University of Alberta

Microfluidics in Blood-Based Diagnostics

by

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Abstract

The aim of this research is to further the advancement of micro total analysis systems in the area of blood-based diagnostics though microfluidics. This thesis focuses on two main areas of diagnostics. The first is the use of microfluidics to improve DNA mutation detection. A method of on-chip DNA self-assembly that enables the combination of heteroduplex analysis and single strand conformation polymorphism is presented.

The other focus of this thesis is in the area of microfluidics-based blood typing. The use of antibodies for agglutination-based microchip blood typing was first explored. A method for mobility-based blood typing for the ABO blood group was then investigated. Finally, a potential technique utilizing electrokinetic focusing for the typing of A1 and A2 blood types was developed. Overall, the methods developed in this thesis may act as building blocks toward further improvements and growth in miniaturization of blood-based diagnostics.

Acknowledgements

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Chapter 1

Introduction

1.1 Microfluidics

A move towards the development of miniaturized total analysis systems (μTAS) has become an area of great interest in recent years. This interest is due to their ability to integrate multiple processes and large reductions in cost, labour, reagent consumption and time [1]. Examples include techniques such as capillary electrophoresis and microfluidic chip based analysis. To date, on-chip polymerase chain reactions (PCRs), microchip based capillary electrophoresis, enzymatic assays and immunoassays as well as detection and separation of biological and chemical components have all been accomplished on microfluidic devices [2-5]. Examples include the detection of certain cancer markers using microchip-based immunoassays [6], and the detection of antibodies achieved by using a blood clotting protein complex [7]. Other devices such as small glucose sensors and detectors for various electrolytes and blood gases are also currently available commercially.

Microfluidics involves the manipulation of solutions and analytes in micro-sized channels for analysis. It offers various unique characteristics compared to conventional macroscopic technology. The smaller dimensions mean dramatically decreased surface to volume ratio leading to a much faster thermal diffusion time. The small quantities of reagents used also means a large reduction in molecular diffusion time. The laminar fluid flow in the small channels also means that any mixing of the fluids is achieved by

diffusion. These characteristics of microfluidics offer potential techniques of analysis that could not be achieved on a macroscopic level.

The incorporation of microfluidics with medical diagnostics is at the forefront of research, and work is being done to move towards the use of this technology to develop less invasive testing methods. One area of research is the attempt to derive information from blood samples. Much of the current diagnostic testing involves the use of blood, ranging from the extraction of genetic information to blood cell and composition analysis. In many cases the isolation and use of blood cells for testing is a critical aspect of the diagnostic. For other cases, it is the antibodies and other proteins in the blood that are of interest.

Several methods that we have developed, which could aid improvements in blood-based diagnostics are presented here. Our focus in this thesis was on blood typing and red blood cell analysis. However, the development of a method for rapid on-chip DNA denaturation and self-assembly for improved mutation detection is also presented. This was a result of initial work on more established on-chip DNA mutation detection aimed at better understanding the microfulidic chip platform. The following provides a background into working with microchips and some of the issues involved.

1.1.1 Pressure Driven Flow

Although microfluidics offers various potential advantages over macroscopic methods, there are also issues that are more prominent or unique to fluid flow in microchannels. One such issue is the sensitivity of microchannels to pressure driven flows. Microfluidic analyses are often carried out in microchips, small networks of wells or reservoirs connected via microchannels. Due to the small volumes used in microchips, minute variations in volumes between two wells represent a larger percentage of the total volume of the wells than when larger volumes are considered. Thus, microchips are more sensitive to pressure driven flow caused by minute volume variations between wells. Pressure differences are then translated into fluid movement in the channels towards equilibrium. Such fluid movement is most often undesirable during analysis. Thus, special attention must be taken to prevent even minute volume differences that may usually not be of concern under macroscopic conditions.

1.1.2 Electroosmotic Flow

In addition to pressure driven flows, electroosmotic flow (EOF) is also present in microfluidics. Electroosmotic flow involves the bulk fluid flow induced by the application of a voltage in a microchannel. A charged double-layer forms at the wall-fluid interface when the channel walls become charged (Fig. 1.1). This charge arises from the ionization of the silanol groups on the surface of the fused-silica glass that leaves the channel walls negatively charged [2, 8]. The double layer is comprised of a rigid layer of adsorbed positively charged ions that is immobile as well as a diffuse layer that consists of mobile ions. The application of an electric field attracts the positive ions from the diffuse layer towards the cathode. This attraction causes the diffuse layer to migrate and drag the bulk fluid along. The flow profile during EOF is different than pressure driven flow in that it is flat instead of parabolic (Fig. 1.2).

The fluid movement caused by EOF is very important in microfluidics. Any time tracking of analytes is required, EOF must be taken into account since it causes a shift in analyte movement. This shift may increase, decrease or even reverse analyte velocity if large enough. EOF is especially significant when accurate velocity measurements are of interest to the final results. Increases in field strength and pH may lead to increases in EOF due to increased ionization of the silanol groups and greater force on the charged particles applied by a larger field. Thus, effective measure and control of EOF are required for many applications.

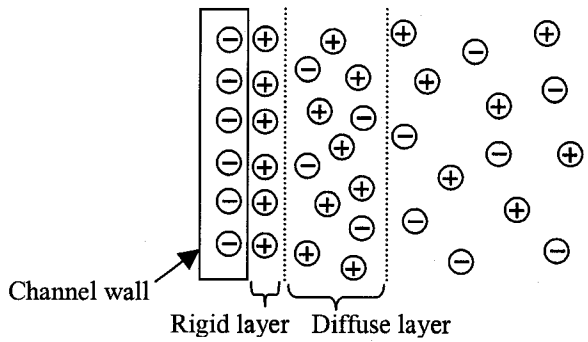


Fig. 1.1: Electrical double-layer formation at channel wall

(a) (b)

Fig. 1.2 Flow profile of EOF (a) compared to pressure driven flow (b)

1.1.3 Laminar Flow

Another issue that must be taken into consideration is that of laminar flow, the typical turbulence-free flow inside microchannels. This flow occurs at low Reynolds (Re) numbers, usually in channels where either the width or height is less than 200 μm. The Re number is a ratio of the inertial to viscous forces in a fluid flow configuration and is given by

$$\mathrm{Re} = \frac{\rho v w}{\mu} \tag{1.1}$$

where ρ is the fluid density, v is the mean flow velocity, w is the characteristic dimension of the of the flow geometry and μ is the fluid viscosity [9]. Low Reynolds numbers, less than 2000 [10] indicate laminar flow. Numbers equalling approximately 1 or lower are considered to be superlaminar and are characteristic of most fluids in microchannels. At low Re number, streams flow with sharp boundaries and mixing occurs primarily due to diffusion between streams and may be maintained up to lengths in the centimetre range. This characteristic may complicate microchip work if fluid mixing is required. Conversely, movement of particles from one stream to another via diffusion only may also be a useful tool for separation of small particles from a sample.

1.1.4 Wall Passivation

In order to manage electroosmotic flow, the conditions of the channel walls are very important. As mentioned above, the ionization of silanol groups is the main cause of EOF. Thus, the treatment of the channels to prevent or reduce ionization would be an effective method for reducing or eliminating unwanted EOF. In addition, this wall passivation is also useful in reducing analyte-wall interactions. Certain biological

analytes, especially proteins, have a strong tendency to adsorb to microchannel walls [11].

Several methods exist for reducing EOF and protein-wall interactions in glass channels. The lowering of the analyte solution pH to below 3 leads to protonation of the silanol groups and leaves the walls neutral [12]. Increasing the pH renders both the walls and proteins negative, reducing their electrostatic interactions [12]. Although effective to a degree, it must be noted that low pHs tend to lead to high conductivity while high pH values give rise to unstable EOF respectively.

Alternatively, static or dynamic wall passivation or coating may also be used to reduce protein-wall adsorption and control EOF. Static passivation involves the incorporation of certain chemicals or additives during fabrication to neutralize the channels or prevent ionization. This often translates into time consuming and rather complex fabrication processes. However, permanent coatings do not require regeneration and hundreds of runs may be done without degradation to the capillary performance [13]. In dynamic coatings, the compounds are physically adsorbed to the surface of the channels instead of covalently bonded. These additives act to both increase viscosity and/or shield the surface charges on the channels. Coating of the channels is achieved by the introduction of additives to the running buffer, performing an initial rinsing step, or both. This means that complicated bonding steps during fabrication can be avoided with less dependence on the exact coating process [14]. However, coating regeneration is required and the interaction of other components in the buffer with the coating on the wall or with the coating additives in the buffer may cause contamination problems during analysis.

Neutral and charged polymers are used for permanently coating glass channel walls. Two commonly used polymers are polyacrylamide and poly(vinyl alcohol) (PVA) [15-17]. Zhang *et al.* [18] used both polyacrylamide and PVA for channel coatings to effectively separate protein on a microchip. Charged polymers are also used, but tend to alter the pH of the medium leading to EOF shifts. Thus, they are usually not used alone, but more commonly combined with other neutral polymers to control EOF. Despite the effectiveness of coatings, their use is often time consuming, complicated and difficult to reproduce [14, 19].

The three main types of buffer additives used for dynamic coating include amines, polymers, and surfactants and salts. Many of the polymers used for permanent coatings are also used for dynamic coating [11, 12]. Solution additives such fibrinogen and bovine serum albumin (BSA) have also been used [16].

The addition of salts was one of the earliest methods used to reduce protein-wall interactions [20-22]. It was believed that the introduction of salts provided the ions to compete with proteins with interaction with the charged channel walls, thus reducing protein-wall interactions [23]. Commonly used cations include Cs^+, Li^+, K^+, and Na^+ [20, 21]. Ionic salts are less effective compared to other additives and also contribute to high currents and Joule heating.

To avoid the drawbacks associated with ionic salt additives, different surfactants with low or no conductivity can be used. Although cationic and anionic surfactants work to reduce adsorption of the same charge proteins, they pose a problem for the adsorption of the oppositely charged proteins and are also strongly denaturing. For these reasons, zwitterions and non-ionic surfactants are preferred over ionic surfactants. Zwitterions are neutral molecules that have both positive and negative regions. They are capable of blocking the binding sites on the capillary walls and act as competing agents for the positive regions on the proteins, reducing both protein-wall interactions as well as protein-protein interactions [22, 24]. Some well known non-ionic surfactants include Tween 20, betaine, Triton X-100, poly(*n*-undecyl-α-D-glucopyranoside) (PUG) and (Brij-35) [19]. In Castelletti *et al.*'s work, Triton X-10, Brij 35 and Tween 20 were all tested with the Tween 20 found to be the most effective [15].

There exist a variety of wall passivation methods where the choice of method is often based on the particular application. In addition to wall passivation via the addition of an additive, a variety of different channel conditioning methods may also be employed. For the analytes of blood components of interest, numerous reports of wall conditioning methods exist. In the electrophoresis of human serum proteins by Colyer *et al.* [25] pre-conditioning of the microchip channels with 1 M NaOH, water, and borate buffer was carried out. Chiem *et al.*, used tricine buffer with Tween 20 and NaCl directly with no pre-conditioning of the chip [26]. In the on-chip immunoelectrophoresis performed by Ichiki *et al.* [27], commercially available veronal buffer with 0.1% gelatin

(GVB) was used. GVB is a buffer used at times with biological analytes [28] and contains NaCl, sodium barbital or barbitone and barbituric acid, was able to suppress EOF and allowed for successful immunoelectrophoresis of sheep red blood cells with antibodies. In another work where human antibody detection was performed, the chip was conditioned by first flushing with 0.1 M NaOH, and then rinsing with water and flushing with 20 mM TAPS/AMPD containing Tween 20 [29]. In Vrouwe's work with the detection of lithium in whole blood, either a permanent coat of polyacrylamide or a buffer additive of 0.01% (hydroxypropyl)methylcellulose (HPMC) was used for reducing EOF [30]. In our work, we used the addition of a low concentration polymer to limit EOF. It was believed that the polymer would add both viscosity to the buffer and help coat the channels to reduce EOF. The polymer was chosen for its compatibility in terms of inertness and versatility in use with biological analytes of both blood components and genetic material.

1.2 Cell Movement Methods

In order to successfully analyze blood and its components for diagnostic testing, the ability to effectively manipulate and control bloods cells on a microchip is critical. Various methods exist for the movement and separation of cells in a microchip. Each has their advantages and disadvantages and the appropriate choice of method depends largely on the purpose of analysis. Several of the commonly used methods are described below.

1.2.1 Electrophoresis

A common electrical method for separation and analysis of biological compounds is the technique of electrophoresis. Electrophoresis has been a valuable tool widely used in the analysis of nucleic acids [31, 32] and proteins [11, 33, 34]. The advent of capillary electrophoresis (CE) has helped to enable microchip-based CE [35, 36]. Capillary electrophoresis has been very successful in the separation, detection, and analysis of proteins, DNA and other nucleic acids.

Various studies have also been done on the subject of red blood cell electrophoresis. Li [37] and Minerick [38] have both electrophoresed red blood cells

(RBCs) while Tsuda *et al.* have detected RBCs on a single cell level [39]. Kitagawa *et al.* and Omasu have both measured the electrophoretic mobility of RBCs [40, 41]. These reports suggest that distinct differences in RBC mobility may be potentially measured. Red blood cells are negatively charged at relatively neutral pH, a fact that has been well established [42]. The negative charge is attributed to the sialic acid content of the cell membranes [43]. These acids are associated mainly with the glycolipids and glycoproteins. Bulai *et al.* analyzed the sialic acid content of A, B, AB, and O RBCs and found that the majority of the sialic acid was bound with the glycoproteins [44]. They also found that the distribution of the sialic acid between the glycoproteins and glycolipids on the red cell membrane were different between the different blood types of the ABO group. This information provides some insight into cell charge and should be noted if cells were to be exposed to an electric field.

The principle behind electrophoresis is the movement of ions through an electric field. The velocity of particles due to the electric field when there is assumed to be no pressure gradient and no EOF (discussed below) is given by [45]

$$v = \mu_e E \tag{1.2}$$

where v is the velocity of the particle, μ_e is the electrophoretic mobility and E is the applied electric field. For cases where Joule heating effects are negligible such as in microchips where the surface-to-volume-ratio is large and where the medium is homogenous, the electrophoretic mobility is considered to be constant for a given set of electrophoresis conditions. For the case where the Debye length, the distance at which a particle is shielded by positive and negative ions, is large compared to the particle, the electrophoretic mobility may be approximated by

$$\mu = (2/3)\, \epsilon\zeta/\eta \tag{1.3}$$

where ϵ is the dielectric constant of the fluid or medium, ζ is the zeta potential or the electrical potential between the diffuse and rigid layers measured from the capillary wall, and η is the viscosity of the liquid or medium. It can be seen that the electrophoretic mobility is dependent on the size of the particle with the larger particles moving slower than the smaller. In cases where the Debye length is small compared to the particle size such as cells, the electrophoretic mobility is approximated by

$$\mu_e = \epsilon\zeta/\eta \tag{1.4}$$

Human red and white blood cells as well as platelets have mobilities ranging from 0.75 μm cm/Vs to 1.10 μm cm/Vs when cells were electrophoresed using CE using a capillary with 0.45 mm diameter in 0.012 M N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonicacid (HEPES) buffer [46]. The difference in mobility allows for the separation of different species to be achieved during electrophoresis. If each of their velocities is constant, then as two species with different mobilities travel down a channel during electrophoresis, they would eventually separate into two separate groups given that the distance of travel is sufficiently long. To enhance the mobility differences or separation resolution that is dependent on size differences, a sieving matrix may be added to further impede movement of the larger particles.

The overall mobility a particle experiences during electrophoresis is not only due to its electrophoretic mobility, but also due to any EOF that may be present. Thus, the overall mobility of a particle is the sum of its electrophoretic mobility (μ_{ELE}) and the electroosmotic mobility (μ_{EOF}). Since electroosmosis may cause the analyte to move in the same or opposite direction as the electrophoretic movement, the overall perceived movement of the particle would be a summation of the two velocities. Similar to the electrophoretic velocity, the field specific electroosmotic velocity of a channel with no pressure gradient is also given by

$$v_{EOF} = \mu_{EOF} E \qquad (1.5)$$

where v_{EOF} is the electroosmotic velocity, and E is the electric field. This is the same as equation 1.2, but the electrophoretic mobility is replaced by the electroosmotic mobility μ_{EOF}.

1.2.2 Dielectrophoresis

Unlike electrophoresis, dielectrophoresis (DEP) is a method for polarizing and controlling particle movement through the use of non-uniform electric fields [47-50]. The use of DEP to create funnels, traps, and particle micromanipulators have all been developed [50-52]. Particles are suspended in a liquid medium where they may move freely and an AC electric field is applied to induce polarization of the particles (Fig. 1.3). The polarizability of the particles depends on its dielectric properties, the conductivity of the particles and suspension medium, and the frequency of the electric field [49].

Although impractical in the macro sense, the high electric field strength requirements in DEP are readily achievable in microdevices.

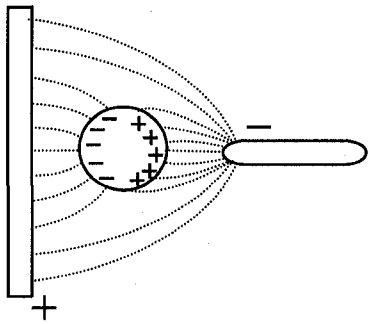


Fig. 1.3: Polarization of particle with non-uniform electric field

Under dielectrophoresis, the induced dipole moment of the particles may align with the electric field and be attracted to the areas of high field strength. This is known as positive DEP. Negative DEP occurs when the moment aligns opposite to the electric field and the particles move toward areas of low fields. Alignment along the electric field is due to the particle being more polarizable than the medium, whereas a medium more polarizable than the particle results in negative DEP. DEP is a very sensitive method for sorting different particles. This may be advantageous because the electrode, field and frequencies used may be set to attract a specific particle. However, if one requires a population of particles with inherent variations to behave in the same manner under DEP, the sensitivity may become problematic. In our case, the sensitivity of DEP to small changes in parameters such as a cell age difference of one or two days may be undesirable. In any given sample of cells, the age may vary from 1 to 120 days, thus the sensitivity of DEP may make it difficult to achieve specific control and manipulation of groups of cells. Negative DEP is usually preferred over positive DEP for the manipulation of cells since no contact with the electrodes are made, reducing the stress on cells [51, 52]. Changes in frequency of the AC field applied may also cause particles experiencing one type of DEP to shift to the other and is a common method for applying the desired DEP forces. This cross-over frequency is also specific to different particles and even same particles of different sizes [49].

DEP allows for "trapping, focusing, translation, fractionation, and characterization of particulate mineral, chemical, and biological analytes within a fluid suspending medium" [49]. It is due to the versatility and wide applicability of DEP that is has become widely used in the handling of many biological components such as cells, beads, bacteria, viruses and molecules. DEP has been successfully used for the separation of viable and non-viable red and white blood cells [53], cancerous and normal blood cells [49], as well as qualitative analyses of red blood cells [54].

1.2.3 Filters

Mechanical filtration or separation systems have been used on microchips for the separation and sorting of cells. Work on the trapping of both white blood cells and other cells in general have also been done [55, 56]. Various filtration methods exist ranging from axial and lateral percolation [55] to simple weir-like or dam structures [57-59]. A series of weir type filters have also been used to trap WBCs [59] along with a lattice of varying channel lengths [56]. Several dam-like structures designs including micropost array, comb-like filters and various filter beds have been investigated as filtration devices. Although the dam structure is simple in design, it is limited by the formation of microchannel blockages caused by trapped cells and also adds stress to the cells due to increased hydrodynamic forces.

It appears that successful isolation of blood cells using mechanical filters in microfluidic devices is very much dependent on the size and deformability of the blood cells since mechanical filters work on the principle of size based separation. If a cell was highly deformable as in the case of red blood cells, and the filter is meant to keep the cell out, then the spacing in the filter would have to accommodate for the cell's ability to squeeze through smaller openings than its actual size. The deformity of RBCs is highly variable based on the concentration of cells, their age, the pressure applied to them, and the viscosity of the suspension medium. These factors must all be taken into consideration. Biological factors such as pH and the amount of energy available to the red cells also contribute to their level of deformability. These factors make mechanical separation of the blood cells from plasma complicated especially when the prevention of hemolysis, destruction of the cells, is important.

1.2.4 Laminar Flow

An alternative to electrically driven systems would be the use of hydrodynamic fluid control. The phenomenon of laminar flow has been used in various analyte detections including the detection of compounds in blood [9, 60].

The lack of turbulence in laminar flow means that mixing between two or more fluids does not readily occur and is only achieved through diffusion. This property has been utilized by many for the extraction and detection of specific analytes in samples through the use of an H-filter (Fig. 1.4). In an H-filter, two parallel streams of sample and detection fluids are simultaneously injected through a channel. The particles diffuse across the streams producing diffusion reactions zones or areas where diffusion occurs between the fluids. Detection is usually based on colour change or fluorescence of the reaction between the indicator and analyte solutions. A third reference fluid stream can also be added with known concentrations of analyte for detection with self-referencing capabilities (Fig. 1.5) [9]. Here, the indicator solution flows in parallel, down the middle between the sample and reference solutions. The analyte concentration is usually determined through the ratio of a property such as fluorescence intensities between the reference-indicator and analyte-indicator reaction zones.

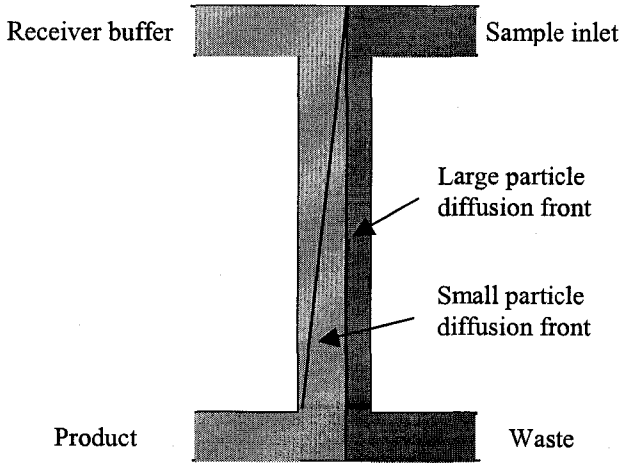


Fig. 1.4: H-filter used to extract small particles from solutions using laminar flow

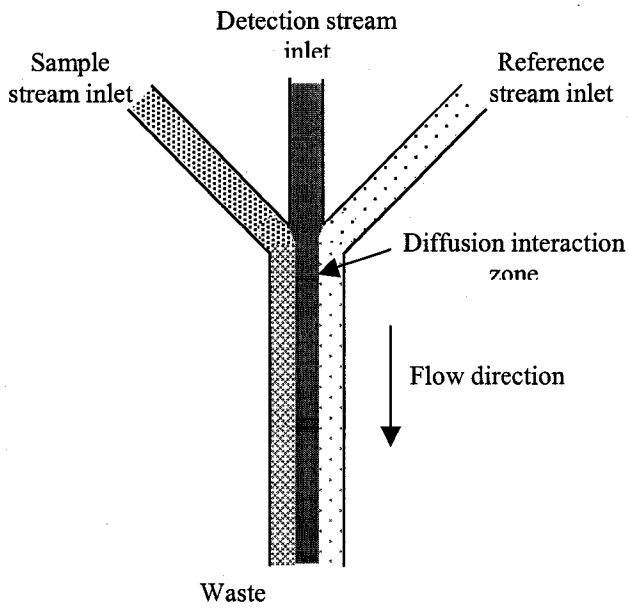


Fig. 1.5: Three parallel stream for self-referencing detection

Blood cells have diffusion rates approximately three orders of magnitude slower than that of H^+ and Na^+ in phosphate buffer [61, 62]. Therefore, the smaller ions and other blood components may potentially be removed from whole blood through diffusion during laminar flow. Among others, T-sensors or T-shaped channels for the determination of blood pH [9, 61], and calcium and albumin levels have been achieved [61, 63]. The use of laminar flow to pattern cells in capillary networks has also been reported [64]. Oakey *et al.* [62] used optical trapping techniques to isolate the particles of interest and move them from the sample stream to the detection one. An H-filter was used for the detection of various drugs from blood [63, 65]. These all demonstrate the usefulness of laminar flow in separation of analytes as well as work with blood. Laminar flow appears to be used mostly for the extraction of small particles from a stream while larger particles such as cells require additional trapping methods other than diffusion. Thus, laminar flow may be effective for the removal of smaller unwanted particles from a blood sample for purification or for extraction of certain smaller particles or interest. However, the isolation of cells will not likely be achievable with the use of laminar flow alone.

1.2.5 Immunomagnetic Beads

A final method for the isolation of cells of interest is the use of immunomagnetic beads. These superparamagnetic beads bind to the target cells and can be isolated with the application of a magnetic field. The beads are several micrometers in diameter and are conjugated to various antibodies that target specific cells of interest. Immunomagnetic beads have been used in immunomagnetic separation (IMS) or magnetically activated cell sorting (MACS) for various applications including the isolation of fetal cells in maternal blood [66], detection of cancerous cells [67], and the capture of a variety of other cells such as stem cells [68] and eosinophils [69] from blood.

The magnetic manipulation of cells enables handling of samples with ease and efficient protocols. Leading manufacturers Miltenyi Biotec (Auburn CA) and Dynal Biotech Inc. (Oslo, Norway) provide available beads for use and complex sample preparation steps may be avoided. Viable cells with high yields and purity can be obtained even at very low cell concentrations. The lack of a lingering magnetic charge once the magnet is removed and the tendency of the beads to not agglomerate make them easy to control and suitable for automated systems and microfluidics. However, the need for a release agent to separate the analyte from the beads means the extraction and washing of the cells is necessary. This would be difficult and complex to implement on-chip and may require off-chip processing.

1.2.6 Summary of Methods

Overall, there are several methods for the manipulation and movement of cells. Among these, electrophoresis appears to be a possible method for the movement of cells from one location to another. DEP and filters act more to trap cells and isolate them rather than to necessarily transport them. The advantage of DEP over electrophoresis is that the particles of interest do not need to be charged and DEP is also sensitive to a wide range of properties including composition [48]. Both electrophoresis and DEP would reduce complications associated with conventional pumping and valving on microfluidic devices. The use of filters may be potentially employed for initial purification of a sample such as a whole blood to remove the large white blood cells. A combination of

magnetic beads and filters may also be used to help isolate components of interest such as red blood cells.

1.3 Blood Typing

Transfusions have become a vital component in modern health care that are used to treat trauma, cancer, and surgical patients as well as those born with blood abnormalities. Although various works are being done on the miniaturization of blood-based tests on microfluidic platforms, little has been done in terms of blood typing. Thus, blood typing was chosen as a focus for this thesis.

Blood transfusions are done for several reasons. A major use of collected blood is in the treatment of anemic patients, those whose red blood cell (RBC) count is insufficient for carrying the oxygen requirements of the body. Apart from red (erythrocytes) and white (leukocytes) blood cells, blood is also composed of platelets and plasma. The platelets are colourless cells that conglomerate to form clots and prevent blood loss. The plasma is comprised of 92% water with the remaining being mostly proteins along with minerals, vitamins, sugars, hormones, and enzymes [70].

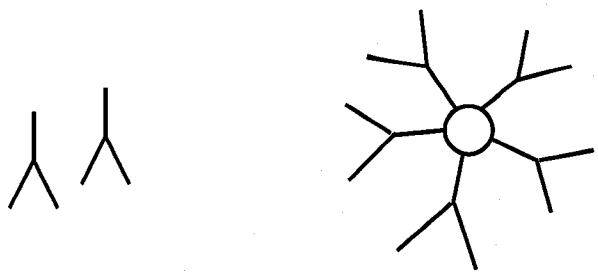
The determination of donor and recipient blood type compatibility is a fundamental aspect of transfusion medicine. This compatibility is based on the existence of different blood groups. Individual blood groups are the result of the presence or absence of various antigens or markers on the surface of red blood cells. The presence of particular antigen(s) renders the individual positive for a specific blood type. For example, two blood groups that are always tested in transfusion medicine are the ABO and Rh blood groups. The presence of either the A or B antigen means the individual is type A or B. The presence of both antigens gives rise to type AB while the lack of both gives type O blood. Similarly, the antigen associated with a positive Rh result is the D antigen. An individual with any particular antigen does not have the corresponding antibody. An antibody is a protein that identifies foreign substances inside the body via specific markers or antigens. If antibodies in the bloodstream are exposed to their respective antigens, they recognize them as foreign. White blood cells are then elicited to attack and destroy the foreign entities as part of the immune response. The strongest

blood type-based antigen-antibody reactions are associated with the A, B, and D antigens. The immune response includes massive red cell destruction, kidney failure and general inflammatory responses that may be fatal. Thus, these two blood groups are the most significant to transfusion medicine. Due to the large scope of this topic, this thesis focuses on potential methods for miniaturizing typing of the ABO group only.

Currently, the Canadian Blood Services (CBS) collects, processes, stores, and distributes blood for the purpose of transfusion within Canada except the province of Quebec. Compatibility testing is done followed by stringent checks and balances during the entire blood processing procedure. However, despite the many checks, blood type incompatibility is still currently the largest source of transfusion-related errors. In a review of transfusion related fatalities from the USA, United Kingdom, and France, approximately 40% to 50% of transfusion related fatalities were the result of clerical errors [71]. The main cause is due to a lack of the ability to confirm the correct blood type at the bedside. The improved portability of μTAS would be able to greatly improve the point-of-care testing.

1.3.1 Current Macroscopic Blood Typing Methods

The standard method for ABO testing is based on agglutination, the clumping of red blood cells when blood group antigens are exposed to their corresponding antibodies. Agglutination is generally strong enough to be observed by the naked eye and serves as a convenient and quick method for establishing ABO type. The types of antibodies involved in these interactions or immune responses to blood group mismatches are primarily IgG and IgM [72]. IgG antibodies are the smaller of the two while IgM antibodies are made up of five IgG antibodies (Fig.1.6). Agglutination occurs more readily with IgM antibodies than IgG due to their larger size.



(a) (b)

Fig. 1.6: IgG (a) and IgM (b) antibodies

Conventional ABO typing may be done in several ways. The simplest and most commonly used for pre-donation testing is the tile method. In this method, a bioplate or a small tray is used to mix drops of donor blood with various anti-sera (solutions containing known antibodies). This method is effective in giving a positive or negative reading, but does not provide a very sensitive method for rating the strength of agglutination. The gold standard test for blood typing is a combination of the forward test and reverse test. In the forward test, a drop of unknown red blood cells (RBC) is mixed in a test tube with a drop of anti-sera (i.e. known antibodies) and briefly centrifuged to facilitate agglutination. The resultant pellet of RBCs is gently re-suspended and observed. The level of agglutination is graded on a scale from zero to four. This forward test reveals what antigens are present on the RBC. In the reverse test, plasma is mixed with known commercial RBCs. Centrifugation and re-suspension are also done the degree of agglutination is again rated. The reverse test is done for confirmation of the blood type and reflects the antibodies present in the plasma. The commercial RBCs used here have known antigens on their surfaces and any plasma that binds with these RBCs will test positive for the corresponding antibodies. Both the forward and reverse tests are done to provide redundancy to ensure that an accurate typing is obtained.

Some other methods of testing include the gel method of red cell affinity column technology, and solid phase adherence assays [8]. For the affinity column, red cells bind with a matrix containing known active antibodies. The presence of various antigens can be determined based on whether or not the cells bound with antibodies. The adherence

assays identifies specific antibodies in patient plasma by reacting the patient sera with red blood cell membranes bound to the surface of small wells. In this case, the cells contain known antigens and binding occurs only if the corresponding antibody is present in the sera. The main advantage of these methods over the tube and tile tests is that the results are more stable with time and do not need to be read immediately. However, the basic principle behind all the various forms of testing is still agglutination. This dependence on agglutination limits these methods in speed and processing time.

The testing of all donors for other blood groups in addition to ABO and Rh, presents a challenge in terms of the large number of samples and time required. Due to the large volume of blood that is processed every month, approximately 1 000 000 units (donations consisting each of roughly 450 mL) per year in Canada [73], and the need for accuracy and consistency, a move towards automation has been the trend in recent years. Various techniques and automated systems are available. Some systems such as the ABS2000 (Immucor, Norcross, GA) are capable of sample preparation including washing, incubation and reagent addition as well as crossmatching where the donor cells are tested against the recipient plasma to ensure there is no agglutination or antibodies present [74]. The standard currently used by the CBS is the Olympus PK7100 and PK7200 systems (Melville NY) that are capable of ABO and Rh typing, and these are capable of handling 240 samples per hour. Although current automated systems have improved throughput for ABO and Rh typing, they still require pre-loading preparations such as sample labelling and centrifugation. They are also expensive and designed for high throughput, limiting availability, portability and practicality in smaller laboratories.

1.3.2 RBC and Antibody Electrophoresis and Immunoelectrophoresis

Various studies have been done on RBCs and their movement and detection in a microfluidic environment. Most electrophoretic methods of cell detection or separation involve the use of immunoreactions to help determine changes in electrophoretic properties of the analytes [27, 75] or to immobilize particular cells [64]. Many macroscopic diagnostic tests are currently conducted using immunoassays, where the antibody-antigen binding process is used for the detection and quantification of specific antibodies or antigens [7]. Thus, attempts to transfer these tests onto microfluidics

platforms have also led to assay based techniques on-chip [76, 77]. Much work has been done with blood serum components as well as their separation and analysis [27, 78, 79]. Many of the reports on blood cells involve their isolation or suspension in a buffer such as the commonly used phosphate buffered solution (PBS) [27, 37, 38].

Cells have also been subjected to both capillary electrophoresis and on-chip electrophoresis. The electrokinetic transport for the manipulation of cells was demonstrated by both Li and Minerick [37, 38]. Li's work showed that yeast cells, canine red blood cells, and Escherichia coli cells were readily transported and lysed on-chip. Minerick's work demonstrated that charges on capillary walls, pH gradients, the tonicity (salt and mineral concentration) and the chemical composition of the cell suspension all play roles in the surface charge of the cells and affect their movement during electrophoresis. Tsuda *et al.* [39] used both laser induced fluorescence (LIF) and direct observation with a microscope to detect single RBCs during CE. Zhu *et al.* also demonstrated successful CE of RBCs of various species with reproducibility [80]. However, this report only focused on the difference between RBC mobilities of different species and did not provide the actual percentage variation between individuals for human RBCs. Morpholino-ethane sulphonic acid (MES) and HEPES buffers were used for the various species tested. The addition of hydroxypropylmethylcellulose (HPMC) was also used to reduce adsorption and aid with reproducibility.

More recently, work has been done with cells for microchip-based electrophoresis. Kitagawa *et al.* demonstrated that single cells could be pulled into and out of micro-reservoirs by the application of an electric field [41]. Parallel single cell CE on-chip was also shown by Munce *et al.* [81]. More complex analyses such as combined cell lysis, PCR amplification and electrophoretic analysis of cells on a microchip were carried out by Waters *et al.* [82]. In addition, the electrophoretic mobility of RBCs has also been studied on several occasions. Omasu *et al.* measured the mobility of sheep RBCs and were able to demonstrate the use of microchips to measure RBC electrophoretic mobilities [40].

In addition to the manipulation of cells alone, the use of CE-based immunoassays (CEIA) has become popular. Immunoassays use the ability of antibodies to bind to antigens to identify certain substances and enables separation and selective detection of

the antigen and antibody-antigen complexes [83]. CEIA combines the benefits of immunoassays with miniaturization, rendering it very attractive. CEIA has been used for the determination of both the presence and the concentration of analytes. Antibodies or antigens are mixed with the analyte and the detection and quantification of their corresponding antigen or antibody is made, usually via fluorescence. To date, the development of CEIA covers a wide range of traditional immunoassays. For example, CEIA was used by Su *et al.* to detect the hormone estrone in serum [84]. Jackman *et al.* also analyzed the performance of antibodies as a means for the capture of specific proteins of interest in an immunoassay. Another method, called immunosubtraction, was also introduced by Paquette *et al.* to detect the presence of specific antibodies [85]. Other works include those done by Mi *et al.* and Tseng *et al.* in the detection of morphine and albumin respectively. The thyroid hormone, thyroxine, was also detected in serum using a competitive immunoassay by Schmalzing *et al.* [86].

Another report of interest was that of the detection of human IgG and IgM antibodies on a microchip by Abad-Villar *et al.* [29]. Detection of the antibody, and antibody antigen complexes was achieved on-chip using an immunoassay. They were able to show that the microchip method gave strong signals and could be accomplished in approximately 1 minute on-chip as compared to under six minutes for conventional CE. This reduction in time was a good demonstration of the speed attainable when using microdevices. It also showed that microchip based CE may be just as effective as conventional CE for the detection of antibodies in certain cases. Histamine has also been shown to be detectable using electrophoresis when bound with IgG antibodies in whole blood [87]. Off-chip mixing and reaction of histamine with IgG was allowed to occur prior to loading onto a microchip for detection.

More specific to RBC electrophoresis, Ichiki *et al.* were able to successfully bind IgG antibodies to target sheep red blood cells in a microchannel during electrophoresis [27]. A mobility difference between the bound and unbound cells was observed. Thus, detection of the target cells was achieved through antibody-cell interactions. The commonly used surfactant Tween 20 was also used to reduce the EOF and/or adsorption of the antibodies to the capillary walls.

The above works demonstrate the potential for complex cell manipulation and analysis on-chip. Although electrophoretic movement of cells has been established in both capillaries and microchannels [37, 38, 88], a standardized method for cell electrophoresis on-chip does not currently exist. Experimental conditions and reagents used vary from one another based on purpose investigation.

1.3.3 Current Miniaturization in Blood Typing

Among the devices available today, some are capable of analysis of whole blood directly. An example is the commercially available device Biosite Triage Cardiac System for the detection of several muscle tissue proteins in whole blood [76]. Other devices for measuring blood sugar levels have also been made readily available. Despite these innovations and ongoing research, the analyses are limited to mostly the detection of small particles and gases. Techniques or devices capable of more complex blood analysis or the combination of multiple analyses are not yet available. Such devices would be very useful in reducing cost and time, and in providing new diagnostic methods. Their development is thus considered to be an important aspect to improving medical diagnostics and provided motivation for this project.

Initial developments of a microdevice for rapid blood typing have been achieved. Recently, reports of devices that are capable of doing partial typing with the use of off-chip sample pre-processing have emerged. Kim *et al.* have developed a device that uses pressure driven flow and micro-filters to detect agglutinated RBCs [89]. In that work, blood cells were first isolated off-chip and prepared for use before being passed through the chip (under pressure) into micromixers that allowed the cells and antibodies to react. The cells were subsequently passed through the filters where agglutinated ones were trapped. Although this device is capable of detecting agglutination, it still requires the pre-processing that exists in current macroscopic test methods. In addition, pumping mechanisms and valves, which are complicated to implement are required.

Another recent report is of a portable blood type detection device of interest is presented by Aebishcher *et al.* This device is capable for reading both the forward and reverse typing results [90]. Sample and test reagents are loaded onto the card for both forward and reverse tests and read by the presence and location of specific bands.

However, this method still requires pre-mixing of the cells and antisera as well as manual loading of the samples. It functions mainly as a portable detection tool. These devices also do not offer quantification of the results, something that is currently available with conventional methods. Regardless of the typing test, whether conventional or the newer miniaturized methods, they still rely on agglutination as the basis for their detection. Although a proven method, improvements to sensitivity of detection may be limited when using agglutination.

Apart from the conventional use of agglutination, Lu *et al.* investigated the mobility of different types of human red blood cells in the ABO group using CE and determined that there was a significant and detectable mobility difference between the four blood types of the ABO group [91]. The RBCs were electrophoresed in phosphate buffered saline (PBS) with a 6% sucrose and pH of 7.4 under 300 V/cm at 23°C. The sucrose was added to both maintain cell osmotic pressure and to prevent cell sedimentation. Different mobilities for the ABO types were detected even when the results from six individuals were analyzed for each blood type were combined. A minimum variation in the average velocity of the RBCs of 2% was observed between the different ABO types. For each of the blood type tested, the variation within one blood type for the six individuals tested was approximately 1.5%. Although these differences were detected in this report, a 2% variation between types is still quite small and is a challenge for on-chip detection. Lu's report strongly suggests that mobility differences between different blood types of the ABO group could also be detected on-chip.

There are very few reports on the determination of mobility differences of RBCs using microchip-based methods. However, a report from Lu *et al.* demonstrates the ability to detect mobility differences of both human erythrocytes compared to other species as well as between the different human ABO blood types. Although limited, these works suggest that the ability to blood type based on mobility differences may be possible.

1.5 Mutation Detection

In addition to tests for assessing the compatibility of blood for transfusions, the CBS also conducts genetic tests to ensure blood quality. Mutation detection in the deoxyribonucleic acid (DNA) or genetic code is associated with many diseases. Various forms of mutations may occur such as the addition of extra strands of genetic information or the deletion of parts of the DNA. However, over 90% of the disease-causing mutations in humans are a result of single nucleotide polymorphisms (SNPs) [92]. DNA is made up of a sequence of nucleotides or building blocks. The four nucleotides form into two pairs where adenine pairs with thymine, and guanine pairs with cytosine. The normal double helix form of DNA is made up of two strands of complementary sequences of nucleotides. SNPs occur when one nucleotide is deleted, inserted, or switched during DNA synthesis.

The detection of mutations in DNA may be achieved in two different ways. The first is through sequencing where every nucleotide in a gene or DNA region of interest is determined and mapped out. This method is the gold standard for the detection of mutations, but is labour intensive, time consuming and costly. This method is also not practical for mutation screening purposes. Thus, various other methods have been developed to enable mutation detection without sequencing. Among these, two methods of interest are heteroduplex analysis and single strand conformation polymorphism. Both methods utilize electrophoresis and the conformation of DNA as the basis for mutation detection. These methods may be used on a microfluidic platform to improve cost and time required for mutation detection. An improved technique for the combinational use of these two methods on-chip is presented in this thesis.

1.6 Scope of Thesis

This thesis includes the use of electrophoretic methods on a microfluidic platform for improving blood-based diagnostics. This includes the use of on-chip electrophoresis for improving mutation detection as well as the development of methods potentially to be used for on-chip blood typing. The initial intent was to first work with mutation detection followed by blood typing. This would enable potential integration of the DNA

and cell work as well as incorporation of blood typing with genotyping or pathogen detection.

Microchip-based electrophoresis was chosen as the platform for the development of these methods due to the fact that it is a proven method for mutation detection in DNA and does not possess many of the complications associated with the other methods of blood component manipulation. The use of electrokinetic manipulation of analytes such as cells and antibodies eliminates the complicated valving and pumping associated with pressure driven flows for on-chip work as well as microchannel clogging caused by filters and dams. There is no need for complex DEP designs, which are extremely specific and may not be practical for use to control several different analytes or one that is high in variability in its characteristics. Thus, this thesis tries to determine whether the one method of microchip capillary electrophoresis may be used to both improve SNP detection as well as develop methods that may be used for blood typing.

The first portion of this thesis is focused on the development of a method for combined on-chip analysis of DNA in both its single and double stranded forms. This was achieved through the successful on-chip denaturation method developed in this work. The other focus is that of microchip-based methods for improving blood typing. The ABO blood group was used during development. An appropriate set of reagents for blood and RBC work were first determined. Both the mobility and immunoreaction of the RBCs were used for the determination of blood type. In addition, a method for determination of the A blood subgroups of A1 and A2 using a microchip was also established.

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Chapter 2

Mutation Using Controlled DNA Self-Assembly

2.1 Introduction

The first aspect studied during this thesis was DNA mutation detection. Mutation detection is an integral part of blood-based diagnostics and may offer insight into genetic diseases. The successful detection of certain sequence specific strands may also be useful in pathogen detection as well as genotyping where specific genes in DNA are identified.

Various methods for mutation detection such as microarray, denaturing high performance liquid chromatography (DHPLC), single strand conformation polymorphism (SSCP), and heteroduplex analysis (HA) all exist and have been used. However, each of their success rates for detection of mutations is approximately 90% [1, 2]. The combination of methods of SSCP and HA has been shown to give mutation detection rates of 100% [3]. Until recently, the integration of HA and SSCP on-chip has been hindered by the inability to control DNA denaturation and hybridization on-chip. However, we have developed a method of denaturation and rehybridization of DNA on-chip that may be used to enhance mutation detection.[1]

The human DNA is comprised of two sets of genetic information stored in the double helix form. Each set of genetic information is referred to as an allele. The wildtype refers to any given genetic sequence that does not contain any mutations. The two copies of genetic information or two alleles may be either identical (homozygous) or

[1] A version of this chapter has been published. Zheng, Y., Footz, T., Manage, D., and Backhouse, C.J., Controlled and Rapid Self-Assembly of DNA on a Microfluidic Chip. Journal of Nanobiotechnology, 2005. 3(2). YZ performed the experimental work with assistance from TF; DM attempted further protocol refinement; CB provided overall direction. All authors contributed to the writing of the paper.

different (heterozygous). The double helix of the DNA may be separated or denatured under conditions such as high temperature or in the presence of certain chemicals. Similarly, low temperatures would facilitate the formation of the double stranded DNA from single strands. The single strands bind with each other to form either completely complementary pairs (homoduplex) or pairs or nearly complementary pairs (heteroduplex). It is this heteroduplex form that often indicates the presence of genetic mutations. DNA strands are made up of a sequence of four units called nucleotides. The four nucleotides are capable of forming into two pairs (base pairs) where each has a complementary nucleotide. Mutations are often in the form of single nucleotide polymorphisms (SNPs) where there exists a change in the basepair match or an insertion or delation of a nucleotide. These SNPs lead to heteroduplex DNA strands where at the location of the SNPs there is a bulge or bubble in the DNA. These bulges alter the shape of the entire DNA molecule, leading to changes in electrophoretic mobility during electrophoresis. The heteroduplex strands typically migrate slower than the homoduplex strands.

In HA, the DNA remains in its double stranded (dsDNA) form and electrophoresis is done to determine mobility shifts between the duplexes. In SSCP, the single stranded DNA (ssDNA) is analyzed. These single strands fold upon themselves forming unique conformations that have mobility differences between different ssDNA or sequences of nucleotides. These conformations also affect the mobility of the DNA during electrophoresis, which enables the presence of the different strands to be detected. Electrophoretic conditions are set for both HA and SSCP so as to enhance the mobility differences between different duplexes for analysis.

The integration of mutation analysis on-chip with other diagnostic methods such as PCR and CE could greatly improve medical diagnostics. Various works have been done on HA [4-6] and SSCP [7] where most require pre-processing to achieve dsDNA and ssDNA for on-chip HA and SSCP analyses. Here, we present a method for on-chip DNA denaturation using formamide that enables both HA and SSCP analysis. The degree of the denaturation is also controlled, ranging from almost entirely dsDNA to almost entirely ssDNA. This method was applied to the mutations H63D, S65C, and C282Y that are most commonly associated with hereditary hemochromatosis.

Hemochromatosis is a condition where excessive iron accumulates in the body, leading to eventual organ failure and death if not treated. Often times, symptoms do not exist until much of the damage is already done. Thus, the ability to economically and rapidly screen for high risk individuals would be very useful in preventing unnecessary deaths.

2.2 Materials and Methods

2.2.1 Samples

DNA was extracted from samples obtained from donated lymphocytes. The DNA was extracted and purified using phenol-chloroform-isoamyl alcohol extractions [8] or the QIAmp DNA Blood kit from QIAGEN (Mississauga, ON) and stored at 4 °C in Tris-EDTA buffer (TE, pH 8.0). The mutations tested were the H63D, S65C, and C282Y mutations found on the HFE gene. The genotype or genes present were confirmed using a commercially available ABI Prism 377 Slab Gel Sequencer from Applied Biosystems (Streetsville, ON). PCR was performed on the mutations to multiply the DNA and provide sufficient quantities for testing. The PCRs were performed with 5 μL of 30 ng/ μL of genomic template DNA, 2 μL of 5 μmol/L of either HEX-HFE-2F primer or H63DR primer, 2 μL of 10 mmol/L dNTPs, 0.75 μL of 50 mmol/L of $MgCl_2$, 2.5 μL of 10x PCR reaction buffer and 0.5 μL of Platinum Taq DNA Polymerase giving final DNA strands of approximately 250 kbases.

2.2.2 Reagents

The reagents used for PCR included buffers, polymerases and primers, and were obtained from Invitrogen (Burlington, ON). GeneScan® polymer from PE Applied Biosystems (Streetsville, ON) was used in the channels of the chip during electrophoresis. The polymer was diluted to a concentration of 5% from the stock solution of 7% and 10% glycerol (Sigma, Saint Louis, MO) was added to the polymer (5GS10G) to help enhance peak separation during electrophoresis. The buffer used was Tris Borate (Fisher Scientific, Fairland, NJ) EDTA (Merck KGaA, Darmstadt, Germany) buffer (TBE). The running buffer was 1xTBE with 10% glycerol added (1xTBE10G)

was made from 10xTBE and glycerol. The buffer used in the sample well was 0.1xTBE1G diluted from the 1xTBE10G. Deionized formamide with a concentration of 99.7% from Sigma (Saint Louis, MO) was aliquotted and frozen until use.

2.2.3 Microchip Electrophoresis

The simple 4-port microchip purchased from Micralyne (Edmonton, AB) was used for the first part of the experiments (Fig. 2.1). The shorter arms attached to the sample and sample waste wells are the injection channels while the long channel from the intersecting point of the channels is referred to as the separation channel. The channels were 20 μm deep and 50 μm wide. Another 8-port Y-chip was used for on-chip mixing and denaturation of the DNA and is shown in Fig. 2.2.

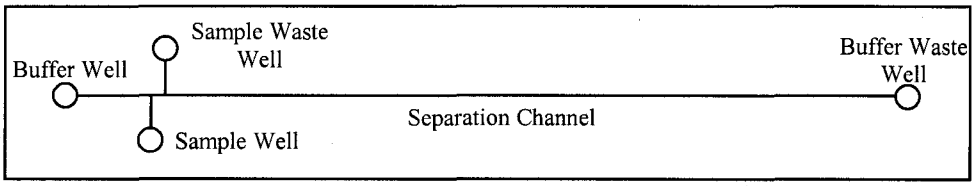


Fig. 2.1: 4-port microchip used for part of the experiments

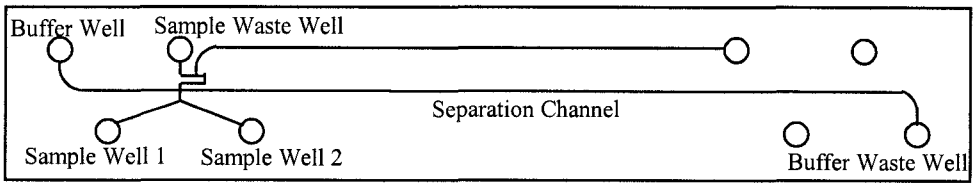


Fig. 2.2: 8-port Y-chip used for on-chip denaturation and mixing of DNA

The experiments were conducted using a Microfluidic Tool Kit (μTK) purchased from Micralyne. The μTK consists of a power source and electrodes for conducting electrophoresis on-chip. Laser induced fluorescence (LIF) was the detection method used during testing with an excitation wavelength of 532 nm and detection at 578 nm. The sampling rate of the signal was 200 Hz while the μTK was controlled via a PC using a compiled LabVIEW interface also supplied by Micralyne. During anlaysis, the

microchip was placed on a stage and platinum electrodes were lowered into the wells. The laser was then used for excitation while a photomultiplier tube detected the signal for analysis. Further details may be found in previous work [6].

The microchip channels were loaded with 5GS10G. The sample well on the 4-port chip was loaded with 0.4 μL of DNA sample with 2.6 μL of 0.1xTBE1G. The remainder of the wells were filled with 1xTBE10G running buffer. For the 8-port chip, the first sample well was filled with 0.4 μL of wildtype DNA with 2.6 μL of 0.1xTBE1G while the second sample well was filled with 0.4 μL of homozygous mutant and 2.6 μL of 0.1xTBE1G. If denaturation was intended, then 1.5 μL of the sample was removed from each of the sample wells and replaced with 1.5 μL of formamide.

Electrophoresis was performed according to Table 2.1. Here, the samples were first injected from the sample well towards the sample waste well by the application of a positive voltage at the sample waste well. A sufficient amount of time was allowed for the establishment of a sample stream down the injection arms. The appropriate amount of time was determined based on the velocity of the DNA sample calculated from the time it took the DNA to travel down the separation channel from the intersection. The voltages were then switched from the sample and sample waste well to between the buffer and buffer waste well. This way a "sample plug" was injected into the separation channel because the sample inside the intersection is the only DNA injected into the separation channel. This is the standard sample injection-separation that is used throughout this thesis. A similar protocol is use for the Y-chip (Table 2.2).

Table 2.1: Electrophoresis Protocol for DNA Mutation Detection and Denaturation

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	60	Ground	Float	400	Float
2	180	Float	Ground	Float	6000

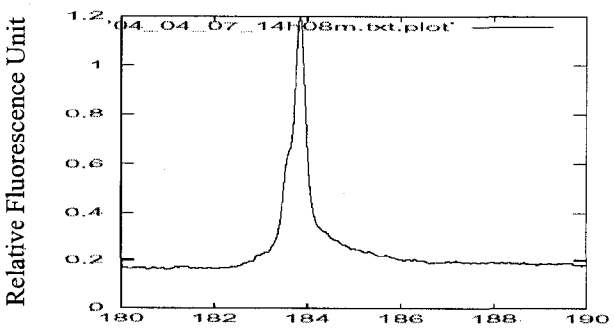
Table 2.2: Electrophoresis Protocol for DNA Detection and Denaturation on Y-Chip

Step	Duration (s)	Sample Well 1 (V)	Sample Well 2 (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	60	Ground	Ground	Float	400	Float
2	180	Float	Float	Ground	Float	6000

2.3 Denaturation Results

2.3.2 Heteroduplex Analysis

Detection of the H63D mutation was first performed on the 4-port chip without any denaturation. This was to establish that the three wildtype and mutated DNA could all be detected. It was found that all three cases of wildtype, homozygous mutant, and heterozygous mutant showed different peak profiles in the electropherograms using our method (Fig. 2.3). This was the case for three consecutive runs conducted for each load and reproducible for all loads tested. There was a shift in the time of arrival for the homozygous mutant where it arrived approximately 3 s faster than the wildtype DNA. This shift was consistently seen and enables the detection of the homozygous mutant from the wildtype. A smaller peak that appeared to follow for the homozygous mutant was also present. There was a more defined second peak associated with the heterozygous sample due to the presence of both homoduplex and heterodupolex strands.



(a)

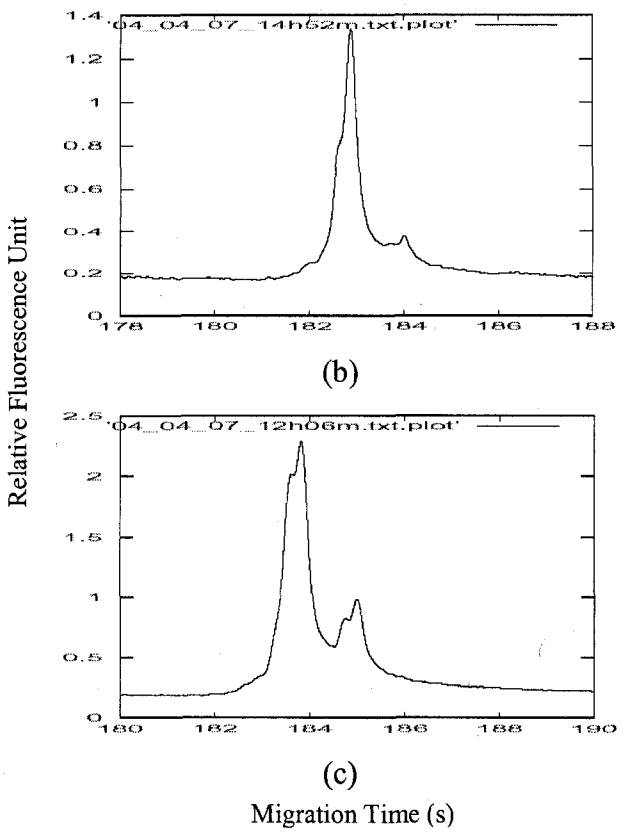
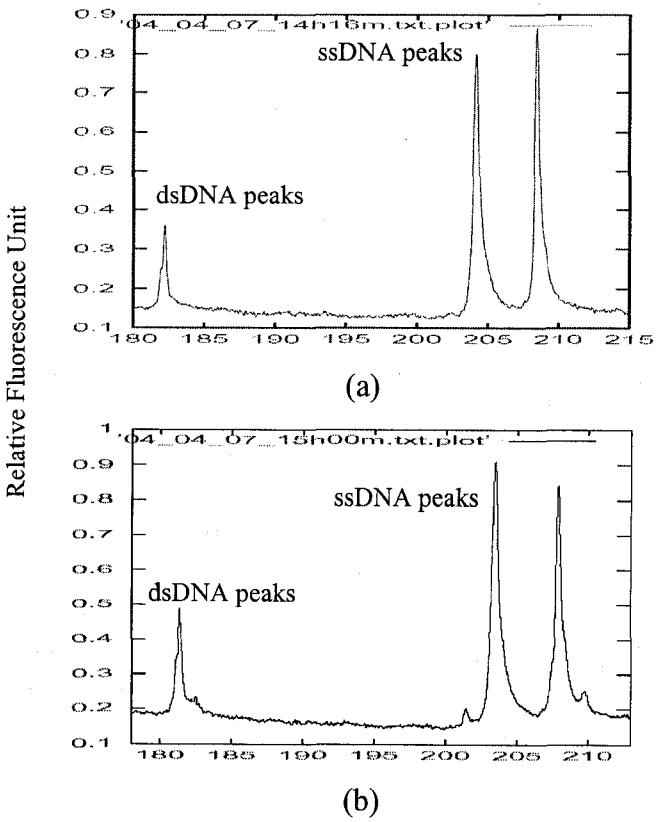


Fig. 2.3: Detection of wildtype (a), homozygous mutant (b), and heterozygous mutant (c) for H63D mutation

2.3.3 Denaturation and Combined HA and SSCP Analysis

Once the non-denuatured sample testing was complete, denaturation using formamide was tested. It may be seen from the results that there are two sets of peaks for each sample tested (Fig. 2.4). The first set of peaks is the double stranded DNA, a result of the incomplete denaturation. Similar peak profiles of the first set of peaks in Fig. 2.4 are identical to those seen in Fig. 2.3 when no denaturation occurred. The second set of peaks is the ssDNA that have been denatured. The peak arrival times of the homozygous mutant ssDNA peaks are once again slightly faster than those of the wildtype ssDNA strands. More notably is the clefting of the second ssDNA peak associated with the heterozygous sample. Thus, the three different samples may once again be deciphered from one another using only the ssDNA profile, effectively constituting SSCP analysis. Further testing of the on-chip combined HA and SSCP analysis was also done by testing

two other mutations associated with hereditary hemochromatosis, C282Y and S65C.[2] The same denaturation protocol was applied and similar results were obtained where both the dsDNA and the ssDNA were observed. The presence of both the dsDNA and the ssDNA as well as the distinctiveness of the profiles of the three samples also demonstrate that a combined HA and SSCP analysis may be achieved on-chip using the formamide denaturation method used here.



[2] A version of this data has been published. Manage 2005. Microfluidics and Nanofluidics. 1(4): 364-372.

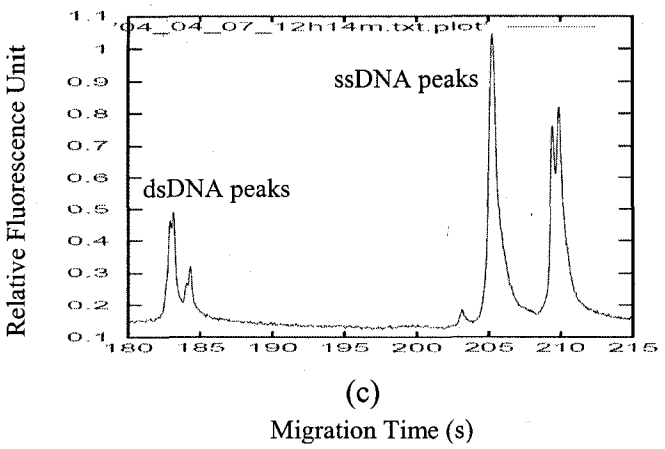


Fig. 2.4: Electropherograms of DNA denatured into dsDNA and ssDNA for the wildtype (a), homozygous mutant (b), and heterozygous mutant (c) for H63D mutation

2.4 DNA Reassembly

It was hypothesized that not only does this method of on-chip denaturation enable the disassembly of dsDNA into ssDNA, but it also allowed the DNA to recombine into dsDNA following denaturation. The recombination would occur once the DNA moved out of the sample well during electrophoresis while the neutral formamide would remain inside the well. In the absence of the formamide's denaturing effects, the DNA could potentially recombine to form their original double strands. To test this theory, 0.2μL each of the H63D wildtype and homozygous mutant DNA were loaded into the sample well and then inject down the separation channel. One run was done with no formamide added.

If no recombination of the two samples occurred and only partial denaturation of the dsDNA was present, then the peaks detected following the addition of formamide would only show profiles associated with the wildtype and homozygous mutant. However, the profile detected was the same as that seen in Fig. 2.3 of the double-stranded heterozygous mutant (Fig. 2.5). This result indicated the presence of a mixture of homoduplexes and heteroduplexes that could only be a result of the single strands of wildtype combining with the single strands of the homozygous mutant. The re-annealing of the single strands likely occurred in the channel where no formamide existed once the sample was extracted from the well. Thus, this formation of heteroduplexes clearly

demonstrated that the presence of complete denaturation and reassembly of the DNA was occurring on-chip.

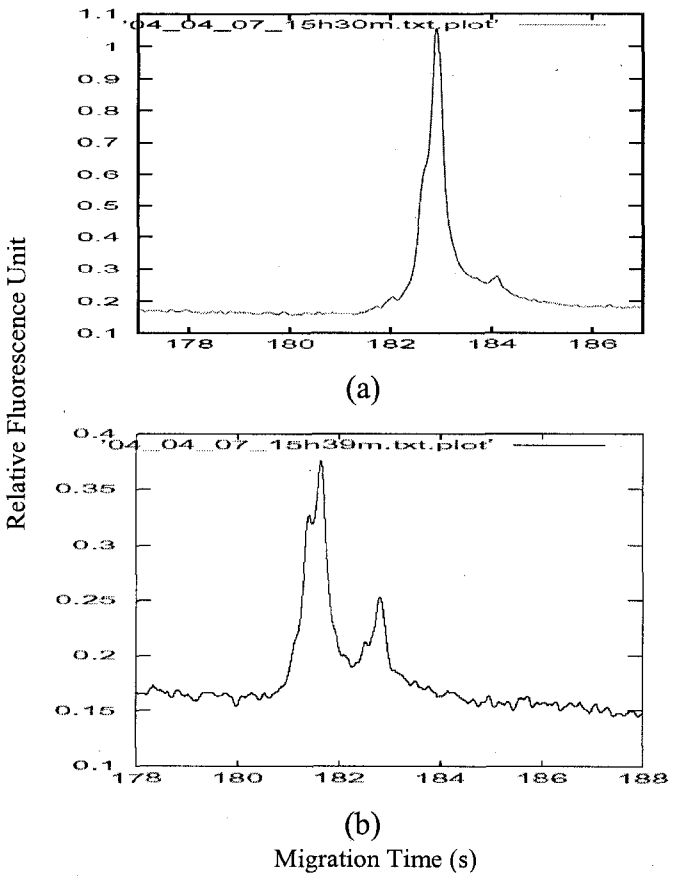


Fig. 2.5: Peak profile of the mixture of wildtype and homozygous H63D mutant prior to (a) and following denaturation with formamide (b)

To further demonstrate the re-annealing process, the Y-chip was used to separately denature the wildtype and homozygous mutant on-chip for reassembly. This would not only demonstrate that the reannealed strands gave the heteroduplex dsDNA, but would also demonstrate that the reannealing did occur inside the channels. The wildtype and homozygous H63D samples were each loaded into separate sample wells on-chip. Once formamide was added for denaturation, the samples were extracted from the wells and electrophoresed down the separation channel according to Table 2.2. The results are shown in Fig. 2.6. The profile resembled that of the heterozygous sample following treatment with formamide, demonstrating the mixing of the two types of

homozygous mutant and wildtype. This suggests that the method developed is a powerful tool for comparing samples in both the same or separate sample wells. The three samples of wildtype, homozygous and heterozygous mutants could all be injected to test for different combination with and without denaturation without reloading the chip. This would greatly improve the throughput.

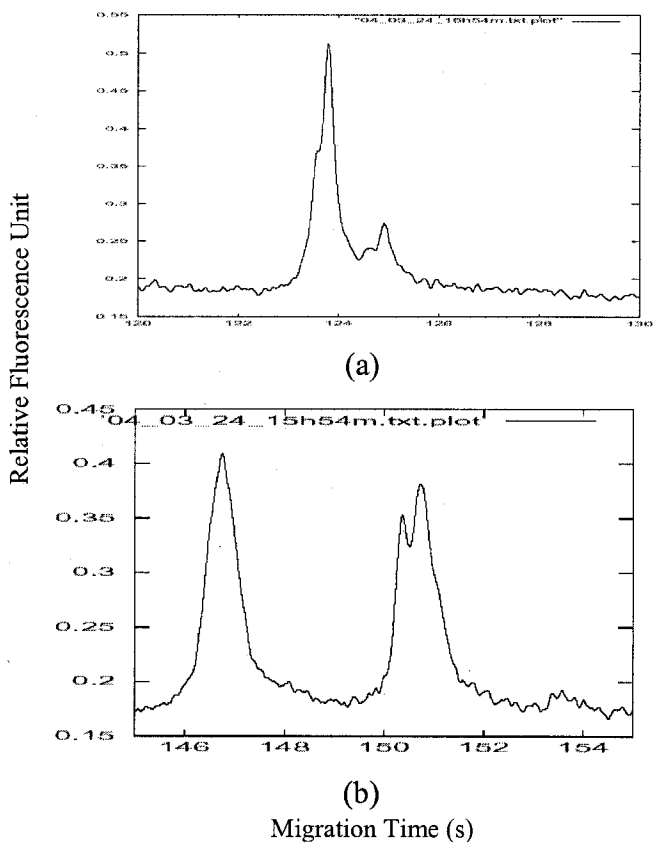


Fig. 2.6: Profile of dsDNA (a) and ssDNA (b) of wildtype and homozygous H63D mutant denatured in two separate wells and then recombined on-chip

It was obvious from the denaturation and re-annealing experiments that the quantity of re-annealing varied with time. It was of interest to investigate how the timing and potentially the sequence of sample extraction would affect the degree of rehybridization. To determine the effect of time on the degree of rehybridization, additional sample from the injection channel between the sample well and the intersection were injected into the separation channel for detection. This was achieved by doing three consecutive runs with 10 s injections of sample into the intersection (Table

2.3). The electrophoresis protocol was repeated three times for the three runs. A lower voltage of 100 V was applied to ensure that no fresh samples from the sample well would be injected into the intersection during the two subsequent runs following the initial run. The time for the sample to reach the intersection from the sample well at this voltage was calculated to be approximately 53 s. The time required for DNA to arrive at a specified detection point, under the same electric fields used for injection were first determined. The DNA velocity was then calculated and used to determine the time it took fresh DNA from the sample well to arrive at the intersection since the injection arm channel length was known. Therefore, during the injection of the three consecutive samples, there was insufficient time for fresh samples from the sample well to arrive at the intersection. Thus, the 10 s injections ensured that samples tested were those that had remained in the channel.

Table 2.3: Electrophoresis Protocol for Rehybridization Testing

Step	Duration (s)	Sample Well 1 (V)	Sample Well 2 (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	10	Ground	Ground	Float	100	Float
2	180	Float	Float	Ground	Float	6000

A sample of heterozygous H63D mutant was thus tested. The results show that throughout the series of three runs show the successive decrease in the ssDNA peaks matching an increase in the dsDNA peaks (Fig. 2.7). The quantity of the dsDNA and ssDNA may be varied depending on the timing of electrophoresis. Future optimization of the microchip geometry to vary the lengths of channels could also be used to control the desired quantity of dsDNA and ssDNA.

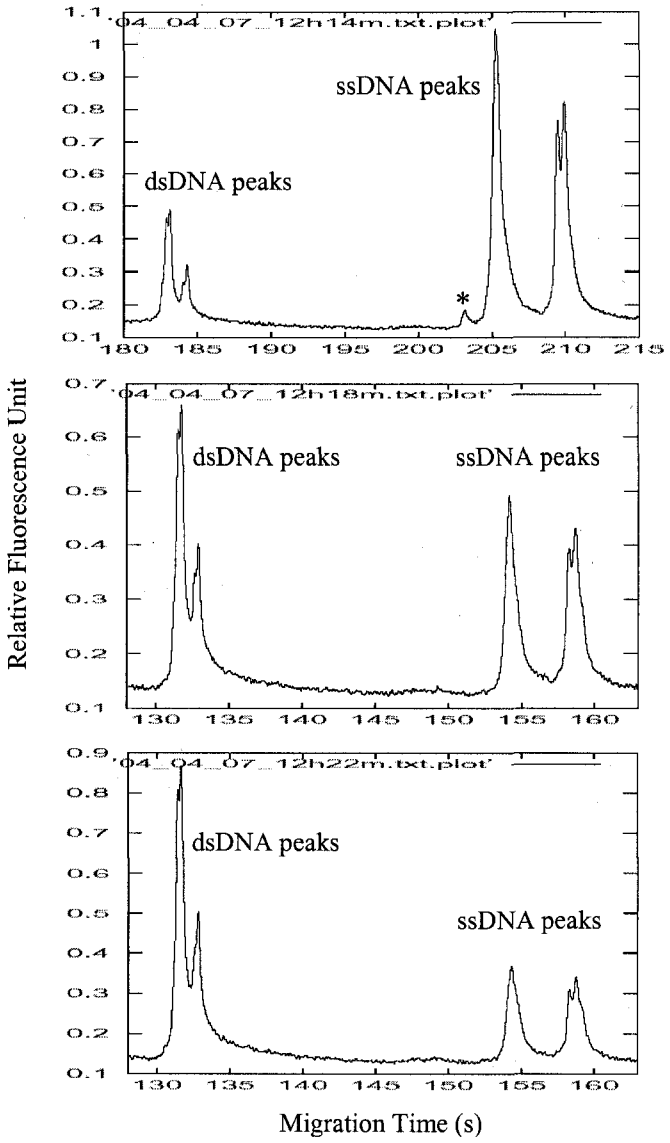


Fig. 2.7: Series of consecutive sample detections of heterozygous H63D mutant left in channel showing a variation in the quantity of dsDNA and ssDNA

Another interesting observation was that of a transient peak associated with the wildtype, homozygous and heterozygous mutants of the H63D and S65C mutations (marked "*" in Fig. 2.7a, 2.8). This peak only appeared following the injection of fresh samples newly denatured from the sample well into the separation channel (Fig. 2.7a, Fig. 2.8). Samples remaining in the channel did not show this peak. This peak was also clefted on the S65C mutation, providing a potential method for determining the between

the two mutations. In addition, this intermediate state may also be used for investigating the dymanics of the rehybridization process.

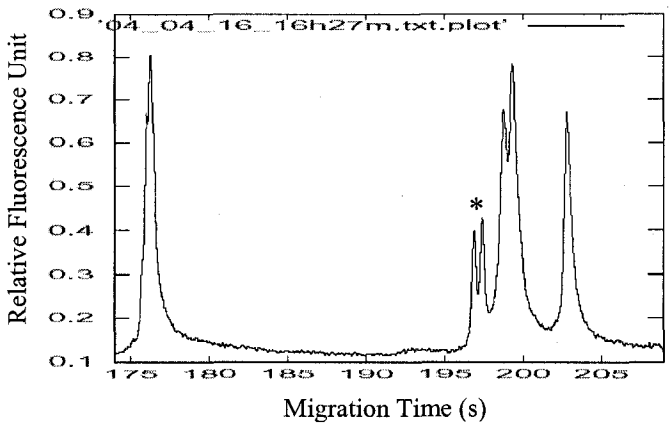


Fig. 2.8: Peak profile dsDNA and ssDNA of S65C heterozygous mutant following denaturation

A potential cause of the transient peak may be the pairing of the PCR primer with ssDNA strands. Artefacts associated with primer-ssDNA complexes have been reported by various groups [3, 9-11]. These reports demonstrated that during SSCP extra peaks could be detected with samples with no further treatment following PCR compared to those that had the primers removed. Reductions in primer concentration also acted to minimize these peaks. Kozlowski and Krzyzosiak suggested that the appearance of these peaks was is due to the primer-ssDNA strands having different mobilities compared to the other ssDNA strands due to changes in mass. Overall, these peaks may be either removed to obtain simpler profiles of the results or used to enhance SSCP sensitivity of detection.

2.5 Conclusions

A rapid method of controlled DNA denaturation and re-assembling on a microchip was developed. This method enables both on-chip heteroduplex analysis and single strand conformation polymorphism analysis offering also control over the relative quantity of ssDNA and dsDNA.

The effectiveness of this method was tested by performing HA and SSCP analysis on several mutations of the HFE gene associated with hereditary hemochromatosis. Both HA and SSCP methods tested using this method were able to distinguish between the wildtype, homozygous mutant, and heterozygous mutant of the mutations tested. The quantity of ssDNA and dsDNA could be controlled by varying the amount of time the DNA spent in the channel without the formamide denaturant. Thus, variations in the chip channel lengths may also be employed in the future to better control the desired quantity of ssDNA and dsDNA. This combined with the rapidity of on-chip denaturation and re-annealing also enables investigation into the mechanisms of DNA reassembly. Short-lived conformations of DNA were detectable with this method, which could provide additional information allowing for a better understanding in reassembly.

Due to the ability of the method to enable both on-chip denaturation and re-annealing of DNA strands, the use of HA and SSCP analysis may be achieved simultaneously on-chip with no need for reloading the chip. This provides improved testing capabilities as well as great advantage in integration. Apart from the HFE mutation samples, other mutation such as BRCA1 and BRCA2 associated with breast cancer have also been shown to be detectable by either HA or SSCP [6, 12, 13]. Thus, this method could be extended to a variety of different mutations and may be a viable method for use with integrated microchips for medical diagnostics.

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Chapter 3

Reagents for Erythrocyte and Antibody Manipulation

3.1 Introduction

The appropriate choice for reagent is critical for the successful manipulation of analytes and analysis in microfluidic chip analysis. Reagents required include a suitable buffer for the maintenance of current and voltages [1] and typically a sieving matrix for separation of analytes depending on the desired task. The choice of buffer affects EOF, adsorption, and analyte charge and behaviour during electrophoresis [2-4]. In addition, wall passivating agents may also be included for controlling EOF on chip as well as reducing interactions of the analytes with the channel walls [2, 5-8]. Wall-analyte interactions or the adsorption of proteins to channel walls is a major problem facing microchip electrophoresis [2, 4, 9] that must be addressed in this thesis due to the likely use of antibodies.

Due to time limitations, a comprehensive analysis and study of all potential buffers could not be achieved. Hence, brief testing of a limited number of buffers was done to evaluate their suitability for CE work with red blood cells and antibodies. Suitability was defined as buffers that did not cause large alterations cell surface structures and functionality of antibodies. The best buffers in this regard would be those used commonly at CBS. However, the buffer also had to be CE compatible. This meant the ability to maintain stable currents during electrophoresis for a specified amount of time necessary to gather sufficient data. Thus, the main goal of this chapter was to first determine whether or not one of the commonly used buffers at CBS was CE compatible and could be used for our purposes. However, other buffers were considered in the even

that the CBS buffers did not yield a suitable candidate. The suitability of the buffers was first assessed by their ability to reproduce similar results to those obtained in our previous work with DNA (Chapter 2) using antibodies. This comparison was based on the stability of currents during electrophoresis, peak detection and signs of adsorption and flow. The following is a discussion of the various buffers and reagents tested for the use in blood-based analysis with particular attention paid to antibodies and red blood cells.

3.2 Buffers

There is a variety of buffers available for use in electrophoresis and capillary electrophoresis. The choice of buffer is often dependent on the analyte and purpose of analysis. These two factors affect the choice of type of chip where glass chips are used more often than polymer chips in the case of proteins [3, 6, 10, 11]. Compatibility of buffer with analytes is another area of concern and varies depending on the analyte. Chip conditioning methods (Chapter 1) are also required to reduce adsorption effects when electrophoresing proteins

Work relating to the electrophoresis of red blood cells and antibodies has been somewhat limited and currently a standard set of reagents for CE of antibodies and red blood cells does not exist. However, various works related to antibody and protein electrophoresis have been done. Malonate buffer was used for on-chip detection of histamine in whole blood [12] and 2-(N-morpholino)ethanesulfonic acid (MES) was used to detect lithium in whole blood [13]. Phosphate buffer was also used by Lim *et al.* to prepare histamine samples. Colyer *et al.* used borate buffer in their work with human serum proteins [10] while Chiem *et al.* chose to use tricine for work with antibodies and theophylline [14]. For both Schmalzing *et al.* and Koutny *et al.*, N-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid/acrylamide, 2-amino-2-methyl-1,3-propanediol (TAPS/AMPD) was the buffer of choice for their work in the immunoassay of thyroxine and serum cortisol [15, 16]. More closely related to our purposes, Ichiki *et al.* used gelatin veronal buffer and phosphate buffer for immunoelectrophoresis of sheep red blood cells and antibodies [17]. They were able to move and bind the cells with the

antibodies on a glass chip and detect the reaction. Abad-Villar *et al.* used TAPS/AMPD buffer for the on-chip detection of IgG and IgM antibodies.

From the literature surveyed, glass chips were mostly used for protein electrophoresis. Vrouwe *et al.* and Lim *et al.* also used a poly(methyl methacrylate) (PMMA) chip [12, 13]. Passivation in glass chips often involved rinsing with NaOH and HCl prior to electrophoresis or the use of a dynamic coating during electrophoresis. Tween 20 or acrylamide additives were found to be the chemicals of choice for these ppurposes [3, 5, 14, 18]. For the PMMA chip a different treatment of 5% Nafion was used followed by a 4 hours rinse with the running buffer.

The electric fields applied for on-chip electrophoresis ranged between 100 V/cm and 1200 V/cm with the majority of the fields at around 500 V/cm. The use of blood cells also corresponded to the use of lower fields in the above-mentioned range. The chip dimensions were found to also be similar to those used in our previous experiments, being approximately 20 μm in depth and 50 μm in width. The method of detection was also mostly laser induced fluorescence with the exception of the PMMA chips, where detection was achieved using other optical methods. Thus, the use of glass chips for our purpose appeared to be the best option in terms of existence of background literature as well as compatibility with the analytes.

From the literature it may be seen that a variety of buffers is used for CE of biological analytes. The buffer used was often dependent on the purpose and specific conditions required in each particular case. There does not currently appear to be a standard buffer for use of on-chip electrophoresis of antibodies and RBCs. Similarly, a standard buffer for the on-chip reaction of RBCs with antisera for blood typing also does not appear to exist. However, potential buffers that may be suitable for RBCs with antibody electrophoresis that are also compatible with blood typing may be potentially derived from existing literature. Thus, testing of the suitable buffer and other reagents for on-chip electrophoresis of RBCs, antibodies, and/or proteins had to be done to determine the best choice for the purposes here.

Several buffers were tested for suitability. They were chosen based on the literature that most closely matched our purposes as well as on how well the reagents performed in on-chip CE. In addition to these, buffers commonly used at the CBS for

blood typing were also tested as initially mentioned. If so, they would be ideal for use with our analytes. The main goal for the buffer testing was to find a buffer that remained compatible with the biological analytes, and was suitable for on-chp CE, rather than to perform an exhaustive comparison or to determine relative effectiveness of each buffer. The performance of the buffers was evaluated based on several factors pertaining to suitability as mentioned above. The first is the buffer's effectiveness in maintaining stable currents and voltages for a sufficient period of time during CE to allow for analysis. The time may vary depending on the actual method of analysis. However, adequate buffering capacity would likely have to be sustained for over 100 s based on past experimental experience (Chapter 2). The second factor is the compatibility of the buffer with red blood cells and antibodies in that it does not significantly alter the cell or antibody surface structure and function.

The main point of testing the buffers used commonly at CBS was to determine if these common buffers could be transferred to the CE realm since their compatibility with RBC and antibodies has already been demonstrated. This involved testing the CBS buffers under the typical CE conditions that have been established in the previous chapter when working with electrophoresis of DNA with some minor adjustments in voltages used. If the CBS buffers proved to yield similar results as those seen with the proven tris borate buffer for DNA testing, then they may be candidates for the purposes here. The CE results obtained using the tris borate was thus set as a standard for comparison. Similar testing of a few other potential buffers used in literature was also achieved in the same manner.

3.2.1 Veronal Buffer

The first buffer tested was gelatin veronal buffer (GVB). This buffer was chosen due to its use in Ichiki's work [17] where sheep RBCs were electrophoresed with target antibodies in a microchip. Reaction of the cells and antibodies was shown to readily occur on-chip in this report when the chip was filled with GVB in all the channels and wells. It was believed by Ichiki *et al.* that the gelatin in the buffer had a passivating effect on the channels, resulting in the reduction of EOF to the point where the mobility difference between the cells bound and not bound with antibodies was detectable. In

Ichiki's work, the cells and antibodies were loaded into separate wells and moved down the channel by the application of a positive voltage at the well containing the antibodies and setting ground to the well containing the cells. They found that the antibodies were positively charged in the GVB while the cells were negatively charged based on their direction of movement. This meant that the two analytes met in the separation channel during electrophoresis as they migrated in opposite directions. The meeting of the two species in the channels was sufficient in allowing the antibodies to bind to the cells. The cells bound with antibodies were found to be slower in velocity than those not bound to antibodies. Cells bound with antibodies travelled between 50 μm/s to 150 μm/s slower than unbound cells depending on the concentration of antibodies, which ranged from 20 μg/mL to 100 μg/mL. This type of immunoelectrophoresis demonstrated that not only could both antibodies and RBCs be electrophoretically moved in a microchannel, but that they could also retain their reactivity and bind on-chip. Such a technique would be extremely useful in the forward and reverse testing that would be required for on-chip blood typing.

Thus, GVB was purchased from Sigma (St. Louis, MO) and tested. A Cy3 fluorescently labelled anti-human IgG from Sigma (St. Louis, MO) was tested using the microfluidic toolkit (μTK) for movement and detection on-chip. Various concentrations of the antibody, 0.1 μg/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL, 30 μg/mL, and 100 μg/mL, were tested to determine the optimal concentration evaluated on the signal strength and shape, and reproducibility. Sample movement was achieved by the application of voltages at different wells in the chip as described below. The concentrations tested were chosen based on existing literature. For example, Abad-Villar *et al.* [18] used 10 μg/mL for their on-chip detection of human IgG and IgM antibodies. Lower concentrations than what Ichiki used were tested here due the more sensitive detection LIF method.

The antibodies were mixed in the GVB directly and loaded. The chip used was the same 4-port chip used in Chapter 2 with the DNA testing (Fig. 2.1). The channels of the chip were loaded with GVB and the wells were filled according to Table 3.1. Two electrophoresis protocols were used for the detection of the antibodies (Table 3.2a, 3.2b). The first electrophoresis protocol was to determine whether the arrival of the antibodies could be detected. A positive voltage was applied at sample waste well while the

detection point was placed in the sample well injection arm of the chip, approximately 125 μm from the intersection. The second protocol was used to determine whether a sample plug of the antibodies could be detected as it passed down the separation arm of the chip. Here, the analytes were first injected from the sample to the sample waste well by the application of a positive voltage at the sample waste well and setting the sample well to ground. The duration of time here allowed for a steady stream of analytes to be established in the short channels near the intersection. Once this was accomplished, a voltage was applied at the buffer waste well while the buffer well was set to ground. This effectively pulled a plug of sample at the intersection into the separation channel for analysis. Detection was made 10 mm from the intersection down the separation arm.

Table 3.1: Reagents in chip wells for antibody detection

Reagent	Sample Well (μl)	Buffer Well (μl)	Sample Waste Well (μl)	Buffer Waste Well (μl)
GVB	-	3	3	3
Ab diluted in GVB	3	-	-	-

Table 3.2a: Electrophoresis Protocol for Sample Arrival Testing

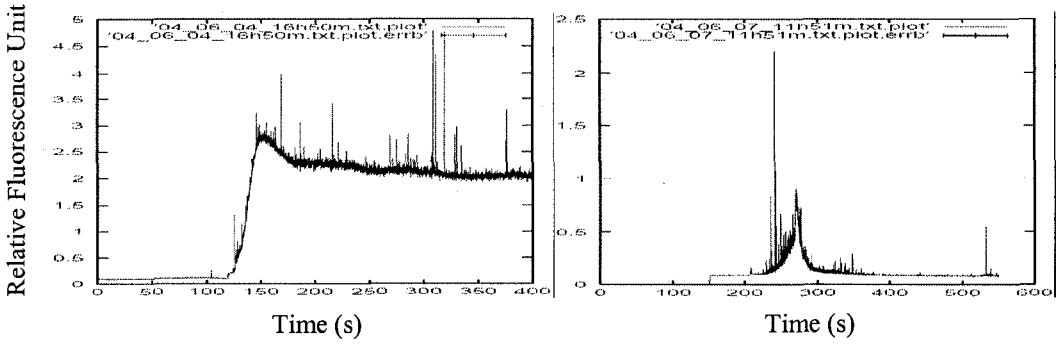
Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Ground	Float	100	Float

Table 3.2b: Injection-Separation Protocol for Sample Plug Detection

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	150	Ground	Float	100	Float
2	400	Float	Ground	Float	6000

It was found that the antibodies could be detected using both protocols. The antibodies could be detected arriving in the injection arm of the chip (Fig. 3.1a) as they migrate toward the sample waste well. This suggested that at least not all of the antibodies were adhering to the channel walls, and that the antibodies are being successfully moved by the application of a positive voltage. Both the actual charge and the adsorption of the antibodies to the channel walls had been the primary concerns in terms of deterring antibody movement prior to testing. The concentration of antibodies arriving at the detection point also appeared to be constant since there was no significant variation in the signal intensity. The signal profile is not that of a perfect step function where there is a sharp upward spike followed by a flat straight line as one may expect. The initial higher peak seen before the signal flattened out was attributed to the transient aspect before a steady state was reached. Another potential cause may be the phenomenon of sample stacking where the analytes at the front of the stream of analytes during electrophoresis accumulate higher in concentration and are detected as an initial spike in signal intensity [19]. Sample stacking is primarily due to the differences in the concentration of the analyte compared to that of the electrolyte in the buffer. The electrolyte concentration difference leads to changes in the analyte velocity as it travels from one region to another such as from the sample well into the channel. Another observation made during testing was that there were spikes in signal during the runs. These were attributed to potential aggregates of antibodies that occurred due to the high concentration used, subsequently causing increases in signal intensity.

The detection of a sample plug injected into the separation well was also achieved with the antibodies because a large single peak was detected (Fig. 3.1b).



(a) (b)

Fig. 3.1: Antibody arrival with detection at the sample well injection arm (~ 50 μm from intersection) with the application of a positive voltage as described in Table 3.2a (a) and antibody peak observed when using injection-separation as described in Table 3.2b (b). The same gain of 0.8 was used on the μTK.

The application of the opposite voltage via a switch of the voltages applied at the buffer and buffer waste wells yielded no signal detection (Fig. 3.2). This was also reproduced over two consecutive loads each with three consecutive runs. Thus, under the specific conditions and in the GVB, the antibodies appeared to be negatively charged since they only appeared to respond to a positive voltage application.

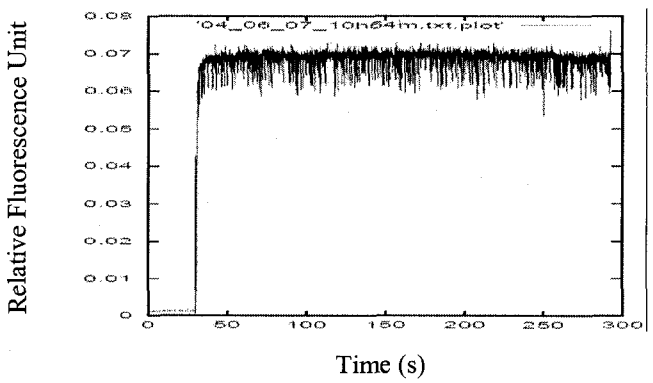


Fig. 3.2: The application of a negative voltage at the buffer waste well yielded no antibody detection as shown on this zoomed in plot of signal baseline. Detection was at 10 mm from the intersection down the separation channel as in Fig. 3.1b.

Overall, it was found that the 10 μg/mL concentration of antibodies gave the most consistent results in terms of reproducibility and detection of antibody peaks (Fig. 3.3a). Ten loads were tested separately on various days for the injection testing according to Table 3.2a. Out of the ten loads, the first run of seven loads showed results similar to that

seen in Fig. 3.1a. An additional five loads were also done using the injection-separation protocol shown in Table 3.2b. Three of these five loads showed results similar to Fig. 3.3b on the first run. However, none of the loads had reproducible results for the second runs. The remainder of the loads for both cases yielded low signal or irregular peak profiles that were not reproducible. Lower concentrations such as 5 μg/mL and 1 μg/mL were also tested, but showed either no or inadequate signal strength while concentrations higher than 10 μg/mL gave overly intense signals and poor peak definition (3.3b, c). The adsorption of analyte to the channel walls likely contributed to both the peak broadening for higher concentrations and the lack of signal for lower concentrations [18]. The low concentration likely resulted in most of the antibodies adsorbing to the channel walls with very little to none arriving at the detection point. Higher concentrations similar to Ichiki *et al.*'s 20 μg/mL yielded extremely high and irregular peaks (Fig.3.3c) and were likely a result of saturation of the channel walls. Thus, both cases remained susceptible to adsorption, resulting in fluctuations in the baseline and inconsistent signal detection. In general, the consistency of the results obtained using GVB did not match that of the standard tris borate buffer used in the previous DNA testing, and GVB was deemed inadequate for our purposes.

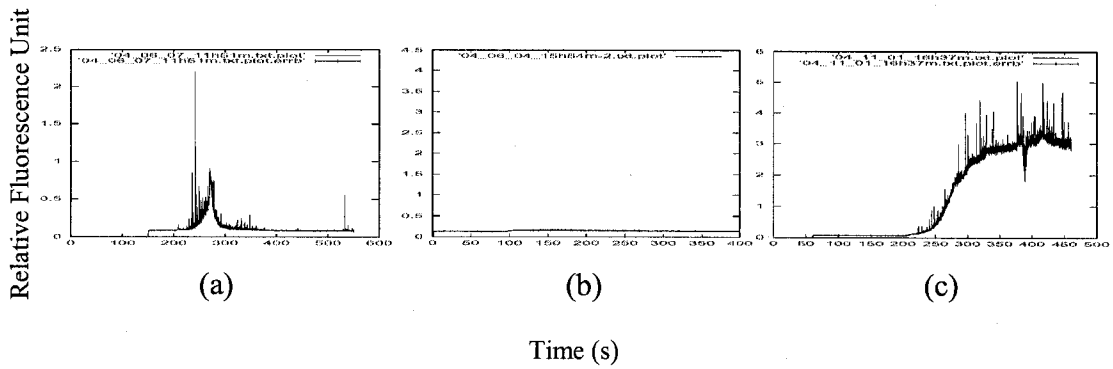


Fig. 3.3: Detection of antibody peak using 10 μg/mL (a), 5 μg/mL (b), and 30 μg/mL (c) of antibodies where only a baseline with no discernable signals was detected for concentrations lower than 10 μg/mL

EOF appeared to be a moderate problem with the GVB in that the differences in arrival times of the peaks on different runs, which were used to gauge the variations caused by EOF between runs, were consistently between approximately 240 s and 260 s. This equalled an approximate 8% variation. This suggested that some EOF was observed

with consistent shifts in fluid that did alter the arrival times of the antibodies. When compared to the tris borate standard used during DNA testing, the EOF observed with the GVB was greater. It should be noted that for the sample arrival testing the signal did not arrive at a maximum intensity and remain at that height throughout the remainder of the electrophoresis run, similar to a square wave, as one may expect (Fig. 3.2a).

Although the GVB showed some promise as a potential microchip CE buffer, the results obtained were not consistent enough and close enough to the tris borate standard to demonstrate that it was viable as a CE buffer for cells and antibodies. EOF, broad peaks and abnormal signal peak shapes following the first run of each load were observed. Thus, it was determined that GVB alone could not be used for CE of cells and antibodies for our purposes.

An attempt to reduce the wall interactions was then made by the addition of Tween 20 to the GVB. Tween 20 is one of the most commonly used surfactants for wall passivation and have been used with various buffers such as PBS, Tris-acetate, Tris-boric acid, and TAPS/AMPD [3, 14, 18]. The wall passivation may reduce both EOF and analyte adsorption effects and could potentially improve the performance of the GVB to a point where it may be used for our purposes. However, the addition of the Tween 20 did not appear to lead to any reductions in adsorption effects such as improved signal intensity or reproducibility. This was true for the addition of 0.01% Tween, a concentration commonly used in literature for reducing adsorption effects of biological analytes when using glass chips. Increasing the concentration of Tween 20 to 0.05%, 1%, and 3% also showed similar results to when no Tween was added. Thus, the GVB was decided to be unsuitable for use as a microchip CE buffer for RBC and antibodies.

3.2.2 Low Ionic Strength Saline

Low ionic strength saline (LISS) is a commonly used buffer in blood banking for washing and storing red blood cells, and was tested next. LISS was one of the CBS buffers tested to see if it could be substituted as an on-chip electrophoresis buffer for RBCs. If LISS were a suitable electrophoresis buffer then its use would reduce a lot of potential problems such as cell lysis and alteration of functionality of antibodies that may occur with other buffers. The ratio of LISS to blood was 1:1 ratio based on standard CBS

usage. Both EDTA and gelatin are also commonly added to the LISS for CBS use. Thus, the addition of 0.12% (w/v) of EDTA (Sigma, St. Louis) as an anticoagulant was made following standard CBS protocol to give a LISS buffer with EDTA (EL). Gelatin was also added to the LISS (EGL) and prepared as described in the text book "Dacie and Lewis: Practical Hematology" [20] and summarized in Table 3.3.

Table 3.3a: Low Ionic Strength Saline

Chemical	Mass (g)	Final Concentration (mmol/L)
NaCl	1.8	30.8
Na_2HPO_4	0.21	1.5
NaH_2PO_4	0.18	1.5
Glycine	18.0	240
Filtered Water	Top to 1 L final volume	-

Table 3.3b: Quantity and Concentration of Gelatin Added to 100 mL LISS

Percentage Gelatin	Mass Added (g)
0.5	0.5
1	1
3	3
5	5

Since the presence of the gelatin in the GVB did not appear to have a large effect in reducing EOF and wall adsorption, Tween 20 was added to the EL buffer and tested for suitability in wall passivation. This was done due to literature support of Tween 20 as an effective passivation agent in various different buffers. Concentrations of 0.1%, 0.3%, 0.5%, 1%, and 3% of Tween 20 were tested. However, no noticeable improvements in signal reproducibility and resolution were observed. A variation of the antibody concentration of 1 μg/mL, 5 μg/mL, and 10 μg/mL was also tested. However, no or only weak signals were detected inconsistently in the EL at the same concentration of 10

μg/mL where the GVB showed the most promise. The lower concentrations of 1 μg/mL and 5 μg/mL also yielded no signals. The chip was loaded according to Table 3.1 using EL. The same electrophoresis tests used for GVB and listed in Table 3.2 were performed. The Tween 20 should not have had a denaturing effect on the antibodies based on previous reports by Abad-Villar *et al.* [18] and Chiem *et al.* [14]. In these reports IgG and IgM antibodies were successfully detected, and antibodies were also reacted with target drugs respectively in the presence of Tween 20. Thus, the Tween 20 should not have altered the antibodies in a way that should have affected their detection.

Initial testing of the EL with Tween gave poor results. Signals could not be detected in 70% of the runs tested. When they were detected, there was little peak definition with most of the signals seen as mere slight increases in the baseline (Fig. 3.4). There was also no reproducibility of signals. Previously suspected antibody aggregates as high intensity spikes observed with the GVB were also seen here. Further testing was not done because it appeared that the EL with Tween was inadequate as a CE buffer.

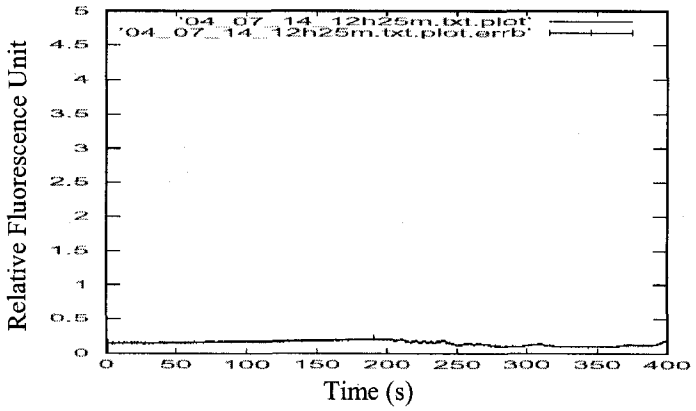


Fig. 3.4: Detection of anti-human IgG antibodies in EL where a sample plug was injected

Due to the lack of signals detected on most of the runs with EL and Tween, it appeared that adsorption may be playing a larger role here than with the GVB tested since the same sample concentrations were used. Thus, it appeared that Tween 20 was not sufficient in reducing the adsorption of the antibodies for the EL. It was then decided that gelatin would be tested as a passivating agent based on its success as suggested by Ichiki *et al.* [17] in their immunoelectrophoresis of RBCs and antibodies. EL with gelatin (EGL) was made with 0.5%. 1%, 1.5% and 3% gelatin and tested. The same 10 μg/mL

concentration of antibodies were used for all of the different gelatin concentrations. When the antibody arrival was tested according to the electrophoresis protocol in Table 3.2a, an increase in the baseline resembling a large bump stretching over 3 separate runs was observed Fig. 3.5. Antibody aggregates represented by the signal spikes appeared to be present on this large bump. Although the shape of the initial peak did not resemble the sharp rise in baseline as seen with the GVB, it did still indicate distinct sample arrival with the large change in baseline. The overall peak profile observed over three consecutive runs is similar to that described by Backhouse *et al.* where the analyte peak profile over a period of hundreds of seconds of injection took on a similar shape [21]. The decrease in intensity could be explained by a depletion of analyte in the sample well. Six loads of EGL were tested in total with 4 of the 6 loads showing a similar profile as that shown in Fig. 3.5. In terms of the reproducibility of the signal peak, the EGL electropherogram was better than that seen with the GVB. (A signal is reproducible if the following criteria are met: each time that a signal peak with an amplitude greater than 0.02 V (gain 0.8) is detected, the same number of peaks is seen in each electropherogram; the signal intensity does not vary more than 20%; the resolution does not vary more than 10%; and the times of arrival of the peaks do not vary more than 10% between runs.) The signals seen with the EGL were only detected in approximately 67% of the runs of the 6 loads tested. This was still much lower than that of the standard TBE where signals were seen each run of each load tested and the reproducibility of signal peaks was 100%. Thus, the EGL was not investigated further as a potential buffer for the antibody and RBC work

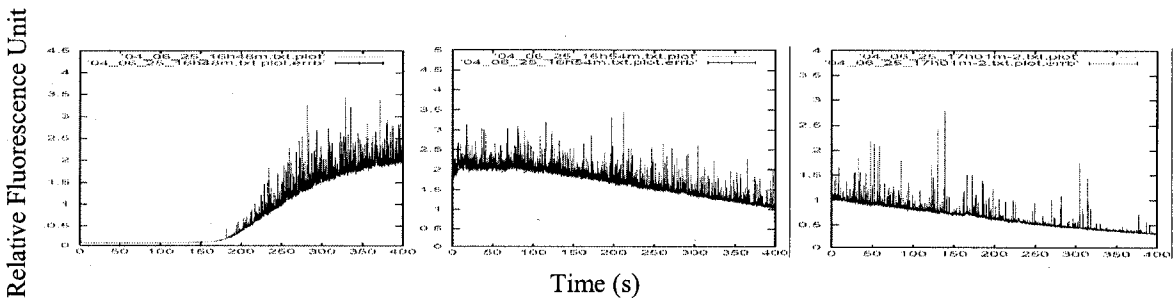


Fig. 3.5: Consecutive runs of injection of anti-human IgG in EGL with 0.5% gelatin concentration. Injection of sample was achieved by the application of a voltage at the sample waste well.

3.2.3 Phosphate Buffer

One of the most commonly used buffers for biological analytes is phosphate buffer [1, 17, 22]. The use of phosphate buffered saline for the electrophoresis of red blood cells has also been reported and shows potential to be used for both proteins and cells [23]. Phosphate buffer was used directly as the electrophoresis buffer in Ichiki's work with immunoelectrophoresis, but yielded poor results in controlling EOF compared to the GVB that was also tested in the same glass channels [17]. Although the same channel conditioning method of rinsing with 0.1 mol/L NaOH followed by 0.1 mol/L HCl was used for both buffers, the EOF was found to be larger by Ichiki *et al.* when using the phosphate. Sodium phosphate buffer is used widely in conventional electrophoresis and has also been used for on-chip capillary electrophoresis with concentrations varying between 50 mM to 150 mM [24, 25]. However, due to the concerns with EOF and pressure driven flows associated with using only a buffer, it was decided that phosphate buffer would be tested with a sieving matrix where the increased viscosity may reduce these effects. The sieving matrix may also have some passivating effects on the channel walls. Other polymers such as poly(ethylene oxide), hydroxyethyl cellulose, and poly(vinylpyrrolidone) polymers have all been used as additives to coat channel walls and reduce EOF [26]. The addition of the sieving matrix GeneScan® was chosen here based on its effectiveness when used with our platform in our previous DNA work [27, 28]. GeneScan® also did not show any reactivity with biological analytes in our previous work, suggesting that it may also not react with the RBCs and antibodies. The testing of the phosphate buffer was another attempt to see if a conventional buffer suitable for working with cells and antibodies could be transferred to the microchip CE realm and produce similar results to the standard ones seen with tris borate. The addition of the sieving matrix makes the direct comparison of the effectiveness of the phosphate buffer to GVB and to LISS inappropriate since the GVB and LISS did not contain any sieving matrices. However, it is the comparison to the final results that is of interest here. These results were compared to the desired goal of a defined single peak with little or no evidence of EOF and adsorption. Thus, the inability to directly compare the different buffer results to each other was not of great importance.

The GeneScan® polymer (7%, P/N:401885) from Applied Biosystems (Streetsville, ON, Canada) was added and yielded a final concentration of 5% as previously done in Chapter 2. A 40 mM phosphate buffer was made by combining 0.400 g of NaH_2PO_4 with 0.400 g of Na_2HPO_4 into 100 mL of water (pH 6.38). GeneScan® polymer (7%, P/N:401885) from Applied Biosystems (Streetsville, ON, Canada) was diluted to a final concentration of 5% with the addition of the phosphate buffer. This was achieved using the same method as previously used with the DNA analysis (Chapter 2), but phosphate buffer was used instead of TBE. During electrophoresis, it was found that the current could be sustained for no more than approximately 150 s before large fluctuations of as approximately 5 μA began. Even prior to these variations, smaller fluctuations in current of approximately 1 μA to 3 μA may also be present. A lowering of the voltage to 1000 V during step 2 of the sample plug electrophoresis protocol (Table 3.2b) did not remove or reduce these fluctuations. The inability of the PBS to maintain constant current for made it non-ideal as the running buffer because it did not offer the amount of robustness the system will likely require when working with the RBCs and antibodies. This is especially true if RBCs were to be combined on-chip for any immuno-reactions. In Ichiki's work [17], their immunoelectrophoresis required approximately 100 s to 300 s. The phosphate buffer would not be able to sustain adequate current for the entirety of the process. In addition, if accurate mobility measurements were required, any fluctuations in current would affect the mobility of the analytes, leading to inaccurate results. This suggested that phosphate buffer would not be an appropriate buffer for use here. During detection, no distinct antibodies peaks were detected using the phosphate buffer as well. Commercially available phosphate buffered saline (PBS) purchased from Hyclone (Logan, UT), commonly used for the washing and suspension of cells for analysis at the CBS, was also tested. The PBS showed similar results as the phosphate buffer where little to no signals were detected. Thus, it did not appear that either the phosphate buffer or the phosphate buffered saline would be appropriate choices for the electrophoresis of antibodies.

3.2.4 Citrate Buffer

Sodium citrate is used as an anti-coagulant in blood collection by the CBS and does not damage the cells, allowing them to function normally. Sodium citrate buffer has also been used for CE of various proteins from serotonin to peptides associated with Alzheimer's disease [29-32]. Although the most often used citrate buffer concentration in CE was 20 mM, Benturquia *et al.* used 80 mM citrate for the analysis of serotonin [32]. Pre-conditioning of the capillaries was also required in this case where the capillary was flushed with 0.1 mol/L NaOH followed by ultrapure water and running buffer for 15 min each. Conditioning of the capillaries did not appear to be an issue for the other reports where citrate was used. In most literature surveyed, CE using citrate was performed on commercially available systems under field strengths at around 250 V/cm. This field was close to what had been tested thus far in our work. Therefore, citrate appeared to be a potential buffer candidate for electrophoresis without damaging biological analytes.

Varying concentrations of 20 mM, 50 mM, 100 mM and 500 mM of citrate buffer with a pH around approximately 5 were made and tested. Each was used to make a 5% GeneScan® dilution as done during phosphate buffer testing as well as in Chapter 2 with citrate replacing the phosphate or TBE. Both 100 mM of citric acid (Sigma-Aldrich, St. Louis, MO) and sodium citrate (Sigma-Aldrich, St. Louis, MO) were made. This was done by the addition of 1.0505 g of citric acid monohydrate to 50 mL of distilled water and the addition of 1.4705 g of sodium citrate tribasic dihydrate to 50 mL of distilled water respectively. The 20 mM citrate buffer was made by combining 10 mL of 0.1 M citric acid with 10 mL of 0.1 M sodium citrate in a total volume of 100 mL of distilled water. Similarly, 500 mM citric acid and sodium citrate were made by the addition of 5.2525 g of citric acid monohydrate and 7.3525 g of sodium citrate tribasic dihydrate each to 50 mL of distilled water. A 50 mL of citrate buffer was then made by the addition of 7 mL of sodium citrate to 5 mL of citric acid. The 7 to 3 ratio was chosen to give conductivities between 2 mS/cm to 5 mS/cm as an arbitrary standard to provide a level of consistency throughout the testing. The pH of the solution using this ratio was approximately 5.2.

The 50 mM buffer was found to yield the best results in that it gave the best signal detection. The 50 mM also resulted in very little cell lysis with lysis at approximately zero. This was established by performing a hematocrit test with the citrate buffer and GeneScan®. A hematocrit test is a commonly performed test used in blood banking to determine the percentage of red blood cell volume to total volume of a red blood cell and plasma mixture. The solution is centrifuged and the volume of packed red cells is determined by comparing the size of the packed cell region in the test tube to a predetermined chart. Thus, the hematocrits of a sample in a particular medium initially and following a certain amount of time are measured to determine whether cell lysis had occurred. Polymer with 50 mM citrate (0.05C5GS10G) was made by the combination of 0.71 g of 7% GeneScan® polymer stock solution (Applied Biosystems, Streetsville, ON) with 0.09 g deionized water, 0.1 g of 500 mM citrate buffer, and 0.1 g of glycerol (Sigma, Saint Louis). Both the citrate buffer and the C5GS10G yielded intiial normal percentages of approximately 45% and 49% hematocrit respectively. The percentage hemolysis or the percentage of red blood cells that had lysed after a two-hour incubation time was zero for both polymer and buffer.

Testing with citrate included not only the monoclonal antibodies, but also Alexa Fluor 546 labelled polyclonal anti-human IgG antibodies from Molecular Probes (Eugene, OR). It was found that in addition to being able to detect both types of antibodies, a lower concentration of 1 μg/mL of antibodies could be used with the citrate instead of the 10 μg/mL required when using GVB, the best results obtained thus far throughout the buffer testing. Both the monoclonal and polyclonal antibodies were detected and showed similar results in that a relatively distinct peak could be seen (Fig. 3.6). There also appeared to be less aggregation of the antibodies, suggested by fewer signal spikes observed per run. The GVB showed greater than 10 signal spikes on the peak per run while the citrate showed fewer than 5 per run. The fewer peaks observed made sense because with the decrease in antibody concentration one would expect the formation of fewer aggregates. The polyclonal antibody had a peak profile that was broader than that of the monoclonal. This is not unexpected since polyclonal antibodies do not come from one source as in the case for monoclonal antibodies, and is likely to have a larger variation in mobility within a population. Thus, the larger variation in the

polyclonal antibodies would lead to a larger variation in mobility within the population. This larger variation may manifest as a broader peak observed during electrophoresis due to the cells arriving at different times (Fig. 3.6b). Electrophoresis was carried in the standard injection-separation method as listed in Table 3,2b, but with an injection of 30 s instead of 150 s. The injection time was changed to 30 s because from testing, it was observed that 30 s was sufficient in moving an adequate quantity of antibodies to the intersection for separation and detection. Overall, 16 different loads were tested with a total of 60 runs conducted. The signal peaks were present as per definition above (p. 64) on only 26% of the runs. For the remainder runs, the signals were very weak, approximately less than 0.02 V in amplitude, with a lack of a convincingly distinct peak. This was likely a result of the poor buffering capabilities of the citrate, enabling only one electrophoretic run per load. Antibody adsorption may have been another factor contributing to poor signal reproducibility. The reduction in peak intensity seen with the citrate buffer was similar to those seen in the past when adsorption was encountered such as in the case with GVB (Fig. 3.3b). Although citrate showed more sensitive detection than the other buffers tested thus far, it was still inadequate in performance when compared to the reproducibility of the standard TBE. Thus, the citrate was also eliminated as a potential buffer to be used for RBC and antibody CE on our system.

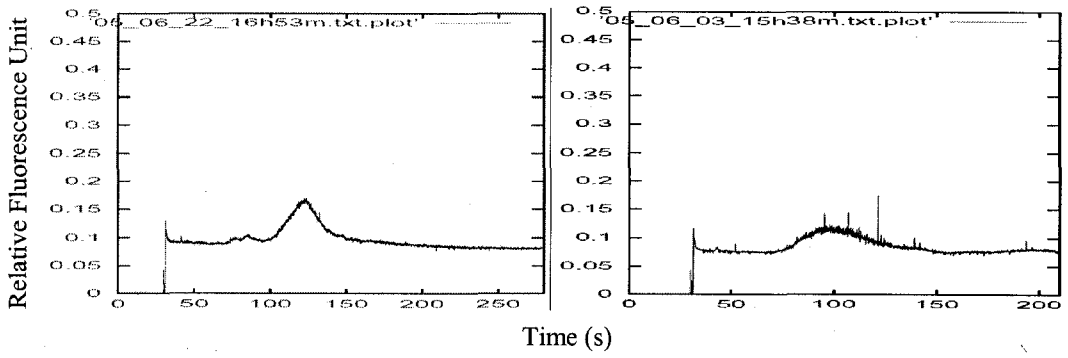


Fig. 3.6: Injection-separation runs of monoclonal (a) and polyclonal (b) anti-human IgG according to Table 3.2a and 3.2b respectively. The chip was loaded according to Table 3.1 with 1 μg/mL concentration of antibodies used for both cases.

3.2.5 Tris Borate Buffer

Following the testing of the citrate buffer, the testing of the initial group of buffers that were known to be compatible with biological analytes was completed. Based on the results obtained, this group did not appear to yield any viable buffer for our purposes. Thus, it was decided that the TBE would be tested as a potential buffer to be used with out analytes. The capability of the TBE to support the CE aspects that were required has already been demonstrated in our previous work (Chapter 2). TBE is commonly used for microchip electrophoresis and is known for its strong buffering capabilities [33-37]. This buffer was used successfully for on-chip HA and SSCP analysis (Chapter 2) and previous work involving on-chip electrophoretic mutation detection [34]. Tris has also been used in the separation of proteins [11] while tris borate buffer was used by Whitesides' group for testing of rapid prototyping in PDMS for amino acid separation [33]. Ma has demonstrated the successful used TBE for yeast and Escherichia coli cell work [28]. Thus, the testing of tris borate buffer appeared to be appropriate for the purposes here. The addition of EDTA was not expected to affect the antibodies since it is a commonly used anticoagulant for blood collection [38].

The 1xTBE with 10% (w/w) glycerol buffer was made as previously described in Chapter 2. Glycerol was kept in the buffer to act as an osmoregulator [39]. The osmolality, the amount of solute per kilogram of solvent, of a solution is important to cells because it will affect the osmotic pressures experienced by the cells. The osmolality of the TBE was approximately 1400 mOsm/kg, higher than the 330 mOsm/kg [40] that would normally be required for maintaining the integrity of the blood cells to prevent lysis. However, the glycerol was kept in the buffer because the cells would have a better chance of tolerating a higher than physiological osmolality in the presence of an osmoregulator. Thus, the glycerol was kept in the reagents for testing of the antibodies to determine whether or not it would affect the antibodies. If the glycerol affected the antibody detection adversely, an alternative osmoregulator such as sucrose would then have to be tested [41].

The chip was loaded as described in Table 3.3.1 and electrophoresis was conducted as described in Table 3.2b. It was found that at a concentration of 1 μg/mL, both monoclonal and polyclonal antibodies were detected with symmetric peaks and

good signal intensity (Fig. 3.7). These results were reliably reproducible in that they could be reproduced on three consecutive runs of the same load and were reproduced for over ten separate loads. The superimposed results of three consecutive runs for two arbitrarily chosen loads are shown in Fig. 3.7. It may be seen that the peak profiles of the runs are very similar for each load and that the results appear reproducible. The TBE was also able to detect the antibodies at 10 times lower concentration than the GVB, providing more sensitivity. The TBE also did not appear to adversely affect the antibodies in any way. A quick test was also done by mixing RBCs in TBE and observing for cell lysis. Cells were incubated in 10 μL of TBE for one hour. The numbers of cells prior to and after incubation were counted and found to vary less than 2% where 54 cells were counted initially and 53 cells were observed following incubation. The one cell loss is not abnormal and may have been caused by a variety of different things such as cell age and handling through pipetting and centrifugation. A tentative conclusion was reached here that such a test for cell lysis is adequate. It was noted that if this test was not sufficient, results in later experiments may be affected. Electrophoresis of RBCs using TBE was then tested. The TBE appeared to be compatible with our biological analytes and gave the most consistent results during CE out of all the buffers tested. TBE was able to detect both antibodies as well as RBCs with no apparent adverse effects to both. Thus, TBE was deemed to be the best candidate among the buffers tested for our purposes.

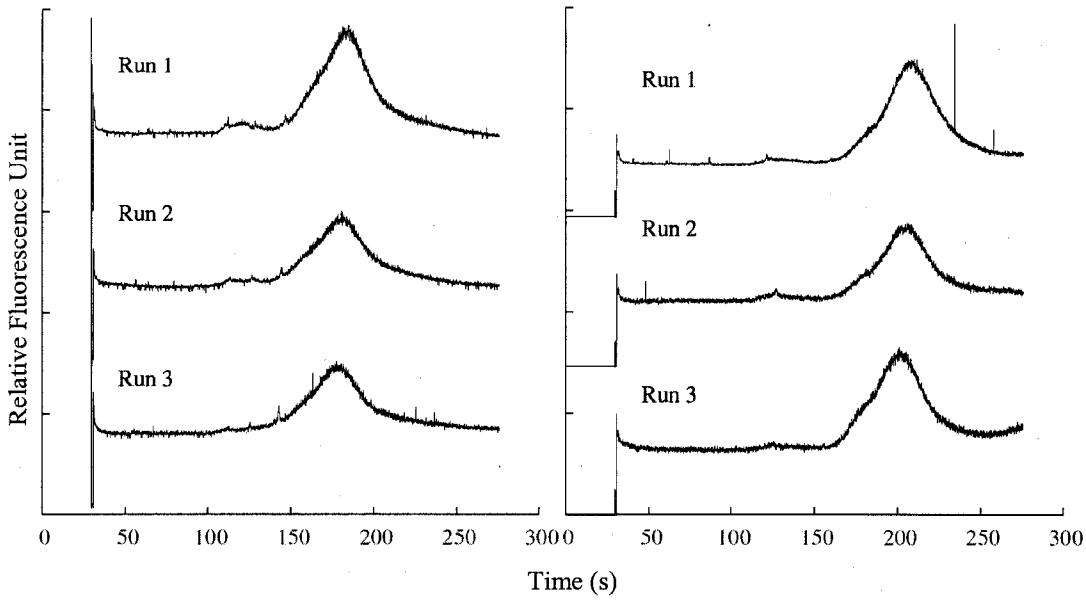


Fig. 3.7: Separate detection of a sample plug with sample concentration of 1 μg/mL monoclonal (a) and 1 μg/mL polyclonal (b) antibody in TBE buffer. Electrophoresis protocol followed Table 3.2b and detection was done at 30 mm.

3.3 Conclusions

The overall goal here was to establish a buffer that could be used for microchip CE with minimal effects on antibody and RBC function and structure. This would ensure that reactions associated blood typing could still potentially take place on-chip. Thus, it was decided that buffers used at the CBS along with some other commonly used buffers used for biological analytes would be tested for their compatibility with microchip CE. If these buffers were suitable, it would mean their use for analyte CE would be ideal due to their compatibility with our analytes. This was anticipated as not an easy task since few reports were found that showed their use in the microfluidic platform. The subsequent results obtained from our testing confirmed this initial hypothesis. The buffers were deemed inadequate for microchip CE when compared to the results obtained with the standard CE buffer of TBE used with our system. However, it was found that the TBE could, instead, be used with the antibodies and RBCs without any apparent significant biological effects.

Overall, among the buffers tested, only the GVB as a buffer alone was able to detect any antibody signal. The GVB was expected to be able to detect the antibodies due to the fact that Ichiki *et al.* used the buffer for their immunoelectrophoresis. Although the GVB showed strong signals, the presence of the gelatin was not adequate in preventing antibody adsorption on the channel walls. This was the case even when the chip conditioning method followed that reported by Ichiki *et al.* Among the buffers tested, only the GVB and PBS had been used previously for purposes similar to those we are proposing. Thus, a comparison to the existing literature could only be made for these buffers. Although the antibodies were detectable with the GVB, adsorption of the antibodies was still observed and prevented reproducibility in peak detection. The GVB also appeared to be susceptible to EOF with a signal arrival time variability of 8%. This was much larger than the 2% variation between types reported by Lu *et al.* [43]. Thus, when using GVB the sensitivity would not be sufficient to detect the smaller 2% changes required for typing. Overall, the GVB also did not match the CE standards in terms of stable electrophoresis and reproducibility set by the TBE buffer during the genetic testing.

In the case of the phosphate buffer, no signals were detected compared to detectable signals reported. In determining the effectiveness of the other buffers as well as assessing the effectiveness of each buffer, comparisons to the typical peak profiles of one distinct peak for plug injections and a constant increase in baseline associated with constant analyte injections were used. This was done because the goal was to determine an effective method for moving antibodies and cells regardless of how effective each buffer is under the same conditions.

The LISS and citrate were chosen due to their use in blood banking while the citrate was also chosen for its use in conventional electrophoresis. Thus, the inadequate performance of these two buffers for microchip electrophoresis was not surprising. Attempts were made toward improving the electrophoresis conditions with this buffer following existing literature, such as the addition of different surfactants and the variation of the surfactant concentrations. However, the extremely poor results obtained with the LISS buffer with and without the modifications suggest that much work was required to even match the results obtained with the TBE or GVB. Since the main purpose of testing

the buffers was to choose an adequate set of conditions so that antibodies and RBCs may be electrophoresed in a microchip instead of a focus on characterizing and optimizing different buffers, the TBE was decided as the buffer of choice. TBE was chosen based on the fact that it possesses strong buffering capabilities as well as being close to physiological pH. The fact that is was used successfully for cells without lysis [28] also demonstrated that it does not significantly affect cell membrane integrity, an important point if the antibody binding sites for antigenic sites are to remain active.

The buffers were not only chosen based on their suitability for working with antibodies, but consideration was also given on use with other blood components. These include mainly red blood cells and other proteins in blood such as albumin, which are abundant in blood. It would be useful for any method developed to use the same buffers so as to ensure ease of integration.

The use citrate and TBE buffers in GeneScan® gave the most sensitivity compared to the other buffers tested where antibody signals were detectable with 10 times lower sample concentration. The higher sensitivity may be due to the fact that the GVB and other buffers had a suppressive effect on the signal strength. A similar effect may have occurred with the GVB, phosphate and LISS buffers that showed poorer sensitivity. In our previous work with DNA using TBE (Chapter 2), a 1/10 dilution of the running buffer was used in the sample well to avoid this signal suppression effect. However, when the various buffers were tested here, no such lowering of concentration was done. This lack of dilution was to try to maintain more uniformity in buffer throughout the chip so as avoid any potential alterations to the cell and antibody due to changes in osmolality. Another cause may be that the amount of adsorption of the antibodies on the channel walls in the absence of the GeneScan® was much greater in the case of the phosphate buffer and GVB, and thus a higher concentration was required for signal detection. Nonetheless, the exact causes of the variations in signal intensity and profile were not the intent of the research and are beyond the scope of this thesis.

The TBE delivered more stable currents and showed more reproducible results than the GVB and LISS. It was not logical to attempt to modify the GVB and LISS to reduce the adsorption issues associated with these two buffers. Adsorption problems are commonly seen when working with microchannel electrophoresis. As previously

mentioned in Chapter 1, various methods exist for reducing adsorption effects. However, no one distinct method for reducing adsorption in all microchannels exists. Thus, each research group tends to optimize its own methods for their reagents and needs. Since the methods suggested by literature for the GVB (Ichiki *et al.*) and other common adsorption reduction methods (addition of Tween 20 and gelatin) for the reagents used here were already tested with unsuccessful results, further testing would have entailed the trial and error of many other methods as well as further characterization of the channel properties. Although the continued optimization of the buffers tested in our work may be a topic of further research, the focus here was to establish a method for moving and reacting RBCs and antibodies. Further testing would have deviated from this focus, especially when an appropriate set of reagents in the TBE and GeneScan® appeared sufficient. In addition, the use of TBE and GeneScan® has been shown to work successfully and reliably for microchip electrophoresis in our laboratory and the attempt to establish a new set of reagents with the same reproducibility appeared redundant.

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Chapter 4

Microchip-Based Erythrocyte Electrophoresis

4.1 Introduction

The movement of red blood cells using electrophoresis has been previously demonstrated [1-4]. This includes the CE of RBCs and the subsequent detection of single cells [1] and the electrophoresis of cells on-chip in microchannels with detection and velocity measurements [2]. Having an isoelectric point of approximately 3.5 [5], the RBCs are negatively charged at physiological pH. In order to successfully use and control RBCs on-chip using electrophoresis for potential analysis, the cell's movement and behaviour in our reagents had to be determined. This was required due to unsuccessful attempts in transferring similar methods from the literature that most closely matched our purposes. An appropriate electrophoresis method or protocol had to be determined that could be reliably used for RBC manipulation. The main focus of this chapter was determining the movement of RBCs on-chip and the establishment of a reproducible RBC manipulation protocol.

Apart from reliably controlling the RBC movement during electrophoresis, another focus here was on the manipulation of red blood cells for blood typing. Minute mobility differences of RBCs have been documented. For example, observed mobility differences due to alterations of cell surfaces between Alzheimer patients and normal RBCs were observed [6]. Diabetic red cells have also been shown to exhibit different mobility from normal cells [7]. The differences between normal and diabetic RBCs were not specifically stated. This was also the case for normal and Alzheimer RBCs in the

second report. Both reports focused more on the changes in mobilities of the abnormal cells associated with morphological or physiological changes associated with the respective diseases. These reports act as a reminder that the mobility of RBCs is dependent on many different factors. Lu *et al.* [8] had demonstrated the detection of mobility differences of a minimum 2% in the ABO group using CE. They were able to find a significant difference in the retention times between the A, B, O, and AB RBCs where differences as small as 0.18 min (2.2%) were detected. A goal here was to determine whether we could reproduce similar results on a standard simple-cross shaped four-port microchip with a much shorter distance compared to the conventional CE. The shorter distances make it more difficult to detect minute differences in mobility of the cells compared to longer separation distances. The reason lies with the method of sample detection on a microchip. The injection of a small quantity of analyte into the separation channel via a sample plug means that there is a minimum distance (L_{req}) that is required for separation given by [9]

$$L_{req} = L_{sample} (\mu_B/(\mu_A - \mu_B)) \qquad (4.1)$$

where L_{sample} is the length of the sample plug and μ_A and μ_B are the mobilities of the two components that are being separated. If L_{req} is set, the sample plug length must not be longer than L_{sample} if separation is to be obtained.

Another issue associated with microchip electrophoresis that is not a problem on conventional CE is that sample leakage may occur from the sample and sample waste wells into the separation channel [10]. The presence of samples leaked into the separation channel may cause false readings of the results. The smaller volumes used on chip are also more prone to evaporation and pressure effects. Unlike conventional CE where the buffer sources are large, the reservoirs on-chip are limited to small volumes. Thus, continuous electrophoresis for periods longer approximately 10 min is difficult without either reloading the chip or replenishing the buffer. The shorter channels of microchips also make them more prone to pressure effects that would affect the mobility of the analytes as well as possibly contributing to sample leakage. A sufficient method for detection of the cells was also required. The existing LIF that had been used for DNA and antibody detection may require modification in that the cells may not pass through the centre of the channel and would escape detection.

4.2 On-Chip Movement of Red Blood Cells

In order to successfully manipulate blood cells on-chip, the behaviour of the RBCs on-chip using the reagents of interest needed to be determined. It was found in the previous chapter that antibodies were best electrophoresed in the polymeric medium of GeneScan® compared to the other buffers tested. The two buffers that appear to be most suitable for use with the antibodies were found to be citrate and tris borate EDTA buffer (TBE). Thus, these two buffers were also tested to determine which would be most suitable for on-chip electrophoresis of the RBCs.

4.2.1 RBC Electrophoresis

The polymer used during electrophoresis was 5% GeneScan® with 10% glycerol (Sigma, St. Louis, MO) and buffer. The buffer was either 50 mM citrate or 1xTBE. The running buffer consisted of 10% glycerol added to the buffer tested to give 0.5C5GS10G made with citrate buffer or 1xTBE10G made with standard 1xTBE. The glycerol was kept in the medium as an osmoregulator to maintain cell integrity [11]. The normal pH and osmolalities were approximately 5.2 and 1300 mOsm for the citrate buffered reagents, and 7.5 and 1350 mOsm for the TBE buffered reagents. Although the physiological osmolality of the cells is approximately 300 mOsm, the presence of the glycerol enabled the cells to survive in the higher osmolality of 1350 mOsm of the TBE. The reagents were monitored through periodic measurements for any changes associated with age to ensure consistency throughout testing. RBCs used were panoscreen reagents cells used in blood typing by the CBS and were obtained from Immucor (Norcross, GA). The concentration of cells used throughout experiments was 1% v/v of packed cells diluted in buffer. This was achieved by the addition of 1 μL of packed cells to 9 μL of running buffer and then taking 1 μL of that mixture and adding to another 9 μL of running buffer. Prior to making this dilution, the reagent cells were first washed in commercially purchased 1x phosphate buffered saline (1xPBS) from Hyclone (Logan, UT) by centrifuging for 5 min at 4000 rpm. This washing and centrifuging process was done twice. The supernatant was then removed leaving the packed cells for use. Freshly washed cells and new working dilutions were made for every load of the chip to maintain

consistency of the reagents tested. By using freshly washed cells, any unforeseen effects the buffer may have on the cells are maintained the same for all the tests, thus avoiding any effects specific to duration spent in the buffer.

A standard 4-port chip was loaded with 3.4 μL of sample cells in the sample well and 3.4 μL of running buffer in the remaining wells. The volumes in the wells were increased from 3 μL to 3.4 μL to combat evaporation effects during testing on the fluorescent microscope, a Zeiss Axiovert 200 Inverted microscope (Göttingen, Germany). Higher evaporation rates were observed on the microscope compared to the μTK due to the heat from the microscope light source. The electrophoresis protocol varied with different detection methods. Several methods were tested in an attempt to determine accurately the cell mobility. Initial methods involved using a stopwatch to manually detect the cells as they arrived at the designated detection point. However, it was found that this method was insufficient in accuracy due to difficulties with manually tracking and recording the times for multiple cells that often arrived within 1 s or 2 s of each other at the detection point. Sequential pictures of sections of channel were also taken at known time intervals to determine the distance the cells moved during the set time intervals. These pictures enabled the tracking of several cells at a time as they moved through the separation channel. However, due to the cell interaction in the channel with the polymer, and occasionally each other, the positions of the cells with respect to one another may change during electrophoresis. This made it difficult to track each cell's individual movement if images were taken between long time intervals. Thus, four images were taken once every 10 s. The total distances the cells travelled during the 30 s were in the order of millimetres and were much smaller than the 50 cm used in Liu's report where CE was used to determine cell mobility differences. Thus, it would be very difficult to achieve similar results when attempting to determine cell velocities with these two distances even if all other aspects were the same. Thus, a detection method that enabled the cells to travel farther down the channel would be needed. The shifting of the cells' locations with respect with one another led to confusion when determining where each cell moved during each time interval, also limiting the number of cells that could be tracked during one run. The third method of detection was to take an image at the detection point following electrophoresis of the cells. This image may be taken at any

point along the separation channel, allowing for versatility in the variation of separation distances. The image taken was able to capture all the cells that had been injected on a particular run. From the picture and the software supplied for the Zeiss Axiovert microscope, the distance the cells travelled from an initial set starting point could be read. Thus, if the starting points were set at the same location for each run, then the distances travelled for each cell between runs and loads could be compared. This was the method of choice when it came to determining the mobility of the cells.

Some testing was done with GVB and flow appeared to be present in the chip. During electrophoresis of the cells down the channel, it was observed that the cells continued to move in the same direction following the removal of the electric field. If the cells had been moving only under the force of the electric field, then with the field's removal the cells should stop. However, their continued movement down the channel, although much slower, suggested that pressure driven flow might have been present. The flow may have been caused by a very slight tilt in the chip. Another cause may have been slight volume imbalances in the two wells connecting the channel that could have resulted from initial variations in pipetting or unequal evaporation. Any measurements taken with this flow present would not have been an accurate representation of the cell mobilities. Thus, it was decided that an increase in viscosity may be able to reduce the system's sensitivity to minute pressure variations.

4.2.2 Electrophoresis in Citrate Buffer

Initial tests of RBC movement during electrophoresis were done with citrate buffer because citrate is used more commonly with biological analytes and would likely have less of an effect in altering the cells than the TBE. Detection was done on the μTK to determine whether the cells could be detected via light scattering and how the cells moved in the given reagents. The standard 4-port chip was used and electrophoresis (Table 4.1) was done with a 1% cell concentration loaded in the sample well while the remaining wells loaded with running buffer. The buffer waste well was set at 2000 V giving an electric field of approximately 240 V/cm along the separation channel. This field was chosen as the approximate maximum field strength to apply to the cells during

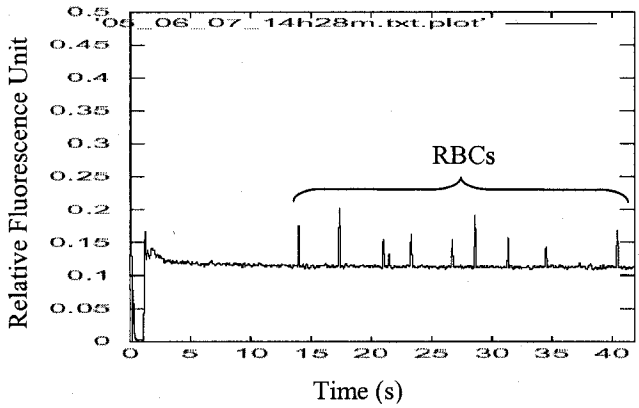
separation because existing literature usually does not exceed approximately 250 V/cm due to potential damage to cells [2, 8, 12, 13].

Table 4.1: Electrophoresis Protocol for Testing RBC Movement Using Citrate Buffer

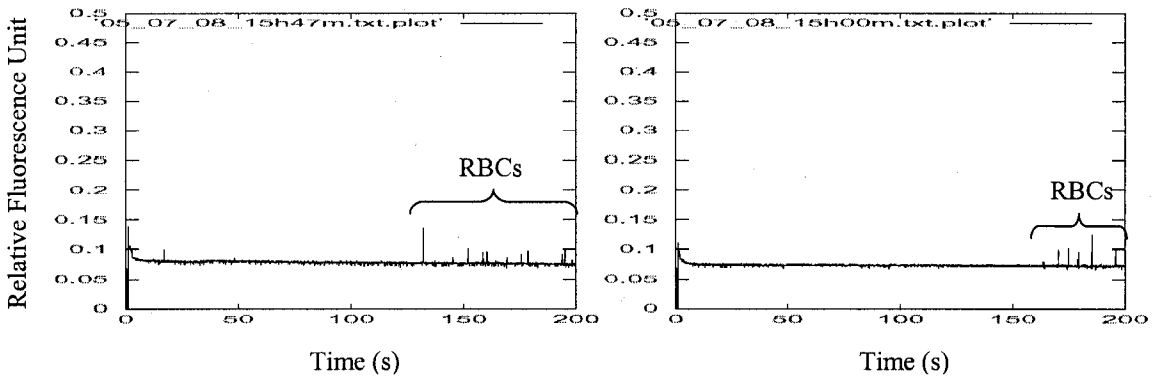
Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Ground	Float	±400	Float

Upon application of the electric field, the cells responded by moving into the separation channel. The cells were electrophoresed using the μTK and detection was done at 10 mm down the separation channel measured from the intersection of the chip. The cells were negatively charged as expected. They moved toward the anode and were detected as sharp peaks in the electropherogram without the need for fluorescent labelling (Fig. 4.1a). The reflected light from the cells appeared to be detectable by the μTK. The cells appeared as narrow peaks where the variations in the peak intensity depended on the amount of light scattered, which in turn depended on the location the laser hit the cells during detection. This was visually observed by watching the cells pass through the 10 μm diameter laser spot and observing changes in scattered light depending on the position of the cell in relation to the laser. When no cells were present during the electrophoretic run, no such narrow peaks were observed. During several runs it was found that the arrival times of the cells at the detection point varied from 130 s to 165 s (Fig. 4.1b). This suggested a variation in the velocity of the cells due to either variations in electrophoresis conditions or due to the variation in cell mobility. Drops in the current during the electrophoretic runs were also noted (Fig. 4.2). This was likely due to the inability of citrate to maintain buffering capacity throughout the entire 400 s of electrophoresis since the current was constant for approximately the first 100 s of electrophoresis. This was empirically observed for at least three loads. This current drop may also lead to cell mobility changes. The volume of the buffer used could not be increased significantly to improve buffering capacity due to limitations of the well sizes, which could hold no more than approximately 3.5 μL. Any large increases in buffer volume by enlargement of the wells such as the addition of micropipette tips to serve as

larger reservoirs would also increase potential pressure driven flow. This is due to the fact that the larger volumes of buffer would be harder to pipette accurately than the smaller 3.4 μL volumes. For example, if the pipette is accurate within 2% for both volumes of 10 μL and 3.4 μL, then the variation in actual volume would be 0.2 μL and 0.068 μL respectively. Thus, pipetting the larger volume would give a larger difference in well buffer volumes between two wells than the smaller volume of 3.4 μL. The larger volume variation would in turn lead to larger pressure driven flow. Thus, if accurate mobility detection were important, then citrate buffer would not be the ideal buffer of choice.



(a)



(b)

Fig. 4.1: Detection of RBCs in electropherogram using citrate buffer with labelling of cells. Continuous injection of cells was done according to Table 4.1

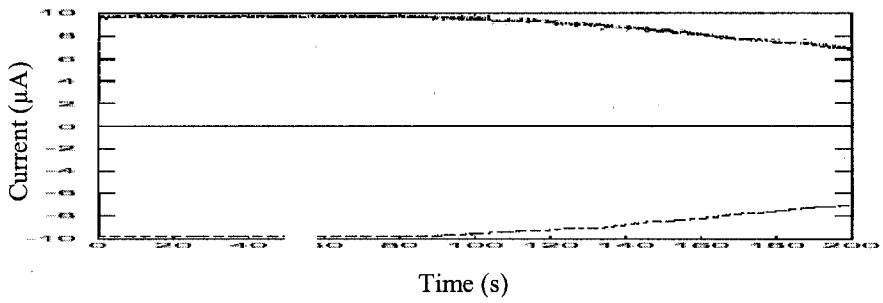


Fig. 4.2: Current measurements at buffer and buffer waste wells (top and bottoms halves of figure) during electrophoresis of RBCs using citrate buffer following electrophoresis protocol of Table 4.1. Decrease of current may be observed beginning at approximately 100 s at both wells. Note, the negative values are representative of direction of current. (File: 05_07_08_15h47m.txt)

In addition to the current variation causing cell mobility changes, the use of the μTK may have also led to inaccurate results. In order to determine the cause of the apparent variation in cell arrival times, the setup was first investigated. It was noted that not all the cells that pass through the detection point may have been detected during electrophoresis because some could have been missed by the 10 μm laser spot if they passed along the edge of the microchannel. Thus, in the 50 μm wide channels, it is likely that the RBCs may have been missed by the laser spot. Therefore, the time it took the cells to arrive at the detection point may not have been accurately measured if one or more initial cells were missed. In order to more accurately determine the arrival times of the cells, which may be used to determine the cell mobility, a change to testing cell movement on the Zeiss Axiovert microscope was made. Due to the fact that the microscope uses a CCD camera to capture images of the channels, all cells that passed through the detection point would be detected. The electrophoresis followed the same protocol used with the μTK testing (Table 4.1). Tracking of cells visually was found to be difficult due to the high speed the cells travelled at under the 2000 V application. Thus, the electrophoresis voltage was reduced to 1000 V from 2000 V to both slow down the cell movement and to further reduce stress on the cells due to the force from the electric field. This change to a smaller voltage is supported by the fact that the majority of the literature that uses fields closer to 100 V/cm than 200 V/cm. An appropriate detection method also had to be found for use with the CCD camera on the microscope. In an attempt to achieve a more accurate measure of the cell mobility during

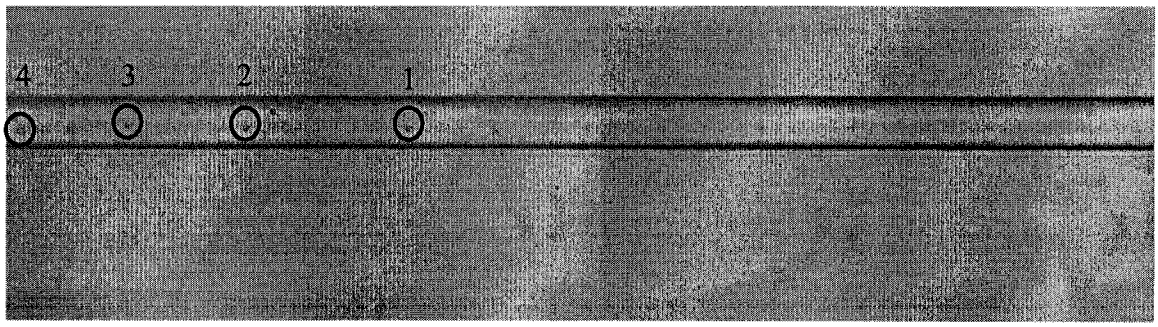
electrophoresis, the use of manual detection with a stopwatch was not used. Once cells arrived in the separation channel, three periodic field applications (separations) lasting 10 s with 35 s intervals between each were made (Table 4.2). The acceleration and deceleration of the cells were assumed to be instantaneous based on their observed movement in the channel. Once the electric field was applied, the cells began moving immediately and stopped instantaneously once the field was removed. This was to precisely control the amount of time the cells were electrophoresed and to enable time to take pictures of sections of the channel as the cells travelled. The combination of the two allowed for calculation of the velocities of individual cells. The application of both 1000 V and 500 V for electrophoresis were done to determine whether a change in the field strength would have any effect on the cell mobility. The microscope was set up to take pictures along the same section of channel between electrophoresis so the distances the cells travelled could be read off the pictures.

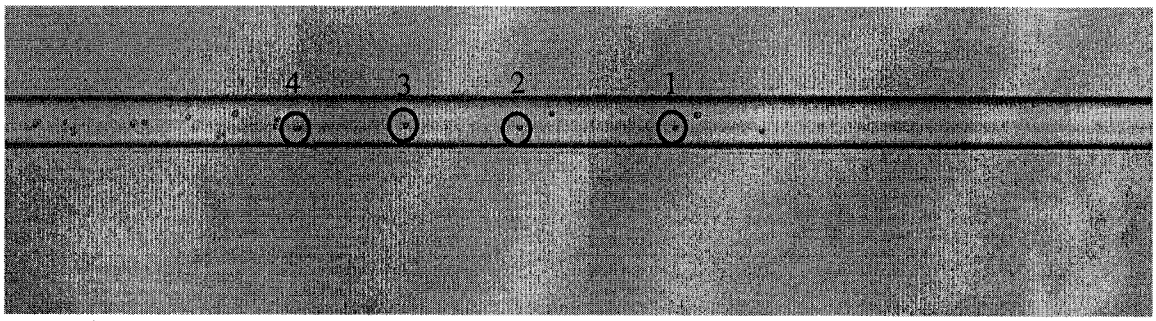
Table 4.2: Electrophoresis Protocol for Periodic Field Applications

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	200	Ground	Float	Float	1000
2	10	Float	Ground	Float	1000
3	35	Float	Float	Float	Float
4	10	Float	Ground	Float	1000
5	35	Float	Float	Float	Float
6	10	Float	Ground	Float	1000

Due to the cell interactions with the channels and other cells, it was difficult to track a large number of cells. Thus, four cells were tracked near the lead of the pack in each run (Fig. 4.3). The location of the cells did not change a great deal between 10 s intervals and could be tracked based on their location relative locations. Cells chosen were of average size and were usually not too close to other cells where it would be difficult to identify them. Any cells travelling near other cells where interaction may occur or those agglomerated with other cells were not chosen. The average velocities for

each of the cells when electrophoresed using 1000 V varied between approximately 90 μm/s to 60 μm/s while the velocities when 500 V was applied varied between 20 μm/s and 30 μm/s. The velocities of the cells were approximately half when 500 V were applied, suggesting that the velocities were proportional to the field strength and that the higher field strength did not appear to have any additional effect on the cells. The lower voltage applied led to lower currents during electrophoresis, which also led to a slowing down of cell velocity. This suggests that the lower voltages were associated with lower cell velocities. Currents varied between 4.5 μA and 5.1 μA when 1000 V was applied and varied between 2.4 μA to 2.7 μA when 500 V was applied. This supports the previous theory that unstable currents during electrophoresis in citrate buffer may affect the cell velocities, leading to inaccurate results if electrophoresis was carried out beyond the buffering capacity of the citrate.





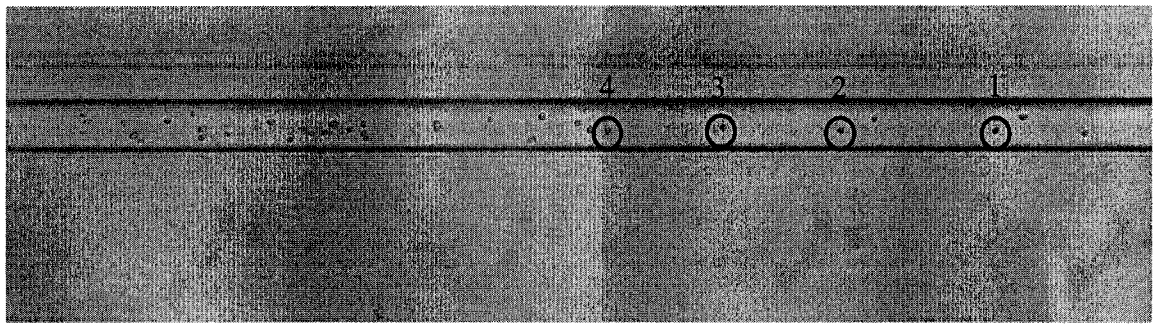


Fig. 4.3: Series of pictures taken along separation channel during separate separations of RBC electrophoresis during one run, enabling tracking of several cells (1-4)

One issue that affected the accuracy of the results obtained in the above method is the large number of cells that are injected into the separation channel. The constant injection of cells from the sample well to the buffer waste well could have introduced a large enough change to the composition of the electrophoresis medium that may lead to changes in current, and thus cell mobility. As more cells are injected into the channel, larger alterations would occur. In an effort to improve the protocol used, an alternative electrophoresis method was then tested. Thus, a move to change the electrophoresis protocol to the injection-separation used for DNA in Chapter 2 was utilized here. This would also allow for a random sample of cells to be use for analysis and eliminate any potential bias associated with using the leading cells that arrive during injection for testing. The cells were injected towards the sample waste well and then a sample plug was injected into the separation channel. The velocities of the cells in the injection plug were then observed. Initially the detection method remained the same. Periodic separations were made while pictures were taken between the separations. Optimization was done and the final electrophoresis protocol used is described in Table 4.3. Step 1 establishes a flow of cells from sample to the sample waste well while step 2 injects the sample plug directly from the sample well into the separation channel. This direct injection enables more cells to be injected than otherwise possible with a sample plug injection between the buffer well and the buffer waste well. The number of cells injected per sample plug varied between 4 to 10 cells. Step 3 allows the cells to establish a steady flow in the separation channel prior to taking the pictures for cell velocity determination in steps 4 through 9.

Note the voltages applied at the sample and sample waste wells during separations in steps 3, 5, 7, and 9. These voltages were applied to pull any residual cells in the shorter injections arms towards and into the sample and sample waste wells so as to prevent cells from leaking into the separation channel during the separation steps. They were dubbed "pullback" voltages because they were used to pull the cells back towards the sample and sample waste wells. Pullback voltages were also used to ensure no cells were present in the short channels due to any pressure driven flows that may be present while images were taken in steps 4, 6, and 8. Some flow was noticed at the intersection where cells flowed into the separation channel during the separation following the initial sample plug injection. This was attributed to slight pressure differences between the sample and sample waste wells. Although no specific determination of the effects of the pullback fields on the cells, the main thing was that the parameters were kept consistent throughout testing. Thus, all the cells would be affected in the same way and comparison between the different loads and runs may be made.

Table 4.3: Electrophoresis Protocol for Using Injection Plug and Citrate Buffer

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	130	Ground	Float	100	Float
2	3	Ground	Float	Float	1000
3	100	120	Ground	120	1000
4	35	50	Ground	50	Float
5	10	120	Ground	120	1000
6	35	50	Ground	50	Float
7	10	120	Ground	120	1000
8	35	50	Ground	50	Float
9	10	120	Ground	120	1000

The results of the cell velocities appeared more consistent with less variation. Types A1, B and O reagent cells from Immucor (Norcross, GA) were tested with a sample of results shown in Table 4.4. It appeared from the results that type O cells had

the largest mobility and type B cells were the slowest. However, the number of cells tested and the standard deviations calculated showed that the velocity differences between the different blood types did not meet a difference of two standard deviations, suggesting no statistical significance in the velocities of the different cell types.

Table 4.4: Velocities of A1, B, and O cells Using Protocol from Table 4.3

Cell Type	Load	Run	Average Velocity (μm/s)	Standard Deviation (μm/s)	Number of Cells Tested
A1	1	1	62.3	0.9	5
A1	1	2	61.9	3.2	5
A1	2	1	62.0	2.3	8
A1	2	2	59.9	2.0	9
A1	3	1	60.0	4.0	4
A1	3	2	64.6	2.3	6
B	1	1	54.8	2.3	4
B	1	2	54.1	2.9	4
B	2	1	56.7	3.1	6
B	2	2	59.7	3.5	5
B	3	1	58.7	0.2	1
B	3	2	58.3	3.7	7
O	1	1	61.9	2.2	4
O	1	2	65.6	1.8	3

In an attempt to improve accuracy of the results obtained and potentially distinguish between the different blood types based on mobility as demonstrated in Lu's work using CE [8], a change in the detection method was made. The current detection method only measured the cells during a total travel time of 30 s. This meant a total distance travelled of approximately 1800 μm. To increase the distance of separation, the detection point was moved to 15 mm down the separation channel from the intersection and detection of cell arrival times was manually achieved using a stopwatch. By

increasing the total distance travelled, any variation in things such as reaction time associated with manually measuring arrival times with a stopwatch would be reduced. The differences in time required to travel a longer distance for different blood types would also be enhanced, making it easier to detect. In addition, the error associated with different starting positions of the cells would also be reduced. This error arises from the fact that the microchannels are 50 μm wide. This meant that cells in a given group could have starting positions any where within that 50 μm width. Thus, total distances travelled by each cell would differ from each other depending on their starting point. A percentage error of D_e (Equation 4.2) was thus introduced.

$$D_e = (D/D_T) * 100\% \qquad (4.2)$$

where D is the distance travelled and D_T was the total separation distance. A change of the detection point to 15 mm meant that a percentage variation of only 0.3% was introduced compared to the 1% introduced with the detection point at 5 mm. A modification of the electrophoresis protocol was made again (Table 4.5). The cells were first injected from the sample well to the sample waste well to establish a steady stream of cells travelling past the intersection. A separation step was done to move the cells past the detection point. The final step was to prevent any cells from flowing into the separation channel prior to subsequent runs. The same 4-port chip and chip loading procedure were used where the sample well was filled with 3 µL of 1% RBC while the remainder wells were filled with 3 µL of buffer.

Table 4.5: Electrophoresis Protocol of Injection-Separation of RBC

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	130	Ground	Float	100	Float
2	300	120	Ground	120	1000
3	100	50	Ground	50	Float

Table 4.6: Summary of Velocity Results with Detection at 15 mm

Type	Total Average Velocity (μm/s)	Standard Deviation of the Mean (μm/s)	Number of Cells Tested
A2	64.2	2.1	14
B	61.4	1.4	12
O	62.7	1.9	17

The overall results from this change in protocol once again gave no significant differences between the three blood types (Table 4.6). The velocities of the cells were averaged and the standard deviations of the mean (SDOMs) were calculated. Although A2 cells were used here, the velocities of the three cells types still showed an overlap in velocities when the SDOM were taken into account. However, it may be that an even longer separation distance along with further decreased D_e would allow for larger separation of differences between the three blood types. Current fluctuations of 0.5 μA were observed as well during testing, which may have been large enough to contribute to variations in the mobility of the cells that led to increased error within the results. Despite the increases in separation distances, the current fluctuation made it difficult to obtain accurate and comparable results between loads in citrate buffer. Longer separation times would likely lead to even larger current fluctuations. Thus, a switch in buffer was made to allow for both longer separation time and less current fluctuation. Determination of the required separation distance and final distances used is described below in section 4.2.3.

4.2.3 Electrophoresis in Tris Borate Buffer with EDTA

Although the protocol modifications improved the reproducibility of the cell velocity results, a switch to TBE buffer was made to allow for a longer separation time with more stable current. Previous testing with DNA had shown the TBE buffer to have strong buffering capabilities in on-chip electrophoresis [14-16] The running buffer was made with standard 1xTBE and the addition of 10% glycerol (1xTBE10G) as was done with the citrate buffer. Initially, a similar protocol to that shown in Table 4.5 was used to

test the cell movement in TBE. During this testing, a comparison of the current stability could be made since both protocols were the same except for the change in buffer. While current fluctuations of the citrate yielded values of 0.5 μA, the current variations with the TBE remained no more than 0.1 μA (Fig. 4.4). This fluctuation was attributed largely to the instrumentation limitations, where an accuracy of ± 0.1 μA is stated in the manual for the power supply and current monitoring software Labview (Micralyne, Edmonton, AB). It was also noted that cells in the injection arms near the intersection would at times drift into the separation channel following injection of the sample plug. In order to minimize or remove this, another step was added to the electrophoresis protocol where the remaining cells in the injection arms were pulled back towards the sample and sample waste wells immediately following injection of the sample plug (step 4). This allowed the cells in the injection arms near the intersection to be moved far enough away from the intersection to ensure no cells drifted into the separation channel. The pullback voltage during injection of cells into the separation channel was also maintained (Table 4.7). The cells were first injected into the separation channel with a pullback voltage applied at the same time for 2 s in step 2, following establishment of cell flow between the sample and sample waste wells (step 1). The two 5 s pauses where no voltages were applied (step 3, 5) were put in place so that images of the intersection could be captured prior to and following the pullback of cells away from the intersection in the short channel arms (step 4). These pictures were effective in showing any movement of the cells in the sample plug due to flow during the cell pullback. This was one method for monitoring flow, which is further described below in section 4.3. It was also determined that setting a constant time for separation was the best method because it was easier to use the instrumentation to control the time precisely rather than attempt to find a method for accurately determining the time of arrivals of the cells at a known detection point. The distances the cells travelled were read from a series of pictures taken near the detection point following separation. There did not appear to be any pressure driven flow during separation because the cells remained in the same locations in the separation channel several minutes following electrophoresis. This was determined by observing the cells following the removal of the separation current. Cells were observed for up to 5 minutes with no movement inside the channels following electrophoresis.

Table 4.7: Electrophoresis Protocol for Cell Mobility Determination Using TBE Buffer

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	130	Ground	Float	100	Float
2	2	120	Ground	120	1000
3	5	Float	Float	Float	Float
4	20	100	Ground	100	Float
5	5	Float	Float	Float	Float
6	600	Float	Ground	Float	1000

Types A1, B, and O cells were all tested using the TBE buffer. The results are summarized in Table 4.8. The velocities of the cells were compared with a 1000 V and a 500 V voltage application at the buffer waste well. At 500 V, the velocity of the cells was approximately half of that at 1000 V. This suggests that there is a proportional relationship between the velocities of the cells and that of the voltage applied where a higher voltage leads to a higher velocity. However, to determine all of the mechanisms at play, further investigation would be required.

The variations of the cell velocities between different loads and the different types were large enough that there was no statistical difference between the mobilities of the different blood types. There was also a large variation between the different loads of the cells of the same blood type. For example, type A1 cells ranged in average velocity of separate loads from 73.5 μm/s to 94.2 μm/s with a range of velocities of greater than 20 μm/s. Although variations in velocity within a population of cells were expected, the 20 μm/s difference appeared to be too large to be from inherent differences in cell moblilities alone. In Lu's report of cell ABO mobility differences observed in CE, the variation in cell mobility of one type did not exceed 1.4% [8]. Therefore, the 24% observed appeared have an additional cause apart from inherent variations of cell mobility within a population. Thus, further testing and adjustments were made to the protocol in an optimization attempt to improve reproducibility and reduce variation.

Table 4.8: Results of Initial Cell Velocity Testing in TBE Buffer

Cell Type	Load	Average Velocity (μm/s)	Standard Deviation (μm/s)	Number of Cells Tested
A1	1	81.9	8.6	6
A1	2	93.5	5.7	9
A1	3	92.0	4.3	13
A1	4	94.2	5.7	18
A1	5	73.5	6.3	11
A1	6	75.1	6.2	12
B	1	84.0	7.1	9
B	2	95.3	6.3	13
B	3	88.2	5.1	5
B	4	77.3	6.1	12
O	1	92.2	13.2	5
O	2	103.4	2.5	3
O	3	101.3	2.9	9
O	4	99.9	5.2	10
O	5	94.8	5.8	10
O	6	88.1	3.3	18

Attempts to determine the cause of the variations led to the realization that several factors affected the cell mobility. Throughout testing, it was determined that cell lysis may be observed during electrophoresis and was indicated by the cell becoming fainter in appearance. This was likely a result of cell membrane deterioration and loss of internal cell contents. The change in cell appearance was accompanied by a large decrease in the cell velocity and the eventual disappearance of the cell (lysis). Thus, the 24% variations seen above may be explained by the beginning of the cells lysis process, which dramatically altered the cell mobility. The charge of the RBCs is due to the sialic acid content on the cell membranes [17-20]. Thus any damage to the cell membrane leads to

changes to the cell surface morphology. This would in turn affect and alter cell charge, leading to changes in mobility during electrophoresis. It was determined from testing that membrane changes may be avoided by only working with cells that have not reached their expiry date. More specifically, cells that are less than three days from their expiry date were not used. The two to three day threshold was empirically determined by analyzing data on cells of various ages. It was found that cells aged between two to three days before their expiration date showed an increase in lysis. Cells were tracked and observed as they travelled down the separation channel. Any signs of colour change (becoming fainter) or slowing down of the cells were noted. The number of cells at the beginning and end of separation were counted to determine if any had lysed. The age of the reagents also played an important role. Increased RBC destruction was observed from virtually no lysis when fresh buffer was used to 80% or 90% when buffers approximately one month old were used. This was likely due to buffer chemical degradation over time. Buffers of different ages were tested on the same cell sample in one day. The number of cells during each run of 6 loads was counted immediately following injection and at the end of separation to determine the amount of lysis. It was found that buffers approximately one month old led to higher cell lysis whereas the buffers fresher than two or three weeks showed almost no lysis. Thus, buffers older than two weeks were not used to keep a larger safety margin and cell lysis was kept to a minimum, approximately less than 2%. The maintenance of buffer temperature was also important. The conductance of the buffer at 6°C was half that of the conductance at room temperature of 23°C, approximately 2.7 mS/cm compared to approximately 4.5 mS/cm). Thus, all working buffers were kept at room temperature for at least an hour following removal from the refrigerator to allow warm up to room temperature before use.

Several changes were also made to the electrophoresis protocol (Table 4.9). To increase the number of cells injected during each run, the injection time was changed back to 3 s and the cells were injected directly from the sample well again. The voltage used during the injection of the cells into the separation channel was also changed to match the electric field the cells experienced during electrophoresis to maintain consistency. The separation time was changed to 599 s from 600 s to maintain the same total time the cells spent in the separation channel following initial injection into the

channel. This enabled comparison between the different electrophoresis protocols tested without introducing the additional variation of a difference in the electrophoresis time.

Table 4.9: Modified Electrophoresis Protocol to Reduce Variation in Cell Velocity

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	130	Ground	Float	100	Float
2	3	Ground	Float	Float	938
3	5	Float	Float	Float	Float
4	20	100	Ground	100	Float
5	544	Float	Float	Float	Float
6	599	Float	Ground	Float	1000

Once the above changes in electrophoresis and reagent handling were met, there was a significant improvement in the results obtained. A sample of the results obtained is listed in Table 4.10. The range of variation of the average velocity is less than 10 μm/s, decreasing by half from the previous velocity of 20 μm/s. The standard deviations were also reduced to smaller than 6 μm/s, and became more consistent. Similar cell velocities for each type were obtained consistently during testing on different days as well as when different lots of the sample cells were used. Thus, a stable and reproducible protocol was established.

Table 4.10: Results with Optimized Electrophoresis Protocol

Cell Type	Load	Average Velocity (μm/s)	Standard Deviation (μm/s)	Number of Cells Tested
A1	1	77.6	4.3	46
A1	2	74.4	4.2	51
A1	3	80.5	5.0	42
B	1	78.5	5.3	69
B	2	77.8	3.6	67
B	3	75.2	2.7	38
O	1	81.8	4.4	37
O	2	83.6	5.7	67
O	3	82.4	4.5	82

This reproducible protocol for determining cell velocity was a unique method developed here. To the best of our knowledge, a method of determining cell mobility for on-chip ABO cell separation has not been reported elsewhere. It allows for the comparison of RBC velocities during electrophoresis with a variation of no more than 2% from EOF and pressure-driven flow on-chip. This method may potentially be extended to cells other than RBCs. If the mobility differences of two groups of cells are larger than 2%, then this method may be used for their electrophoretic separation based on mobility alone.

4.3 On-Chip Flow

In order to accurately determine the movement of RBCs during electrophoresis, a measure of the flow in the chip was critical. Initial problems with pressure driven flow were observed. This was observed in the injections arms where flow of the cells was observed when no electric fields were applied. The flow tended towards one direction and was found to be a result of the chip not being level. The cause of the imbalance was discovered to be the electrodes in the wells pushing down on the chip with uneven forces

due to slight variations in lengths. This was resolved by ensuring that both the chip was level and that no electrodes were pushing down on the chip. This required the proper balance between the volume of liquid in the wells and the depth that the electrodes were lowered. The electrodes had to be lowered far enough that the evaporation of the buffer or sample did not cause the electrodes to lose contact with the chip. However, care had to be taken to ensure that the electrodes did not touch the bottom of the chip to prevent any unwanted pressure. Once these were achieved, the pressure flow was almost complete removed.

The other contributor to flow on-chip was EOF. An accurate method for determining the EOF had to be established to ensure that the results obtained between different loads of testing could be compared. This is particularly important when comparing cell velocity between different runs or loads. The method for determining on-chip EOF that is most often used is the use of a fluorescent neutral dye in the channel during electrophoresis of the analyte [21]. The main problem with using this method was that it was difficult to determine how the various dyes would interact with the RBCs. They may bind and affect the surface charge of the RBCs. Depending on how the the dyes interact with the RBCs, the degree of interaction between the different blood types may also differ, therefore affecting them and their velocities differently. The velocity of the cells may be indirectly affected by any alterations the dye may have on the cell membrane and charge. Although a dye could be used separately from the RBCs, it would not be able to determine the EOF during the specific run with the RBCs present. Omasu *et al.* [2] used neutral beads with the RBCs during electrophoresis to determine EOF. However, extensive searches were not able to find the neutral beads used by Omasu *et al.* or a potential replacement. The main issue was that very few manufacturers make beads that are neutral. Beads that were available were not guaranteed to maintain a constant charge during electrophoresis. This would have defeated the purpose of using the beads. The charge of beads was also often only specified for certain buffers or media and that did not match the buffers used in our experiments. Thus, an alternative to neutral beads and dyes needed to be found.

It was decided that a calibration that most closely resembled the conditions the RBCs were exposed to would be the best in determining the amount of flow the cells

were exposed to. Instead of using a fluorescent dye, DNA would be used to measure the movement. Since work with DNA and the behaviour of the size standard GS 500 (Applied Biosystems, Streetsville ON) had already been well established through previous work, it was decided that this would be a suitable sample for use. The chip for the calibrations were loaded with 2.7 μL of running buffer with 0.3 μL of the GS 500 size standard while the remainder of the wells were loaded with 3 μL of running buffer as was done during calibrations for DNA work. The chip was then electrophoresed according to the same electrophoresis protocol used for the RBCs (Table 4.9). The detection point was situated at 40 mm, sufficient to observe all the size ladder peaks. The arrival time of the peak corresponding to 200 bp was noted during each run of the calibration (Fig. 4.4). Change in velocity between the first and second calibration runs for the DNA was calculated and assumed to be due to EOF since in the absence of EOF the arrival times should be identical. EOF data from the three days were compared, and it was found that there was a maximum velocity variation of 1.9% when runs within a load were compared. When the different loads were compared, the maximum velocity variation was 2.4%. These variations appeared randomly, but within a particular range and did not fluctuate from this range during the three days of data gathering. There also did not appear to be any notable patterns in terms of increasing and decreasing EOF. Thus, a similar velocity variation would apply to the RBCs during mobility determination where the maximum variation due to EOF would not exceed 2.4%.

Changes in chip were negligible because continuous calibrations were done throughout a day and found that the chip did not change for 16 consecutive runs. Fig. 4.4 is a representative figure of the calibrations done in that day. The amount of variation in flow between two consecutive days was similar to those observed within one day of only calibrations runs.

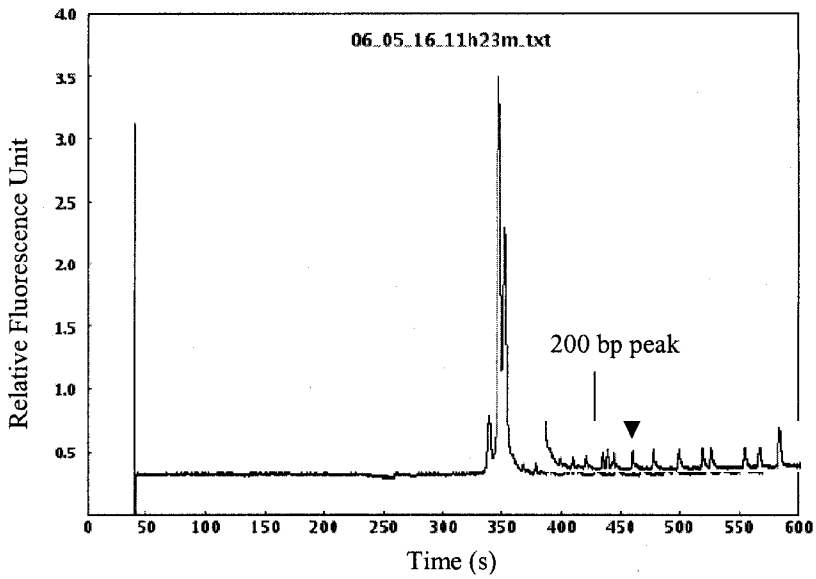
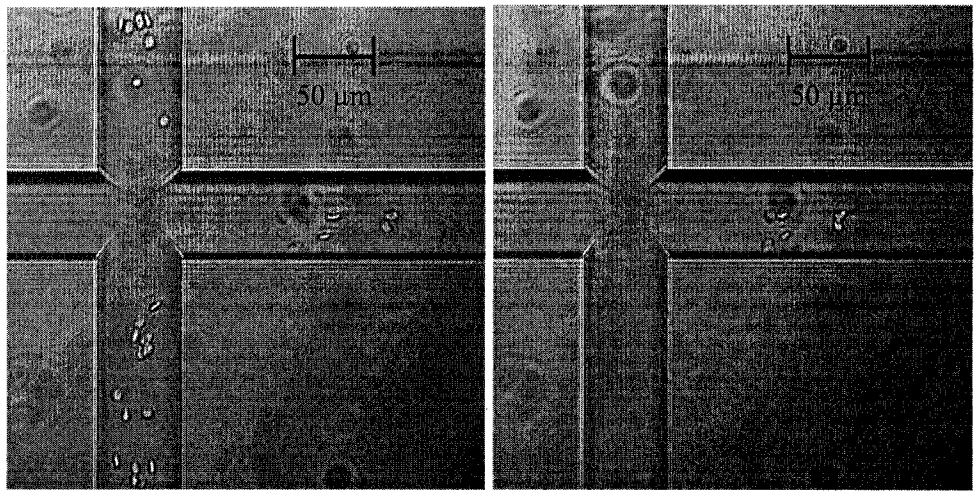


Fig. 4.4: Sample electropherogram of calibration test prior to testing of RBC mobility

Cell movement during the pullback step was also observed since ideally the cells should not move at all. Any movement during this time within the separation channel would alter their initial starting position, and increase variation in the results obtained. It was noted that the cells in the plug did exhibit some movement back toward the intersection during the pullback step. However, this was small where less than a maximum of 200 μm of movement was observed (Fig. 4.5). Thus, this did not contribute much to the overall flow in the chip and only contributed to a maximum of approximately 0.5% of the overall variation in the cell velocity results when Equation 4.2 is applied. Monitoring of this flow was done because it may be indicative of the overall flow effects on the chip. The exact correlation of this flow to EOF during separation is uncertain, but a larger flow during the pullback would suggest that there is more unintentional movement during separation. Thus, the monitoring of the flow of the cells during injection also served as an indicator that the chip performance may be changing or degrading. Subsequent calibrations may then be done to determine whether the chip required rejuvenation.



(a) (b)

Fig. 4.5: Sample plug cell movement prior to (a) and following (b) pullback step of electrophoresis protocol

Once the cells moved into the separation channel approximately no movement due to flow was observed. This was determined by removing the electric field and stopping the cells at various locations along the channel to observe the cell movement. No movement in the cells were observed for several minutes. This allowed sufficient time for the data gathering to occur. Thus, cell movement to following electrophoresis did not introduce any additional variations in velocity.

Prior to data gathering, calibrations were done between loads in addition to only at the beginning of day of testing to determine if there were any changes in EOF between loads on the same day. The variations due to EOF were similar to those observed with calibrations done at the beginning of the day and did not exceed the 2.4% maximum stated above. Thus, calibrations were only done at the beginning of each day of testing during data gathering to reduce the time consumption. If the difference between the calibrations of various days did not exceed approximately 2.5%, then the chip performance was considered adequate and no chip rejuvenations were required.

4.4 RBC Mobility Differences

4.4.1 Mobility Based ABO Testing

The main goal of establishing a reliable electrophoresis protocol for determining the mobility of the RBCs was to determine whether mobility differences between the ABO blood types could be observed on-chip. This may then lead to typing of the ABO group based on mobility differences of the RBCs. The conditions that would lead to variations in results such as flow were noted and quantified. Thus, the developed protocol described in section 4.2.3 was used to test the mobility of types A1, B, and O RBCs. The results of the RBC mobilities were determined from three days of consecutive testing and data gathering. Three consecutive days of testing was used to minimize any changes to chip performance or sample and reagent aging. Data were gathered for each blood type over three days to allow for a sufficient quantity for analysis and minmize any bias introduced by the particular day.

A plot of the data of the velocities of the three blood types showed that there was a large amount of overlap between the three blood types and that none of the types showed a distinct velocity peak profile from the other two (Fig. 4.6). However, the type O cells appeared to be faster than the A1 and B cells whereas the velocities of the A1 and B cells were very similar. The higher velocity suggests the O RBCs were more strongly attracted to the anode, thus were likely more negatively charged than the A1 and B RBCs. This may have been a result of the lack of A and B antigens on the surface of the O cells. The more negative charge may be due to the sialic acid content or their position on the O RBC surface that differs from types A1 and B. The sialic acid gives the cell its negative charge and is found bound to the glycoproteins and glycolipids on the cell membranes. The acid's distribution between the two vary for the different blood types of the ABO group [17]. Thus, the presence of the A and B antigens may be affecting the binding and/or distribution of the acid on the membrane, thus affecting the charge the cells.

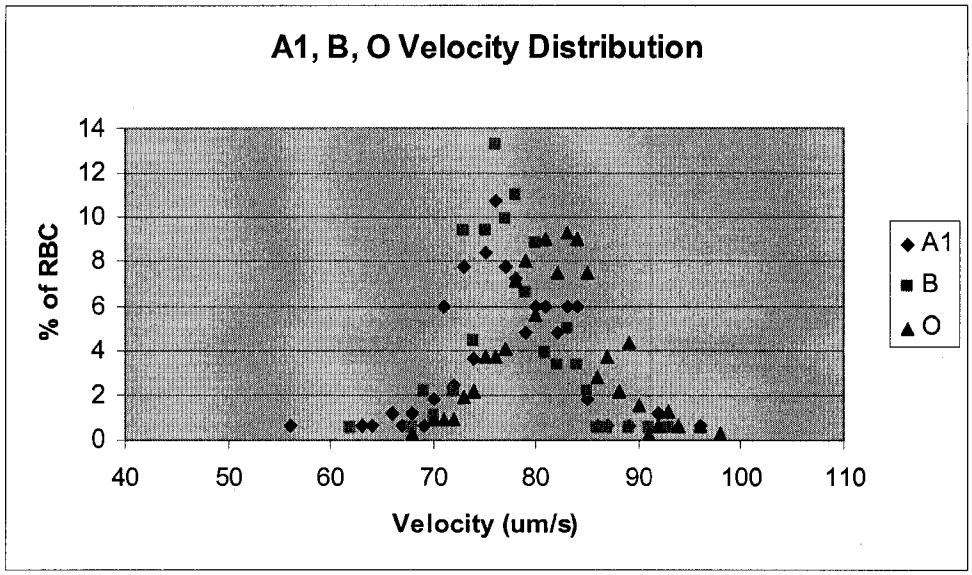


Fig. 4.6: Plot of velocity distributions of types A1, B, and O RBCs

Although no distinct differences were seen between the three groups of cells velocities, tests were done to determine whether there was actually any difference between the three groups of velocities. The distributions for each of the individual types were first tested for normal distributions by plotting a probability-probability (PP) plot of velocities of each blood type using the data shown in Fig. 4.6 and Table 4.10. These plots were done using Statistical Package for the Social Sciences (SPSS) software (Chicago, IL) and found to be linear, indicating the velocities were normally distributed. The t-test was then performed ($P < 0.05$) to determine whether there was a significant difference between the three groups of velocities. While types A1 and B showed no significant differences from each other, a comparison of types B and O cells as well as A and O cells both showed that both types B and A had velocities that were significantly different from type O. Although these differences were seen, mobility shifts were not large enough to show a distinct separation of the velocity distribution of the A and B from O cells. The separation between the three velocity distributions was also less than two standard deviations of separation. Thus, the velocity differences between the three cell types were too small to be used for distinguishing the different cell types during electrophoresis.

In an effort to determine if the electrophoresis voltage used and the time for electrophoresis did not significantly alter the results, tests were done with both a lower

field as well as a shorter electrophoresis time. It was believed that the shorter time would not improve separation of the different blood types since this would mean a shorter separation time. The reduction in electrophoretic voltage, however, may improve separation to a small degree. It was believed that the reduction in voltage reduced the force and stress on the cell membrane to further minimize any damage to the membrane that may occur during the long electrophoresis time. This was based on the fact that there is less force acting on the cell, supported by smaller shape changes or stretching of the cell observed under the lower electric field. The lower speeds at which the cells travel through the polymer also means a gentler passage through the pores of the sieving matrix, again reducing potential damage to the cell membranes. Reduced changes in the membrane may lead to less variation in charge of the cells and therefore, less velocity variation. Cell lysis during electrophoresis was not a problem because all the cells that were injected in the sample plugs were detected at the end of the run for all runs during data gathering. The electrophoresis protocol was first altered from Table 4.9 by changing the voltage in step 6 to 500 V from 1000 V. The results obtained are summarized in Table 4.11 and reflect a reduction in velocity to approximately half of what was observed when 1000 V were used. The standard deviations also reduced by approximately half or slightly more than half from those seen with 1000 V. This suggests that the cell velocity is proportional to the current or voltage applied during electrophoresis as earlier observed with the citrate buffer. The type O cells appeared once again as the fastest cells whereas the A1 and B cell were the most similar in velocity. The A2 cells were found to be slower than the O cells, but faster than the A1 and B cells. The differences were not large enough, as seen with the amount of overlap of velocities between cell types, to be used along to separate the four types from each other. Thus, the change to 500 V for the separation voltage did help to improve slightly separation of the different types in the ABO group as expected, but not to a degree that this method for mobility measurement could be used for on-chip ABO typing.

Table 4.11: Velocity of A1, A2, B, and O RBC with Electrophoretic Voltage of 500 V

Cell Type	Load	Average Velocity (μm/s)	Standard Deviation (μm/s)	Number of Cells Tested
A1	1	40.8	1.6	65
A1	2	38.2	1.5	55
B	1	38.6	1.9	23
B	2	39.6	1.8	47
O	1	44.0	1.3	37
O	2	40.5	1.2	57
A2	1	42.0	1.2	65
A2	2	39.4	1.0	66

When a shorter electrophoresis time of 400 s was tested, the velocities were found to be comparable to when the longer time of 600 s was used. However, none of the types were significantly different from each other. The standard deviations were similar to those obtained when using the longer time. This suggests that there were no significant variations in the velocities of the cells, but the shorter separation time also made it more difficult to decipher between the types. Thus, the separation time of 600 s was more adequate.

4.4.2 Individual Versus Mulitple Donor Testing

In an attempt to determine whether the type of sample used affected the velocity distributions of the different cells in the ABO group, tests were conducted using Biotest reagent cells (Denville, NJ). The Immucor cells that had been used comprised of 6 to 40 donors per type. This potentially introduced a larger variation in the velocity distribution compared to if only one donor was used. The Biotest cells were obtained from only one donor. Thus, a comparison of the results with those previously obtained (Fig. 4.10) would help to determine the contribution of the variability due to variation among individuals.

The same method was used to test the Biotest cells as those used for the initial Immucor cells (Table 4.9). The Biotest cells were found to have smaller standard deviations compared to when pooled donor cells were tested under 1000 V (Table 4.12). The standard deviations of the pooled donor cells varied between 2.7 μm/s and 5.7 μm/s while the standard deviation of individual donor cells varied between 1.9 μm/s and 3.4 μm/s. This supports the idea that the larger variation in cell velocity of the pooled donors was in part due to population variation. However, the variation in the population among different individuals does exist and any method used for typing must be able to accommodate this variation.

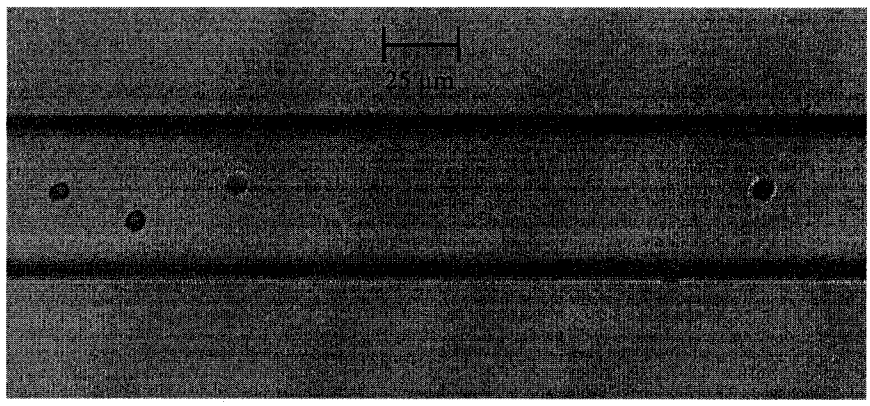
Table 4.12: Velocity of Single Donor A1, B, and O RBC

Cell Type	Load	Average Velocity (μm/s)	Standard Deviation (μm/s)	Number of Cells Tested
A1	1	87.9	3.4	32
B	2	85.1	2.6	27
O	1	86.5	1.9	24

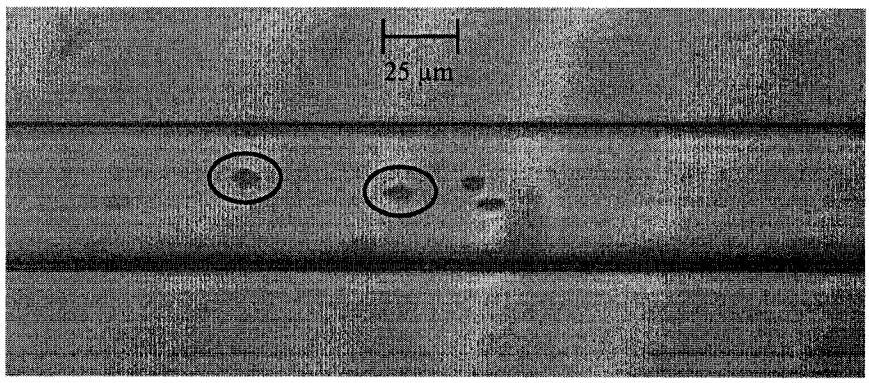
4.5 Electrophoresis and Cell Shape

During the course of testing of the movement of cells in electrophoresis, several interesting phenomena were observed. The first was the cell shape in the electric field. The cells, being normally biconcave in shape, exhibited shape changes during the application of an electric field. Although not all, many cells exhibited a shape change from round to a more oval (top view) one when an electric field was applied (Fig. 4.7). The cells changed from the round or flat disk like shape, depending on the observed view, to a more elongated shape that resembled a "torpedo". This change in shape was not necessarily associated with a change in velocity. At times, the torpedo-shaped cells moved faster than those that did not exhibit much change in shape. Other times though, it was the cell that did not change shape that moved faster than those in the torpedo shape. This suggests that the cell mobility is dependent on the charge of the cell, which is not

necessarily altered by the shape of the cell. The change in shape was likely due to the cell responding to a force applied on it. Required to move through tiny capillaries in the human body, the RBCs are extremely deformable. Thus, an applied force on the cell would likely be able to stretch the cell, resulting in the change in shape. Any physical changes to the cell membrane during electrophoresis may also alter their deformability and result in a change in shape back to the round or bi-concave shape. Thus, although the force during electrophoresis may be constant as expected, the cell may still change shape due to physical changes in its membrane that may have been brought on by electrophoresis. This may be an area for further investigation in the future that could provide insight toward controlling cell movement through changes in shape.



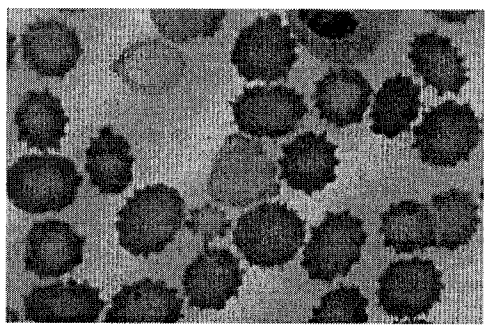
(a)



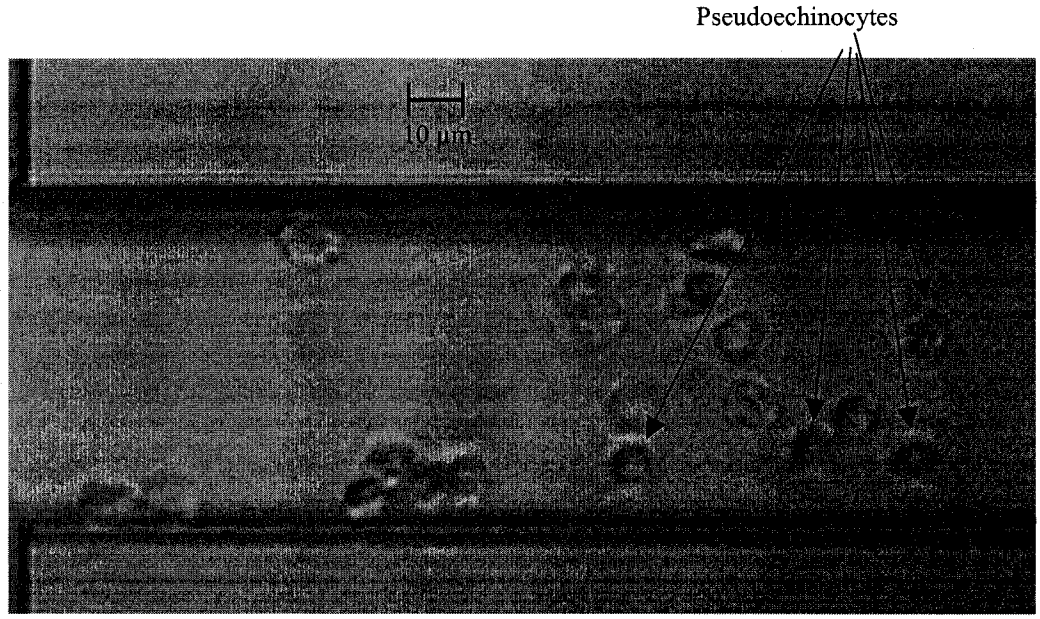
(b)

Fig. 4.7: Shape of RBC under no (a) electric field and cell shape during electrophoresis under an applied electric field during separation according to Table 4.9 (b)

Another change in the cell shape that was observed was the appearance of jagged edges of the cell member, similar to those seen on echinocytes (Fig.4.8). These "pseudoechinocytes" were observed mainly between the injection and separation phases of electrophoresis. They may have been a result of alterations in the cell membrane due to the application of an electric field. Normally, echinocyte formation may occur due to a variety of different conditions such as dehydration, energy depletion, increases in pH, and electrolyte imbalances. Thus, it is conceivable that the presence of an electric field may have temporarily affected the RBC in such a way that induced an electrolyte imbalance, which led to temporary echinocyte formation. The spikes on the cell membrane did not immediately disappear following the removal of the electric field since some were observed to be in this shape after the field removal. This suggested that the change may more permanent than a mere stretching of the cells. Alterations may have been happening to the cell membrane that would eventually lead to permanent changes in the membrane structure or integrity since the time it took the cells to regain normal shape also varied. Some of the pseudoechinocyte changed back after a time of approximately 20 s or less while others took closer to 1 minute or more. The cells tended to take longer to regain shape at the end of the electrophoretic run than at the beginning. If the changes were due to electrolyte imbalance then it may be that the imbalance was larger at the end of electrophoresis and it took a longer time to regain balance.



(a)



(b)

Fig. 4.8: Image of echinocytes taken from www.godenvetlab.co.za/photo_gallery.htm (a) and picture of "pseudoechinocytes" or RBCs following injection (b). Clearer images were not obtained since the pseudoechinocytes were most often observed between the injection and separation phases of electrophoresis where less than ample time was available to capture a clearer image. Their existence was also not predictable enough to be readily reproduced that before and after images could captured to better illustrate the change in the cells.

Another possibility is that the change in shape may be another type of physiological response. This was suggested by the fact that the cells displayed the change into pseudoechinocytes more readily at the beginning of electrophoresis during initial sample plug injection. Approximately 90% of the pseudoechinocytes observed were seen following the initial plug injection as shown in Fig. 4.8b. The change back to the normal cell shapes also occurred more readily following initial injections of the cells into the separation channel than at the end of separation during electrophoresis. This may be due to the fact that the cells are essentially alive when first introduced into the chip and changes in shape due to a physiological response occurred more readily rather than a purely physical response. The faster response of the cells to the application and removal of electric fields may be due to their ability to respond more readily to the electric field at the beginning of the electrophoresis than they are at the end. On occasion, the cells changed shape during electrophoresis. However, this was only a rare occurrence and may also be explained by the same mechanisms already mentioned. A similar

physiological change may also be occurring when the torpedo-shaped cells were observed. However, the almost instantaneous response of the cells to the electric field when changing between biconcave and torpedo shapes corresponding to application and removal of the field suggests that this response is more physical than physiological.

4.6 Conclusions

One purpose of the work in this chapter was to determine RBC movement in an electric field and establish reliable control of that movement on-chip. This began with testing of RBC movement on the μTK. The RBCs were found to be very responsive to the applied electric field. When the optimized protocol was used, the cells were negatively charged and moved toward the anode when the field was applied and stopped when the field was removed. The detection method using the μTK, however, was not ideal for the electrophoresis of the cells in the tested chips. The diameter of the cells and the laser spot diameter being both approximately 1/5 the width of the channels meant that the cells could travel down the channel past the detection point without passing through the laser spot. Although the scattered light from the cell could be used for the detection of the cells, not all cells were detected. This meant that data points could be missed, making the LIF detection using the μTK unreliable. Thus, the move for detection on the Axiovert microscope was made. A reliable protocol for measuring the mobility of the cells was achieved using TBE buffer due to its better buffering capabilities that enabled longer sustained electrophoresis over the citrate buffer, despite the fact that the cells moved readily in both.

During the protocol development, the issue of flow in the chip had to be overcome. Slight pressure driven flows were present during near the injection arms due largely to the electrodes pushing down on the chip. Adequate EOF monitoring was also required to minimize uncertainty error when comparing results obtained from different loads of the chip. An appropriate measurement of EOF was developed using the standard GS-500 size ladder to calibrate the chip during testing to ensure no significant EOF was present. Overall, approximately less than 3 % of the uncertainty in the results was due to flow.

Once reliable RBC movement on-chip was established, extended testing and modifications were made to achieve a protocol that could be used reliably for accurate determination of the cell velocities. The development of the protocol was an attempt to establish a method for potentially typing the ABO blood group on-chip as previously reported by Lu *et al.* using CE. Much effort was put into establishing a reliable protocol that had the minimum uncertainties possible when determining the cell velocity. Once this protocol was achieved, testing was done to determine if mobility shifts of the different cell types of the ABO group could be used to distinguish each type from one another. It was found that although the type O cells were generally faster than the type A1 and B cells, the quantity of overlap of the three velocity distributions was too great for the method to be used for the accurate on-chip typing of the ABO group.

It must be noted that the samples used in our tests were from pooled donors containing 6 to 40 different individuals that would broaden the velocity distribution, making it more difficult to separate the different blood types. Although the Biotest cells with one donor per type did display a narrower distribution than when the pooled samples were tested, they still did not show large enough mobility differences that they could be used for typing. A longer separation distance may have been able to produce better separation of the three types tested. A longer separation time would enable the cells to travel farther. This increase in separation distance would further reduce any uncertainties associated with minor variations in cell starting position and movement during pullback as previously mentioned. The inherent variation associated with the microchip and platform used here was found to be approximately 3%. This is larger than the 2% variation that was required to distinguish between the ABO types reported by Lu *et al.* Thus, it is not surprising that adequate mobility differences could not be detected using our method. Lu *et al.* also only used six individuals per blood type during testing. It may be that these single donor samples coupled with the much longer separation distance with CE led to their ability to distinguish the ABO blood types. Nonetheless, if mobility differences were to be use to type the ABO blood group, a method to enhance mobility differences would have to be used. This means the use of an antibody to bind to the RBC or the use of an alternative method for altering cell mobilities and enhancing the differences between different types.

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Chapter 5

Isoelectric Focusing

5.1 Introduction

In addition to the commonly used capillary zone electrophoresis, other methods or variations of electrophoresis also exist. These include methods such 2-D polyacrylamide gel electrophoresis (2-D PAGE), isotachophoresis [1-3], and isoelectric focusing [4-8]. Among these different separation methods, isoelectric focusing is an important method of electrophoretic separation that is not only used alone, but is also combined with other methods such as in 2-D PAGE.

Isoelectric focusing (IEF) is based on the ability to alter the charge of analytes by varying the pH of their suspension solution. The charge of analytes may be shifted from positive to negative by increasing the pH of the solution. The pH value at which the analyte is neutral in charge is the isoelectric point (pI) of the analyte [9]. At pH levels below the pI, the analyte is positively charged while values above the pI render the analyte negative. IEF uses the fact that different analytes have different pI values as the basis for separation. Ampholites, which small molecules with both acid and base groups, are first electrophoresed through the sieving matrix to establish a pH gradient. The analytes are then electrophoresed as usual. Each analyte will be charged and continue to move along the separation medium until they reach the pH that matches their pI. At this location the analyte becomes neutral in charge and will no longer migrate under an electric field. Thus, different analytes will stop at different locations along a separation medium with a pH gradient and be separated. In the case of capillary IEF, the method offers one of the highest resolving powers among electrophoretic methods. Currently,

applications of capillary IEF include antibody characterization [10], analysis of hemoglobin [11, 12] and various proteins [13-17], and physiological fluid analysis [18, 19]. Microchip-based IEF for DNA and protein analysis and the development of on-chip 2-D PAGE have also been achieved [6, 20].

Since different pI values have been found for proteins and used in their separation through IEF, it was possible that RBCs and the antibodies would also have different pIs. Upon investigation, RBCs were shown in existing literature to have a pI of approximately 3.5 [21] while IgG and IgM antibodies have pIs between 4.25 to 9.95 and 5.5 to 6.7 respectively [22]. If an appropriate pH could be established between the pI of the RBCs and antibodies where the two are of opposite charge, then the application of an electric field would lead them to move in opposite directions. This would allow for an elegant method of separation that is extremely useful in the separation of RBCs from antibodies and possibly other proteins when working with whole blood. The isolation of either cells or antibodies would be useful in a variety of situations such as the isolation of RBCs for blood typing, the isolation of white blood cells for analysis and PCR, and the detection of chemicals or components of interest in blood plasma. Work was done here to first establish the pI of the antibodies of interest, those associated with blood typing. The feasibility of pH adjustment of the buffer to render antibodies and RBCs of opposite charge was then investigated. Optical observation of antibody movement during electrophoresis was also conducted here for a better understanding of their movement.

5.2 Isoelectric Point Determination

In order to potentially use the isoelectric points of the cells and antibodies for control of their movement, possibly for separation, the determination of their isoelectric points needs to be achieved. This will not only be used in determining how the movement of the analytes may be controlled, but also whether or not there is a sufficient difference between the isoelectric points of red blood cells and antibodies in whole blood to separate the two based on charge differences. Literature has reported the pI of red blood cells to be approximately 3.5. However, the pI of IgG and IgM antibodies did not have specific values, but may vary. Thus, the pI of the antibodies used in blood typing

needed first to be experimentally determined to narrow the range of possible pH values before proceeding to determine whether a pH value could be found for RBC and antibody separation.

5.2.1 Materials and Methods

The determination of the pI for anti-human antibodies used in Chapter 3 was achieved by using a commercially available Mini-PROTEAN 2-D electrophoresis kit obtained from Bio-Rad (Hercules, CA, USA)[1]. This kit enables IEF in microcapillaries. IEF protein standards from Bio-Rad with known isoelectric points (catalog number 161-0310) in the range between 4.45 and 9.6 were used during IEF to determine the pI of the unknown proteins. The IEF was done using polyacrylamide gels prepared according to the instruction manual of the Mini-PROTEAN 2-D kit. The reagents used were purchased from EMD Chemicals (NJ, USA). In the initial experiments, the Cy3 conjugated monoclonal anti-human IgG developed in goat from Sigma (St. Louis, MO) was used to give a representation of what the antibody pIs would be in serum. The IgG was chosen also due to the fact that it is fluorescently labelled and may be used with the LIF with the μTK for detection. This is important in determining whether the charge assignment was achieved or not when separation from blood cells is attempted based on different polarity of charge. An additional Novaclone monoclonal anti-A antibody from Dominion Biologicals (Dartmouth, NS, Canada) was also used to help estimate the potential pI range of serum antibodies and to determine the adequate antibody concentration required for IEF. Once the protocol was established and the pI of the fluorescent antibody established, the pIs of anti-B and anti-D antisera were also determined using IEF. This would determine whether or not one buffer alone with a specific pH could be used to render the anti-A, -B, and –D antibodies required for ABO and Rh typing a charge with different polarity than the RBCs to enable electrophoretic separation based on charge difference. The Alexa Fluor 546 labelled polyclonal anti-human IgG antibody was obtained from Molecular Probes (Eugene, OR) and tested to obtain information about whether the variation among individuals would affect the

[1] Sample preparation and testing for the determination of pI values were achieved collaboratively with Amanda Dahlseide, project student to Dr. Jason Acker at the Canadian Blood Services.

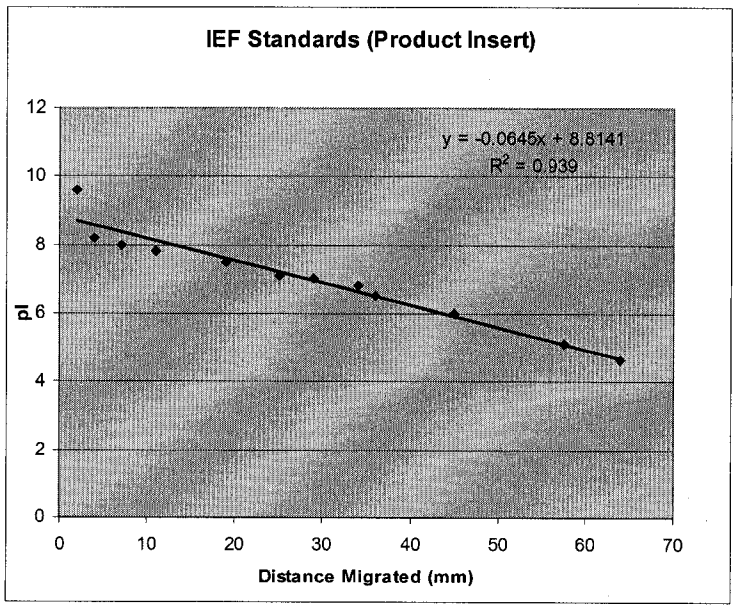
required buffer pH. A fluorescent bovine serum albumin (BSA) from Molecular Probes (Eugene, OR) and a non-labelled BSA (Sigma-Aldrich, St. Louis, MO, USA) were also tested to mimic the effect of serum proteins. All samples were stained with Coomassie blue following a standard protocol [23] for detection after IEF was completed.

Isoelectric focusing of analytes was conducted as follows. A pre-electrophoresis step was first achieved to establish a pH gradient in the microcapillaries. This was achieved by running the gels for 10 min at 200 V with the two ampholytes, Biolyte 5/7 and Biolyte 3/10 from Bio-Rad to establish a pH gradient. Once the gradient was established, the gels were loaded with 10 μl of sample and electrophoresed at 500 V for 10 minutes. The gel samples were then electrophoresed at 760 V for a final separation time. This separation time was varied between 3.5, 4.0, 4.5 and 5.0 hours during four separate trials to determine which gave sufficient time for the determination of the pIs. It was found that 4.0 hours gave the best results in that it allowed for the best separation of the analytes in the gel for analysis with the shortest time. Thus, 4 hours was used for all the experiments. In the case of the anti-human IgG antibodies, 500 ng of antibody gave the clearest banding and was used for the experiments. For the anti-A antibodies, due to the fact the manufacturer does not give out specific concentration details, the antibodies were diluted to various concentrations of 1/20, 1/25, 1/33.3, 1/50 and 1/100 to determine which would be the best for IEF. The same dilution may be used for the other antisera based on the assumption that they are similar in concentration. This is so because the tube test for blood typing is conducted with the standard quantity of antisera to blood for both the forward and reverse tests. The different antisera need to have the same or very similar concentrations to maintain consistency. It was found that the 1/33.3 dilution gave the most consistent results in terms of visible, distinct bands, and this was the concentration used for the other types of antibodies as well.

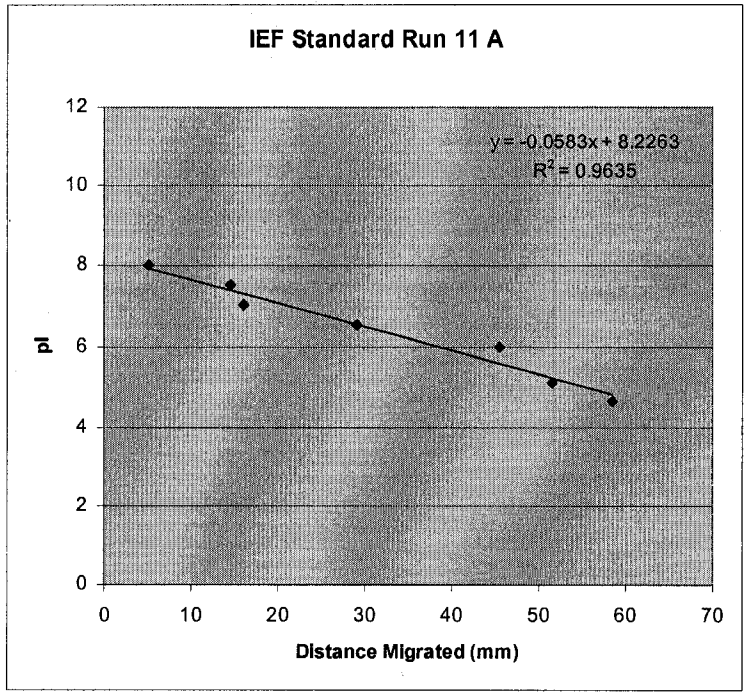
5.2.2 Results of Antibody pI Determination Using IEF

The data for the IEF of the anti-human IgG was gathered on three consecutive days. Each day, three runs of the antibodies were conducted where each run consisted of six gels. Similarly, six gels containing the protein standards were also run in parallel. The three clearest gels for each of the protein standard and the antibodies were selected

and anlayzed. The pIs of the total nine results were averaged to give a final pI value for the IgG antibodies. Standard curves were constructed using the distances the protein bands travelled in millimetres and the known pI of the protein standards provided in the product insert. The distances obtained experimentally were matched with the distances shown in the product insert to determine the corresponding pI values. Although not all the proteins were observed due to lower resolution of the microcapillaries compared to the standard slab gels used by the manufacturer, a standard curve could be plotted using the observed bands. The curve of distance migrated versus pI values for the experimental results (Fig. 5.1b) were compared to that stated in the product insert (Fig. 5.1a). Both showed linear curves as expected with R^2 values of 0.9635 and 0.939 respectively. The experimental and standard curves also matched closely in slope with values of -0.0583 mm^{-1} and -0.0645 mm^{-1} respectively. This suggests that the pI values obtained using microcapillary IEF were correctly matched to the proteins. Thus, if protein standards were run with each set of gels, then pI values of the test proteins may be obtained by comparison to the standards' curve.



(a)

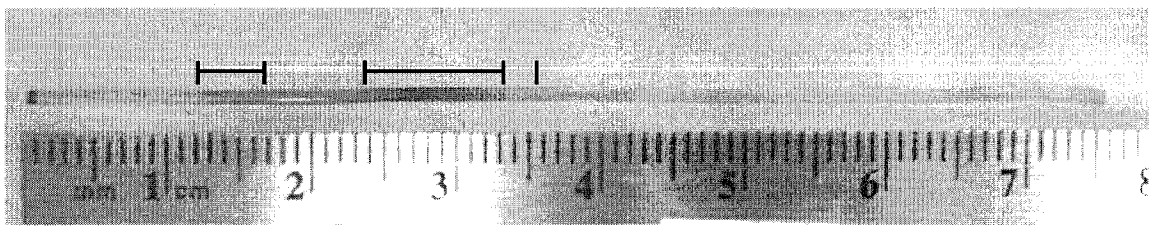


(b)

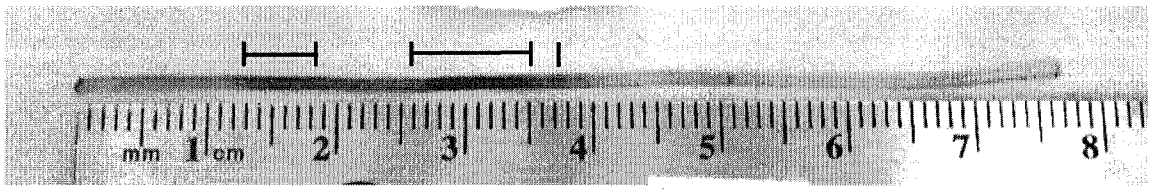
Fig. 5.1 Plot of pI and distance travelled for protein standards using information obtained from product insert (a), and experimental results (b)

Standard curves were constructed for each run and the points were based on the average of the three best gels chosen. A linear line was determined from the standard graphs using Excel and used to determine the pI of the antibody for that particular run.

Initial testing with the anti-A and the anti-human IgG showed both had three bands (Fig. 5.2). This suggested three populations of proteins each with a different pI. This is not unusual due to inherent population variations present within each of the two types of antibodies. These populations may have arisen from fragments of the antibodies that may be present in the solution. The presence of other small quantities of serum protein (as suggested by the manufacture) may also be contributing to the multiple peaks seen. From the gels, the distances both antibodies travelled are very similar, suggesting that the pIs are also very similar. The anti-human IgG band showed slightly farther migration down the gel than the anti-A (left to right), indicating a lower pI since the IEF was conducted from a high pH to a lower one. Thus, the use of the IgG as an approximate standard was acceptable since it would provide a lower limit.



(a)



(b)

Fig. 5.2 Micro gels of anti-A antisera (a) and anti-human IgG (b)

The overall average pIs of all three bands of the anti-human IgG were calculated. The pH of both the beginning and the end of the bands were calculated according to their corresponding proteins standards. The three bands seen had different pI values at the beginning and end of each band with the lowest pI being 5.9 (Fig. 5.3). The pH corresponding to the beginning and end of each band was calculated based on their distances from the beginning of the gel. The last band was small enough that it had the same pH for the beginning as well as the end. The results from three runs were averaged with the resulting pI values shown in Fig. 5.3. It appears that if the IgG antibodies are in a buffer with a pH of higher than 5.9, then the antibodies should be negatively charged while a pH lower than 5.9 would render them positively charged.

Fig. 5.3 Diagram representation of anti-human IgG with the average pIs of the three bands

IEF experiments were then done for the anti-A, -B, and –D antibodies as well as the polyclonal IgG and the BSA. The BSA was tested because it may play a role when attempting to separate the polyclonal antibody from other analytes. The BSA may also

have an effect on the pIs determined for the monoclonal IgG antibodies because their stock solutions contained 1% BSA as preservative. Thus, the pI and subsequent charge of the BSA was of interest. The proteins standards were run with all the samples and the isoelectric points for the pIs were determined where the lowest pH for each band was used as the pI for that specific band. All of the bands observed are summarized in Table 5.1. The pI values were all within the expected range for antibodies reported by literature above of 4.25 to 9.95 [24]. The two BSA samples only had one single band. Overall, the lowest pI value was 5.4. Thus, theoretically a buffer with a pH lower than this pI would render the antibodies and proteins listed in Table 5.1 positively charged.

Table 5.1 Isoelectric Points of Anti-A, -B, -D, Polyclonal, and BSA

Antibody/Protein	pI		
	Band 1	Band 2	Band 3
Anti-A	6	5.8	5.4
Anti-B	5.9	5.6	5.1
Anti-D	6	5.9	5.4
Polyclonal	-	6.1	5.9
Fluorescent BSA	-	-	5.5
Non-labelled BSA	-	-	6.3

5.3 Antibody Charge Determination

Once the pI of the antibodies and proteins were determined, attempts were made to see if the charge of the antibodies of interest could be controlled. Citrate buffer was used for testing due to the fact that it has several different pKa values of 3.13, 4.76, and 6.40 at 25 °C [25]. The pKa value is defined as the negative log of the acid dissociation constant Ka, which measures the ability of an organic compound to donate protons. Effectively, the buffering capacity of a buffer is highest at a pH matching its pKa. The buffering capacity of a buffer is its ability to counteract the effects (maintain pH) of electrolysis where dissociated ions OH^- and H^+ begin to alter pH of the electrophoresis medium. Buffers are usually comprised of a weak acid and its conjugate base or less

commonly a weak base and its conjugate acid. The addition of weak acids and bases to a solution does not significantly alter the pH of that solution. The buffers act to absorb the strong bases or acids (OH^- and H^+) introduced into a solution by reacting its weak base with the strong acid or its weak acid with the strong base. In this reaction, either the weak acids or weak bases are released into the solution respectively. However, since weak acids and bases do not significantly alter the pH of the solution, very small or negligible changes in the pH of the solution occur. Hence a buffer is able to maintain the pH of a solution.

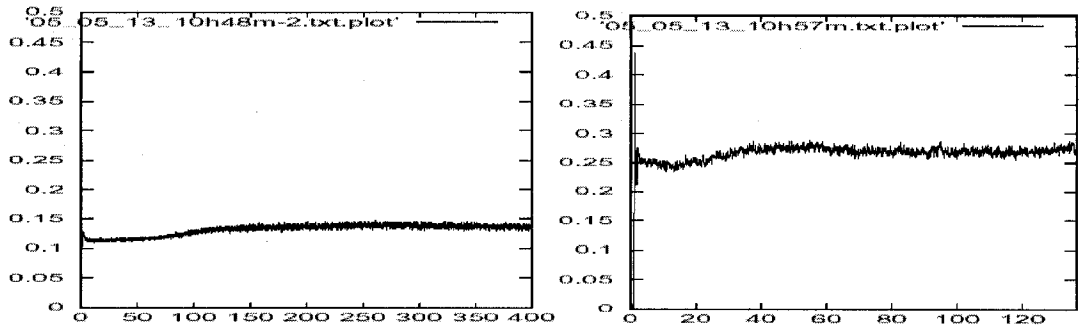
The citrate with three pKa values has three pHs where it functions well as a buffer. This enabled electrophoresis to be carried out at three different pH values to test the effect of pH on the charge of the antibodies.

Since the lowest pI of the antibodies tested was 5.4 and the pI of the RBCs is approximately 3.5 [21], then the target pH lay between the two. It would be within this pH range that the RBCs may be negatively charged while the antibodies are positively charged. Thus, the first buffer tested had a pH of approximately 5.3 to maintain a pH as close to physiological as possible without exceeding the range of pH values between the two pI values. The buffer was made according to the method outlined in Chapter 3 (section 3.2.4). The chip channels were loaded with C5GS10G made from 5% GeneScan® polymer (Applied Biosystems, Streetsville, ON, Canada), 10% 50 mM citrate buffer (made in house) as well as 10% glycerol (Sigma-Aldrich, St. Louis, MO, USA). The sample well was filled with 3 μl of 1 μg/ml concentration of monoclonal IgG antibodies diluted in running buffer. The running buffer consisted of 50 mM citrate buffer with 10 % glycerol (Sigma-Aldrich, St. Louis, MO, USA). Electrophoresis was conducted according to Table 5.2 where a continuous stream of antibody sample is sent down the short channels of the chip. Detection of the signal was done near the intersection of the chip, approximately 125 μm towards the sample well from the intersection. This point was chosen due to increased sensitivity of detection at this location to smaller volumes. If smaller volumes than usual are sent down the injection channel, the usual "sample plug" that is sent down the separation channel may contain too low a concentration of the sample to be successfully detected.

Table 5.2 Electrophoresis Protocol for Antibody Movement in Charge Determination

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Ground	Float	±400	Float

In this detection, a peak profile resembling a step signal similar to that shown in Fig. 3.1 was expected. Since the pH of 5.3 was very close to but below the pI of the antibodies, it was believed that a smaller portion of antibodies may be negatively charged due to some inherent variations in the antibodies. The number of antibodies that are negatively charged at a pH of 5.3 may be less than when a pH much lower than 5.3 is used, meaning when a pH further away from the pIs of the antibodies is used. The negatively charged antibody would move when a positive voltage was applied. When a positive voltage was applied at the sample waste well, only a very small change in the baseline of the electropherogram was seen (Fig. 5.4a). This indicated a very weak signal, suggesting that a very small volume of antibodies was present at the detection point. Due to the fact that the pH was close to the pI of the antibodies, it was very likely that a small portion of the antibodies in the sample was still negatively charged as suspected. The antibodies seen during IEF testing with pI values near 5.3 may still be positively charged. A move of the detection point closer to the sample well to eliminate potential problems with adsorption loss of antibodies to channel walls also yielded similar results. Only a slight increase in the baseline of the electropherogram was detected when a positive voltage was again applied (Fig. 5.4b).



(a) (b)

Fig. 5.4 Electropherograms of monoclonal antibody detection with a buffer pH of 5.3 and the application of a positive voltage (a) and an effective negative voltage (b) at the sample waste well. Standard 4-port chip was used with detection was done at approximately 125 μm from the intersection towards the sample waste well.

Next, an effective negative voltage of 400 V was applied at the sample waste well. It was found that apart from an initial small signal, the remainder of the electropherogram did not show any signals detected (Fig. 5.5). This was tested on the same chip load immediately following testing with a positive voltage. Thus, the results suggest that no new antibodies moved into the channel with the application of the negative voltage. The initial signal detected may be the remaining antibodies at the detection point in the channel. These antibodies likely moved back towards the sample well with the application of the reverse (negative) voltage. That movement would have explained the presence of only the small amount of antibody detected in Fig. 5.5. No abnormal EOF was detected through monitoring as previously done with calibrations runs at the beginning of testing. Similarly, no other flow was observed in the chip. Since, the same protocol was used for testing at the lower pH values as was done at the higher values, there should not have been any additional flow that was not observed previously. Similar results were observed for three consecutive runs. Further testing with a fresh chip and detection at the new location could help reveal whether or not the rise in the baseline was due to the initial antibodies being injected into the separation channel. This would have to be reproduced more than once to ensure accurate results and to eliminate the possibility of artefacts.

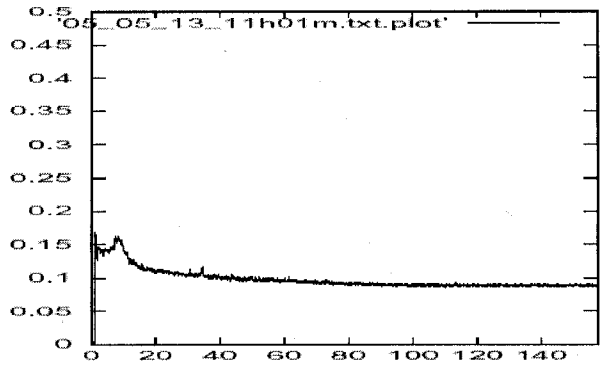
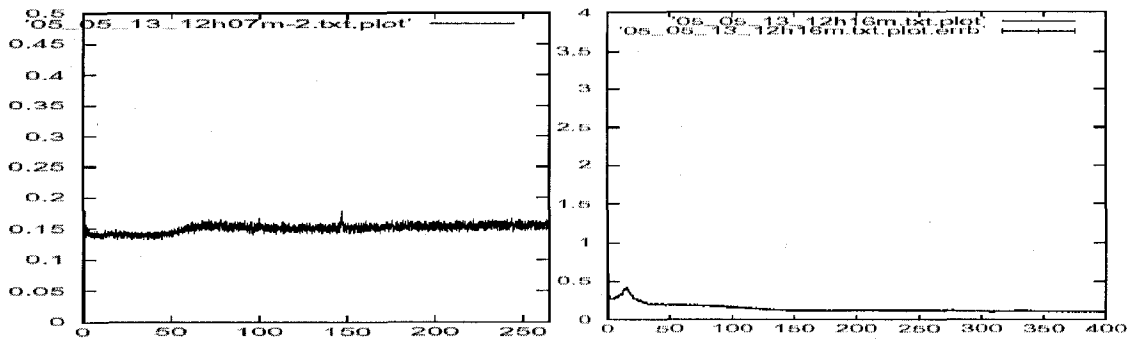


Fig. 5.5 Only a signal is detected at the beginning of the run when a negative voltage is applied at the sample waste well according to Table 5.2 in a buffer with a pH of 5.3. Standard 4-port chip was used with detection at approximately 125 μm from the intersection towards the sample waste well.

The weak signal also did not appear to be a result the lower injection voltage applied here. The same 100 V was applied previously during buffer testing in Chapter 3 and yielded a much larger antibody signal. The conditions during testing were otherwise identical. Adsorption also did not appear to play a large role in this testing based on its past performance (Chapter 3) and because the baseline remained stable with no noticeable increases during the run. Thus, the low signal intensity was concluded to be due to a lack of antibodies present and not a result of other chip conditions. Therefore, it appeared that the pH of 5.3 was not sufficient to render the antibodies positively charged and a lower pH was required.

A lower pH of 4.5 was tested next to determine whether a lower pH, further from the pI of the antibodies would lead to more antibodies being positively charged. Similar results to when a pH of 5.3 was used were obtained (Fig. 5.6). There was a small rise in the baseline suggestive of antibody arrival when a positive voltage was applied at the sample waste well. This meant that a minute amount of antibodies was still negatively charged. Upon application of the negative voltage, a signal was detected at the beginning of the run followed by no signal. The antibodies still did not appear to be more positively charged than at a pH of 5.3 as hypothesized, and a small number of antibodies were still negatively charged based on the slight rise in baseline (Fig. 5.6a). It appeared that the small percentage of the population that may have been positively charged could not be detected.

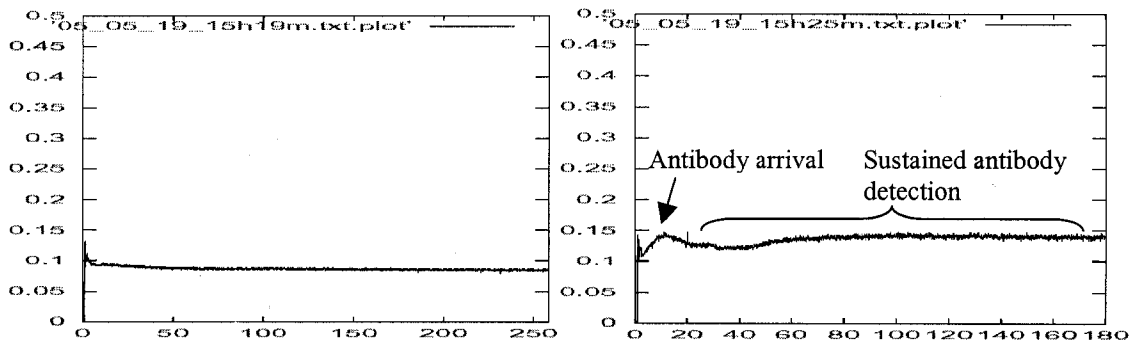


(a) (b)

Fig. 5.6 Monoclonal antibody signal detection using a lower pH of 4.5 with the application of a positive voltage (a) and an effective negative voltage at the sample waste well (b)

An even lower pH of 3.7 was then tested to determine whether or not the antibodies could become more positively charged. Here, the application of a positive voltage did not yield any signal detection (Fig. 5.7a). However, an effective negative voltage applied at the sample waste well showed a small, sustained increase in the signal (Fig. 5.7b). This suggested that at the pH of 3.7, the quantity of antibodies being positively charged became large enough that there was a more constant stream of antibodies during injection. This apparent increase in the amount of positively charged antibodies suggests that as the pH is lowered further from the pI of the antibodies, the antibodies were becoming more positively charged. However, there still appeared to be weak negatively charged antibodies at a pH of 3.7. A cause for these negatively charged antibodies may be attributed to slight changes in the pH within the channel that could potentially occurred during the application of an electric field. The changes in pH is largely due to electrolysis [26, 27]. An accumulation of H^+ and OH^- ions at the anode and cathode respectively leads to their migration into the channels through diffusion and electrophoresis. If the buffering capacity of the running buffer is insufficient, pH variations will start to occur inside the channel. Such shifts in pH have been documented [28] and may have led to enough of a change in charge of the antibodies during electrophoresis to cause them not to be electrophoresed as readily as theoretically expected based on the pI values. Lower pH values than 3.7 were not tested because a pH of 3.7 was already very close to the pI of 3.5 of the red blood cells. Even if the antibodies were to become positively charged at a pH lower than 3.7, the RBCs would be

either neutral or positively charged as well. This would defeat the goal of obtaining positively charged antibodies and negatively charged RBCs for their electrophoretic separation. Thus, a similar problem in maintaining the red blood cells negatively charged would likely be encountered if lower pH were used for increasing the positive charge of antibodies. Even if the charge of the cells was not an issue, the quantity of antibodies positively charged at this pH is still too small for sufficient separation of antibodies and RBCs. Therefore, the attempt to find a pH between the pI of the antibodies and the RBCs to render the two of opposite charge was not successful. The separation of the two pI values was too small for a sufficient quantity of antibodies to become positively charged.



(a) (b)

Fig. 5.7 Antibody detection with a buffer pH of 3.7 and the application of a positive voltage (a) compared to an effective negative voltage (b)

5.4 Visual Observation of On-Chip Antibody Movement

Movement of antibodies on-chip were attempted with the monoclonal and polyclonal antibodies on-chip and observed using a Zeiss Axiovert 200 Inverted Microscope (Göttingen, Germany). This was an attempt to determine the movement of the antibodies to better understand their movement and charge during electrophoresis. Due to the decreased sensitivity of the fluorescent microscope, which uses a CCD for fluorescence image capture, it was found that a higher concentration of antibodies was required to be able to observe the signal. Citrate buffer with a concentration of 50 mM was used for the testing. Antibody concentrations of 3 μg/ml, 10 μg/ml, and 30 μg/ml were tested with the 30 μg/ml concentration to be the one giving the best signal strength.

The other concentrations gave weak signal strengths that made the comparison between behaviour of different concentrations not possible. The channels of the chip were filled with 5% GeneScan® polymer (Applied Biosystems, Streetsville, ON, Canada) containing 10% glycerol (Sigma-Aldrich, St. Louis, MO) and made with an end concentration of 50 mM of citrate buffer (C5GS10G). The pH and osmolality of the polymer and buffer were monitored throughout testing between days. Neither varied much with the pH staying at approximately 5.2 while the osmolality ranged between approximately 1400 mOsm to 1560 mOsm. The sample well of the chip was filled with antibodies diluted in running buffer while the remainder of the wells were filled with running buffer. All wells were filled with a volume of 3 µl. The running buffer was made of 50 mM citrate buffer with 10% glycerol. Electrophoresis was conducted according to Table 5.3.

Table 5.3 Electrophoresis Protocol for Antibody Movement Using Microscope Detection

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Ground	Float	200	Float
2	200	Float	Ground	Float	6000

The first sample tested was the monoclonal antibodies. Here, when the voltage was applied, the antibodies were moved out of the sample well and travelled as one band (Fig. 5.8). The band was quite faint with the total width marked by the line shown in the figure. The weakly fluorescent image is due to the poorer sensitivity of the CCD compared to LIF. The band is also not very sharply defined due to slight diffusion of the antibodies at the two ends of the band. The bright dot seen in the channel was either an aggregate of the antibodies or a small contaminant. The movement of the monoclonal antibodies as one band down the channel was expected since the charge of the monoclonal antibodies would be the same within the population and there is no established pH gradient as in IEF. Any broadening of the band was likely caused by slight adsorption of the antibodies as they travelled down the channel due to the high concentration used.

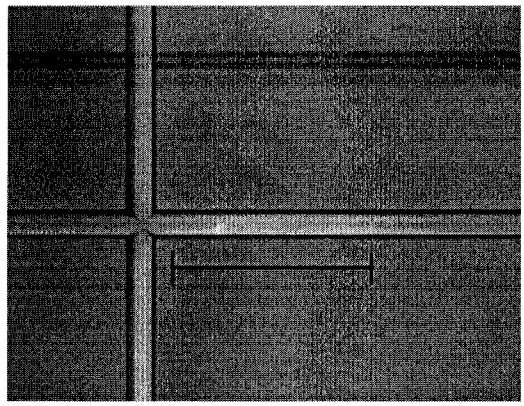


Fig. 5.8 Monoclonal antibodies travelling as one band when injected down the separation channel

The polyclonal antibodies were then tested following the same protocol as the monoclonal antibodies. Various bands were observed in the separation channel as the antibodies travelled down the channel. Fig. 5.9 displays sequential pictures of a sample plug of antibodies as it travelled down the separation channel. The plug of sample appeared as one band and then began to separate into several fluorescent bands of antibodies beginning at approximately 5 mm to 10 mm from the intersection and continuing down the channel as separated bands until the applied voltage is removed. A close up of the banding may be seen in Fig. 5.10. These results were reproducible for at least three runs in one load and also reproducible in at least three different loads suggesting that it is not an artefact. The banding is likely due to the variation in population among the antibodies since it is polyclonal in nature. Electrophoresis down the separation channel may have led to the separation of these different populations due to variations in mobility. This offers a potential for on-chip separation of antibodies based on mobility shifts.

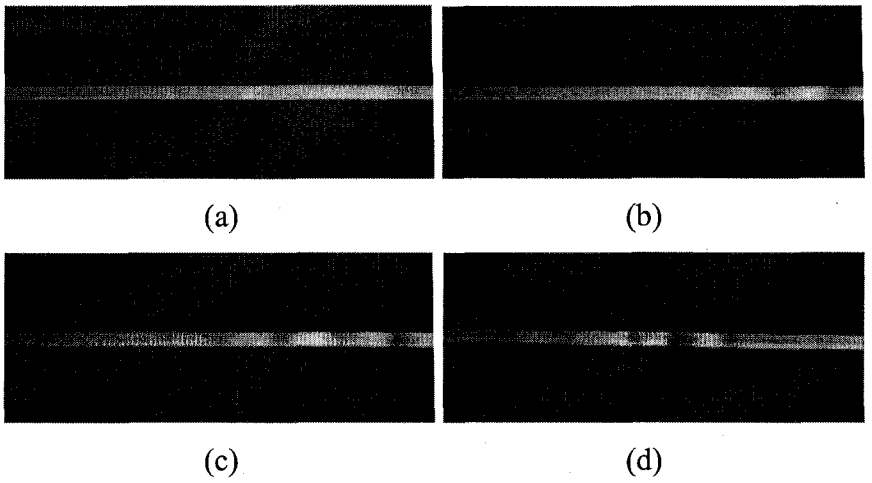


Fig. 5.9 Banding of polyclonal antibodies as a sample plug travels down separation channel. A concentration of 30 μg/mL was used.

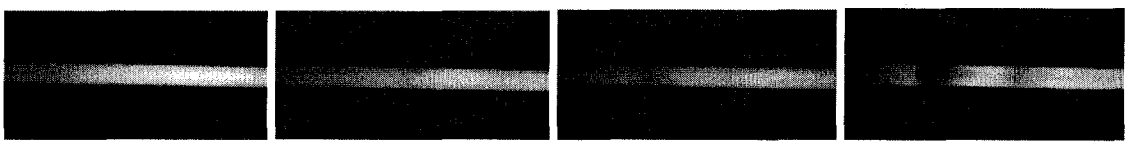


Fig. 5.10 Close up of banding phenomenon seen with the polyclonal antibodies during electrophoresis in separation channel. A concentration of 30 μg/mL was used.

Another interesting phenomenon observed was the presence of more brightly fluorescent dots within an antibody band (Fig. 5.11). These bright dots are though to be antibody aggregates. This corroborates the results obtained with the μTK where large spikes were seen on occasion on top of the antibody peaks. The buffers and polymers used were the same for both cases. The large peaks were believed to be antibody aggregates and the presence of the bright fluorescent dots from the Zeiss microscope images supports this theory. The formation of aggregates was not unusual, especially with the high concentration 30 μg/mL antibodies used. This concentration was used due to the lower sensitivity on the microscope that required a high enough concentration of observing the fluorescence. Following the observation of what appeared to be antibody aggregates on the microscope, it was believed that a reduction in antibody concentration would likely lead to a reduction of aggregates. The reduction and near absence of the antibodies spikes seen when a lower concentration of 1 μg/mL was used during work on the μTK suggested that the lower concentration does reduce the amount of aggregation. However, a lower concentration was not tested here due to sensitivity of detection limits

of the fluorescence microscope. The presence of the aggregation also did not appear to have any effect on the antibody movement, charge, or detection. Since the main purpose of testing using under the microscope was to observe antibody movement and charge, no attempts were made to reduce antibody aggregation. The application of the electric field to move the antibodies could have acted to mix them and facilitate aggregation. The increased viscosity due to the presence of the polymer and glycerol could have also increased the chances of antibody agglomeration compared to a buffer only suspension where the antibodies may be able to move around more freely. Further studies would be required to determine the exact cause of the aggregation.

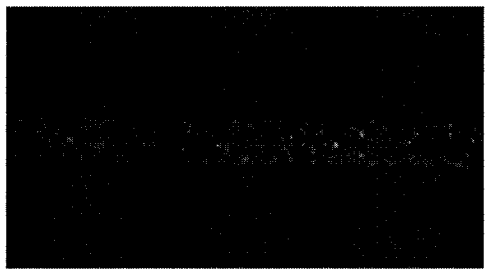


Fig. 5.11 Bright fluorescent dots believed to be antibody aggregates were observed in an antibody band during electrophoresis. A concentration of 30 μg/mL was used.

The banding of the antibodies appeared to be an indicator of potential separation of the antibodies during electrophoresis. However, the exact basis of that separation was not determined. If determined, this may become a useful technique for antibody separation. Similarly, further study of the aggregation phenomenon may reveal information regarding antibody aggregation that would be useful for future antibody work.

5.5 Conclusions

Work in this chapter was inspired primarily by the idea that antibodies and red blood cells could potentially be electrophoretically separated based on a charge polarity difference. It was believed that an appropriate pH could be found to be between the pI of RBCs (approximately 3.5) and antibodies such that the antibodies would be positively charged while the RBCs remained negatively charged. IEF experiments were first

conducted to determine the pIs of the analytes of interest. These included the anti-human monoclonal and polyclonal antibodies, the various antisera that would be used for ABO and Rh typing as well as BSA that would be abundantly present in serum.

A commercially available microelectrophoresis kit was used in experiments and the lowest pI value for the various analytes tested was approximately 5.4. This meant that a pH of between 3.5 and 5.4 would have to be used to render the antibodies positively charged and the RBCs negatively charged. The fluorescently labelled monoclonal and polyclonal antibodies were used during the testing of different pH values to determine which pH would render the antibodies positively charged. They were chosen because they were readily detectable. Although the IEF experiments showed pI values higher than 5, during actual experimental testing the antibodies did not all become positively charge when placed in a buffer with a pH lower than 5. The most antibody detection achieved was with a buffer pH of 3.7. Even at this pH, the signal strength of the antibodies was still not as strong as when they were negatively charged where a buffer pH of approximately 6 was used. It is uncertain at this point the exact cause of these results. Further investigation is required to fully explain the observed results. Buffer pH values lower than 3.7 were not tested since this value is too close to the pI of RBCs. Overall, the window of available pH values to ensure the cells and antibodies are of opposite charge was found to be too small. The percentage of antibodies positively charged was deemed too low to be of practical use. Such low pH values may also have adverse effects on the functionality of the antibodies since they are designed for physiological pH, approximately 6.8 – 7.4 [29]. Such potential alterations in functionality would adversely affect the sensitivity of any binding of the antibodies with the RBCs, which may render any attempts at forward and reverse testing futile. Therefore, although separation of serum antibodies and RBCs based on charge polarity differences using electrophoresis would have been an elegant method, it does not appear to be feasible under the conditions tested. An alternative method for separation would have to be found.

In addition to the IEF testing, the movement of the fluorescent antibodies was further investigated using an inverted microscope to capture optical images. Both monoclonal and polyclonal antibodies were tested. The monoclonal antibodies were

found to move together down the separation channel as expected. Any broadening of the band as it travelled was attributed to potential adsorption of the antibodies on the channel walls due to the high concentration used. Unlike the monoclonal antibodies, the polyclonal antibodies did not travel as one band, but separated during electrophoresis into various bands. This separation was likely caused by the mobility variations in the population of the antibodies due to their polyclonal nature. This banding may be indicative of on-chip separation of antibodies based on mobility shifts. However, further work is required to more precisely control this potential separation so that it may provide practical use. In addition to banding, bright fluorescent dots were seen during certain runs with the antibodies. These were believed to be antibody aggregates and are consistent with results obtained with the μTK where large spikes were seen during antibody electrophoresis. These spikes were believed at the time to be antibody aggregates, and are shown here under the microscope to be present.

Overall, antibody charge in varying pH buffers was determined while their movement under electrophoresis was also better understood with optical observations. It was determined from the results that the use of opposite charges of RBCs and antibodies by variation of pH will not be achievable under the tested conditions and alternative method for will have to be established. In addition the movement of antibodies during electrophoresis was optically observed using an inverted microscope. Valuable information regarding the charge of the antibodies and their movement and behaviour under microchip electrophoresis was obtained, which may be used in future work. Further investigation into the banding of the antibodies on-chip also provides an interesting avenue for potentially better controlling the phenomenon that may be useful for antibody separation work.

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Chapter 6

On-Chip Erythrocyte and Antibody Interaction

6.1 Introduction

Although some success was met with both the antibody and the RBC mobility testing for blood typing, the development of one method for ABO blood typing was still not achieved. It was believed that the use of the antibodies and RBCs on-chip together would enable better detection and typing methods. There are limited reports of RBC and antibody reactions in current literature. Although Ichiki *et al.* [1] has reported the reaction of sheep red blood cells with antibodies on-chip, and others [2-4] have demonstrated that binding of antibodies and cells in microchannels is possible, no one standard technique currently exists.

The focus here was the on-chip binding and manipulation of RBCs and antibodies at the same time using our existing platforms. If on-chip forward and reverse typing were to be achieved, the successful binding of the antibodies and RBCs would be required. The next thing that would be need was the successful detection of that binding. The main problems facing the use of antibodies and RBCs on-chip appear to be those associated with electrophoresis of antibodies on-chip. They include adsorption of antibodies to the channels and the presence of the antibodies affecting the electrolyte concentration and altering current during electrophoresis. The ability of the cells and the antibodies to bind was also a major concern. It was difficult to predict the effects the electrophoresis medium had on the cell membranes and the functionality of the antibodies. Thus, it was recognized that some pre-processing of the samples may be required. In addition, the ability to do partial typing was also considered. Although the ideal method would enable

typing of the complete ABO group, the identification for certain antigens may also be useful. The main focus of this chapter was to set up the initial foundation for antibody and RBC interaction on-chip. This includes the determination of the interaction between the RBC and antibodies on-chip, whether the antibodies bind to the target cells, and if so how that binding may be detected. Two types of detection for antibody and RBC binding may be used. The first is a change in the fluorescence signal intensity or peak profile while the second is the mobility of the cells. In addition to the cell-antibody interaction, their lack of interaction was also of interest. Any mobility differences between the two may potentially be used for their separation, a feat that would be very useful in on-chip blood analysis. The basic ability to detect the RBCs and antibodies using LIF required investigation.

6.2 RBC and Antibody Electrophoretic Separation

In order to conduct tests using both antibodies and cells from blood samples, the initial separation of the two is required. Prior to attempting to separate the RBCs and the antibodies, testing was done to determine the behaviour of the RBCs and antibodies in the microchips. It was believed that the RBCs and the antibodies may both be detected using LIF based on previous testing (Chapters 3 and 4). The RBCs were seen as narrow peaks while the antibodies were detected as a change in baseline leading to a broad peak. Thus, it was logical to assume that if the two did not interact, they may both be detected as a change in baseline and narrow peaks separately or overlapping depending on their respective mobilities.

The RBCs were first tested with the polyclonal antibodies from Molecular Probes (Eugene, OR) to determine if the presence of an antibody that was not specific for the particular cell would have an effect on the cells. Testing was done using either the citrate or Tris Borate EDTA (TBE) buffered GeneScan® (Applied Biosystems, Streetsville, ON, Canada) (C5GS10G or 5GS10G) as described in previous Chapters. The corresponding running buffers were 50 mM citrate or 1xTBE with 10% glycerol (0.05C10G or 1xTBE10G). Reagent cells used in blood typing by the CBS were RBCs obtained from Immucor (Norcross, GA). The concentration of cells used throughout experiments was

1% cell volume compared in 99% buffer. Prior to making this dilution, the reagent cells were first washed in commercially purchased 1x phosphate buffered saline (1xPBS) from Hyclone (Logan, UT) by centrifuging for 5 min at 4000 rpm twice. The supernatant was then removed leaving the packed cells for use. Freshly washed cells and new working dilutions in running buffer were made for every load of the chip to maintain consistency and minimize effects of being suspended in the running buffer.

The cells were first electrophoresed using the μTK. The channels were filled with C5GS10G and the sample well loaded with 3.4 μL of either cells alone in the running buffer or cells and fluorescently labelled polyclonal anti-human IgG antibodies obtained from Molecular Probes (Eugene, OR). The concentration of cells used was the usual 1% whereas the concentration of the antibodies tested was 1.5 μg/mL. The velocities for both sample types were tested using the same electrophoresis protocol. The cell mobilities were of interest and the cells were observed through the μTK eyepiece. It was found that the cell mobility was not affected by the presence of the antibodies. The RBC arrival time at the designated detection point was approximately 1 min ± 1 s for both cases when antibodies were present and when antibodies were not present. This was encouraging in that it suggested that the antibodies that were not specific (targeted to bind) for the RBCs also did not bind to them. Even if some antibody binding were present, the extent was small enough not to be detected. Thus, non-specific binding did not appear to be a problem at the tested concentrations. This meant that the antibodies and cells could potentially be separated from each other without having to break any bonds that may have formed between the two.

Previous attempts at separating the antibodies and cells based on different isoelectric points to render the antibodies and cells of different charge polarities were not effective (Chapter 5). Although this meant that the antibodies and cells could not be sent in different directions electrophoretically, it did not rule out the fact that the two had different mobilities. The idea was to first determine if the cells and antibodies had mobilities that were sufficiently different that their separation could be achieved by sending the two down different channels following a period of electrophoretic separation. Tests were conducted using the μTK. Cells and the fluorescently labelled monoclonal antibodies (Sigma, St. Louis, MO) were loaded in the sample well with a concentration of

1% cells and 1 μg/mL antibodies. The remainder of the wells were loaded with the 1xTBE10G running buffer while the channels were filled with 5GS10G. The TBE buffer was chosen due to its superior buffering capabilities over the citrate since a longer separation time may be required for electrophoretic separation of the antibodies and cells. Electrophoresis was carried out according to Table 6.1. There appeared to be a distinction between the arrival of the cells and antibodies. The cell arrival was shown as narrow peaks previously seen in Chapter 3 on the electropherogram. Although there appeared to be an increase in the baseline, suggesting potential antibody arrival due to similarity in peak profile to that observed in Chapter 3 (Fig. 6.1), this arrival was not consistent. Although not shown, the increase in baseline that suggested antibody arrival was observed after the 120 s window shown in Fig. 6.1b. A noticeable increase in baseline of at least 0.1 relative fluorescence units in amplitude from the maximum peak height had to be present to be deemed a result of antibody arrival. The inconsistency may have been caused by the presence of the RBCs. Repeated pipetting of any specific quantity of packed RBCs leads to an inherent small variation because the cells are more viscous than water and the exact same volume is not expected each time it is pipetted. The buffer concentration inside the sample well would also be different with the presence of the RBCs compared to just the antibodies. This alteration may lead to slight variations in the charge of the antibodies, leading to a variation in mobility. The negatively charged RBCs could have also competed with the antibodies as they were electrophoresed down the channel. These different factors may have all contributed to the inconsistent analyte arrival times during electrophoresis. Another cause of this baseline increase may be the agglomeration of the cells or antibodies. However, this is not likely because the concentrations of the antibodies and cells were the same that were used in Chapters 3 and 4 where no such behaviour was exhibited. The chip performance was also not at question due to the fact that calibrations were done at the beginning of testing each day to ensure no degradation had occurred. Although the same protocol was used, the arrival times of the antibodies and cells varied by as much as 40 s. This inconsistent arrival times may have been due to the RBCs missed by the laser detection point due to the large width of the channel as previously observed in Chapter 4. The peak height of the antibodies also varied and could not be consistently detected at a reproducible intensity. The beginning

of antibody arrival was designated as the first small bump preceding the large antibody arrival peak that was consistently seen with the antibody detection. Increases in concentration of the antibodies did not appear to improve increases in signal intensity or improved distinction of antibody arrival point. Overall, the RBCs tended to arrive earlier than the antibodies in four runs out of six. However, the separation was not distinct and reproducible enough that it could be reliably used to separate the antibodies and cells by sending them down different channels after electrophoretic separation. Thus, an alternative had to be found.

Table 6.1: Electrophoresis Protocol for RBC and Monoclonal Antibody Separation

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Ground	Float	200	Float
2	3	Ground	Float	Float	2000
3	400	Float	Ground	Float	2000

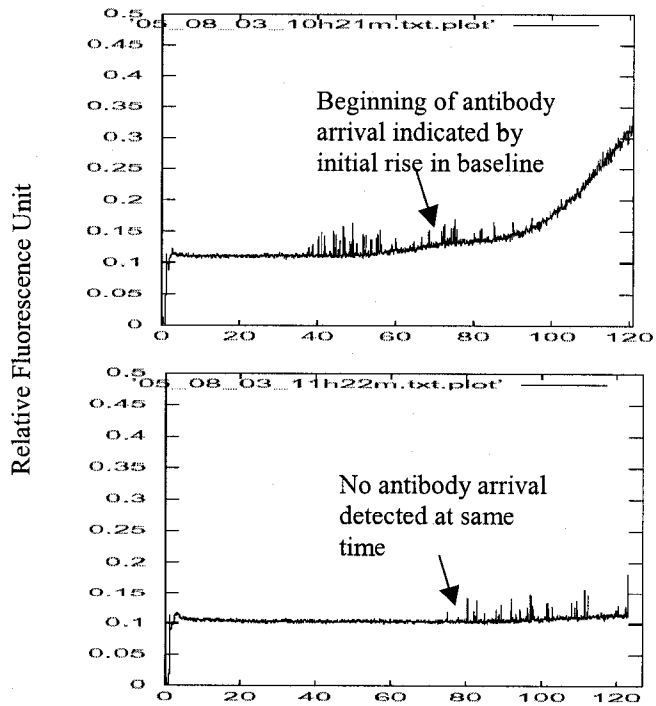

Fig. 6.1: Electropherogram of RBCs and antibodies electrophoresed together on two separate loads. For each load, the sample well was filled with 1% RBC and 1 μg/mL of antibodies and electrophoresis was conducted according to Table 6.1.

6.3 Antibody and RBC Binding

It had been noted in previous testing (Chapter 3, 5) that the antibodies adsorbed to the channel walls just as other proteins have been reported [5-12]. The antibodies were observed in Chapter 5 to adsorb to the polymer and/or channel walls during electrophoresis. This occurred even in the presence of a polymer, an attempt at dynamic passivation. It was thus hypothesized that a sufficient amount of antibodies could adsorb to the polymer during electrophoresis to effectively separate the antibodies from the cells since the cells readily passed through the channel without any adsorption.

Prior to any separation attempts, the quantity of antibody adsorbed onto the polymer during electrophoresis had to be determined. The switch to use of antisera was done to see if they would also adsorb to the polymer as the fluorescently antibodies had since both are antibodies. In addition to determining whether the antisera would adsorb to the polymer, the switch may also provide a method for identifying the quantity of antisera that adsorbed to the polymer. This could be tested by running antibodies through the same channel as their target cells and observing for any signs of agglutination or changes in cell behaviour. Since previous tests had been done to determine the concentration of antibodies and cells required for micro-agglutination, an estimate of the concentration of the antibodies adsorbed by the polymer could be obtained. If the antibodies adsorbed did not bind with the cells as they passed through, the presence of the antibodies may still be altering the mobility of the cells due to their presence in the electrophoresis medium. Thus, any changes to cell behaviour were noted.

6.3.1 RBC Agglutination

Due to the small quantities of antibodies used on-chip any loss of analyte, in particular antibodies since they exist in smaller quantities in blood than RBCs, may significantly alter analyte detection and reaction. Thus, it was important to determine the lowest concentrations of RBC and antisera that could be used and still elicit a noticeable reaction. Tests were conducted and showed that in order for micro-agglutination to occur, the lowest concentration of RBCs required was 1/8 dilution of 5% concentration of cells mixed with a 1/128 dilution of stock concentration of Novaclone antisera (Dominion

Biological Ltd., Dartmouth, NS, Canada). The lowest dilution of antisera required for a 5% concentration of cells was 1/128 dilution. Thus, initial tests used a 1/60 dilution of antisera with 1% concentration of RBCs was used. The antibody concentration was set higher than the lower limit to account for the fact that not all antibodies will be adsorbed and be used in binding with the cells. The citrate buffer was used for testing because it is more commonly used for biological cells than TBE buffer so that is likely better at maintaining the physiology of the cells.

In order to test the reactivity of the antibodies and cells on-chip, the antibodies were loaded in the well usually designated as the sample waste well while the cells were loaded in the sample well. All the wells were filled with 3.4 μL of reagents. Both the cells and antibodies were loaded with 50 mM citrate running buffer. The chip channels were filled with citrate buffered GeneScan®, 0.05C5GS10G. The antibodies were first allowed to travel through the separation channel and then the cells were electrophoresed through the same channel (Table. 6.2). The antibodies were first electrophoresed down the channel to utilize their tendency to adsorb to the channel walls to our advantage. It was hypothesized that the adsorbed antibodies would react with cells as they were subsequently electrophoresed down the channel.

Initial observations seemed to suggest that the cells were travelling slower when antibodies are first passed through the channel. Thus, the time it took the cells to move across the field of view in the μTK was approximately measured by visually observing the cell movement and timing using a stopwatch. These brief tests were done to provide an initial idea as to whether the presence of the antibodies would alter the cell mobility. It was hypothesized that the antibodies, at least a percentage of those that travelled down the separation channel, would be able to react and bind to the cells and alter their mobilities. Three runs were done with both the cells alone and cells with antibodies together. It was found that for each run of the cells with antibodies present, the time it took them to move across the field of view was approximately twice as long as those that travelled without antibodies. It took the cells approximately 2 s to move across the field of view when no antibodies were present and 4 s when antibodies were present. This was seen when antibodies specific for the cells tested were flushed down the channel first. The cells were also observed at the same designated location down the channel.

Table 6.2: Electrophoresis Protocol for RBC and Antisera Binding

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Float	Float	Ground	2000
2	400	Ground	Float	Float	2000

In addition to altering cell mobilities, the presence of the antibodies also appeared to contribute to cell aggregation. The same procedure used for testing cell mobility above was then used to test RBC aggregation. The concentration of antibodies required for aggregation was also varied between a 1/60 dilution and the stock solution. Antibodies were first electrophoresed down the separation channel according to Table 6.2. This allowed for antibodies to adsorb to the channel or polymer and potentially react with any cells travelling down the same channel in subsequent runs. RBCs were then electrophoresed down the same channel using the same electrophoresis protocol. Agglomeration or potentially agglutination of the cells in the channel was observed when target antibodies were first flushed down the channel (Fig. 6.2). The minimum concentration of antibodies required for reproducibly showing clearly distinct antibody-induced aggregation was a 1/10 dilution of the antisera. The amount of cell aggregation appeared to be greater when target antibodies and RBCs were present compared to when no antibodies were present (Fig. 6.3). Further investigation of this observation yielded the following results. Although slight cell aggregation may be observed in the absence of any antibodies, the number of cells aggregated is different than that seen when antibodies were present. Comparison of the amount of cell aggregation showed that when no antibody was present, the maximum number of cells observed in any aggregate did not exceed 4 cells. However, as seen in Fig. 6.2, the number of cells in an aggregate is larger than 4 cells. Thus, the criteria for determining an antibody-induced aggregation were set to aggregates with a minimum of 5 cells. Any cell aggregates of greater than 4 cells were designated as a positive while aggregates less than 4 cells were designated as negative. The number of cells in the antibody-induced aggregates was usually approximately 10 cells but may reach as high at 20 cells, much higher than the designated threshold. The aggregates were observed within a specific section of the channel following each run.

Testing with non-target antibodies was also done to ensure that the increased aggregation when target antibodies were used were indeed due to the antibody binding to the cells. It was found that a degree of non-specific binding induced aggregation was also present (Fig. 6.4). However, the number of cells in the aggregates did not exceed 7 cells, compared to the approximate 10 cell aggregates that were observed when target antibodies were used. This suggested that some non-specific binding did occur. However, the amount of cell aggregation was still less than that of the target antibodies. Another threshold was then set to 7 cells for non-specific binding.

Overall, 18 aggregates were observed for each of three scenarios: antibodies with target cells; antibodies with non-target cells; and no antibodies present. For antibodies with target cells, 6 of the aggregates were observed to be greater than the threshold of 7 cells. For the non-target cells, 6 aggregates were above the 4-cell threshold while all aggregates were below the 4-cell threshold for the case where no antibodies were present. In the presence of target antibodies, the number of cell aggregates above the 4-cell threshold was approximately 30%, making the presence of target antibodies apparently distinguishable from when no or non-target antibodies were present. Although this number may be improved upon, it still demonstrates a distinct difference compared to when no antibodies were present. This method was only intended as a start in determining whether the antibodies in the channels may bind with cells passing through. Thus, this method shows potential to be used for the identification of blood cell antigens through the binding of antibodies and their target cells. This may be achieved following further optimization and refinement of the method and may lead to blood typing. Lower concentrations of antibodies than what was tested here may lead to aggregates with fewer cells. This would add difficulty in identifying positive antibody induced aggregation. This was hypothesized due to the lower cell aggregation quantity associated with lower antibody concentration observed during initial testing of the minimum concentrations of antisera and reagent cell concentration needed for agglutination. However, further testing is required to verify this and to determine the actual quantity of reductions of aggregation.

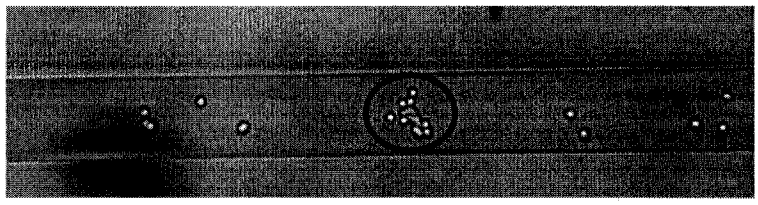

Fig. 6.2: Antibody-induced aggregation of RBC with target antibody flush (anti-A antibodies with A RBC)

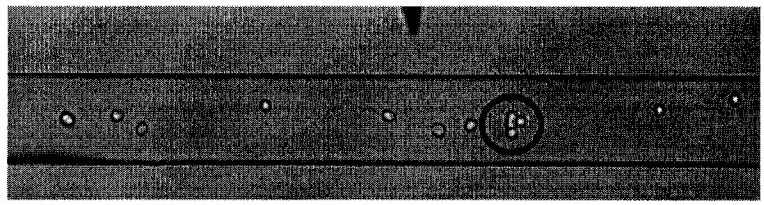

Fig. 6.3: Normal occasional aggregation of A RBC with no antibodies present

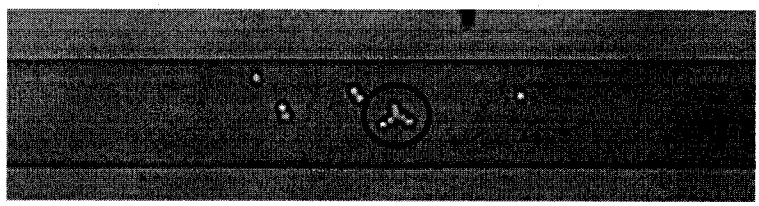

Fig. 6.4: Non-specific antibody-induced aggregation of RBC (anti-B antibodies with A RBC)

6.3.2 RBC Mobility Shifts

Another theory that was tested was the hypothesis that the mobility of cells travelling down the channel could be an alternative method for the detection of the presence of the antibodies. This was supported by the fact that during testing of the potential agglutination of cells when no cell aggregation was present, the mobility of the cells was still different in the presence of antibodies. The change in velocity was likely not due to changes in EOF because cells were tested in the absence of antibodies in the same channel following runs with antibodies and still travelled faster than when antibodies were present. If the change in the channel surface led to the slowing down of the cells, one would expect the cells to remain slower in velocity. However, this did not appear to be the case. Thus, it was suspected that the presence of the antibodies played a role greater in altering the cell mobility than just from an EOF perspective.

The ability to use cell mobility to determine type would be a more sensitive method because it required a lower concentration of antibodies than that needed for aggregation of the cells. The amount of shift in the cell mobility could also be used to quantify the amount of antibody binding. This would offer a more standard method for quantification of the level of agglutination than presently available with the standard macroscopic method for blood typing.

Tests of cell mobility with the addition of antibodies were done using the Zeiss Axiovert 200 Inverted Microscope (Göttingen, Germany). The first tests were done using the three period separation method described in Chapter 4.2.2. Pictures of a specific section of the separation channel were taken so that the movement of cells down the channel during the three separations could be tracked (Table 6.3). Both 1000 V and 500 V for the separation voltage tested. The lower voltage was tested in an attempt to maintain a more stable current during testing.

Table 6.3: Electrophoresis Protocol for Cell Tracking Using Consecutive Separations

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Float	Float	Ground	2000
2	200	Ground	Float	Float	1000
3	10	Float	Ground	Float	1000 or 500
4	35	Float	Float	Float	Float
5	10	Float	Ground	Float	1000 or 500
6	35	Float	Float	Float	Float
7	10	Float	Ground	Float	1000 or 500

Preliminary results showed that the velocity of the A and B RBCs appeared slowest when tested with their respective target antibodies compared to when non-target and no antibodies were present (Table 6.4). The velocities without antibodies were also slightly faster than either specific or non-specific antibody binding. This concurs with the agglutination results obtained previously where cell aggregation was the largest with target antibodies, less so with non-target antibodies, and the least when no antibodies

were present. It makes sense that the increased quantity of antibodies on the surface of the cells may have led to decreases in the cell velocity. This is due to the fact that the presence of the antibodies may act to block the negative charges on the cell surface or at least affect the charge of the cell by altering the cell surface.

Reproducibility in terms of obtaining the same currents during each load was an issue when testing the cell mobility variations. Although the currents were stable during electrophoresis for each run and load, the currents varied between loads by as much as 30%, approximately one in every three loads. This made it difficult to compare all the loads with one another since the currents affected the velocity of the cells as previously noted in Chapter 4. Thus, only the data from loads with the same currents were gathered and compared (Table 6.4). Statistical Package for the Social Sciences (SPSS) was used to conduct an analysis of variance test (ANOVA). It was found that the velocities involving type A1 cells were significantly different from each other. However, the type B cells in each of the three groups with no antibodies, target antibodies, and non-target antibodies were used did not show a significant difference form each other. The velocities of the B with anti-A and B with anti-B cells showed significantly different velocities from each other. However, the type B cells alone did show a difference from when either of the antibodies was present. These results suggested that the presence of the antibodies did affect the velocity of the cells. Although the three cell velocities did not all show significant differences from each other, an optimization of the antibody concentration may increase the differences. Further investigation may be able to reveal a more optimal concentration of antibodies that a larger difference in velocities will be shown.

The current variation observed during testing may have been primarily caused by the weak buffering capacity of the citrate buffer as previously seen in Chapter 3. This could have left it more susceptible to slight variations in RBC and antibody concentration between loads, leading to the variations in separation current between loads. A lowering of the separation voltage to 500 V from 1000 V was also tested, but similar variations still remained. The results listed in Table 6.4 have the same currents for each of the two separation voltages so that the velocities were readily comparable for each voltage.

Table 6.4: Preliminary Results of Mobility of RBC with Antibody Testing

Cell Type	Antibody Tested	Separation Voltage	Average Velocity (μm/s)	Standard Deviation of the Mean (μm/s)
A1	None	1000	62.6	0.9
A1	A	1000	54.1	1.2
A1	B	1000	59.4	2.1
B	None	500	27.4	1.2
B	A	500	25.4	1.7
B	B	500	21.1	1.1

6.3.3 Antibody Binding in TBE

In an attempt to improve reproducibility by stabilizing the current during electrophoresis, the TBE buffer with stronger buffering capabilities was then tested. The intentions were to use the same cell mobility detection methods used in Chapter 4. However, antibodies would also be used here in an attempt to enhance the mobility differences between different blood type RBCs. Before that could begin, both the adsorption of the antibodies in the polymer buffered with TBE and the reaction of the antibodies with the RBCs in TBE buffer had to be first tested.

Initial tests were conducted using 5GS10G and 1xTBE with 1% RBC and 1/60 dilution of antisera. The electrophoresis protocol was the same as that shown in Table 6.2. In these initial tests, the amount of RBC aggregation when target antibodies were present was similar to that seen when no antibodies were present (Fig. 6.5). This apparent lack of aggregation when only the buffer was changed suggested that the binding of the cells and antibodies occurred less readily in TBE buffer compared to citrate. However, the lower amount of aggregation did not necessarily translate into a complete lack of binding of the antibodies with the RBCs. Further investigation was required.

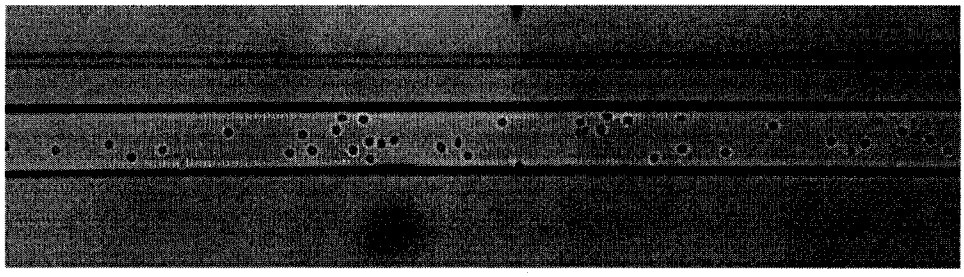

Fig. 6.5: Initial testing of RBC aggregation using 1/60 dilution of asntisera in TBE buffer

Attempts were then made to test the adsorption and binding of the antibodies and cells in TBE buffer using the testing method optimized for the citrate buffer. This provided a higher concentration of antibody that may induce more cell aggregation. The concentration of the antibodies used was a 1/10 dilution of the antisera and the cell concentration was 1%. The detection method was also changed to that of RBC mobility shift method developed in Chapter 4. This method provided a more accurate measure of the cell mobility due to the longer distance the cells were allowed to travel prior to detection. The longer separation distance also minimized the errors associated with variations in starting points of the sample plug of RBCs during initial injection at the intersection as calculated by Equation 4.2. The higher accuracy also offered a more sensitive test of any mobility shifts the cells may be exhibiting that could not be detected before using the three consecutive separation detection method.

In order to combine the two methods, a change in chips had to be made. An additional sample well was needed in order to allow for the injection of a sample plug down the separation channel. A move to using an already available 8-port chip was made (Fig. 6.6). In addition to the two sample wells at the head wells, this chip also had additional wells connected at the tail end. However, apart from the usual designated buffer waste well, the other wells were not used during electrophoresis. They also did not introduce any problems such as flow issues during electrophoresis as attested by the periodic calibrations done at the beginning of testing each day that showed two consecutive runs with shifts in peak arrival times within the allowable 2% error margin. This margin was set according to the results obtained in Chapter 4.

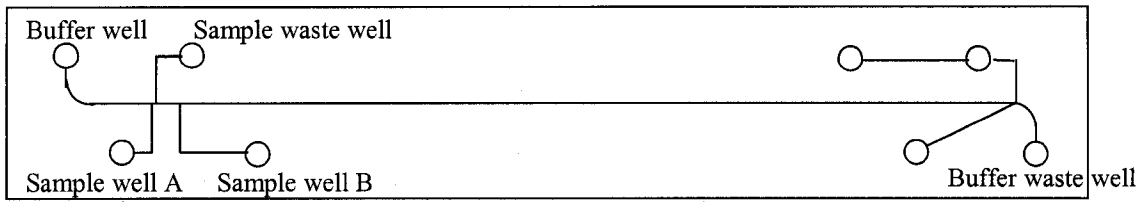

Fig. 6.6: Diagram of chip used for testing RBC and antibody binding using TBE buffer

One sample well was filled with 3 μL of 1% RBCs diluted in running buffer from packed cells washed in PBS as previously done up to this point. The other sample well was filled with 3 μL of a 1/10 dilution of antisera, diluted with running buffer from the stock solution. The remainder of the wells were filled with 3 μL of running buffer. The electrophoresis protocol is described in Table 6.5. This protocol combines what was used for the cell mobility testing and the optimized protocol for the binding testing in citrate buffer. The variations in voltage used were to maintain the same electric fields since the chip has slightly different dimension than the usual 4-port chips that had been used thus far. The different voltages applied at step 5 were to ensure that the cells experienced equal electric fields from both sample and sample waste wells during this pullback step. In this step, the remainder cells in the short channel arms close to the intersection are pulled back towards the sample and sample waste wells to prevent their leakage into the separation channel during electrophoresis.

Table 6.5: Electrophoresis Protocol for RBC and Antibody Binding in TBE Buffer

Step	Duration (s)	Sample Well A (V)	Sample Well B (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	400	Float	Ground	Float	467	Float
2	130	Ground	Float	Float	100	Float
3	3	Ground	Float	Float	Float	927
4	5	Float	Float	Float	Float	Float
5	20	84	Float	Ground	119	Float
6	5	Float	Float	Float	Float	Float
7	599	Float	Float	Ground	Float	500

The tests initially showed encouraging results. There was a detectable slowing down of the RBC in the presence of their target antibodies. The average velocity of type B cells without any antibodies was found to be 39.8 μm/s with a standard deviation of the mean of 1.2 μm/s. When antibodies were present, the velocity of the cells reduced to 34.9 μm/s with a standard deviation of the mean of 1.4 μm/s. These two velocities are different from each other by nearly two standard deviations, suggesting that the presence of the antibodies was able to alter the velocity of the RBCs. Tests were done on fresh chips on two separate days with a total of two loads, and three runs for each case of type B cells with and without antibodies. However, these were only initial test results and had to be reproducible to be considered reliably real and not only valid for this set of data. Attempts to reproduce these results were met with little success. Further measurements of RBCs with and without antibodies showed little difference in their velocities. This was true on at least five separate occasions. The cause for the discrepancy was not apparent during investigation. The chip conditioning and reagents used were also the same for the initial testing and the attempts at reproducing those results. It was concluded that the initial encouraging results may have been obtained under very specific conditions that were not readily apparent and controllable. Further investigation would be required to determine the actual cause of the variation and whether it is controllable or not.

In addition to the type B cells, testing was also done for type A cells and the velocities of the cells with and without antibodies were very similar and could not be used to distinguish the type. The increase of antibody concentration to a 1/3 dilution from the 1/10 dilution also did not increase the velocity difference. This suggests that the additional antibodies may not have increased or improved binding of the antibodies to the RBC to show any visible differences in velocity shifts.

There appeared to be very little binding of the antibodies to the RBCs when electrophoresed in the TBE. No reports were encountered that used TBE as a buffer for the reaction of antibodies and cells. This may suggest that TBE is not suitable for such a purpose. However, as previously mentioned many different buffers were used for antibody and RBC electrophoresis with no one standard. Thus, the lack of a report does not rule out the possibility that TBE may be used for antibody and RBC reaction.

Various factors may have contributed to both the lack of binding as well as the inconsistent results. Conditions such as the age of the RBCs may have affected binding. The set of data that showed a difference in the velocity of the cells with antibodies used a different set of cells than the rest of the tests. This set of cells was close to their expiration date and thus was only used in one test before fresh cells were then used. When the older cells were used they were three days from the expiration date for the reagent cells while the closest the fresh cells came to the expiration date during testing was eight days. Any small changes that may have occurred to the cell membrane or surface due to aging in this difference of five days may have affected the binding of the antibodies. Although likely small, the already weak binding of the antibodies and the RBCs in TBE may have been thus further weakened to a large enough extent that a difference in velocity was noted. Future testing with cells closer to their expiration date may be done to try to determine whether this age difference did play a role in the velocity differences. The age of the cells alone should not have altered the velocity since previous testing in Chapter 4 had already ruled that out. The buffer itself should not have been an issue unless the duration of one day also makes a difference in affecting the binding of the antibodies. The same buffer and freshly made buffer were both tested, but there did not appear to be any differences in velocities between the two buffers.

A more likely possibility is that the amount of adsorption of the antibodies when TBE was used was much less than that in the citrate. This is supported by the repeatable results observed with TBE during antibody testing in Chapter 3. Thus, TBE does not appear to be ideal if antibody adsorption is desired. Overall, the binding of antibodies to their target RBCs appears possible, but occurs less readily in TBE than citrate buffer. For reproducible on-chip aggregation results, citrate should be used.

6.4 A1 and A2 Typing

Although the use mobility shifts for the determination of the typing of the complete ABO blood group could not be achieved on-chip, the ability to partially determine the group was investigated. Apart from attempting to react the RBCs and antisera on-chip for overall blood typing, efforts were also made to improve testing for

the two major type A subgroups of A1 and A2. The A1 and A2 subgroups are two variations of type A. Both have the A antigen, but subgroup A2 has approximately 4 times fewer antigens than the A1 subgroup. The ability to determine A1 from A2 is important in blood typing because A2 RBCs react more weakly with the A antibody. This means a weaker immune response overall when A2 donor platelets are used during minor incompatibilities. An automated method that is capable of rapidly determining the subgroup would be extremely useful in the areas of organ transplantation and platelet transfusions. Here, two methods for distinguishing A1 and A2 were tested. The first was the same mobility-based separation that was tested with the typing of the complete ABO group (Chapter 4). The second was based on LIF using the μTK combined with electrokinetic focusing.

6.5.1 Mobility-Based Separation

The idea behind using mobility shifts to distinguish between A1 and A2 RBCs was the same as that for ABO typing. Previous testing (Chapter 4) had already shown that the velocities differences between A1 and A2 cells were not significant enough to be used for distinguishing them. Thus, it was decided that pre-processing of the cells would be done to help enhance potential differences. In blood banking, the type A cells are labelled with a fluorescent lectin that is specific for the A antigen. Since there is a much larger number of A antigen on A1 cells, those cells would be labelled with more antibodies and thus fluoresce brighter. The cells are then passed through a flow cytometer and the level of fluorescence determines the presence of A1 cells. The increase in the fluorescence of the A1 cells are due to the higher number of A antigens on their surfaces compared to the A2 cells. Here, the hypothesis was that the larger quantity of lectin on A1 cells would affect their charge and render their velocities significantly different than that of the A2 cells. The method for testing was the same as that described in Chapter 4. The usual 4-port chips were used for testing. The RBC was loaded in the sample well at the concentration of 1% while the remainder of the wells were loaded with the 1xTBE10G running buffer. The RBCs were labelled according to the cell labelling

method described in Roback's work [13][1]. A PE-labelled dolchos biflorus lectin purchased from from Biomeda Corporation (Foster City, CA) was used to bind to RBCs. A 1:30 dilution of the lectin was first made with PBS. Whole blood was centrifuged for 10 minutes at 3000 RPM to separate the blood cells from plasma. The RBCs were then isolated and made into a 4% suspension in PBS by volume. A 50 μL volume of lectin was then mixed with 25 μL of the 4% cells in a test tube. The tube was shaken briefly to mix and then set at room temperature for five minutes to incubate. Once incubated, 200 μL of PBS was added to the tube and mixed with the contents before the entire tube was centrifuged for 1 min. The supernatant was then removed and another 200 μL of PBS was added to the tube to wash the cells a second time. Following the mixing and centrifugation the second time, the supernatant was again removed and the cells were ready to be tested. If the cells were not used immediately, another 1000 μL of PBS was added to the tube to suspend the cells until they were used. In this case the mixture was again centrifuged to remove the supernatant when ready to be tested. All samples were used on the same day they were prepared. Testing using a flow cytometer was done to ensure that the samples did not degrade and the lectin were still bound to the cells. It was also determined that a suspension of cells in the running buffer, TBE, did not appear to affect the binding compared to suspending the cells in PBS. This was done by testing two groups of cells where one was suspended in TBE while the other in PBS. The flow cytometry results showed no noticeable differences when the two groups were tested. The channels were filled with 5GS10G. The electrophoresis protocol used was the same as that used for testing the mobility of the ABO group cells (Table 6.6) and testing was done on the Zeiss Axiovert 200 microscope.

[1] Sample preparations were made by Erika Dempsey, project student under the supervision of Dr. Jason Acker at the Canadian Blood Services in the summer of 2006.

Table 6.6: Electrophoresis Protocol for Testing A1 and A2 RBC Mobilty

Step	Duration (s)	Sample Well (V)	Buffer Well (V)	Sample Waste Well (V)	Buffer Waste Well (V)
1	130	Ground	Float	100	Float
2	3	Ground	Float	Float	938
3	5	Float	Float	Float	Float
4	20	100	Ground	100	Float
5	5	Float	Float	Float	Float
6	599	Float	Ground	Float	500

The mobility of cells A1, A2, A1 bound with lectin, and A2 bound with lectin were all tested. The mobilities all appeared very similar with small differences less than two-sigma (Table 6.7). The presence of the lectin did not seem to alter the velocity of the cells either in increasing or decreasing its velocity during electrophoresis. Since the A1 cells have 4 times more antigen sites that the A2 cells, it was expected that there would be a larger amount of lectin bound to the A1 cells. This was supported by the higher fluorescence intensity exhibited by the A1 cells when passed through a flow cytometer. Since the velocity of the A1 and A2 RBCs both bound with lectin did not appear to have any large differences, it suggests that the quantity of the lectin bound also did not have a detectable effect on the velocity of the cells. Thus, the use of mobility shifts of the RBCs bound and not bound with the lectin could not successfully determine A1 from A2 cells. Thus, an alternative method was tested.

Table 6.7: Results of Mobility Testing of A1 and A2 RBCs

Cell Type	Lectin Presence	Average Velocity (μm/s)	Standard Deviation (μm/s)
A1	No	40.5	1.2
A1	Yes	39.5	1.6
A2	No	40.3	1.2
A2	Yes	41.2	1.1

6.5.2 Electrokinetic Focusing

Since the attempt using RBC mobility did not work, a move to use a method more similar to the macroscopic testing procedure was decided upon. Instead of using the Axiovert microscope for testing, detection was moved back to LIF on the μTK for more sensitive detection. A modification to the usual fluorescence detection method on the μTK had to be made because of the issue of missing cells as they pass outside the circumference of the laser spot as previously mentioned. If only a small percentage of the cells passing through the channel were detected, the results would not be accurate, affecting the final number of detected fluorescent cells. Since approximately 50% of the cells are expected to be fluorescent when bound with the lectin based on the flow cytometry results, it is important to determine an accurate percentage of fluorescent cells if fluorescence was to be used to determine the A subgroup. Electrokinetic focusing was thus employed to ensure the cells passed through the centre of the channel to enable detection.

Electrokinetic focusing has been utilized by various groups to focus analytes into a stream for improved detection [14, 15]. The typical 4-port chip is used where focusing voltages are applied at the shorter arms so that the analytes can travel down the centre of the detection channel. A similar protocol was employed here[2], and the electrophoretic protocol used is described in Table 6.8. Step 1 in the protocol was to establish a sufficient flow of cells down the separation channel. The well designated the sample well where the cells were usually placed was not filled with cells. Rather, the cells were placed in the reservoir usually designated as the buffer well. Due to the fact that these reservoir designations have been used throughout this thesis, they will remain as such to avoid confusion. Once the flow of cells is established down the separation channel, two additional voltages were applied at the sample and sample waste wells to focus the cells into a narrower stream down the separation channel.

The usual 4-port chip was used with the sample loaded in the buffer well instead of the usual sample well (Fig. 6.7). This was done to enable the application of the focusing voltages at the usual sample and sample waste wells.

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[2] Ken Wong, research assistant at the Canadian Blood Services, assisted in the literature search and initial protocol development on electrokinetic focusing.

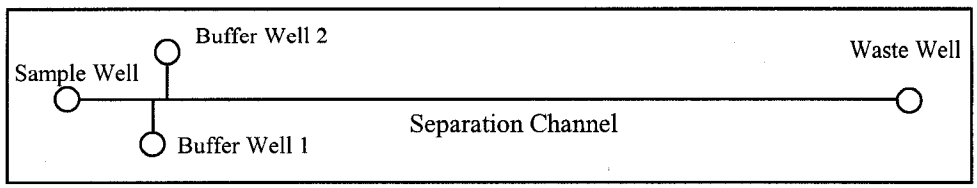

Fig. 6.7: Diagram of chip used during electrokinetic focusing of type A cells

A designated number of cells were set as the minimum requirement for the acquisition of sufficient signal to determine the type of cell electrophoresed. Since the mobility of the cells was not critical and the number of cells could be visually observed during electrophoresis, the duration of electrophoresis and separation carried out for a designated time giving approximately 100 cells or sample points. The detection of the cells was made in the separation channel as previously done. The focusing was achieved following the electric fields applied by Schrum *et al.* Locations at 0.25 mm, 0.5 mm, 1.0 mm and 3.0 mm from the intersection were also tested. The distance of 0.5 mm from the intersection was decided to be the best detection point because it gave the most consistent signals in that the signals were approximately 0.1 V high in amplitude and there appeared to be little deviation of the cells from the centre of the channel. Detection at 1.0 mm and 3.0 mm were too far from the focusing point that the cells began to deviate from the centre of the channel. The detection point of 0.25 mm gave more signal variation between 0.1 and 0.2 V of the fluorescence intensity. The RBCs were labelled with the lectin as previously done during the cytometry and mobility testing. The samples were also tested on the same day they were prepared.

Table 6.8: Electrophoresis Protocol for Electrokinetic Focusing of A1 and A2 RBC

Step	Duration (s)	Buffer Well 1 (V)	Sample Well (V)	Buffer Well 2 (V)	Buffer Waste Well (V)
1	200	Float	Ground	Float	1000
2	150	10	Ground	10	1630

Initial tests were done with A1 bound with lectin, A1 alone and A2 bound with lectin. It was hypothesized that the lectin bound with A1 would show a distinctly larger

fluorescence than the other two samples. Even though a certain amount of binding with the A2 cell should occur, the quantity of lectin should be much less. Thus, it should lead to a much lower fluorescence signal.

The results of these initial tests matched the hypothesis that there was a distinct increase in the fluorescence of the A1 cells bound to lectin compared to unbound A1 cells as well as the A2 cells. The A1 cells not bound to lectin showed little fluorescence beyond the scattered light (Fig. 6.8), and the amplitude of their signals did not surpass 0.1 V. The signals of the A2 cells bound and not bound with lectin were also low and did not exceed 0.1 V in amplitude. A dropout of signal may be seen in Fig. 6.8c for the case of A2 cells not bound with lectin. This was a random noise signal that occurs at times with the μTKs and did not represent any meaningful observation. The increase in the fluorescence signal of the A1 bound with lectin was thus concluded to be due to the fluorescent lectin. Thus, it was also decided that 0.1 V in signal amplitude would be a suitable threshold since the A1 cells bound with lectin were expected to fluoresce higher than both the A2 bound with lectin and cells not bound with lectin. The A1 RBCs bound with lectin showed signals with approximately 30% of the cells showing fluorescence with amplitude greater than 0.1 V during initial testing. The number of fluorescent cells above the threshold for the other three cases of A1 and A2 not bound with lectin and A2 bound with lectin was 0% for all. This gave a total percentage difference of approximately 30% between lectin bound A1 cells and all other cases. These results were obtained on four loads with their first runs. They were reproduced on the second runs of each of the loads. Similar results were reproduced on two other loads (1 run each) with both giving a percentage of approximately 30% for lectin bound A1 cells. These initial positive results obtained on the μTK were similar to those obtained using the flow cytometer. In the cytometry results, approximately 45% of the lectin bound A1 cells fluoresced above the set threshold for the highest percentage. The lectin bound A2 cells had a maximum 10% of cells with fluorescence above the threshold out of the 5 runs tested. This showed a 35% difference in the number of cells with higher fluorescence levels for the A1 cells compared to the A2 cells, suggesting that determination of A1 cells was possible using cytometry. Since a similar difference of approximately 30% was seen

with the μTK, it appeared following these initial tests that the A1 bound with lectin could be identified from the A2 using the microfluidic platform.

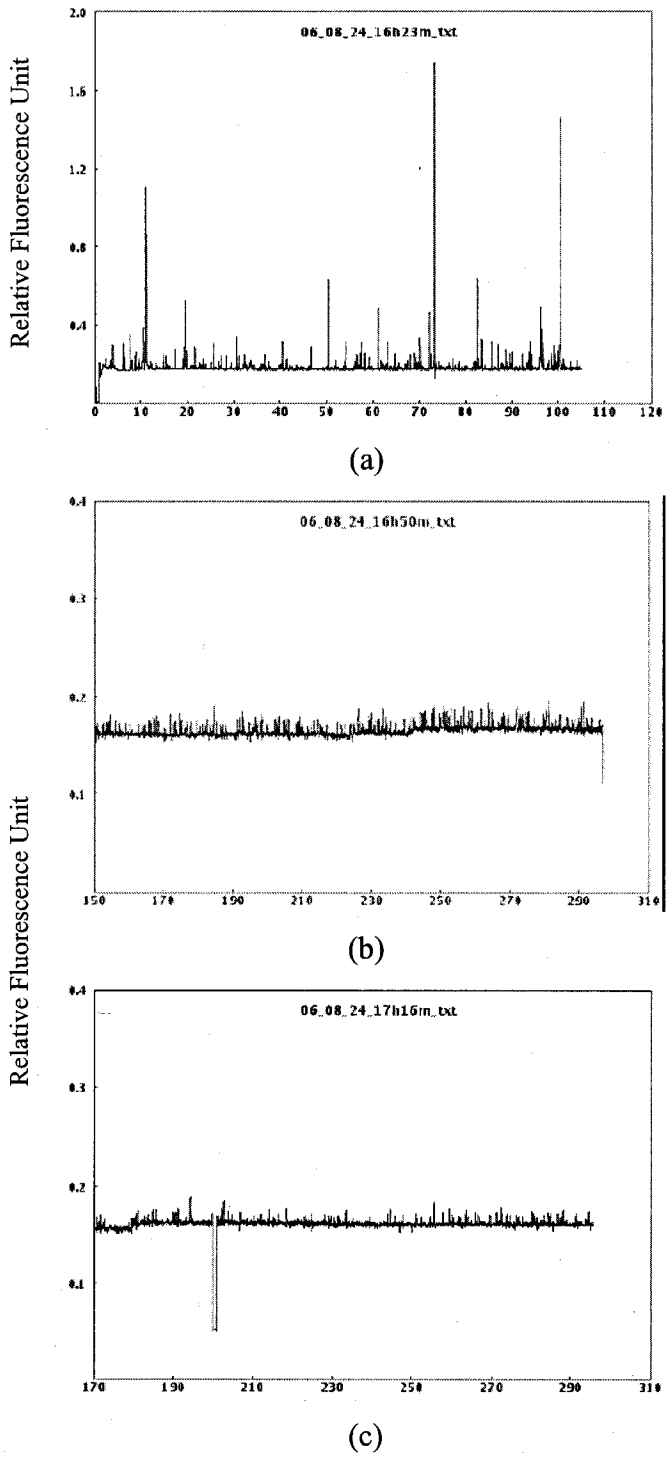

Fig. 6.8: Electropherogram of A1 RBCs bound with fluorescent lectin (a), A1 RBC alone (b), and A2 RBCs bound with lectin (c)

In order to further determine the reproducibility and reliability of the above method for the determination of A1 cells, blind sample testing was then done. The samples included type A1 and A2 cells not bound with lectin, and A1 and A2 cells bound with lectin. The signal amplitude of 0.1 V was used as the threshold as was done during initial testing for determining whether the signal was positive for fluorescence or negative. A positive result would mean that the cells were A1 bound with lectin whereas a negative result corresponded with the remainder of the cases. The percentage of positive signals required for a positive identification of A1 cells bound with lectin was then set at greater than 10% to allow for a larger safety margin. This threshold was used with 0.7 gain on the μTK as previously done, and tested on two different μTKs to determine whether results were system specific. Similar results were obtained on both systems tested.

The cells were electrokinetically focused and their fluorescence captured. The total number of cells and the number of cells above the threshold were then counted. The percentage of RBC above the threshold was then calculated based on the counts. The results of the blind sample testing showed percentages of cells above the threshold to be lower during positive results compared to the initial tests. A1 cells bound with lectin were successfully determined from A2 cells in 33% to 50% of the cases tested. The remaining unsuccessful cases were often due to inconclusive results where the percentages of positive signals did not reach above the set 10% of total signals. These results may have been due to not testing the cell samples immediately after preparation. A minimum of approximately 1.5-hour delay occurred on the three different occasions of testing. The elimination of this lag time may reduce any adverse effects of the TBE on both the lectin and the binding of the lectin to the RBCs, which have led to weak fluorescence. Future tests may be conducted with various incubation times of the prepared samples in TBE prior to electrophoresis on the μTK to determine its effects on the fluorescence signals. If indeed the lag time caused problems then the preparation and testing of the RBCs should be coordinated such that the samples are tested immediately following binding with lectin. Once the effect of the TBE is determined, then further testing can be done in attempts to reproduce the results obtained during the initial testing for the blind samples. Only once the blind samples are reproducibly identified, could this

method be considered for clinical uses. Depending on the number of cells detected above and below the threshold, the set requirement of 10% above the threshold may be lowered. The number of cells required for the count may also be lowered if there is sufficiently small variation in the maximum percentage of positive cells occurring in negative samples. This would enable a reduction in the percentage of signals required to be positive to obtain a positive A1 result. Thus, a reduction in the number of cells and time required for could also be achieved. Further data gathering along with the testing of donor cells would be required to determine the final optimized testing protocol.

6.6 Conclusions

The focus of this chapter was on the control and manipulation of antibodies and RBCs on-chip. The idea was to see if the two could be reacted on a microchip so as to provide methods to improve blood based diagnostics on-chip. The main focus was the use in blood typing. The first step was the determination of appropriate conditions for the antibodies and cells to bind on-chip. The two buffers used in our microfluidic systems were citrate buffer and Tris Borate EDTA buffer. These two buffers were chosen based on the testing of various suitable buffers done in Chapter 3. The suitability of the buffer for work with biological analytes was assessed, specifically their suitability for working with antibodies and RBCs. As previously noted (Chapter 3), various different buffers have been used for work with proteins and antibodies in CE and on-chip. Although several such as PBS and citrate were used more often than others, there was no standard buffer for use for antibody and RBC reactions on-chip. The difficulty in finding an appropriate buffer for our purposes was further enhanced by the need for sufficient buffering required by on-chip electrophoresis.

Tests showed that the binding of antibodies and RBCs occurred readily in the citrate buffer. The adsorption of antibodies in the polymer filled channels also provided a method for immobilization of antibodies to be used for binding with RBCs. The chip was first "flushed" with antibodies and then RBCs were passed through the same channel. Binding of the antibodies to the cells was observed and determined through the mobility of the RBCs. It was hypothesized that the presence of the bound antibodies acted to slow

down the cells during electrophoresis. This was demonstrated by the slowing down of RBCs in the presence of target antibody binding. Thus, this method of introducing antibodies to cells in a microchannel could be a viable method for potential forward blood typing.

The use of TBE buffer, although allowed for a more stable current and longer separation times, showed less success due to a lack of binding of the antibodies to the cells. In order to determine more quantitatively the amount of binding and to accurately use cell mobility to determine antibody binding, a buffer that allows for antibody-cell binding such as citrate but with better buffering capability is required. Thus, a better understanding of which buffers is more suited for allowing for both sufficient electrophoresis and antibody-cell binding is a topic for future research.

The use of antibody binding to enhance mobility shifts in RBCs also requires further optimization. More stringent controls on the quantity of antibodies adsorbed are required. This may involve methods such as the rejuvenation of the chip prior to each load so that no residual antibodies are present. The use of disposable chips may also be considered if the chips are to be used for typing in the future. The fluctuation in the quantity of antibodies adsorbed as well as the quantity that bound with the RBCs was also too large. This led to problems with reproducibility and quantification. Further testing and protocol optimization to improve these issues are required to determine whether this is a viable method for determining mobility shifts using antibody binding and potentially blood typing.

This mobility method was tested as a potential method for separating A1 cells from A2 cells was also tested. However, similar issues with insufficient amounts of mobility difference between the two cell types to enable proper distinction were seen as those as with mobility based ABO typing. A reliable method for the determination of A1 and A2 cells was achieved using electrokinetic focusing of the cells on-chip coupled with LIF detection. Pre-processing was required with this method, but offered results similar to those currently used on the macroscopic scale for A1 determination. Further testing with donor samples would aid in demonstrating the reproducibility and reliability of this technique. This method could potentially be use for rapid determination of the A1 subgroup, a feat that would be extremely useful in organ transplants and transfusions.

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Chapter 7

Conclusion

The many advantages associated with miniaturization of diagnostic methods have fuelled research for this thesis. The reductions in reagent consumption, time, cost, labour and the potentials for portability and integration have all provided motivation. The methods to potentially improve on-chip blood based diagnostics were developed and are briefly summarized here.

The use of microchip based electrophoresis for improving mutation detection was the first technique investigated. A method for on-chip chemical denaturation of DNA was developed. Formamide was used a denaturant of double stranded DNA into single stranded DNA. This method enabled not only the denaturation of the DNA, but also allowed for the recombination of DNA during electrophoresis away from the denaturant. Thus, both heteroduplex analysis (HA) and single stranded conformation polymorphism (SSCP) analysis were enabled on-chip. This denaturation technique provided a quick and simple method for the combination of HA and SSCP that, in combination, may improve the mutation detection rate to close to 100% compared to only 90% for either HA or SSCP alone.

Next, investigation into on-chip antibody and red blood cell manipulation was accomplished. Here, various buffers and electrophoretic protocols were tested to find appropriate reagents and methods for the successful and reliable on-chip manipulation and movement of cells and antibodies. Effective methods to overcome electroosmotic flow and analyte-wall interactions, and improve reproducibility were developed.

Successful binding of red blood cells with antibodies on-chip within microchannels was also achieved.

Finally, the improvement of on-chip blood typing was investigated. Attempts were made to conduct the forward blood typing test on-chip. Efforts were focused on the use of mobility shifts between red blood cells of different blood types. It was discovered that too much variability existed within a population of cells for each of the ABO blood types tested for this method to be reliably used on-chip. Mobility enhancement, possibly through the binding of the blood cells with selective antibodies, appears to be effective in increasing the mobility shifts to a significant level for successful typing to occur. This serves as a strong starting point for the next step in developing a method for on-chip forward ABO typing based on red blood cell mobility.

Although ABO typing using a microchip-based method was not accomplished, a novel method for the distinction between the A1 and A2 subgroups of type A was developed. This method used off chip binding of a fluorescent A1 sensitive lectin with on-chip LIF detection coupled with electrokinetic focusing. The increased signal intensities of the A1 cells were distinctly different than those of the unbound A2 cells, enabling determination of the two blood types.

On-chip denaturation to improve DNA mutation detection by enabling simultaneous HA and SSCP was successfully achieved. This achievement combines a large reduction in the time required for both types of analyses through improved integration capabilities. Work with blood components led to the discovery of a set of reagents and conditions that may be easily used for reproducible on-chip electrophoresis of red cell and proteins. A potential method for the determination of A1 from A2 cells using a microfluidic platform was also developed that may be used towards future miniaturization in blood typing. The use of electrophoresis as the primary mode of analyte manipulation on-chip enables the future integration of the techniques developed here with more ease. Electrophoresis also adds flexibility for their potential integration with other existing on-chip methods that also employ electrokinetic movement. The work here provides new techniques for furthering lab-on-a-chip advancements. Overall, the techniques developed in this thesis may help to build a strong basis for the future development of blood-based diagnostics in microfluidics.