentation velocity, \( v_s \). At steady state, \( F_c \) is balanced by a drag force, \( F_d \) which can be calculated by Stokes law if the particles can be considered as small isolated spheres and have a Reynolds number less than unity:

\[
F_d = 3\pi v_s \mu D_p
\]

in which \( \mu \) is the fluid viscosity. By equating the forces in equations 5.1 and 5.2, we derive the sedimentation velocity for stokes flow:

\[
v_s = \frac{d_p^2(\rho_p-\rho_f)\sqrt{R^2\omega^2+g^2}}{18\mu\pi}
\]

As we see from equation 5.3, this basic theory of sedimentation indicates that WBCs, RBCs, platelets and bacteria have large differences in their sedimentation velocities due to differences in particle diameters despite having similar densities. Because \( R, \omega, g, \) and \( \mu \) are constant in an experiment, the relative sedimentation velocity of a red blood cell compared to a bacteria is \( \sim 30 \). In the same manner, relative sedimentation velocity of a white blood cell compared to a bacteria is \( \sim 96 \). This shows that in the spinning disk, red and white blood cells will move much faster than the bacteria and will quickly sediment into dense layers close to the disk wall. So, if the spinning speed and spinning time and disk diameter are carefully selected, we can manipulate the value of the sedimentation velocity in order to maximize the separation of the bacteria from red cells.

This chapter presents our data on finding the optimum spinning conditions to separate bacteria from blood cells in human blood. We considered various disk sizes and spinning velocity profiles. Furthermore, the shape of the weir and the bowl affected the amount of the plasma recovered, which had a direct effect on bacteria recovery.
5.2 General

5.2.1 Various Shapes of the Spinning Object

Based on the literature review, we determined that separating bacteria from the blood cells would be possible in a centrifugal field. Most of the published research articles\textsuperscript{21-22} on centrifugal systems that we reviewed used microfluidic volumes of the blood, so we had to design a spinning object that had the capacity of holding blood in milliliter volumes. Our first design was a hollow sphere that had a collection trough around its equator that could hold 7 ml of blood while spinning. As the sphere stopped spinning, the plasma drained to the bottom of the interior of the hollow sphere (bowl) and was collected with a pipette. The successful separation of the bacteria from the rest of the blood cells proved that the centrifugation technique was possible. To improve the sphere stability on the motor and to reduce the weight of the object, we changed the shape of the spinning object into a hollow disk, and a compact disk (CD) motor was scavenged from a discarded boom box and bolted to a table. Hollow disks were printed with 3-D printers. A platform that attached the hollow disk to the motor shaft was also printed. Initially the spinning speed of the CD motor was controlled manually; but with further refinement we designed the control systems described in sections 4.4 and 4.5.

5.2.2 Baffles

A baffle is a vertical triangle-shaped fin placed in the vestibule. Baffles were added to the disk design since it was found qualitatively in preliminary experiments that the presence of the baffles helped fluid to flow down from the weir more uniformly, perhaps helping by decreasing shear mixing at the interface between the plasma and the cell pack, and definitely helping by wicking plasma fluid—guiding it down from the vestibule into the bowl of the disk as the
spinning speed slowed to a stop. We hypothesized that if we increased the number of the baffles in the disk design, we might increase the volume of the recovered plasma. To check this hypothesis, we designed three 12-cm disks. One had 32 baffles, the other had 16 baffles, and another had none. Because baffles displaced blood volume, the volume of the liquid blood and PBS inside the chamber was carefully measured for various disk designs so that the interface between plasma and air (while spinning) contacted the weir in exactly the same place, allowing comparison of the results.

These experiments revealed that the number of the baffles had a significant effect on the volume of recovered plasma, $V_P$. However, statistical analysis revealed that bacteria concentration recovery ($C_{c,bact}^*$) was not affected by the number of baffles. Further statistical analysis was done to study the correlation between the number of baffles in the 12-cm disk and red cell recovery. Table 5-1 contains mean averages for $V_P$, $C_{c,bact}^*$ and red cell recovery $C_{RBC}$ for the discussed disks. Table 5-2 contains the $p$ values for comparisons between these disks.

### Table 5-1: Mean average results for disks with various number of baffles

<table>
<thead>
<tr>
<th></th>
<th>No baffles</th>
<th>16 baffles</th>
<th>32 baffles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered Plasma Volume (mL)</td>
<td>3.51 ± 0.21 mL</td>
<td>3.8 ± 0.09 mL</td>
<td>3.37 ± 0.33</td>
</tr>
<tr>
<td>Bacteria Concentration Recovery (%)</td>
<td>65.08 ± 9.69</td>
<td>68.73 ± 4.11</td>
<td>74.29 ± 14.51</td>
</tr>
<tr>
<td>Red Cell Recovery (%)</td>
<td>3.67 ± 2.87</td>
<td>7.91 ± 0.99</td>
<td>8.2 ± 2.23</td>
</tr>
<tr>
<td>Bacteria Total Recovery (%)</td>
<td>32.75 ± 5.66</td>
<td>37.37 ± 2.45</td>
<td>35.76 ± 8.66</td>
</tr>
</tbody>
</table>

As shown by Equation 5.3, the main parameters that were hypothesized to have significant effects on red cell and bacteria recovery were spinning speed and spinning period. The initial experiments on the 12-cm disk were all done at 3000 rpm and a spinning period of 60
seconds. These values were selected based on the spinning speed and period reported by Amasia and Gorkin for their microfluidic spinning disk\textsuperscript{22-23}. Our preliminary experiments showed that if 7 ml of blood was spun for 60 seconds at 3000 rpm, its plasma would separate from the red and white cells and the bacteria would remain mainly in the plasma. The reason for this physical phenomena is explained by the difference between the sedimentation velocity of the WBCs, RBCs and the bacteria. As shown in equation 5.3, sedimentation velocity changes by the second power of $\omega$, rotational angular velocity. This suggests that any change in the angular velocity would result in a considerable change in the sedimentation velocity which then would affect the bacteria and red cell recoveries.

| Table 5-2: $p$-values for comparisons between disks with different number of baffles |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | No baffles vs. 16 baffles | No baffles vs. 32 baffles | 16 baffles vs. 32 baffles |
| Plasma Volume                  | 0.013            | 0.0851          | 0.0168          |
| Bacteria Concentration Recovery | 0.5              | 0.36            | 0.465           |
| Red Cell Recovery              | 0.0067           | 0.01            | 0.03            |
| Bacteria Total Recovery        | 0.14             | 0.56            | 0.72            |

Spinning period is another important parameter that directly effects red cell and bacteria recoveries. As mentioned above, the physical theory behind our spinning device is that if 7 ml of blood containing bacteria is spun for sufficient time, bacteria would remain for a longer time in the plasma while red cells and white cells would form a pack against the wall of the disk. If the spinning time was not sufficient, the separation of the red cells from the plasma would not occur and the red cell recoveries in the collected plasma would be higher than desired at the end of the spinning process. Higher $C_{RBC}^*$ in the recovered plasma is undesirable since these cells interfere
with the subsequent processes, such as filtration. On the other hand, if the spinning period was longer than what was needed, the bacteria would sediment towards the cell pack in the disk and end up impacted upon or trapped in the cell pack layer, resulting in an undesirable low bacteria recovery. So it was desired to find the optional spinning period at a specific spinning speed, based on Equation 5.3 and the data from experiments. The requisite spinning time was found to be proportional to the distance that the bacteria would travel within the disk, as the disk started to spin according to the specific spinning speed profile, an example of which is shown in Figure 4-4.

We considered that the spinning speed varied from zero to a maximum value and back down again. To make a consistent comparison in experiments with different spin profiles, we defined a characteristic length as the distance that a hypothetical 8 micron particle, with the density of a red cell, traveled within the disk from the start of the spinning until the disk came to a complete stop. Characteristic length is a parameter that enables us to compare the bacteria and red cell recovery of the disk with a specific diameter at various spinning speeds and spinning times. Characteristic length, \( a \), was calculated as:

\[
a = \int_0^t v_s \, dt
\]

where \( t \) is the spinning time and \( v_s \) is the sedimentation velocity, given by Equation 5.3 for an 8-\( \mu \)m-diameter sphere of density 1.098 g/mL.

The diameter of the disk is a key experimental parameter that was studied since it appears in equation 5.3, the sedimentation velocity. Four disk designs with diameters of 8 cm, 10 cm, 12 cm and 16 cm were produced. The volume of blood and PBS for each disk was carefully calculated so that these values were scaled such that the blood contacted the weir in the same position of the weir.
The volume of the blood and PBS used for each of the disks with various diameters was calculated according to the design of the disk vestibule and weir reported in Table 5-3.

**Table 5-3: Blood and PBS volume used for each size of the disk and disk measurements for each size of the disk**

<table>
<thead>
<tr>
<th>Disk Size (cm)</th>
<th>Blood volume (mL)</th>
<th>PBS volume (mL)</th>
<th># of experiments</th>
<th>Radius at back wall (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.83</td>
<td>1.25</td>
<td>32</td>
<td>4.925</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>1.5</td>
<td>138</td>
<td>5.925</td>
</tr>
<tr>
<td>16</td>
<td>9.33</td>
<td>2</td>
<td>82</td>
<td>7.925</td>
</tr>
</tbody>
</table>

Our criteria to calculate the needed blood volume for various disk sizes was that the layer of the blood and plasma must touch the outer surface of the weir in the same place. The volume of the PBS for the 12 cm diameter disk was found based on preliminary experiments that suggested adding a diluting agent to the blood might increase the distance between the red and white cells, facilitating the migration of the bacteria within this void space.

Experiments were done for each disk size to study the effect of spinning speed and spinning time on bacteria and red cell recovery. Spinning speed profile was integrated using Equation 5.4 to calculate the characteristic length. So, instead of reporting the change in the bacteria and red cell recovery versus spinning speed and spinning time, the change in these parameters are presented below as a function of characteristic length. Figures 5-1 through 5-3 show total bacterial recovery in % for various disk sizes, speeds and dwell times. Figure 5-5 through 5-7 show concentration recovery for the same experiments.

As explained before, higher spinning speed and spinning time would result in a high value of characteristic length and reduction of RBCs. Similarly, higher spinning speed and
spinning time would cause the bacteria to migrate further toward the cell pack and increase the chance of the bacteria being trapped in the red cell pack. This would lower the total bacteria recovery. Our results from all three disks illustrate that increasing the characteristic length results in lower total recovery of the bacteria. So, the optimal spinning speed and time for higher bacteria recovery is a low value of characteristic length.

Figure 5-1: Bacteria total recovery versus characteristic length for a 10-cm-diameter disk. Spinning speed varies between 1000 rpm to 4000 rpm.
Figure 5- 2: Bacteria total recovery versus characteristic length for a 12-cm-diameter disk. Spinning speed varies between 1000 rpm and 4000 rpm.

Figure 5- 3: Bacteria total recovery vs characteristic length for a 16-cm-diameter disk. Spinning speed varies between 1000 rpm to 3500 rpm.
As shown in Figure 5-4, bacteria concentration recovery (converted to %) decreases as characteristic length increases, and this trend is similar and independent of the size of the disk. Statistical analysis indicates that characteristic length has a significant effect on bacteria concentration recovery ($p < 0.0001$). Bacteria concentration recovery, as explained before, is defined as the ratio of the bacterial concentration in plasma to the bacterial concentration in the whole blood. We expected that the data in the plot of the concentration recovery versus characteristic length would be less scattered compared to the similar plots of bacteria total recovery since concentration recovery does not depend on the volume of the recovered plasma. The plots of concentration recovery versus characteristic length for all three sizes of the disk are shown below in Figs. 5-5 through 5-7.
Figure 5- 5: Plot of bacteria concentration recovery versus characteristic length for 10-cm-diameter disk.

Figure 5- 6: Plot of bacteria concentration recovery versus characteristic length for 12-cm-diameter disk.
As illustrated by the plots, the bacteria concentration recovery data are still scattered. The slope of the average of the data is -0.2942%/mm. A model was created to determine intercepts for the data with this slope, the model being

\[
\% \text{ Recovery} = \text{Intercept} - 0.2942 \times a
\]

where "a" is characteristic length. Table 5-4 gives the estimated intercept values for the disks. The Tukey's HSD analysis indicates that the intercept for the data sets for the 10-cm-diameter disk and the 16-cm-diameter disk are not different \((p > 0.1)\). However, the 12-cm-diameter disk has a lower value of statistical significance, indicating that there is less recovery at low values of characteristic length for the 12-cm-diameter disk.
Table 5- 4: Y-intercept for disks of various diameter

<table>
<thead>
<tr>
<th>Disk Type</th>
<th>Intercept Value for the Linear Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-cm-diameter disk</td>
<td>110.77</td>
</tr>
<tr>
<td>12-cm-diameter disk</td>
<td>97.94*</td>
</tr>
<tr>
<td>16-cm-diameter disk</td>
<td>117.42</td>
</tr>
</tbody>
</table>

*Value is statistically different than other disks ($p < 0.0001$).

In order to make comparisons between the volumes of the recovered plasma between the disks with various sizes, we considered the fact that the volume of whole blood and PBS at the beginning of the spinning process were very different from each other. To quantitate the amount of value recovery, we defined a parameter called recovered plasma fraction, $V_{fr}$, which represents the fraction of whole blood volume recovered as "plasma".

$$V_{fr} = \frac{\text{Recovered Plasma Volume} - \text{PBS Volume}}{\text{Whole Blood Volume}}$$

Statistical analysis to investigate the effect of disk size and characteristic length on recovered plasma fraction showed that both characteristic length and disk type had a significant effect on the recovered plasma fraction ($p < 0.001$).

The slope of the average of the data in Figure 5-8 is -0.00034 /mm. A model was created to determine the intercepts for the data with this slope, as

$$Recovered \ Plasma \ Volume \ Fraction = -0.00034 \ast a + \text{Intercept}$$

The estimated values for intercept for the disks with various diameters are given in Table 5-5. The Tukey's HSD analysis indicated that only the intercepts of 10-cm-diameter disk and the 12-cm-diameter disk were different ($p = 0.027$). The intercepts of 12-cm-diameter and 16-cm-diameter disk were not statistically different from each other ($p = 0.25$). Neither were the
intercepts of 10-cm-diameter disk and 16-cm-diameter statistically different ($p = 0.1$). The 12-cm disk had the highest value of volume recovery.

![Graph](image)

**Figure 5-8**: Plot of plasma volume fraction versus characteristic length.

**Table 5-5: Y-intercept for disks of various diameter**

<table>
<thead>
<tr>
<th>Disk Type</th>
<th>Intercept Value for the Linear Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-cm-diameter disk</td>
<td>0.345</td>
</tr>
<tr>
<td>12-cm-diameter disk</td>
<td>0.380</td>
</tr>
<tr>
<td>16-cm-diameter disk</td>
<td>0.368</td>
</tr>
</tbody>
</table>
5.3 Red Cell Recovery

The average number of red blood cells in the blood of a normal person is between 4.2 to 6.1 million cells per milliliter of blood. One purpose of this separating process is to reduce the number of the red blood cells in the recovered plasma to avoid complications in the subsequent steps of the downstream diagnostic processes. This aim was achieved by entrapping the red blood cells in the trough disk. As the disk starts spinning, blood is thrown into the vestibule. Red blood cells migrate towards the disk wall while plasma moves through the red cell pack in the opposite direction. As the disk continues spinning, red cells form a layer that is rich in the red and white cells. When the disk stops spinning, the plasma drains to the bowl of the disk while the majority of the red cells remain in the trough. Usually the draining plasma pulls down thin red cell streaks. These red cell streaks are responsible for the large portion of the red cell recovery values reported in each experiment. The number of the red cell streaks was recorded by the examiner for each experiment as the disk stopped spinning and was given a number between 1 and 6 to indicate the extent of contamination by red cells pulled down by the streaks. Red cell recovery was quantitatively defined as the ratio of the number of the red cells in the recovered plasma to the initial number of the red cells in the whole blood. As increasing characteristic length was equivalent to increasing spinning speed and/or spinning time, increasing this length increased the sedimentation of red cells along with the bacteria, resulting in lower number of the red cells in the collected plasma, or in other words, lower red cell recovery. This seems advantageous, but we have to consider that as we increase characteristic length, bacteria recovery decreases. Considering the fact that the main aim of this project was to recover as much as bacteria as possible from the whole blood and process it, it is inevitable that we have to consider both bacteria and red cell recoveries along with each other as two main criteria to select the
proper characteristic length for our experiments. The plots of red cell recoveries versus characteristic length are shown below in Figures 5-9 through 5-11.

Figure 5-9: Red cell recovery versus characteristic length for the 10-cm disk.

Figure 5-10: Red cell recovery versus characteristic length for 12-cm disk.
We see from the plots that, as expected, red cell recoveries decreased as the characteristic length increased. However, the trend of change in red cell recovery versus characteristic length can be divided into two sections. The slope of red cell recovery versus characteristic length is much steeper when the characteristic length below 50. But for the characteristic length higher than 50, the slope is much milder. We decided to compare the disks by comparing the part corresponding to characteristic length below 50 to each other for all three disks (10-cm-diameter, 12-cm-diameter and 16-cm-diameter). The other part of data of red cell recovery versus characteristic length corresponding to characteristic length higher than 50 for all three sizes of the disk were also compared to each other. For characteristic lengths less than 50, the 12-cm disk produced RBC recovery that was different from the other disks, with the slopes (change in recovery with respect to characteristic length) being more negative (more sensitive).
5.4 Stability during Deceleration

Our preliminary experiments showed that there may be a correlation between the rate of deceleration and the occurrence of remixing of blood cells into the plasma. Later investigation by another graduate student proved that the deceleration rate near the end of spin-down must be carefully controlled to avoid mixing of the clear plasma and the red cell pack. To precisely meet this demand, the spinning speed was controlled by the Labview system as described in the previous chapter. Because another student did this research, it is not reported herein. The most significant thing to note is that it was possible to maintain separation during deceleration with the 10-cm, 12-cm and 16-cm disk. The 8-cm-diameter disk could never maintain separation; that is why no results are reported in this thesis for the 8-cm disk.

5.5 Miscellaneous Experiments

We performed a number of small experiments to try to increase the volume of plasma that was recovered and to increase the concentration recovery. These experiments are reported below.

Table 5-6: Slopes for red cell recovery vs. characteristic length

<table>
<thead>
<tr>
<th>Disk Type</th>
<th>Characteristic length smaller than 50</th>
<th>Characteristic length higher than 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-cm-disk</td>
<td>-0.00041</td>
<td>-0.02</td>
</tr>
<tr>
<td>12-cm disk</td>
<td>-0.00116</td>
<td>-0.04</td>
</tr>
<tr>
<td>16-cm-disk</td>
<td>-0.00043</td>
<td>-0.017</td>
</tr>
</tbody>
</table>
Table 5-7: p-values for red cell recovery vs. characteristic length

<table>
<thead>
<tr>
<th>Comparisons between the disks</th>
<th>Characteristic length smaller than 50</th>
<th>Characteristic length higher than 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-cm-disk vs. 12-cm-disk</td>
<td>0.015</td>
<td>0.07</td>
</tr>
<tr>
<td>10-cm disk vs. 16-cm disk</td>
<td>0.71</td>
<td>0.96</td>
</tr>
<tr>
<td>12-cm-disk vs. 16-cm-disk</td>
<td>&lt;0.0001</td>
<td>0.018</td>
</tr>
</tbody>
</table>

5.5.1 Roof Height

During the separation of the blood cells from plasma, the fluid that is becoming cell-free starts to form a meniscus with the ceiling of the vestibule. The surface tension between the recovered plasma layer and the roof of the disk overcomes gravity and a small volume of the plasma remains entrapped in the corners, such as corners between the back wall and the roof, and the corners between the back wall and baffles. This adversely affects the recovery of plasma which lowers the total recovery of the bacteria. To solve this issue, we decided to increase the roof of the disk to see if it had any effect on the volume of the recovered plasma. The disk that had a higher roof was designed so that the volume of the upper part of the vestibule had increased while the volume of the trough was kept the same. The volume of the blood and the shape of the trough were kept the same while more PBS was added to compensate for more
volume in the disk vestibule with a higher roof. Our hypothesis was that increasing the roof of the disk will decouple the meniscus in the upper roof from the meniscus in the back of the trough (see Figure 5-11). This might result in a higher volume of plasma draining from the back wall and may increase the volume of the recovered plasma. Increasing the roof of the disk might decouple the plasma meniscus formed under the roof ceiling and cause it to drain. However, our experiments showed that there was no significant difference between the two disks in their volume of the recovered plasma or we did not have enough data \( (p > 0.6) \). This might be attributed to having already placed a fillet in the back corner of the roof (see Figure 4-2).

Moreover, bacteria concentration recovery did not significantly change as we changed the roof height \( (p = 0.08) \). But increasing the height of the roof of the disk increased the red cell recovery significantly. The average red cell recovery for the disk with higher roof was 13.10 ±
3.01% while for the regular 12-cm disk was 7.92 ± 1.25 % (p = 0.0021). Since higher RBC recovery is not desirable, we do not recommend a higher roof.

5.5.2 Extended Weir

Our preliminary experiments suggested that lengthening the outward-facing edge of the weir in the disk design would increase the recovery of the bacteria since it may skim the interface between the plasma and cell pack. We proposed a hypothesis that if we used a more extended weir, (see Figure 5-12), bacterial recovery would increase since we would be scraping the interface where sedimenting bacteria collects. Experiment results show that volume of the recovered plasma increases as a result of the extension in the weir. This results in higher bacteria total recovery compared to the disk with regular weir. Also bacteria concentration recovery and red cell recovery increases when the weir is extended compared to the disk with normal weir (p < 0.01).

![Figure 5-13: Plot of bacteria concentration recovery versus characteristic length comparing two various disk weir designs.](image)
5.6 Conclusion

Experiments showed that spinning blood in the hollow disk successfully separated bacteria rich plasma from the blood cells. To maximize bacteria recovery, we investigated the effect of various mechanical aspects of disk design—such as disk diameter, height, weir, baffles, spinning speed and time of spinning—on the key parameters for bacterial recovery. Our results showed that spinning speed and time had a significant effect on bacteria concentration recovery. Characteristic length was the parameter that we defined to include the changes in spinning speed and time. Independent from the size of the disk, bacteria concentration recovery and red cell recovery decreased as characteristic length increased. Our results show that for a 12-cm-diameter disk, the average total bacteria recovery is 60.66 % at characteristic length between 15 and 30 mm. Comparison between 12-cm and 16-cm disks showed that bacteria concentration recovery is influenced by the disk type and characteristic length. The comparisons between 10-cm disk and 16-cm disk provided similar information. However, analysis of comparisons of 10-cm and 16-cm disks were different and bacteria concentration recovery only depended on characteristic length. Statistical analysis showed that recovered plasma volume decreased as characteristic length increased. Comparison of pure plasma fraction from three disks showed that this parameter was only affected by characteristic length for the 12-cm and 16-cm disks. But the comparison between 10-cm and 16-cm disk revealed that recovered plasma fraction depended both on the characteristic length and disk size. Red cell recovery certainly does depend on the characteristic length. Increasing the characteristic length resulted in lower red cell recoveries similar to bacteria concentration recovery. The influence of disk size on the red cell recovery followed the same trend as the bacteria concentration recovery.
6 CHEMICAL ASPECTS

6.1 Introduction

In this chapter, the effects of various chemical parameters such as anticoagulant type, gender of the blood donors, amphiphilic molecules such as γ-aminobutyric acid, sebacic acid and Pluronic P105 and adenosine diphosphate (ADP) on bacterial recovery were studied. The key dependent parameters were volume of the recovered plasma that drains over the weir as the disk stops and is collected, the concentration of the bacteria in the collected plasma and the concentration of other blood components such as red blood cells and platelets in the recovered plasma.

6.2 Effect of the Type of Gender on the Volume of the Recovered Plasma

As the disk stops spinning, the plasma slowly drains over the weir into the disk bowl where it is collected by a pipette. The volume of the recovered plasma directly impacts the total recovery of bacteria since the bacteria are found in the plasma. Therefore, increasing the volume of the recovered plasma will result in collecting more bacteria. Various chemical parameters were studied to see if they had any effect on the volume of the recovered plasma.

The first parameter studied was the effect of gender of the blood donors on the volume of the recovered plasma. Average volume (and 95% confidence interval) of the plasma recovered (in experiments conducted by 12-cm-diameter disk spinning at 3000 rpm for 60 seconds) for
female and male blood was reported as 3.75±0.16 mL and 3.63±0.18 mL, respectively. Statistical analysis showed that there was no significant difference in the volume of the recovered plasma of female and male blood donors. P-values comparing volumes of male and female for citrated, heparinized and EDTA-treated blood were 0.73, 0.64 and 0.24 respectively. See Figure 6-1.

![Figure 6-1](image-url)

**Figure 6-1**: Plot of volume of recovered plasma versus the gender of the donor and the type of anticoagulant used. M = male; F = female. The mean values and 95% confidence intervals are represented (n > 18).

6.3 Effect of Disk Surface Wettability on the Volume of the Recovered Plasma

The second independent parameter studied related to the volume of the recovered plasma was disk surface hydrophilicity. Visually, we could see that there was a non-wetting contact angle between the plasma and the edge of the weir. This appeared to hold back plasma and might have prevented some plasma from flowing down. Thus we tried to make the surface of the disk more wettable by the plasma in order to increase the volume of the recovered plasma. Surface
wettability or hydrophilicity is defined by the contact angle between the surface and fluid. The surface is called hydrophilic if the contact angle between the surface and a water droplet is below 90°. We desired a "plasmaphilic" surface, which may be related to hydrophilicity of the surface.

To investigate the effect of surface wettability on the volume of the recovered plasma, we conducted qualitative experiments and treated the surface of the disk chemically to enhance its hydrophilicity and wettability; then we compared the volume of the recovered plasma from the chemically-treated disks to untreated ones. The wetting agents utilized to enhance hydrophilicity of the disk surfaces were γ-aminobutyric acid, sebacic acid and Pluronic P105. These chemicals were either sprayed on the surface of the disk, or the disk was soaked in a solution that contained these chemicals. Our qualitative observations indicated that none of these surface treatment methods had any statistically significant effect on the volume of the plasma that was recovered. The $p$-values were all greater than 0.05.

### 6.4 Effect of the Type of Anticoagulant on the Volume of the Recovered Plasma

Another chemical parameter investigated was the effect of various types of anticoagulants on the volume of the recovered plasma. If we take the average of the volume of recovered plasma across both genders, the mean average (and 95% interval) for citrated blood was reported as 3.83 ± 0.14 ml, which is slightly but not significantly higher than the average for the heparinized blood (3.78 ± 0.135 ml). Further statistical analysis showed that there was no significant difference between volume of the recovered plasma in experiments using blood treated with heparin versus citrated blood ($p = 0.59$). The average volume of recovered plasma for the blood treated with EDTA was reported as 3.63 ± 0.11 ml. Similar statistical analysis showed that there was no significant difference between the volume of the recovered plasma in
the experiments using blood treated with EDTA versus heparinized blood \((p = 0.17)\). Statistically there was no significant difference between the volumes of the recovered plasma in experiments using blood treated with EDTA versus citrated blood \((p = 0.08)\).

### 6.5 Effect of the Type of Anticoagulants on Bacterial Recovery

Based on the literature review, EDTA, heparin and citrate were chosen as potential anticoagulants for our experiments. Our results showed that these anticoagulants had a significant effect on measuring bacterial recovery if we did not wash the growth media out of the bacteria sample for the experiments. Bacteria growth was inhibited in the experiments using citrated or heparinized blood if we did not wash the bacteria. However, bacteria growth was not affected in blood treated with EDTA even if the bacteria were not washed. The experiments that used EDTA as the anticoagulant had approximately total bacterial recovery of 40% on average for 12-cm diameter disks at 3000 rpm and 60 seconds. There was a significant difference between the average bacteria concentration recovery of male (recovery = 0.72 ± 0.21) and female (recovery = 0.59 ± 0.16) blood treated with EDTA \((p = 0.003)\). In the experiments that used citrated or heparinized blood, we were not able to measure the CFUs in the recovered plasma either correctly or repeatedly. We think that the bacteria growth inhibition by hemoglobin was exacerbated by citrate or heparin. As a result, we cannot report average bacterial recovery from experiments that used citrate or heparin as their anticoagulant.

### 6.6 Effect of the Type of Anticoagulant on Red Cell Recovery

Due to higher levels of hematocrit for male donors compared to female ones, the number of the red cells in the recovered plasma was higher for male plasma than for female plasma. Red cell recovery in male blood was affected by the type of the anticoagulant used (see Figure 6-2). It
had its highest value in experiments that used EDTA as anticoagulant (RBC recovery approximately 7%) while citrated blood produced lowest number of RBCs in the recovered plasma (RBC recovery ≈ 3%). Statistical analysis indicated that there was no significant difference in red cell recovery of experiments regardless of the type of anticoagulant used prior to experiments (see Table 6-1 and Table 6-2). Red cell recovery in female blood was not sensitive to the type of the anticoagulant used ($p > 0.1$).

**Table 6-1: Average RBC recovery for experiments with various type of anticoagulants**

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>RBC Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA-treated</td>
<td>6.69 ± 0.95 %</td>
</tr>
<tr>
<td>Heparinized</td>
<td>4.6 ± 1.12 %</td>
</tr>
<tr>
<td>Citrated</td>
<td>2.83 ± 0.87 %</td>
</tr>
</tbody>
</table>

**Table 6-2: $P$-values for statistical analysis comparing red cell recovery of three types of anticoagulants**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA vs. Citrate</td>
<td>0.38</td>
</tr>
<tr>
<td>EDTA vs. Heparin</td>
<td>0.76</td>
</tr>
<tr>
<td>Heparin vs. Citrate</td>
<td>0.59</td>
</tr>
</tbody>
</table>

### 6.7 Effect of the Type of Anticoagulant on Platelets in the Recovered Plasma

Our data show that the number of the platelets in the recovered plasma were affected by the type of the anticoagulant used. The number of the platelets in the recovered plasma from the citrated blood was the lowest compared to the samples that had been treated with heparin or EDTA while EDTA-treated blood produced the highest number of platelets (see Figure 6-3). Statistical analysis showed that there was a significant difference between the number of the platelets of the recovered plasma samples of blood treated with EDTA compared to citrated blood ($p = 0.0001$). The number of the platelets in the recovered plasma of citrated blood was significantly lower than heparinized blood ($p = 0.0384$).
6.8 Effect of Gender on the Number of Platelets in the Recovered Plasma

The gender of the blood donors had no significant effect on the number of the platelets in the experiments that used citrated or heparinized blood ($p = 0.06$ and $0.06$ respectively). However, there was a significant difference between the numbers of the platelets in the recovered plasma from male donors with female ones in blood treated with EDTA ($p < 0.001$).

6.9 Effect of ADP on the Number of Platelets in the Recovered Plasma

Adenosine diphosphate (ADP) is reported to trigger platelets to aggregate. If platelets stick to each other, there would be bigger clumps of platelets which would sediment faster than the bacteria. This would result in a lower number of platelets in the recovered plasma. We added various concentrations of ADP (between 0.5 and 1.1 g/L) to the blood samples before spinning the disk to see if it caused the platelets to sediment out of plasma. However, our data show that there was no significant difference between the numbers of the platelets in the recovered plasma as a result of using ADP regardless of its concentration or the type of the anticoagulant used. See Figure 6-3. P-values for citrated, heparinized and EDTA-treated blood are 0.67, 0.35 and 0.67 respectively.

Ault et al have studied the effect of ADP on platelet aggregation in blood treated with citrate, heparin and EDTA. They report that EDTA-treated blood, unlike citrated or heparinized blood, does not aggregate by adding ADP$^{24}$. Mohammad et al. report reversible platelet aggregation by adding 0.1 mL of $10^{-3}$M ADP solution to blood reaching the final concentration to $10^{-5}$M of ADP for citrated and heparinized blood. The time needed for platelet aggregation is reported as 30 to 45 seconds$^{25}$. The concentration of ADP that started aggregation in citrated and heparinized blood for our experiments was 1.1 g/L. At this concentration of ADP, we were able
to filter samples of the recovered plasma collected after spinning for 60 seconds at 3000 rpm without clogging filters with 1µm pores. However, investigating the micrograph images taken from the blood samples before spinning and the recovered plasma showed no difference in the number of the platelets after adding ADP.

![Figure 6-2: Concentration of platelets in the recovered plasma as a function of anticoagulant and gender. The ADP concentration in these experiments ranged from 0 to 1.1 g/L. The mean values and 95% confidence intervals are reported.](image)

6.10 Conclusion

The process of spinning blood in the hollow disk results in a bacteria-rich plasma which is collected to identify the bacteria and possible antibiotic-resistant genes. Increasing the volume of the recovered plasma results in higher number of total bacteria collected, facilitating the subsequent steps of identification. Therefore, increasing the volume of the recovered plasma is very important. Our data shows that the volume of the recovered plasma is slightly but
Figure 6-3: Concentration of platelets in the recovered plasma as a function of anticoagulant and ADP. The ADP concentration in these experiments ranged from 0 to 1.1 g/L. The mean values and 95% confidence intervals are reported.

not significantly higher in experiments using female blood compared to male ones. The type of anticoagulant used also did not affect the volume of the recovered plasma significantly. Our observations suggested that plasma could not drain freely over the weir as the disk stops since the surface of the disk is not very wettable by plasma. To overcome this challenge, various wetting agents such as γ-aminobutyric acid, sebacic acid and Pluronic P105 were used to treat the surface of the disk chemically in order to improve its wettability; but none of the agents significantly improved the average volume of the recovered plasma.

We could not obtain repeatable counts of bacteria in experiments using citrated or heparinized blood; thus we selected EDTA as the anticoagulant for experiments regarding bacterial recovery. EDTA did not affect the growth of bacteria while the others did. The average concentration recovery for a 12-cm disk at 3000 rpm corresponding to characteristic length of 94 mm using EDTA-treated blood was 68%. The average total bacterial recovery for similar
experiments was approximately 40% for experiments in which blood was spun at 3000 rpm with a characteristic length of 94 mm. Bacteria recovery was higher in the experiments that used male blood compared to the ones using female blood. Average bacteria concentration recovery in experiments using male blood was 72.5% while for the ones using female blood was 59.3%.

Red cell recovery was higher with male blood than female blood, and was sensitive to the anticoagulant used in male blood. Experiments using EDTA-treated blood produced the highest number of RBCs in the plasma. The effect of anticoagulant on RBC removal in female donors is not apparent.

Platelets are present in the recovered plasma in high numbers. ADP was used to attempt to aggregate the platelets and increase their relative sedimentation velocity compared to single platelets. However, using ADP did not prove to be effective in reducing the number of platelets in the recovered plasma.
7 DISCUSSION

We have developed a new technique capable of separating bacteria from whole blood for rapid sepsis diagnosis. Our hollow rotating disk is designed to separate the blood cells from the plasma containing the bacteria. Our results show that for a 12-cm-diameter disk, the average total bacteria recovery is 60.66% at characteristic length between 15 and 30 mm. For the 10-cm-diameter disk, this average is 62.37%. Similarly, for the 16-cm-disk, this average is 67.18%. The significance of our method compared to other techniques is that bacterial separation can be done in less than 5 minutes, and this process is capable of handling large volumes of blood.

The difference between our hollow disk and standard bench-top centrifuges can be explained by the thickness of the layer that the bacteria must sediment through. In a bench top centrifuge, the volume of blood is spread in a geometry of a long narrow tube. The bacteria must sediment through the length of the blood tube, which is on the order of centimeters. However, in our hollow disk the same large volume of blood is spread all over the disk vestibule, so the thickness through which the bacteria must migrate is less than two millimeters. For these shorter distances, the sedimentation and separation will be accomplished more quickly. Moreover, the boundaries between separated layers will be more distinct.

Using our hollow disk technique, we were able to concentrate the bacteria to a higher concentration than the initial concentration estimated by simple calculations based on the dilution of blood by PBS. This can be attributed to the bacteria being carried inward toward the
center of the disk by the inward flow of the plasma as the plasma is squeezed out of the red cell pack during consolidation in the vestibule.

Sedimentation velocity is defined as the steady-state velocity of a particle in blood relative to its surrounding fluid. As the disk starts spinning, some of the bacteria moves toward the center of the disk due to convection of the plasma in that direction. However, a few bacteria are entrapped within the red cell pack as the red cell consolidation as the red cells are moving towards the back wall of the vestibule. Our experimented results show that the initial net displacement of the bacteria is toward the center of the disk and increases the bacterial concentration in the plasma, while the concentration of the bacteria trapped in the red cell pack is much lower than in the concentration in the plasma. Adding PBS as a diluent to the blood spreads out the red cells providing more space for the bacteria to escape towards the disk center, probably increasing plasma concentration even more.

Our experiments show that after this initial high bacterial concentration in the plasma, as characteristic length increases, the bacteria concentration recovery decreases. This is attributed to the bacteria sedimenting toward and into the consolidated red cell pack as the characteristic length increases. If the spinning time is too long, the bacteria will eventually move into the red cell pack in the vestibule, resulting in low bacteria recoveries. On the other hand, if the spinning time is too short, the lack of separation of the blood cells will result in a plasma rich in red cells, and even low in bacteria for extremely short or slow spins.

One important discrepancy between the observed data and my initial expectations is the characteristic length at which the red cells consolidated in the vestibule. The data from plots of bacteria recovery versus characteristic length (see Figures 5-5 to 5-7) reveal that bacteria recovery reaches very low values when characteristic length is higher than 250 mm. The data
from RBC recovery (Figures 5-9 through 5-11) show that the recovery decreases until a characteristic length of 50 mm. Considering the fact that the simplified theoretical relative velocity of the red cells is about 30 times higher than the bacteria, we expect the red cell recoveries to have negligible values at these high characteristic lengths. However, RBC recovery never goes to zero. As illustrated by plots of red cell recoveries versus characteristic length (see Figures 5-9 to 5-11), the slope of the line of red cell recovery versus characteristic length is steeper than the slope of the similar line for the bacteria recoveries. This can be explained by the fact that some of the assumptions made while defining sedimentation velocity are not entirely valid. For example, characteristic length has been defined as the distance that an 8-micron-diameter hard sphere particle with the density of the red cell travels as the disk starts to spin until the disk comes to a stop. However, red cells are flexible biconcave discoid soft particles which change their shape as they travel in the disk. Moreover, Stokes law was used to calculate the drag force affecting the particles. The requisite criteria for Stokes law is that there is a single spherical particle which does not interact with any others, and the \( Re < 0.01 \). The fact that the number of the red cells in the blood are very high makes it obvious that the assumption of the single particle is neither accurate for the bacteria nor the red cells. The shape and flexibility of the particles also affect the drag force. These deviations are represented by a correction constant \( K_{total} \) in the following modified Stokes law:

\[
f_{drag} = 3\pi \mu v_s D_p K_{total}
\]

\( K_{total} \) is the correcting parameter that includes all deviations caused due to crowdedness of the red cells (accounted by parameter \( K_{space} \)), their flexibility(\( K_{flex} \)), and shape (\( K_{shape} \)).

\[
K_{total} = K_{flex} K_{shape} K_{space}
\]
Each of these factors will be discussed in turn. A commonly used colloidal science

definition of sedimentation velocity, \( v_s \) is:

\[
v_s = s \cdot R \cdot \omega^2 \tag{7.3}
\]

in which \( s \) is defined as sedimentation coefficient\(^{26} \). \( R \) is the distance between the particle and the
center of rotation, and \( \omega \) is the angular velocity. The sedimentation coefficient \( s \) is the
proportionality constant linking velocity (\( v_s \)) and acceleration (\( R \cdot \omega^2 \)). From physics and force
balances, \( s \) can be calculated from:

\[
s = \frac{m}{f_{friction\_factor}} \left( 1 - \frac{\rho_f}{\rho_p} \right) \tag{7.4}
\]

in which \( m \) is the mass of the particle, \( f_{friction\_factor} \) is friction factor on the particle and \( \rho_p \) and
\( \rho_f \) are the densities of the particle and the fluid. If we combine the two above equations,
sedimentation velocity is calculated as:

\[
v_s = \frac{m}{f_{friction\_factor}} \left( 1 - \frac{\rho_f}{\rho_p} \right) R \cdot \omega^2 \tag{7.5}
\]

Replacing \( f \), friction factor, by modified version of stokes law (Eq.7.1), we arrive at:

\[
v_s = \frac{m}{3\pi \mu D_p K} \left( 1 - \frac{\rho_f}{\rho_p} \right) R \cdot \omega^2 \tag{7.6}
\]

This equation is equivalent to Eq. 5.3 for spheres of density \( \rho_p \) and \( K=1 \)

Now, we attempt to obtain the values for \( K_{total} \). If RBCs are modeled as a solid oblate
spheroids with a diameter of 8 \( \mu \)m and thickness 2 \( \mu \)m, \( K_{shape} \) is 1.17\(^{26} \). However, the biconcave
shape of a RBC is slightly different than an oblate spheroid used in this calculation. Furthermore,
the shape of the RBCs changes from a biconcave to a more streamlined shape as the
sedimentation velocity increases. This is due to the drag forces that act on the RBCs in a centrifugal field that deform the flexible RBCs. \( K_{flex} \) is the parameter that accounts for the flexibility of the RBCs. Experimental results show that \( K_{flex} \) has a value of about 0.45 at 3,000 rpm and radius of 6 cm\(^2\).

As the particles move in a flow field, the fluid surrounding these particles is perturbed by the movement of the particles. Considering the fact that the number of RBCs in the blood is very high, the flow field around 1 RBC is perturbed by the nearness of adjacent RBCs. This increases the drag force and decreases the sedimentation velocity of the particles. Tenneti et al report that the drag forces increase as the volume fraction of the particles in the blood increase. For example, for the hematocrit level of 35, which is calculated for our experiments using diluted blood, the drag correction \( K_{space} \) has a value of about 14. Combining \( K_{space} \), \( K_{flex} \), and \( K_{shape} \) and using the diluted viscosity and density of plasma as 1.12 mPa.s and 1.08 g/cm\(^3\), and using \( R \) distance of 5.8 cm from the center of a hollow 12-cm-diameter disk spinning at 3,000 rpm, we calculate the sedimentation coefficient \( 6.85 \times 10^{-8} \) s. At these same conditions the sedimentation velocity of a RBC is calculated to be about 0.38 mm/s, relative to the surrounding fluid\(^2\).

In a closed space like a centrifuge tube or hollow disk, the migration of the red blood cells away from the disk wall displaces fluid and creates a back flow that is towards the center of rotation. If the volume fraction of the red blood cells is represented by \( \varphi \), the back flow towards the center of rotation is proportional to \((1 - \varphi)\). Consequently, the reduction in the absolute velocity of the red blood cells towards the trough is proportional to \((1 - \varphi)\). This reduces the particle absolute velocity to \( 0.38 \text{ mm/s} \times (1 - 0.35) = 0.247 \text{ mm/s} \). If the physical distance the RBC must migrate is on average 1.5 mm, the RBCs (at Hct = 35) should be cleared out of plasma in approximately 6 seconds at 3000 rpm for the 12-cm-diameter disk in which \( \nu_s = 0.247 \). 
mm/s. But the experiments show that the characteristic length and time (at 3,000 rpm) required to reduce the number of the red blood cells from the plasma is much higher than the time estimated above. This can be explained partially due to the increase in the $K_{space}$ value which increases to $>100$ as the red blood cells start to consolidate into a solid pack. In other words, $v_s$ decreases many fold as RBCs approach the cell pack.

There have been studies on sedimentation velocity of RBCs. Bar et al have measured the sedimentation coefficient of RBCs in diluted blood at various rotational accelerations. If we use the data provided by Bar et al, and adjust the density and viscosity of saline-diluted plasma, the experimental sedimentation coefficient of Bar et al. is corrected to be about $3.5 \times 10^{-8}$ sec and the sedimentation velocity is estimated to be 0.20 mm/s at 3,000 rpm in our disk\textsuperscript{27}. We note that the sedimentation coefficient measured in the experiments of Bar apparently are for the absolute particle velocity, and not the velocity of the particle relative to the adjacent fluid. Thus no $(1-\varphi)$ correction is made to the data of Bar\textsuperscript{27}.

Considering the fact that the main aim of this project is to separate the bacteria from whole blood, it is important to know the parameters that affect the sedimentation of the bacteria. The bacteria and RBCs are generally (within an order of magnitude) the same size; therefore they would have approximately the same $K_{space}$-value. This will lead into similar decreases in the sedimentation velocity increasing sedimentation time in an environment crowded with cells.

As mentioned before, there is another important discrepancy between the observed data and my initial expectations that RBC recovery should go to zero at high values of sedimentation length. It is possible that artificially high readings of hemoglobin in the recovered plasma might be attributed to the higher numbers of sheared red cells releasing their contents to the recovered
plasma. For precise measurements of red blood cells present in the recovered plasma, we should do the measurements as soon as the plasma is collected, which is as soon as the disk stops spinning; but even then, some RBCs may have been sheared during sedimentation and the hemoglobin release may produce an artificially high value of RBC recovery. But we estimate that this correction would be only on the order of 10%, if such a correction is needed.

As explained in Chapter 5, increasing characteristic length results in lower red cell and bacteria concentration recoveries. The main aim of this project was to separate bacteria from the blood cells and collecting them. Lowering the number of the red cells in the recovered plasma is desirable, but in selecting the proper characteristic length we have to compromise the lower red cell recovery to obtain the higher bacteria. Careful analysis of plots of 5-5 to 5-10 suggests that the proper characteristic length lies in the range of 15 to 30 mm depending on the tolerance of the subsequent processes, mainly the filtering processes.
8 CONCLUSION AND RECOMMENDATIONS

8.1 Conclusion

We have developed a technique that successfully separates bacteria-rich plasma from blood cells. If the spinning speed of the disk is reduced carefully, we can avoid remixing in the separated plasma layer and collect the plasma that has a low number of red blood cells. Regardless of the size of the disk, both bacteria concentration recovery and red cell recovery decrease as spinning speed and time increase. Decreasing the number of red blood cells in the recovered plasma is desirable due to less complications in the subsequent process steps. However, lower red cell recovery is the result of the selection of higher spinning speed and/or spinning time which also lead to lower bacteria recovery. Therefore, the end user must select the spinning parameters to obtain the highest possible bacteria recovery with regard to the tolerance of the subsequent steps towards red cells, specifically the tolerance of the filtration process towards the red cells that may clog filters.

The main objective of this process is to separate bacteria from blood and maximize bacteria recovery. The best bacteria concentration recovery ($C^*$) is at the characteristic length range of 15 to 30 which, for 12-cm-disk, is 61%. Considering the fact that the high number of red blood cells in the plasma interferes with the subsequent steps of bacteria identification, we need to select a characteristic length that has lower number of red blood cells. The number of the
red blood cells versus characteristic length does not reduce significantly as characteristic length increases above 50 mm.

Total recovery strongly depends on the volume of the recovered plasma. Our data shows that a portion of the plasma does not drain over the weir and is retained in the disk trough. This problem is attributed to several factors including disk design and wettability of the disk surface. We tried to increase the volume of the recovered plasma by both chemical and mechanical methods. Wetting agents such as $\gamma$-aminobutyric acid, sebacic acid and Pluronic P105 were used to treat the surface of the disk to make it plasmaphilic. However, these chemical methods were not effective. Mechanical methods such as weir extension, increasing the roof height and adding baffles were investigated to increase the volume of recovered plasma.

The number of baffles had a significant effect on the volume of the recovered plasma. Volume of recovered plasma in 12-cm-diameter disk was higher in the disk with 16 baffles compared to the disk with 32 baffles or the disk with no baffles. The best number of baffles for a 12-cm disk is 16. Extending the length of the weir increased the volume of the recovered plasma significantly which increased the total recovery of the bacteria. Our experiments showed that increasing the height of the disk roof results in a more uniform plasma drainage but there is not enough data to discern if the volume of the recovered plasma increased or not. The best volume of plasma was recovered from 10-cm-diameter disk with extended weir.

Three sizes of disks were studied thoroughly to see if there is a correlation between the disk size and bacteria or red cell recovery. Our results showed that the red cell or bacteria recovery versus characteristic length was not statistically different for the disks with various sizes when correlated by characteristic length, and as characteristic length increased, red cell and bacteria recovery decreased. Considering the scatter in the data in the plots of red cell recovery
vs. characteristic length and the plots of bacteria recovery vs. characteristic length, we conclude that there is no significant difference between the three disk sizes.

8.2 Recommendations

As mentioned previously, recovered plasma contains red blood cells that might cause challenges in the subsequent filtration process. To reduce the number of red cells-present in the plasma, we first need to gain a better understanding of the sedimentation process. Mathematical modeling of the red cell sedimentation and backward flow of plasma and bacteria from the red cell pack can provide helpful information which can be used to improve the process and identify the best operational parameters. The models can also be used to predict the sedimentation of platelets.

Investigating the chemical methods enhancing surface wettability is suggested to be done with the prospect of increasing the volume of the recovered plasma, since increasing the volume of recovered plasma directly affects total bacteria recovery.

Current design of the disk results in non-uniform flow of plasma. Investigating the reason for the non-uniform plasma drainage and solving this issue might increase the volume of the plasma.

Scatter in the data masks the point where consolidation of red cells is completed so we can start to reduce the spinning speed without fearing the remixing of red cells into the plasma. Finding the optimal spinning speed and spinning time (optimal characteristic length) may result in higher bacteria recovery.
Bacteria growth was hindered in the experiments that used heparinized or citrated blood. Therefore, we selected EDTA as our anticoagulant. It is recommended to investigate the effect of anticoagulants such as heparin and citrate by measuring the number of the bacteria via filtration since red cell and platelet recovery was lower in the experiments conducted using centrifugation of the blood treated with these two anticoagulants. The use of citrate appeared to reduce the number of platelets.

PBS is added to the blood on the disk before spinning to change the viscosity of the blood. Lower viscosity increases the sedimentation velocity. The effect of various ratios of PBS to whole blood can be studied to find the optimum ratio. Disk design such as slope of the bowl can be investigated to see if it has an effect on recovered plasma volume and find the optimal angle for the disk bowl.

Overall, the designed hollow disks are capable of successfully separating bacteria from whole blood. The improvements mentioned above may increase the efficiency of the separation process.

### 8.3 Publications

The main part of the data presented in this thesis has been published in several journal papers and a patent listed below:


9 BIBLIOGRAPHY


