A Bio-MEMS device for separation of breast cancer cells from peripheral whole blood

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A BIO-MEMS DEVICE FOR SEPARATION OF BREAST CANCER CELLS FROM PERIPHERAL WHOLE BLOOD

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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The Department of Mechanical Engineering

by

Juan Feng
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Abstract

This work demonstrates the application of Bio-Micro Electro Mechanical System (Bio-MEMS) technology for early breast cancer detection and diagnosis. Early breast cancer detection and diagnosis typically uses conventional mammogram screening followed by biopsy, which can be problematic since mammography can only detect highly calcified tumors greater than 1 cm in size. A micro-device was developed to identify and specifically collect tumor cells of low abundance (1 tumor cell among $10^7$ normal blood cells) from circulating whole blood.

By immobilizing anti-EpCAM (Epithelial Cell Adhesion Molecule) antibodies on polymer micro-channel walls by the chemical surface modification of PMMA, breast cancer cells from the cell line MCF-7, which over-express EpCAM on their surfaces, were caught by the strong binding affinity between the antibody and antigen.

To validate the capture of the rare breast cancer cells, three fluorescence markers, each identified by a separate color, were used to reliably distinguish the cancer cells from blood cells. The cancer cells were defined by DAPI$^+$ (blue), CD45$^-$ and the FITC-cell membrane linker$^+$ (green). White blood cells, which will interfere in the detection of the cancer cells, were identified by DAPI$^+$ (blue), CD45$^+$ (red), and the FITC-cell membrane linker$^+$ (green).

Three EpCAM/Anti-EpCAM binding models were used to determine an optimal velocity, 2mm/sec, which should guarantee the binding of the maximum number of cells, a critical binding force, and a maximum throughput. At higher velocities, shear forces (> 0.48 dyne) will break existing bonds and prevent formation of new ones. This detection micro-
device can be assembled with other lab-on-a-chip components for follow-up gene and protein marker analysis.
Chapter 1    Introduction

1.1  The Importance of Early Detection of Breast Cancer

Breast cancer, the most common cancer in women, represents the second leading cause of death in women in the United States after lung cancer. Each year invasive breast cancer is diagnosed in 180,000 women in the US, and more than 40,000 women die from this disease (Patlak, 2001). The occurrence rate of breast cancers in females varies with geography, with the highest frequency of 1 in 4 female cancers in North America and Western Europe, to a low frequency of 1 in 16 in China and Japan (Patlak, 2001).

Before researchers discover new therapies to prevent breast cancer and cure all women patients at any stage of cancer, early cancer detection will be looked upon as the most effective strategy for reducing the burden of this disease. In clinical trials to reduce breast cancer death, some patient cases have shown that the successes of the breast cancer treatments are, as with most cancers, highly dependent on early detection of tumor formation. Treatment in the earlier ages, corresponding to the earlier detection of tumors, is more likely to be effective. Since female patients at early stages of tumor growth and proliferation can be cured when treated properly, earlier detection of tumors holds a wonderful hope for those people. Early cancer detection is the hallmark of successful cancer treatment.

1.2  Conventional Early Breast Cancer Detection

The widespread introduction of national screening programs for breast cancer had led to a significant increase in the number of non-palpable breast lesions detected (Frachebound,
Studies have shown that routine mammography screening can reduce the number of deaths from breast cancer by about 25-30% among women between the ages of 50 and 70 (Frachebound, 1998).

X-ray mammography and ultrasound imaging, commonly used as screening techniques have played important roles in breast cancer detection. Mammography imaging is based on density of the tissues while ultrasonic imaging is based on the acoustic impedance, which is the product of the density of the observed tissue and the velocity of the ultrasound through the tissue. The different principles underlying those two detection technologies, lead to mammography being used more in mass detection while ultrasound is a useful adjunct to mammography and used to determine if the detected abnormalities are benign or malignant.

Although the diagnosis and treatment of breast cancers have noticeably improved the outcome of the disease, reducing the death rate over the last decade, they are not perfect due to the low detection rate. These screening techniques cannot eliminate all deaths from breast cancer because they can only detect highly calcified tumors that are ~1 mm in size. As many as 15% percent of breast cancers may be missed by mammography screening (Patlak, 2001).

For the case of lung tumors, by the time tumors were noticeable by x-ray screening, they had already advanced to a stage that was incurable by spreading beyond the lung. Despite of the effectiveness of detecting small tumors by screening device, it still may not detect the early cancer reliably enough to reduce the number of cancer deaths.

There are two key requirements needed to improve screening tools: sensitivity, which is identification, as correctly as possible, women who have breast cancer; and specificity,
which is to determine the type of the cancer, and how far it has spread. So far, no single imaging method has both high sensitivity and specificity for breast cancer (Moore, 2001).

In addition, breast imaging techniques still need advances in image quality. Interpretation of mammogram images is subjective and can be variable among radiologists. The sensitivity of screening mammography is especially low in younger women because of their higher dense breast tissue (Harris, 1997). The dense tissue interferes with identification of abnormalities associated with tumors, which will lead to a higher rate of false test results in young women.

Mammogram screening yields inconclusive results, which can require patients to undergo other invasive, expensive, and discomforting follow-up programs, such as the surgical biopsy, in which patients have to go through an operation to remove tissue samples from the suspicious area of the breast. Those following programs probably bring out the deleterious effect for some women patients, for example, due to suspicious but insufficient mammogram, three quarters of all breast lesions biopsied turn out to be benign, which means those lesions may never develop into life-threatening diseases.

Another reason to explain the remaining breast cancer mortality despite considerable progress in earlier detection using these screening techniques is the intrinsic metastatic properties of cancer. Conventional tumor screening and staging usually miss subsequent metastasis formation, which is generated with the spread of tumor cells to other sites in patient’s body. Due to this reason, using mammography to detect the tumors in one organ, while missing the target in other sites of the body will never help researchers find a way to treat the cancer entirely. The critical direction for cancer research should be focused on the cellular level cancer, and how and why these genetic errors occur.
1.3 Conventional Early Breast Cancer Diagnosis

Mammogram screening is followed by surgical biopsy due to the inconclusive results. Suspicious tissue is removed and sent to a pathology laboratory for analysis to determine if the detected tumor is benign or malignant. Fine Needle Aspiration (FNA) and Core Needle Biopsy (CNB), as well as surgical biopsy, are used increasingly as the initial diagnostic procedures.

Some types of cancer, like pancreatic carcinomas, cannot be detected until they are far advanced and have invaded neighboring tissues due to their remote anatomical location. However, the breast is anatomically accessible; hence FNA and CNB are used increasingly as the initial breast cancer diagnostic procedures, as well as surgical biopsy.

1.3.1 Fine Needle Aspiration (FNA)

Fine needle aspiration (FNA) is a percutaneous (“through the skin”) procedure that uses a fine gauge needle (22 or 25 gauge) and a syringe to draw sample fluid from a breast cyst or remove clusters of cells from a solid mass (Meunier, 2002). The cellular material drawn out with FNA is sent to the pathology laboratory for analysis. There are two ways to perform FNA, depending on the symptoms. For the first case, if the radiologist or surgeon can feel a breast lump by touch, the needle will be guided into that area of concern by palpating (feeling) the lump. In the second case, the lump is non palpable, image guidance using either mammography or ultrasound is used to accomplish the FNA operation.

By placing the needle on the region of the lesion (abnormality) to create vacuum, the tissue sample is taken out. If the fluid sample extracted from the breast lump appears to be bloody, it indicates cancer and is sent to pathology laboratory for analysis. In most case,
these fluids turn out to be clear, green, white, or yellow, which means that it is typically benign (non-cancerous), and will be simply discarded because there is no medical value gained from further microscopic observation and analysis. According to the breast tissue sample properties, the National Cancer Institute has recommended a standardized uniform terminology to define five categories, unsatisfactory, benign, indeterminate/atypical, suspicious/probably malignant, and malignant (NCI, 1997).

As the fastest and easiest method of breast biopsy, FNA is also the simple procedure for patients to go through due to the fact that no stitches are required and the patients are capable of resuming normal routine immediately afterwards. However, FNA has the disadvantage of incomplete assessment because the small sample volume extracted cannot be evaluated in relation to the surrounding tissue. For example, based on a FNA breast sample, a pathologist may diagnose ductal carcinoma in situ (DCIS), a non-invasive breast cancer, but failed to notice infiltrating ductal carcinoma (IDC), an invasive and more lethal breast cancer, in a near area (Meunier, 2002).

1.3.2 Core Needle Biopsy (CNB)

A core needle biopsy is another percutaneous (“through the skin”) procedure that involves removing small samples of breast tissue using a hollow “core” needle (with the gauge of 16, 14, or 11) instead of the finer gauge needle used in FNA (Smyczek-Gargya, 2002). To guarantee that a sufficient sample of breast tissue is obtained, three to six separate core needle insertions are usually needed. As with FNA, the radiologist or surgeon performs the procedure either by palpation or through image-guidance using mammography or ultrasound. Core needle biopsy (CNB) allows for a more accurate assessment of breast mass
than fine needle aspiration (FNA) because a larger tissue sample is removed for diagnosis. Nevertheless, it is still uncertain that no serious target is missed because CNB can only remove samples of a mass and not the entire area of concern.

Although breast cancer is anatomically accessible using biopsy procedures, due to the fact that breast cancers metastasize in a very early stage, so far these biopsy procedures are not perfect enough to detect all cancerous tumors without error, so there is still a need for improved diagnostic techniques for breast cancer.

The National Academy of Science concluded that there is a long way to go until cancer can be cured. A great deal of work remains to be done, particularly in the field of cancer protein and gene markers. This area holds promise for improving the accuracy of early breast cancer detection and diagnosis (Patlak, 2001).

1.4 Gene and Protein Markers for Cancer Diagnosis

The major objective of breast cancer research is to identify molecular changes in the breast tissue at an early stage. The key to really and entirely beat the breast cancer is to exactly understand the intrinsic nature and causes of cancer, and therefore come up with the effective therapies to control breast cancer at a molecular level. With the knowledge of both specific genes that trigger other mutations in the gene sequence, and lead to development chain of cancer, and characteristic cell-surface proteins referred to as tumor-associated antigens specific to breast cancer cells, researchers can trace down the original cause of the breast cancer more easily and propose effective ways to target them at the molecular level. Recently, significant research work has been focused on those gene and protein markers associated with breast cancer.
1.4.1 Gene Markers

Many cancers, including breast cancer, are suspected of demonstrating chromosomal instability because the multiple mutations in tumor suppressor genes or photo-oncogenes inevitably result in uncontrolled cell growth and metastatic spread (Sidransky, 1992). Breast cancer is a sophisticated disease in which several genetic aberrations occur., Known molecular markers that are particularly important in diagnosing this disease are: BRCA-1, BRCA-2, p53, erbB oncogenes, loss of heterozygosity (LOH), chromosomal aberrations, microsatellite instability, transforming growth factor alpha (TGFα), and the multiple drug resistance (MDR) gene (Dahiya, 1998). The BRCA-1 and BRCA-2 genes, tumor suppressor genes, are identified as defects associated with family inheritance and dramatically increase a woman’s risk of developing breast or ovarian cancer. The knowledge of these genetic changes and the biological consequences related to breast cancer is critical to understand the cause of this disease and help to propose the rational therapy to prevent and treat it.

Over the last few years, numerous techniques have been developed to detect mutations and determine the possible roles each gene marker plays in the formation of malignant tumors. In the work of designing an assay to analyze mutations, several tremendously important factors need be considered, such as inexpensive cost, high selectivity, sensitivity sufficient to detect the small amount of mutant DNA among a myriad of normal DNAs, capability of interferences prevention from the sample matrix. One of the potential techniques for analyzing gene mutations uses micro-electrophoretic systems for size-fractionating DNAs (Effenhauser, 1994, Simpson, 1998), which offers high speed of analysis and high throughput if done in a multi-channel format. The effectiveness of this system is highly dependent on the length of the channel. However, building a long column
on a small footprint is hard to implement. An attractive alternative technique to electrophoretic-based genetic screening are DNA microarrays, which consist of tethered oligonucleotides or genomes attached to planar surfaces (Collins, 1999). Two methods utilized to accomplish the immobilization oligonucleotide or DNA targets on solid supports are the light-generated synthesis method (Pease, 1994), and inkjet technology (Guo, 1994). In most cases, glass is used to act as the support material for high-density micro-arrays. In direct to BRCA-1 gene, high-density hybridization arrays have been constructed for screening the entire coding region for all possible homozygous and heterozygous sequence changes (Hacia, 1996). Although microarrays have an intrinsic advantage, they have inevitable limitations as well. It is difficult to detect frameshift mutations using arrays and they can also be expensive to manufacture.

### 1.4.2 Protein Markers

At the early stages of tumor development, genetic markers in some cases cannot entirely and accurately identify a specific phenotype due to the static nature of the cell’s genome. For this reason, it is desirable to look at protein markers, which are specifically associated with breast cancer as well as the gene markers. To identify tumor cells, these protein markers must be either under-expressed or, more preferably, over-expressed in the tumor compared to the surrounding normal cells. Several tumor protein markers have been used increasingly in recent years as clinical tools for breast cancer diagnosis. Those typical protein markers include, MUC-1 (CA 15.3), Her2/neu, carcinoembryonic antigen (CEA), tissue polypeptide antigen (TPA), Epidermal growth factor receptor (EGFR), and epithelial adhesion molecule (EpCAM).
Her2/neu is a type I membrane protein and has been discovered to be shed into serum by proteolysis of the tumor cell membrane (Slamon, 1987). Unfortunately, only 20-30% of breast cancer patients over-express this protein.

Carcinoembryonic antigen (CEA), another marker for breast cancer, belongs to a family of cell-surface glycoproteins with increased expression in breast cancer.

CA15-3, a high molecular weight (300 - 450 kDa) polymorphic epithelial mucin, has been given considerable attention as a breast tumor marker. It has been proven that CA15-3 antigen is a more sensitive and specific indicator than Carcinoembryonic antigen (CEA) (Vizarra, 1994).

For screening protein expression, two-dimensional gel electrophoresis is commonly used to isolate particular protein types (Alaiya, 2000). This technique involves separating proteins in the first axis according to charge with isoelectric focusing (IEF), followed by separations of the focused proteins along the second axis by molecular weight determination by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The proteins can be visualized by staining with Coomassie Brilliant Blue R250, silver stain, or fluorescent dyes (Gorg, 2000). While being a core technology in protein analysis for several years, 2-D electrophoresis is fraught with difficulties, such as a basic pH limit, long analysis time, and irreproducibility.

An innovative alternate method for protein expression analysis uses protein microarrays. These arrays are formed by printing the proteins onto activated glass surfaces. Then the proteins stained with fluorescence interact via molecular associations with their appropriate counterparts. Cancer research has utilized this technique to obtain molecular
profiles of human cancers for categorizing tumor cells (Liotta, 2000). In the case of breast cancer, several biosensors have been introduced for monitoring different markers. For example, Wang (Wang, 2001) developed a silicon-based ultrasonic immunosensor for the detection of CA15.3 antigens expressed by breast cancers. In his work, CA15.3 antibodies were immobilized on the surface of sensor, and once the antibody is associated with CA15.3 by binding reaction, a mass increase resulted.

1.5 Immunotherapy

Antibodies have been widely used in scientific studies of cancer and cancer diagnosis as well. Monoclonal antibodies have been designed to recognize specific types of cancer cells expressing particular antigens. This strategy has appeared and been successful since the invention of antibodies in the 1970s. The development of monoclonal antibodies (mAbs) directed against epithelial differentiation antigens such as cytokeratins (CKs) and against tumor-associated cell-membrane glycoproteins has opened a diagnostic window to detect disseminated tumor cells as early as primary diagnosis (Schlimok, 1987).

Immunotherapy is a major consideration in cancer therapy, which involves using antibodies against cancer cells or cancer-associated targets. The main goal of immunotherapy is that antibodies can be injected into patients to sieve out the cancer cells, potentially disrupt the cancer cell activities and enhance the immune response against the cancer.

For treatment of certain breast cancers, numerous clinical tests have shown that the antibody trastuzumab (Herceptin) can be useful for the women whose breast cancer cells over-express the HER2/neu protein. Another study (Braun, 1999a) was conducted in ten
patients with advanced breast cancer. They received a single dose of 500 mg of edrecolomab, which is directed against the epithelial cell adhesion molecule (EpCAM) widely expressed on the surfaces of breast cancer cells (Braun, 1999a). Among all of the patients, a phenomenal reduction of the tumor load was monitored by a second follow-up bone marrow aspiration within 5-7 days of edrecolomab antibody treatment. In four of the ten patients, no metastatic cells could be identified after treatment with edrecolomab. Therefore, it could be speculated that the observed disappearance of tumor cells from bone marrow was a consequence of the action of the mAbs (Braun, 2001).

However, immunocytochemical analysis and treatment has its limitations. The actual target cells, like tumor cells disseminated to bone marrow, may express a heterogeneous pattern of the potential target antigens. Tumor antigen heterogeneity may therefore represent an important limitation of the efficacy of monospecific antibody therapy (Braun, 1999b). Also, monoclonal antibodies may have a negative effect on normal body cells, and cause many side effects, like high body temperature, trauma, and low blood pressure.

1.6 Bio-MEMS

Biological Micro-Electro-Mechanical Systems (BioMEMS) has been a topic of growing interest over the past decade. The fabrication of micro-instrumentation using photolithographic techniques has been integrated into molecular biology and offers many advantages over conventional large-scale analysis tools including: reduced analysis time and reagent amount, and increased parallelism of analysis. Due to the small size of the micro-devices, Bio-MEMS also have the potential to be multiplexed easily. Using fabrication techniques, lithographic or molding procedures, many sample processing fluidic networks
can be machined into the device with high packing densities. Another important and appealing advantage is that individual micro-components can be assembled into the device having specific functions. For example, with the combination of assembly and micro machining, various components, such as reaction and detection chambers, micro-pumps, micro-needles, and separation channels, can be fabricated and packed directly onto the instrument platform.

Since emerging in the early 1990s, Bio-MEMS technology has now already been available to fabricate micro-sized mechanical parts, used in the disciplines, like biology and medicine. Harrison and Manz pioneered fabrication of micro-electrophoresis devices in glass substrates using photolithography and wet chemical etching (Harrison, 1992). After this profound contribution, various micro-devices have been constructed and widely used in bioanalytical applications, such as PCR amplification of oligonucleotides used in genomic applications (Cheng, 1996; Belgrader, 2001). Also, the analysis of proteins using either electrophoretic separation (Liu, 2000) or immunological events (Sata, 2000) have been reported.

Due to the optical properties of glass or quartz substrates, all the devices mentioned above were built on them using photolithographic procedures and wet chemical etching. Also, glass substrates allow chemical modification when necessary. However, when considered for machining high aspect ratio features, it is difficult to fabricate channels that are narrow with large depth using wet etching in glass since it is an isotropic etching process. To solve the potential problems inherent with glass-based devices, the fabrication of micro-devices in plastic materials instead of glass has been initiated, such as poly (methylmethacrylate) (PMMA) (Martynova, 1997). An attractive property of transparent
polymer is that they can be easily molded into desired shapes with very high resolution using techniques, such as injection molding, imprinting, hot embossing, laser ablation, or X-ray lithography. Based on the properties of different plastics, like the range of glass transition temperature and thermal conductivity, the right material can be selected for the appropriate system. For example, polycarbonate was selected as the material for PCR due to the high glass transition temperature required for 95°C denaturation zone.

BioMEMS can be also widely applied in Tissue Engineering, drug delivery, management of diabetes, and Bio-MEMS may have many, as yet, unimagined approaches, and no one can predict exactly how widely and effectively this technology will be applied in medical field in time.

1.7 Goal of the Project

This work demonstrates the application Bio-MEMS technology for breast cancer detection and diagnosis. Early breast cancer detection and diagnosis typically uses conventional mammogram screening followed by biopsy, which can be problematic due to the fact that mammography can only detect highly calcified tumors greater than 1 cm in size. A micro-device was developed to identify and specifically collect tumor cells of low abundance (1 tumor cell among $10^7$ normal blood cells) from circulating whole blood.

There were four main objectives set forth for the research. The successful completion of these four goals will aid in the early cancer detection and treatment:

1. Develop a bio-micro-device using micro-fabrication technology and surface chemistry modification to capture tumor cells of low abundance from whole blood. High target cell capture efficiency of this micro-device was the ultimate goal;
2. Visualize the capture of the breast cancer cells and differentiate the target cancer cells from other blood cells in the micro-device;

3. Determine an optimal velocity, which should guarantee the maximum binding of cancer cells, the critical dissociation force and a maximum throughput;

4. Apply a correct blood flow model to estimate the required pressure drops in micro-devices with different types of geometries.

The vision of the complete assembled lab-on-a-chip system would be: a skin patch containing a micro-needle array to draw blood from breast tissue of the patients is put in the bottom of the device; next, the drawn blood flows into the micro-channels designed in this research, the cancer cells trapped on the wall of micro-channels are lysed and sent to PCR (Polymerase Chain Reaction) and other specific assay components to perform the genetic analysis and identification. Each module can be a single use device. The whole objective is to detect early stage breast cancer and analyze the gene and protein markers expressed specifically by tumor cell in order to be able to effectively treat the disease before tumor metastasis.

1.8 Thesis Outline

The rest of this thesis is outlined as follows. Chapter 2 will present a general understanding of different areas including physical, rheological properties of blood, breast cancer cell properties, antibody-antigen binding theory, surface modification of PMMA, Fluorescence analysis and blood fluid dynamics. In Chapter 3, the surface contact area between the single cancer cell and the micro-channel wall will be evaluated, and three EpCAM/Anti-EpCAM binding models will be discussed in detail to determine an optimal velocity, and critical binding force. Also, fluid mechanic parameters present in the blood casson equation and the simulation of the 3-D velocity profile of blood flow in micro-
channel are shown in the rest part of this chapter. Chapter 4 will describe the steps taken to trap and detect the cancer cells, which involve antibody immobilization on the PMMA micro-channel, breast cancer cell line culture, cells fluorescence staining protocols, flow control experiment in micro-channels and detection of cancer cell under fluorescence microscopy. The following chapters will discuss micro-channel geometry effects and cell concentration effects on the cell trap performance. Lastly, Chapter 6 will present the conclusions, and future work.
Chapter 2  Background

Chapter 2 presents a general understanding of different areas including physical, rheological properties of blood, breast cancer cell properties, antibody-antigen binding theory, surface chemical modification of PMMA, fluorescence analysis and blood fluid dynamics.

2.1 Physical, Rheological and Flow Properties of Blood

After blood is drawn from the patients, it will flow through the functionalized micro-channel to sieve out the breast cancer cells. Therefore, a thorough knowledge of the physical and fluid properties of blood is essential for understanding and modeling blood flow in the micro-device.

Human blood is a suspension of cells in an aqueous solution of electrolytes and nonelectrolytes (Fung, 1993). Whole blood can be divided into about 55 volume % plasma, and about 45 volume % cells, or “formed elements” (Cooney, 1976). The hematocrit, a quantity of significant physiological importance, is defined as the volume percentage of cells, and is easily measured by centrifugation of a small blood sample (Cooney, 1976). The plasma is about 90% water by weight, 7% plasma protein, 1% inorganic substances, and 1% other organic substances (Fung, 1993).

2.1.1 Cells Count and Morphology

The cells consist of about 95% (by number) red blood cells (RBCs), or erythrocytes, , while the white cells of various categories and platelets make up less than 1/600 and 1/800 of the cellular volume, respectively (Cooney, 1976; Fung, 1993). In human blood, the ratio
of the number of leukocytes to that of erythrocytes is 1 to 1000. Women have RBC densities of \(4.8 \times 10^6/\mu l\), and men have densities of \(5.4 \times 10^6/\mu l\). White cells account for 0.13% of the total cell count and the number of white cells ranges from 4500 to 11000/µl. The remainder, about 4.9%, consists of platelets. The normal range of platelet density is from 150000/µl to 400000/µl (Cooney, 1976; Reid, 1998).

Human red cells, with a typical diameter of 6-8 µm and thickness of around 2.8 µm have the shape of biconcave disks, which can deform into a bullet-shaped entity during passage through small capillaries. Platelets are much smaller, having a diameter of 1-4 µm (Platt, 1999). The white blood cells are more rounded and include different cell types, including lymphocytes, monocytes, neutrophils, eosinophils, basophils. Eosinophils and band neutrophils are usually 9 to 15 µm in diameter, basophils average 10 to 15 µm in diameter, lymphocytes range in size from 6 to 10 µm, and monocytes range in size from 10 to 30 µm in diameter (Platt, 1998).

Red blood cells are very deformable, and they can take different odd shapes in the flowing blood in response to hydrodynamics stress acting on them. When flow stops, the red cells become biconcave. The red blood cell membrane is peculiar in that it can sustain a large shear deformation (the cell membrane ruptures if the area is changed by more than a few percent). Unlike leukocytes and cancer cells, erythrocytes are non-nucleated.

White blood cells are the minority of cell population, and hence have little influence on blood viscosity. In microcirculation, however, white blood cells may adhere to the blood vessel wall and obstruct the flow due to the small size of the vessel. The obstruction is related to the deformability of white blood cells (Fung, 1993).
Breast cancer cells, with a diameter of 15~30\(\mu\)m, are two or three times bigger than red blood cells, and spherical. At the early stages of cancer, it has very low abundance in the blood (1~10 tumor cell in 1 ml). As adherent cells, breast cancer cells have a tendency to form cell aggregates.

Figure 2-1 shows the morphology and frequency of three types of cells, erythrocytes, leukocytes and breast cancer cells.

2.1.2 Blood Viscosity and Flow Curve

Viscosity is defined as the ratio of shear stress (N/m\(^2\)) to shear rate (or velocity gradient, 1/s) for a fluid. Figure 2-2 displays four different types of models for fluids, Newtonian, Power law, Bingham plastic, and Casson. If the viscosity of the fluid is the slope of the flow curve, the constant-slope lines corresponds to Newtonian behavior, which demonstrates that fluid viscosity, is independent of shear rate. Blood falls into the category of “Casson” curve, which is characterized by a viscosity that depends on the shear rate, and
also means that blood will not flow at all until a shear yield stress \( \tau_y \), or greater has been imposed on it.

### 2.1.3 Effect of Shear Rate on Viscosity

Under conditions of low shear, red blood cells have the tendency to gather, and "rouleaux", which leads to a rise in viscosity (Cooney, 1976). From Figure 2-3, the apparent absolute viscosity of blood decreases with an increase in the shear rate. The aggregated RBCs break down at higher shear rates. When the shear rate exceeds 50 sec\(^{-1}\), no rouleaux exist, and the value of blood viscosity is asymptotic with respect to shear rate.

From Figure 2-3, it is noted that nearly constant viscosity values are reached at shear rates above approximately 30-50 sec\(^{-1}\) (Whitmore, 1968).

![Fluidic models (Whitmore, 1968)](image)

Figure 2-2 Fluidic models (Whitmore, 1968)
2.1.4 **Cell-Free Marginal Layer**

It was observed by Thoma that in a tube flow, red cells tend to move toward the axis of the tube, leaving a marginal zone of plasma near the wall (Fung, 1993). In channels at least 15% wider than the cells there is a migration of the cells to the central axis of a capillary leaving a cell-free layer of plasma adjacent to the channel wall that can be as thick as 4 µm (Fung, 1993). The existence of the cell-free layer has been recorded by high-speed photography of the flow in vessels and glass tubes.

2.1.5 **Tube Diameter Effect**

In vivo, experimental data have shown that the apparent viscosity is a function of tube diameter as well as the shear rate. The dependence of apparent viscosity on tube diameter is known as the Fahraeus-Lindqvist effect. This interesting effect is related to the cell-free marginal layer, or plasma layer, adjacent to the tube wall. Since cells tend to migrate to the center of the tube to form an “axial accumulation”, while no cells are present near the tube
Therefore, the hemotocrit of fluid near the wall will be reduced while that of the core fluid in the center of the tube will be increased (Cooney, 1976). Since the shear rate near the wall is much greater than that at the core, the decreased viscosity near the wall due to cell-free marginal layer outweighs the increased viscosity at the core due to the axial accumulation. Taking into account these two influential factors, the increased viscosity in the center and simultaneously reduced viscosity at the wall, the effective apparent viscosity is smaller than if no cell-free marginal layer occurred. It is worth mentioning that the reduction of the viscosity is much more pronounced for small tubes because the cell-free layer is a large fraction of the whole tube volume.

Pries proposed the human blood apparent viscosities in both vivo and vitr a flows. In vivo, apparent relative viscosity correlations for human blood is (Cokelet, 1997; Pries, 1994),

\[
\eta_r = \left[ 1 + \left( \eta_{0.45}^* - 1 \right) \frac{(1 - H_D)^C - 1}{(1 - 0.45)^C - 1} \left( \frac{d}{d - 1.1} \right)^2 \right] \times \left( \frac{d}{d - 1.1} \right)^2 \\
C = \frac{1}{1 + 10^{-11} d^{12}} + \left( \frac{1}{1 + 10^{-11} d^{12}} - 1 \right) \left( 0.8 + e^{-0.075d} \right) \\
\eta_{0.45}^* = 6e^{-0.085d} + 3.2 - 2.44e^{-0.06d^{0.45}}
\]

Relative apparent viscosity, \( \eta_r \), relative to the plasma viscosity, which ranges from 1.3 to 1.7 cp. \( H_D \) is the hematocrit in the vessel and \( d \) is the vessel diameter (\( \mu m \)). \( C \) is a curve-fitting constant of the correlation.

For vitro measurements, the apparent blood viscosity is a function of tube diameter and hematocrit was expressed by Pries (1992) as:
The hydraulic resistance of a vessel, \( R_e \) is computed with the value of the apparent viscosity correlation,

\[
\eta_r = 1 + (\eta_{0.45}^* - 1) \frac{(1 - H_D)^c - 1}{(1 - 0.45)^c - 1}
\]

\[
C = \frac{1}{1 + 10^{-11}d^{12}} + \left( \frac{1}{1 + 10^{-11}d^{12}} - 1 \right) \left( 0.8 + e^{-0.075d} \right)
\]

\[
\eta_{0.45}^* = 220e^{-1.3d} + 3.2 - 2.44e^{-0.06d^{0.65}}
\]

2.2 Breast Cancer Cell Properties

2.2.1 Comparison Between Tumor Cells and Normal Cells

All cells have many receptors on their membrane surface, which gave communication between cells and their environment. Tumor cells of ectoderm origin differ from normal blood cells, which are mesoderm origin, in their gene expression, and each of these two cell populations has different tissue-specific molecules on its surface (Racila, 1998). Normal breast cells do not have receptors for epithelial adhesion factors, but breast cancer cells do. This means that breast cancers are likely to have arisen from a defect in this epithelial adhesion gene, which causes the receptors to be replicated numerous times on the cell surface.

2.2.2 EpCAM Overexpression on Breast Cancer Cells

Tumor cells will express specific patterns of potential target antigens. Epithelial Cell Adhesion Molecule (EpCAM) is widely expressed on tumor cells of various origins, especially for breast cancer. Breast cancer cells overexpress high levels of the EpCAM
antigen; there are on the order of 280,000 EpCAM molecules per cell. A prostate cancer cell line expressss lower levels of EpCAM, about 25,000 EpCAM molecules per cell. (Chianese, 2002). The EpCAM expression of breast cancer cells makes these cells suitable targets of the Anti-EpCAM antibody.

2.2.3 Epithelial Cell Adhesion Molecule (EpCAM)

EpCAM is identified as a ~ 40-kilodalton (kd) protein in Western blotting (Yemul, 1993). It is also known as epithelial glycoprotein 40 (EGP40), epithelial surface antigen (ESA), GA733-2, KSA, and the 17-1A antigen. Encoded by the GA-733-2 gene, EpCAM is expressed on the baso-lateral cell surface in most simple human epithelial cells. Normal epithelial cells, which may express EpCAM are present in the lower respiratory tract, the lower gastrointestinal tract, the tubules in the kidney, the surface epithelium of the ovary, the exocrine and endocrine pancreas, secondary germ cells of telogenic hair follicles, and secretory tubules of sweat glands in the skin. In addition, all epithelial cells in the thyroid gran and the thymus express EpCAM. The outer cortex and Hassall’s corpuscles have low expression. In the liver, only the bile ducts appear to be positive with anti-EpCAM antibodies (De, 1994). Tumors arising from non-epithelial cells, such as lymphoma, mesothelioma, neuroblastoma, and melanoma do not express EpCAM (De, 1994). EpCAM has attracted attention as a tumor marker due to its expression in the vast majority of carcinomas (Litvinov, 1994).

2.2.4 Tumor Cells in Peripheral Whole Blood

At the early stage of tumorigenesis, tumor cells are shed into circulating blood, which makes it possible to detect cancer cells in the blood stream before the primary tumor is large.
enough to be detected by standard screening examinations (Racila, 1998; Gross, 1995). Individual cancer cells can detach from a tumor, invade the blood vessels, and flow throughout the bloodstream until they find a suitable location, then invade new tissue and form a new tumor. In human blood, the only epithelial cells in the circulatory system are tumor cells, so EpCAM antigen over-expressed by breast cancer cells at the low frequency among whole normal blood cells, will be a precise precursor to detect breast cancer at the early stages of tumor growth and metastasis. Also, the expression of EpCAM by micrometastatic breast cancer cells defines these cells as suitable targets of the Anti-EpCAM antibody.

The challenge of early detection of tumor cells is the extremely low frequency of occurrence in blood, which is 1 tumor cell in $10^6$ normal blood cells (5–10 cancer cells per micro liter of whole blood). The tumor cell frequency is many orders of magnitude lower than the red blood cell count, however, the great differences in gene and protein expression between tumor cells and normal blood cells, tissue-specific molecules on the cell surface can be used to identify and specifically collect tumor cells of low abundance. It is vital to establish a sensitive and reliable method for detecting tumor cells in peripheral whole blood.

2.3 Antibody-Antigen Interaction

2.3.1 Antibody Structure and Function

Antibodies are immune system-related proteins called immunoglobulins. Each antibody is made up of two 15000-MW polypeptide chains, designated as heavy (H) chains, and two 25000-MW chains, designated as light (L) chains (Kuby, 1997). Those four chains join together to form a “Y” shaped molecule. The antibody structure is shown in Figure 2-4.
The variable region, the tips of the “Y”, consists of 110-130 amino acids, and includes the end of both the light and heavy chains. The amino acid sequence in this variable region varies so greatly among different antibodies that each antibody has a specific identity for its binding antigen. Treating the antibody with a protease can cleave the variable region, producing Fab (Fragment Antigen Binding), which retains the “antigen-binding” activity, and an Fc fragment, which is found to crystallize during cold storage.

The constant region determines the mechanism used to destroy an antigen. There are five basic amino acid sequence patterns (μ, δ, γ, ε, and α) for the heavy-chain constant-region sequences (Kuby, 1997). Each of the sequences is called an isotype. The heavy-chain constant-region structure and immune function determine the class of the antibody: IgM, IgG, IgA, IgD, or IgE.

The variable region can be further subdivided into hypervariable (HV) and framework (FR) regions. Hypervariable (HV) regions have a relatively higher ratio of different amino acids than those in the framework (FR) region. HV regions are also referred to as
complementarity-determining regions (CDRs) because this region is directly complementary to the antigen. The FR regions form a beta-sheet structure to serve as a scaffold, holding the HV regions in position to react with antigen.

### 2.3.2 Ligand-Receptor Kinetic Binding Theory

Receptor-mediated cell adhesion plays a crucial role in physiological and biotechnology-related processes. The specific binding of ligands to receptors is widely used in molecular recognition. In the immune system, ligand-receptor binding plays an important role in defending the living mechanism against malignant cells and pathogenic organisms.

The binding of receptor to a ligand is a reversible process, involving non-covalent bonds. Receptors bind ligands with a high-affinity lock-and–key mechanism, and the bonds are weaker than covalent bonds (Chang, 1996). The assumption of receptor-ligand binding is that the cell is brought close enough to the ligand substrate so that receptors on the cell can interact with the ligands on the substrate (Chang, 1999). When they are first brought to this position, any particular receptor is unlikely to have a ligand sufficiently close to be able to form a bond. As one or both reactants diffuse in their respective membranes, opportunities for bond formation will occur (Bell, 1978), and an encounter complex is realized. For the membrane-bound reactant, in general, the reaction can be conceptually separated into two steps. In the first step the reactants A and B simply encounter each other and diffuse on their membranes until they are close enough to form the encounter complex AB. In the second step the binding reaction occurs. The reaction may be written as

\[ A + B \Leftrightarrow AB \Leftrightarrow C \]
Table 2-1  Sample Antibody/Antigen Bindings Parameters

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>$R_T$ (#/cell)</th>
<th>$k_f$ (M$^{-1}$min$^{-1}$)</th>
<th>$k_r$ (min$^{-1}$)</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fcγ</td>
<td>2.4G2Fab</td>
<td>$7.1\times10^5$</td>
<td>$3\times10^6$</td>
<td>0.0023</td>
<td>$7.7\times10^{-10}$</td>
</tr>
<tr>
<td>Chemotactic Peptide</td>
<td>FNLLP</td>
<td>$5\times10^4$</td>
<td>$2\times10^7$</td>
<td>0.4</td>
<td>$2\times10^{-8}$</td>
</tr>
<tr>
<td>TNF</td>
<td>TNF</td>
<td>$6.6\times10^5$</td>
<td>$9.6\times10^8$</td>
<td>0.14</td>
<td>$1.5\times10^{-10}$</td>
</tr>
<tr>
<td>Insulin</td>
<td>Insulin</td>
<td>$1\times10^5$</td>
<td>$9.6\times10^6$</td>
<td>0.2</td>
<td>$2.1\times10^{-8}$</td>
</tr>
<tr>
<td>EGF</td>
<td>EGF</td>
<td>$2.5\times10^4$</td>
<td>$1.8\times10^8$</td>
<td>0.12</td>
<td>$6.7\times10^{-10}$</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Fibronectin</td>
<td>$5\times10^5$</td>
<td>$7\times10^5$</td>
<td>0.6</td>
<td>$8.6\times10^{-7}$</td>
</tr>
</tbody>
</table>

Receptor-ligand binding is a very complicated phenomenon, and it remains far from being clearly understood. Clearly the bond strength, rate of formation and dissociation of the ligand-receptor bindings, and diffusivity on the membrane are key determinants of the adhesion process, and they are the focus of many cell adhesion studies.

Table 2-1 shows sample receptor/ligand binding parameters (Lauffenburger, 1993), including $k_f$ and $k_r$ are the forward and reverse reaction rates respectively, and $K_D$ is the binding affinity.

Here, TNF means tumor necrosis factor; and EGF means epidermal growth factor.

2.3.3  Antibody Affinity

The strength of the interactions between a single antigen-binding site on an antibody and an epitope is known as the affinity of the antibody for that epitope (Kuby, 1997). High-affinity antibodies bind antigens tightly and remain bound for extended times. The interaction between low-affinity antibodies and antigens is weak and the bonds tend to
dissociate easily. The association of antibody-antigen interaction can be described by the equation

\[ Ag + Ab \xrightarrow{k_1} Ag - Ab \]

Where \( k_1 \) is the forward, or association rate constant, expressed as liters/mole/second (L/mol/s), and \( k_{-1} \) is the reverse, or dissociation rate constant, expressed as 1/second. The equilibrium constant \( K \), or affinity is the ratio of the association rate to the dissociation rate. It is as follows,

\[ K = \frac{k_1}{k_{-1}} \]

### 2.3.4 Strength of the Bond Related with Affinity

It was presumed previously that the mechanical strength of adhesion between antibody-antigen complexes is related to the bond affinity. Dembo (Dembo, 1988) made the prediction from a theoretical model that adhesion strength is a function of the logarithm of the binding affinity. Experiments by Kuo (Kuo, 1993) were consistent with the prediction by Dembo.

### 2.4 Fluorescence Technique

Fluorescence techniques are used frequently for cell and identification. It is fast and precise. Since the fluorescence emission signal is shifted in wavelength away from the excitation signal, the detection of very low sample concentrations is possible. This aids in early detection and treatment.
2.4.1 Fluorescence Process

In the fluorescence process, fluorochromes are excited by absorption of radiation, such as ultraviolet light, and then emit radiation, generally at a frequency lower than the exciting frequency. In Figure 2-5, fluorescence is shown as a three-stage energy transmission process.

In stage 1, either the radiation source supplies the energy, \( h\nu_{EX} \), of a single photon. The frequency of the radiation source is \( \nu_{EX} \). Once the fluorophore molecule is hit by the photon, it absorbs the photon energy, and creates an excited state (\( S_1' \)). This stage is defined as the excitation stage.

During the second stage, the fluorophore undergoes conformational change, also referred to as internal conversion or vibration relaxation. The energy of \( S_1' \) is partially dissipated, yielding a relaxed, excited state \( S_1 \). Fluorescence quantum yield is a measure of the quantum efficiency of different fluorophores.

![Jablonski Diagram](image)

Figure 2-5  Jablonski Diagram
Fluorescence Quantum Yield = \frac{\text{number of photons emitted at stage 3}}{\text{number of photos absorbed at stage 1}}

This value ranges from zero for inefficient fluorophores to one for ideal fluorophore.

Stage three is defined as the Fluorescence Emission. A photon of energy $h\nu_{Em}$ is emitted and the fluorophore returns to its ground state, $S_0$. Fluorescence is a cyclic and repeatable process.

Due to the energy dissipation in stage two, the energy of the photon is lower in stage three. The energy difference represented by $h \cdot (\nu_{Ex} - \nu_{Em})$ is called the Stokes shift. Fluorochromes that can be excited with the same wavelength can have different emission wavelengths. In multicolor fluorescence applications, different stokes shifts are required for the same light emission source to eliminate the overlapping bandwidth and cross talk, unless optics are used to filter one fluorescence or the other.

### 2.4.2 Immunofluorescent Staining

Antibodies can be visualized by tagging the antibody molecules with a fluorescent dye, or fluorochrome. Dye can be conjugated to the Fc fragment of an antibody molecule without affecting the specificity of the antibody. Absorbing light at one wavelength, the fluorescein dye emits light at a longer wavelength (Kuby, 1997). The emitted light is viewed with a fluorescence microscope, generally equipped with either mercury or xenon arc lamps, which produce high-intensity illumination powerful enough to image faintly visible fluorescence specimens, and excitation-emission filter cubes.

There are two basic fluorescence antibody staining methods, direct and indirect staining. For direct staining, the specific antibody, which is also called primary antibody is
directly labeled with fluorescein. For indirect staining, the primary antibody is unlabeled and is detected with an additional fluoreochrome-labeled reagent. The most common reagent used is a fluorechrome-labeled anti-isotype antibody. Indirect immunofluorescence staining increases the sensitivity of staining because multiple fluorochrome reagents will bind to each primary antibody molecule (Kuby, 1997).

2.4.3 Photobleaching

The irreversible destruction of an excited fluorophore is photobleaching. Different methods are used to reduce the problem. For example, reducing excitation intensity, scanning for shorter times, using anti-fade reagent.

2.5 Blood Flow Modeling

2.5.1 Blood Flow in Large Vessels

The behavior of blood flow in large vessels, with the diameters ranging from 0.2 cm ~2.5 cm, is Newtonian. Utilizing the Navier-Stokes equations to describe pulsatile blood flow in large vessels is a correct mathematical model.

The Navier-Stokes Equation for incompressible Newtonian fluid are,

\[
\rho(\frac{\partial u}{\partial t} + u \frac{\partial u}{\partial x} + v \frac{\partial u}{\partial y}) = -\frac{\partial P}{\partial x} + \mu(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2})
\]

\[
\rho(\frac{\partial v}{\partial t} + u \frac{\partial v}{\partial x} + v \frac{\partial v}{\partial y}) = -\frac{\partial P}{\partial y} + \mu(\frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2})
\]

The continuity equation gives:

\[
\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} = 0
\]
In Figure 2-6, $y$ is measured in the vertical direction from the bottom to the top. The flow conditions are well-defined as to be fully developed flow, that is, the flow field is independent of $x$; also, it is independent of time. Based on these assumptions, the governing equations are simplified to Poiseuille flow, which describes the steady laminar viscous flow in a channel or tube from high pressure to low pressure.

$$\frac{-\partial P}{\partial x} + \mu \frac{\partial^2 u}{\partial y^2} = 0$$

Applying the lower and upper boundary conditions,

$u=0$ at $y=0$,

and $u=0$ at $y=h$,

The velocity profile is derived, and has the simple parabolic form:

$$u = \frac{1}{2\mu} \frac{\partial P}{\partial x} (y^2 - hy)$$

The maximum velocity occurs at the centerline, i.e., $y=h/2$,

$$u_{\text{max}} = \frac{h^2}{8\mu} \frac{\partial P}{\partial x}$$

The volume flow rate is expressed as
\[ Q = w \int_{0}^{h} u \, dy = -\frac{h^3 w}{12 \mu} \frac{\partial P}{\partial x} \]

where \( w \) is the width of the channel.

The mean velocity, \( \bar{u} \), averaged over the height of the channel, is given by the equation

\[ \bar{u} = \frac{Q}{wh} = -\frac{h^2}{12 \mu} \frac{\partial P}{\partial x} = \frac{2}{3} u_{\text{max}} \]

2.5.2 Blood Flow in Small Vessels

For pulsatile flow in small vessels, there is no single "best" model available. More sophisticated and more accurate measurements were needed to carry out in the future to identify the "correct" form of the stress-strain relations. Among all of the possible models, the Casson equation was a popular one because of its assumption of a yield stress - that is, the necessity of an initial state of stress in order to start the blood flowing.
Chapter 3  Biosystem Modeling

To catch cancer cells on a micro-channel wall, the most important issue is to control the velocity of the blood sample in the micro-channel. Too large a velocity will decrease the time for collisions between the antibody and antigen. Too slow a flow rate will decrease the encounter rate. Therefore, there must be an optimal velocity to guarantee highest binding frequency between an antibody and antigen. In the proposed biosystem, breast cancer cells flow through the functionalized micro-channel and are captured by the anti-EpCAM immobilized on the wall.

Hammer’s theory was adapted for this biosystem to evaluate the optimal velocity that would guarantee the maximum binding rate between cancer cells and antibodies found on the wall. Another key parameter is the critical binding force, which describes how well the cancer cell/PMMA surface association holds up under fluid shear. Both Bell’s kinetic model and Dembo’s affinity model were adapted to estimate the dissociation force. Large flow velocities will lead to a large shear force on the cells bound to the wall of the micro-channel. Comparing the critical binding force on the contact surface area of the fixed cells with the shear force, the critical velocity for breaking down the existed binds can be evaluated.

In the EpCAM-mediated cell adhesion to the anti-EpCAM coated surface under the shear flow field, many chemical and physical factors may play a role. The key chemical parameters, including the EpCAM receptor density on the cell surface, the forward and reverse reaction rate in the EpCAM binding system, and the receptor diffusivity on the cell membrane were the main determinants of the number of receptor/ligand bonds formed during a collision and the binding force in the contact zone. The fluid force and shear stress
exerted on an adherent cell exposed to a shear flow will affect the receptor-ligand binding, and this force will counterbalance with the antibody/antigen binding force. There are also non-specific forces resulting from the colloidal interaction, including van der Waals forces, electrostatic repulsion, steric stabilization were also considered to determine if these non-specific forces significant factors affecting the motion of the cell near the surface.

3.1 Simulation of Anti-EpCAM and EpCAM Antigen Binding Under the Flow in Micro-Channel

Chang Hammer’s model (Chang, 1999) was adapted to illustrate and discuss the relationship between the effective forward reaction rate of anti-EpCAM/EpCAM binding and the relative flow velocity of the antibody and antigen. This was used to model the effective rate of binding as a function of the fluid velocity and to determine the optimal flow velocity, for high capture efficiency in the micro-channel. Figure 3-1 shows a schematic of the MCF-7 cell captured by the anti-EpCAM/EpCAM binding with the relative velocity due to flow through the micro-channel.

![Figure 3-1 Scheme of the MCF-7 Cell Binding in Flow](image)
Table 3-1 lists estimates for parameters of the model.

### 3.1.1 Flow Velocity Effects on the Binding

In this model, the Peclet number, Pe expressed as

\[
P_e = \frac{R_p \times V}{D}
\]

\(R_p\) is radius of the receptor, \(V\) is the relative velocity, and \(D\) is lateral diffusivity. The Peclet number, \(P_e\) is a function of flow velocity and is a measure of the importance of the relative velocity, compared to the lateral diffusivity.

Chang and Hammer assumed that the 2-D convection-diffusion equation.

\[
D \nabla^2 C(r) - V \cdot \nabla C(r) = 0
\]

(1)

with the boundary conditions

\[
C(r) = C_\infty \quad \text{at } r=\infty
\]

(2)

\[
C(r) = 0 \quad \text{at } r = R_p
\]

(3)

The flux \(J\) through the reactive circle of radius, \(a\) (radius of the receptor), is related to the forward rate constant for encounter, \(k_0\), as

\[
J = k_0 C_\infty
\]

(4)
here $k_0$ is given by

$$k_0 = \pi DNu$$

(5)

The Nusselt number can be expressed as a function of the Peclet number (Equation 6)

$$Nu = J/\pi D C_\infty$$

$$Nu = 2 \left[ \frac{I_0(Pe/2)}{K_0(Pe/2)} + 2 \sum_{n=1}^{\infty} (-1)^n \frac{I_n(Pe/2)}{K_n(Pe/2)} \right]$$

(6)

Therefore, the forward encounter rate constant $k_0$ is also a function of the Peclet number.

$$k_0 = 2\pi D \left[ \frac{I_0(Pe/2)}{K_0(Pe/2)} + 2 \sum_{n=1}^{\infty} (-1)^n \frac{I_n(Pe/2)}{K_n(Pe/2)} \right]$$

(7)

Since every encounter does not lead to a binding, the probability, $P$, that a colliding antibody-antigen pair will ultimately react needs to be determined (Moore, 1981). In Bell’s model, it was proposed, that for the membrane-bound receptor/ligand binding, the reaction was conceptually separated into two steps.

$$A + B \Leftrightarrow AB \Leftrightarrow C$$

(8)

In the first step the reactants A and B simply encounter each other and diffuse on their membranes until they are close enough to form the encounter complex, AB. After that, the second step reaction would take place forming the bond. The theory was developed for a static solution, while this application required a dynamic model, in which the EpCAM mediated MCF-7 cell flowed on a surface with Anti-EpCAM immobilized. In the first reaction step, the two reactants combine $A + B \Leftrightarrow AB$; with increased flow velocity, more
anti-EpCAM and EpCAM encounter complexes are formed. However, in the second reaction step, the formation of the bond through reaction, \(AB \Leftrightarrow C\), only takes place when there is sufficient encounter duration time. Too large a flow velocity will take the cells away from the surface before the second receptor/ligand binding reaction occurs.

Defining \(P\) as the probability that a binding event occurs before the antigen has left the reactive circle, yields Equation 9.

\[
P = \frac{k_{on}}{k_{on} + 1/\tau}
\]

where \(\tau\) is the averaged duration of an encounter and \(k_{on}\) is the forward reaction rate of the antibody-antigen pair binding. When \(\tau\), the duration of the encounter increases, the probability of binding increases.

Introducing a dimensionless duration time, \(\Lambda\), defined as:

\[
\Lambda = \tau (a^2/D)
\]

By defining a dimensionless number, \(\delta\), relating the probability of reaction to diffusivity:

\[
\delta = a^2 k_{ow}/D
\]

The probability of binding, \(P\), changes to the form in Equation 13.

\[
P = \Lambda \delta (1 + \Lambda \delta)
\]
Combining the forward rate constant, $k_0$, and probability, $P$, yields the effective reaction rate (Equation 10),

$$k_f = k_0P = \pi D Nu P = \pi D Nu \Lambda \delta / (1 + \Lambda \delta)$$  \hspace{1cm} (14)

For each type of receptor/ligand binding, the dimensionness number, $\delta$, is determined by intrinsic reaction rate. All of the key values are a function of the Peclet number, or flow velocity.

### 3.1.2 Determination of the Optimal Flow Velocity for Binding

From the equation, $k_0 = \pi D Nu$, the forward rate constant, $k_0$, is proportional to the Nusselt Number, $Nu$. Equation 7 expresses the calculated Nusselt number as a function of the Peclet number and is plotted in Figure 3-2. The relationship is nearly linear and mathematically increasing.

The dimensionless duration time, $\Lambda$, is also a function of the relative velocity through the Peclet number, $Pe$. The duration time $\Lambda$ decreases rapidly with increasing $Pe$. Plotting non-dimensional effective reaction rate constant (Equation 11) for different values of $\delta$ yields Figure 3-4.

For anti-EpCAM and EpCAM binding, $\delta = (a^2 k_{iw}/D)$ is a function of the antibody/antigen binding affinity and equal to 250. From Figure 3-4, the curve with $\delta$ equal to 250, the dimensionless forward effective reaction rate constant $k_f/D$ initially increases with $Pe$ (relative velocity) and until it reaches a plateau when $Pe$ is $\sim 1000$. When $Pe (R_p V/D)$ reaches 1000, the effective reaction rate reaches a maximum. The optimal flow velocity is

$$V = Pe \times D/R_p = 2 \text{ mm/s}$$
Figure 3-2  The Calculated Nusselt number as a function of Peclet number.

Figure 3-3  The Dimensionless duration time, $\Lambda$ as a function of $Pe$. 
From Figure 3-2, increased Peclet number, which represents the relative motion between antibody and antigen, leads to a higher forward encounter rate of binding. As the convection velocity increases the encounter rate, the reaction rate of antibody-antigen binding is enhanced.

However, from Figure 3-3, the duration of each encounter between an antibody and antigen pair decreases with the increase of the Peclet number. While increasing the relative velocity increases the encounter rate between antibody and antigen, the duration time for molecules to react with each other decreases.
Equation (10) combines the two factors influencing the effective reaction rate, encounter rate and duration time, and yielding the relationship between the effective reaction rate and the Peclet number. From Figure 3-4, the reaction rate increases with Pe (relative velocity) and then reaches a plateau. This indicates that convection enhances the rate of collision and the antibody-antigen reaction rate (Chang, 1999). As the fluid flow rate increases, the rate of encounter between antibody and antigen rises monotonically, but decreases the collision duration (Chang, 1999). The net effect is that the forward rate constant will rise up to a plateau as these two effects counterbalance each other.

3.2 Micro-Channel Diameter Effect on Viscosity Determination

In low Reynolds number flow, the shear rate can be simply calculated as the ratio of the flow rate to half of the width of the tube. In the micro-channel of 20 µm width at a flow rate of 2 mm/sec, the shear rate is approximately 200 sec\(^{-1}\), greatly above the required shear rate (30-50 sec\(^{-1}\)) for constant blood viscosity. For our design, we can safely assume the constant viscosity. However, we still need to consider the Micro-channels geometries effect on blood viscosity.

In vitro measurement, the apparent blood viscosity as a function of tube diameter and hematocrit is expressed as (Pries, 1992),

\[
\eta_r = 1 + \left(\eta_{0.45}^* - 1\right) \left(1 - \frac{H_D}{0.45}\right)^C - 1
\]

\[
C = \frac{1}{1 + 10^{-11}d^{12}} + \left(\frac{1}{1 + 10^{-11}d^{12}} - 1\right)0.8 + e^{-0.075d}
\]

\[
\eta_{0.45}^* = 220e^{-1.3d} + 3.2 - 2.44e^{-0.06d^{0.645}}
\]

(15)
The equation for the hydraulic diameter is, \( d = \frac{4A}{P} \), where \( A \) is the cross sectional area and \( P \) is the perimeter. Here, \( d \) and Hemocrit, \( H_D \), are defined as 27 \( \mu \)m and 0.4, so the apparent blood viscosity in the micro-channel (15\( \mu \)m \( \times \) 150\( \mu \)m \( \times \) 4mm) is calculated as 2 cP. From Figure 3-5, blood viscosity increases with the increasing hydraulic diameter.

### 3.3 Comparison of Forces

Chang and Hammer (1996) compared the different forces applied to the cell/surface attachment. Figure 3-6 defines the geometry used in this model, with \( a \), the radius of the cancer cell; \( h \), the closest separation distance between the cell and the substrate; \( L \), the bond length; \( T \), the tension of the bond; and \( F \) and \( \tau \) are the external force and torque acting on the cell respectively.
Figure 3-6 Comparison of Applied Forces on a Single Cell/Surface Attachment

At equilibrium the balance of forces in the x-direction gives:

\[ F = T \cos \phi \]  

(16)

The torque balance gives,

\[ \tau = -Ta \cos(\theta + \phi) \]  

(17)

The bond length, L, ranging from 20 nm to 40 nm, is much smaller than the radius of the cancer cell, a, at the average level of 12 \( \mu \)m. Based on this geometry, with a\( >> \)L, the assumption was made that \( \theta \) is small, and \( \phi \) is near to 90°. The detailed calculation of forces comparison is shown in Appendix 2. The relationship between the shear force, F, and the normal force, T, was

\[ F = T \sqrt{\frac{2(L-h)}{a}} \]  

(18)
For a typical value of bond length in average, $L$ is 25 nm, the characteristic separation distance, $h$, is approximately 20 nm (Chang, 1996). Since the mean cancer cell radius is 12 µm, the ratio between the shear force and the normal force was approximately 0.03. The required normal force to break a single bond is approximately 33-times greater than the shear force. The shear force is much more disruptive of the bond than the normal force. Therefore, among all of the forces applied to detach an MCF-7 cell from the surface, the shear force is the most likely to rupture the antibody-antigen bond and the other forces are so insignificant that that they can be ignored in the model.

3.4 Estimation of the Critical Binding Force and the Dissociation Velocity for Breaking the Bonds

Cancer cells captured by the anti-EpCAM/EpCAM binding force on the functionalized PMMA surface are exposed to a flow field relative to the PMMA. This is similar to the situation in which a balloon tied to a string under a strong wind. In the fluid flow field, a larger flow velocity will produce higher shear forces on the captured cells. Fluid flowing through the channels exerts forces on the cells already captured on the micro-channel wall. If the flow rate is too high, the flow disruption will be significant enough to uproot the cell from the wall.

To avoid the cells already caught on the wall from being dislodged by the large shear force, both the Bell kinetic model and Dembo affinity model were adapted to estimate the shear force required for dissociation as well as the critical flow velocity at the forces needed to break the bonds. The objective was to find the critical binding force, shear velocity, and shear modulus.
3.4.1 Contact Area Evaluation

Knowing the number of bonds per unit area and the contact area, the net binding force in a contact area can be evaluated. The area of the contact zone for a smooth, relatively non-deformable particle can be estimated using the geometry of the Cozens-Roberts model (Cozen-Robert, 1990). Applying this model the values for the contact region radius for assumed rigid cell were estimated.

The parameters in the schematic (Figure 3-27) diagram are: a, the radius of cancer cell; h, the separation distance between the cell and PMMA substrate coated with anti-EpCAM, and H, the maximum separation distance for the antibody-antigen binding; and r, the radius of the contact area. From this geometry, the relationship between h, H, and r was derived. Define $h^*$ and $a^*$ as shown in Figure 3-7:

\[
h^* = H - h
\]

(19)

and

\[
r^* = a - h^*
\]

(20)

![Figure 3-7 Contact Area](image)
From the Figure, \( \theta \) and \( r \) are given by:

\[
\theta = \cos^{-1}\left(\frac{r^2}{a}\right)
\]

(21)

\[
r = a \sin \theta = a \cdot \sin\left(\cos^{-1}\left(\frac{a - H + h}{a}\right)\right)
\]

(22)

According to the dimensions of IgG, \( \sim 19 \times 57 \times 240 \text{ Å} \) (Clausen, 1981), the characteristic separation distance, \( h \) and \( H \) are estimated to be \( \sim 100 \text{ Å} \) and \( 400 \text{ Å} \). The representative radius of the MCF-7 cancer cells, \( a \), was taken to be 12 µm. The radius of the contact area was calculated with this model as 0.85 µm (\( H=0.04 \text{ µm} \) and \( h=0.01 \text{µm} \)) based on the above equation. The contact area, \( A \), has a value between \( \pi a^2 \) and \( 2\pi a^2 \), depending on the curvature. Here, the factor of 2 is considered within level of uncertainty. In this model, \( A \) was calculated to range from 2.27 to 4.54 µm² and a mid-range value of 3.5 µm² was chosen.

### 3.4.2 Model I: Binding Force Determination Developed from Dembo Binding Affinity Model

Receptor-mediated cell adhesion is a central phenomenon in many physiological and biotechnological processes. The mechanical strength of the adhesion is generally presumed to be related to the chemical affinity of the receptor/ligand bonds. Antibodies with low affinity bind antigens weakly and tend to disassociate easily, whereas antibodies with high affinity bind antigens more tightly and longer.

In Kuo’s model (Kuo, 1993), it was demonstrated that the adhesion strength varies with the logarithm of the binding affinity, consistent with a prediction from the theoretical
model by Dembo et al (Dembo, 1988). In Dembo’s model, the critical tension, $T_{\text{crit}}$, was defined to be the value required to peel a cell surface from an adhesion substratum.

$$ T_{\text{crit}} = \frac{k_B \theta N_R \ln \{1 + \frac{N_L}{K_D}\}}{1 + \cos \alpha} \quad (23) $$

The variables were $k_B$, Boltzmann’s constant; $\theta$, the absolute temperature; $N_L$, the substratum ligand density; $\alpha$, the front angle between the membrane and the surface; $N_R$, the cell receptor density, and $K_D$, the dissociation constant.

The surface energy for binding between the cell membrane and surface was defined as the numerator on the right-hand side of the above equation is.

$$ \gamma = k_B \theta N_R \ln \{1 + \frac{N_L}{K_D}\} \quad (24) $$

Evans [11] showed that an alternative expression of surface energy in terms of the total force to detach the cell from surface, $F_t$, and the contact area, $A_c$;

$$ \gamma = \left(\frac{F_t}{A_c}\right) \frac{l_b}{2} \quad (25) $$

where $l_b$ is the extent of stretch to reach the critical force to break down the single bond. The above two equations of surface energy gives:

$$ f_c = \frac{2k_B \theta N_R}{l_b} \ln(1 + \frac{N_L}{\eta K_D}) \quad (26) $$

Here, $K_B=1.38\times10^{-23}$ J/K, $\theta=298$K.
Table 3-2  The Parameters Used in Dembo Affinity Model Applied to anti-EpCAM/EpCAM Binding

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>Radius of cancer cell</td>
<td>12 $\mu$m</td>
</tr>
<tr>
<td>$N$</td>
<td>EpCAM molecules number on single breast cancer cell</td>
<td>280,000</td>
</tr>
<tr>
<td>$N_R$</td>
<td>Cell EpCAM receptor density</td>
<td>$1.6\times10^{10}#/cm^2$</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>EpCAM/Anti-EpCAM forward rate constant (Willuda, 1999)</td>
<td>$0.99\times10^5$/mol⋅sec</td>
</tr>
<tr>
<td>$k_r$</td>
<td>EpCAM/Anti-EpCAM reverse rate constant (Willuda, 1999)</td>
<td>$0.3\times10^{-3}$/sec</td>
</tr>
<tr>
<td>$K_D$</td>
<td>EpCAM/Anti-EpCAM binding affinity</td>
<td>$3\times10^{-9}$M</td>
</tr>
<tr>
<td>$N_L$</td>
<td>Substratum ligand density</td>
<td>$3.5\times10^{12}$/cm²</td>
</tr>
<tr>
<td>$l_b$</td>
<td>Extent of stretch to reach critical force</td>
<td>8.8A</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Fitting specificity (Kuo, 1993)</td>
<td>$1.3\times10^{18}$#-liter/cm²-mol</td>
</tr>
<tr>
<td>$A_c$</td>
<td>Contact area</td>
<td>3.5 $\mu$m²</td>
</tr>
</tbody>
</table>

Using the parameters in Table 3-2, the critical force to break one EpCAM-antiEpCAM bond, $f_c$, was $6.7\times10^{-6}$ dyne. If the assumption that cells are relatively non-deformable was made, the critical force, the sum of that all of bonds in the contact zone (3.5 $\mu$m²) was calculated as $3.6\times10^{-3}$ dyne.

3.4.3  Model II: Binding Force Developed from Bell Kinetic Model

In the second model, the same as in the model I, the goal was to estimate the magnitude of the critical dissociation force needed to break down the attached bonds in the contact zone, and further estimate the critical flow velocity at which all of the adherent ligand-receptor bonds would rupture rapidly.

Assume that the breast cancer cell is attached to the PMMA substrate surface by many bonds. To separate the cell, a force is required that will fairly rapidly rupture each bond. If
E₀ is the energy change in binding and r₀, is the range of minimum, then the net force f₀=1.6 ×10⁻⁵ E₀/r₀.

If force, F, is tending to separate the cells, and assuming that each bond is equally stressed, the force per bond is F/N_b and the reverse rate constant are replaced by,

\[ k_p \exp\left( \frac{F\gamma}{kTN_b} \right) \]

(27)

when N₂>>N_b,

\[ \frac{dN_b}{dt} = k_o(N_f - N_b)N_R - k_pN_b \exp\left( \frac{F\gamma}{kTN_b} \right) \]

(28)

A critical force, F_c, which is determined as just sufficient to detach the cell from the PMMA substrate. It is straightforward to show that the critical force per potential bond, f_c (Bell, 1978), is

\[ f_c = \frac{F_c}{N_f} = \frac{kT}{r_o}\alpha_c \]

(29)

where α_c is obtained from:

\[ \alpha_c \exp(\alpha_c + 1) = KN_R \]

(30)

Using the parameter values in the table, the critical force to break down one EpCAM-anti-EpCAM bond, f_c, was 6.7×10⁻⁶ dyne. This value was the critical force to break a single bond and provides a reasonable estimate of the force required to separate a cell from a surface.
Table 3-3 The Parameters Used in Bell Kinetic Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{on}$</td>
<td>Forward rate constant (Willuda, 1999)</td>
<td>$0.99 \times 10^5$/mol·sec</td>
</tr>
<tr>
<td>$k_r$</td>
<td>Reverse rate constant (Willuda, 1999)</td>
<td>$0.3 \times 10^{-3}$/sec</td>
</tr>
<tr>
<td>$K$</td>
<td>Equilibrium constant</td>
<td>$3.3 \times 10^8$M$^{-1}$</td>
</tr>
<tr>
<td>$N_R$</td>
<td>Receptor density</td>
<td>$160/\mu$m$^2$</td>
</tr>
<tr>
<td>$r_0$</td>
<td>Separation distance between molecules at the minimum breaking force</td>
<td>0.5 nm</td>
</tr>
<tr>
<td>$A_c$</td>
<td>Contact area</td>
<td>$3.5 \mu$m$^2$</td>
</tr>
</tbody>
</table>

Assuming that the EpCAM receptors on the cancer cell membrane are uniformly distributed, the number of bond pairs in the contact zone can be expressed as, $A_c \times N_R$, and the total force, $F$, to remove the cell from the contact surface is,

$$F = f_c \times A_c \times N_R = 6.7 \times 10^{-6} A_c \times N_R = 3.7 \times 10^{-3} \text{ dynes}$$

From a lot of experiments, the result shows that about a factor of 3 smaller than the earlier estimate of the force $f_o$ needed to completely dissociate a bond. The critical force will provide a reasonable estimate of the force required to separate two cells or a cell from a surface. The models could also be applied to evaluate the optimal velocity and critical binding force in the capture of any other type of cancer cells.

### 3.4.4 Increased Dissociation Force due to Cell Response to Binding

![Cell Spreading Response to Binding](image)

Figure 3-8 Cell Spreading Response to Binding
Small, round cells recognize and bind to immobilized ligands, adhering to the tissue-specific substrate. After initial contact, the small, round MCF-7 cells flatten and elongate. The enlarged contact area makes dissociation more difficult than predicted by the models, which assumed non-deformable cells. Using a soft lithography-based micro-patterning technique originally developed in Whitesides' laboratory at Harvard, the surface contact area can be measured (Singhvi, 1994). For example, the typical diameter of a hepatocyte, an epithelial cell of the liver, is 20 µm, whereas upon attachment and unconstrained spreading, the cell diameter increases to 30–40 µm. After attachment, cells were observed to elongate in the axial direction upon spreading (Singhvi, 1994).

According to the theory of cell spreading response to attachment, once the cell is adhered to the wall, it will flatten, which results in an increase in the tightness of binding. The deformable MCF-7 breast cancer cell has a larger contact area than the 3.5 µm² predicted in the non-deformable Cozen-Robert model. The surface contact area radius was assumed to be around 12 µm, much larger than 0.85 µm, evaluated in the Cozen-Robert model. This yielded a contact area of 452 µm². The critical binding force to uproot the whole cell from the substrate is the product of the critical force for a single bond and the number of bonds in the contact area. The force was also assumed to be evenly distributed over the bonds in the contact zone, so the critical binding force was linearly proportional to the number of bonds in the contact area. A more accurate estimate of the binding force between a breast cancer cell and the PMMA substrate is,

\[ F = f_c \times A_c \times N_R = 6.7 \times 10^{-6} A_c \times N_R = 0.48 \text{dyne} \]
The whole number of bonds in the contact area was estimated to be $A_c \times N_R = 72345$. The spreading of the MCF-7 cells on the antibody-modified PMMA substrate resulted in an increase in the number of bonds and a more than 100-fold increase in the adhesion strength, which made the detachment more difficult.

Different cancer cell lines, express different numbers of EpCAM molecules on their surfaces, so the critical binding force between EpCAM and anti-Epcam varies among them. For a prostate cancer cell line, 25,000 EpCAM molecules are expressed on the cell surface, with the number of bonds generated in the contact zone is $A_c \times N_R = 6577$. From Figure 3-9, for the prostate cancer cell line, the net dissociation force is 0.042 dyne, more than 10-times smaller than for the breast cancer cells which over-expressed the EpCAM.

Figure 3-9 The Critical Binding Force for different types of Cancer
3.4.5 Dissociation Velocity

From Chang and Hammer’s comparison of different applied forces, it was known that the shear force was the most destructive. To estimate the critical flow velocity required to break the antibody and antigen bonds, it was assumed that when the shear force equaled the total binding force, the bonds would break. A relationship between the critical flow velocity, \( v \), and shear force was determined. When a stationary cell of radius, \( r \), is exposed to a fluid stream of velocity, \( v \), the force on the cell will be given by Stokes law (Bell, 1978).

\[
F = 6\pi \eta rv
\]

(31)

In our model, \( \eta \) is the blood viscosity, 2 cp, and \( r \) is breast cancer cell radius, 12 \( \mu \)m. Comparing this hydrodynamic force with the critical binding force of 0.48 dynes determined from Models I and II, the dissociation velocity, at which the hydrodynamic shear force will uproot the anchored cells from the PMMA substrate can be evaluated.

\[
\nu = \frac{0.48 \times 10^{-5}}{6 \times 2 \times 10^{-3} \times 12 \times 10^{-6} \pi} = 10.6 m/s
\]

This means that the breast cancer cell held by 72345 bonds in the contact area can resist fluid velocities less than or equal to about 10.6 m/sec. The prostate cancer cell, which is held by 6577 bonds, can only resist a fluid velocity of \( \leq 92 \) cm/s.

3.5 Life Time to Break the Bonds

Each bond is reversible and the lifetime of a bond, \( \tau \), is written (Evanse, 1985)

\[
\tau = \tau_o \exp\left[\frac{(E_o - \gamma f)}{kT}\right]
\]

(32)
In which \( t \) is the time to separate cell from surface subject to the force. \( E_0 \) is the bond energy, \( f \) is the applied force per pond, \( \tau_0 \) is the reciprocal of a natural frequency of oscillation of atoms in solids. \( \gamma \) is a parameter determined to account for the structure of the solid and its imperfection. The lifetime is a function of \( f \) and \( T \). If representative values for the antibody-antigen bond are taken as shown in Table 3-4 to be \( \tau (f = 0) \approx 0.01 \) second, then \( \tau_0 = 10^{-8} \) second (Bell, 1978).

Using the parameter values cited in Table 3-4, the values for the bond lifetime are given in Figure 3-10 (Bell, 1978).

Table 3-4  The Parameters Used in Bond Lifetime Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )</td>
<td>Dimension of the binding cleft</td>
<td>0.5nm</td>
</tr>
<tr>
<td>( E_0 )</td>
<td>Bond energy</td>
<td>0.37ev</td>
</tr>
<tr>
<td>( T )</td>
<td>Temperature</td>
<td>310K</td>
</tr>
<tr>
<td>( \tau_0 )</td>
<td>Periodical time for oscillation of atoms</td>
<td>( 10^{-8} ) sec</td>
</tr>
</tbody>
</table>

Figure 3-10 The single bond life time at different applied forces
In Figure 3-10, when there was no applied force, the lifetime for a single bond was 0.01 sec, and the bond lifetime decreased dramatically with the increase in the applied force.

When the applied force was smaller than the integrated binding force in the contact area zone, the cell will remain anchored to the substrate; however, for the applied force larger than the binding force among all the bonds, cells will detach from the substrate rapidly. For the applied forces much larger than the critical force, $f_c$, the time, $T$ to detach and remove the cell away from the surface, was expressed by Bell as follows (Bell, 1978),

$$T = \frac{\exp(-Y_0)}{k_r(1 + Y_0)}$$

(33)

Here, $Y_0 = r_0f / KT$

Figure 3-11 displayed how rapidly the cell detached the surface once the applied force larger than the critical binding force was applied.
From Bell kinetic model and Dembo binding affinity model, the same single binding force, $6.7 \times 10^{-6}$ was obtained for Anti-EpCAM/EpCAM binding. It was shown in Figure 3-11 that when this critical force was applied, the time for an MCF-7 cell to detach from the PMMA substrate would be on the order of 0.1 sec. When the larger forces were applied, the cell would be separated from the surface more rapidly and easily.

### 3.6 Nonspecific Force in Antibody/Antigen Binding

When whole cells or cell membranes are used for receptor/ligand binding, a ubiquitous problem is the occurrence of non-specific interactions, such as van der waals attraction between hydrophobic regions of interacting membranes, electrostatic repulsion between charged cell surfaces, and steric stabilization (Bell, 1978). Generally, cells carry a net negative electrical charge and tend to repel each other because of the repulsive electrostatic force. In addition, the van der Waals force is attractive and has a longer range than the repulsive electrostatic forces. This suggests that cells will repel each other up to a certain distance and then be attracted to each other. In numerical calculations, this separation distance was estimated as around 10 nm. The corresponding non-specific forces needed to separate the cells were estimated to be around $10^{-5}$ dyne per square micrometer of cell area (Bell, 1978). Multiplying this value with the estimated contact area of 452 µm², the resultant force to detach the cell from the surface turns out to be only $4.5 \times 10^{-3}$ dyne, more than 100-fold smaller than the specific binding force calculated as 0.48 dyne. Therefore, it is apparent that specific bonds can cause cells to adhere to the surface much more tightly than the nonspecific forces, and the nonspecific forces can be negligible with respect to the specific
binding force. Also, cells separated by distances of 10 nm should have opportunities for contact and the formation of the specific bond.

Understanding cell adhesion completely requires more quantitative feeling for each basic interaction involved in receptor-ligand binding shown in Appendix 1 (Bongrand, 1984).

3.7 Blood Flow Modeling Using Casson Equation

For blood pulsatile flow in small vessels, there was no single "best" model available. Among all of the models, the Casson equation has been popular because of the assumption of a yield stress - that is, the necessity of an initial state of stress in order to start the blood flowing.

The stress-strain equation was expressed as,

\[ \sqrt{\tau} = \sqrt{\eta} \sqrt{v} + \sqrt{\tau_y} \]  \hspace{1cm} (34)

The yield stress \( \tau_y \) in the above equation was related to the hematocrit of the blood, \( H \).

\[ \tau_y = 0.1 \cdot (0.625H)^3 \]  \hspace{1cm} (35)

The volume flow rate of blood was derived having the same expression as Poiseuille flow except adding the factor of \( F(\epsilon) \).

\[ Q = -\frac{\pi D^4}{128\eta} \frac{dP}{dX} F(\epsilon) \]  \hspace{1cm} (36)

where, \( \epsilon \) is a parameter related to the yield stress \( (\tau_y) \), vessel diameter \( (D) \), and the pressure drop \( (\frac{dP}{dX}) \), and expressed as,
\[
e \frac{dX}{dP} = D \epsilon \cdot \left(-\frac{dP}{dX}\right)^{-1} \tag{37}
\]

Therefore, the pressure in the channel of length L, was shown as below,

\[
\Delta P = \frac{128 \eta L}{\pi D^4} Q F(\epsilon) \tag{38}
\]

where 
\[
F(\epsilon) = 1 - \frac{16}{7} \epsilon^{1/2} + \frac{4}{3} \epsilon - \frac{1}{21} \epsilon^4
\]

For a micro-channel with a hydraulic diameter of 27 µm, the pressure drop was calculated: 7585 Pa=1.1 psi. Solving these equations, the pressure drops for micro-channels with different diameters were calculated.

Figure 3-12 displayed the according pressure drop in micro-channels with different diameters. With the increase of the diameter of the micro-channel, the pressure drop decreased.

The 3-D velocity profile of the blood flow in the micro-channel was displayed in Appendix 3.

<table>
<thead>
<tr>
<th>Table 3-5 The Parameters Used in Blood Casson Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>W</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>L</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>(\tau_y)</td>
</tr>
<tr>
<td>(\eta_{\text{plasma}})</td>
</tr>
<tr>
<td>(\eta)</td>
</tr>
<tr>
<td>V</td>
</tr>
<tr>
<td>Q</td>
</tr>
</tbody>
</table>
Figure 3-12 Pressure Drops for Micro-Channels with Different Diameters (Calculated from Casson’s Equation)
Chapter 4    Experiment

In this chapter, the experimental steps taken to trap and detect the cancer cells were described. It involves antibody immobilization on the PMMA micro-channel, breast cancer cell line culture, cells fluorescence staining protocols, flow control experiment in micro-channels and detection of cancer cell under fluorescence microscopy.

4.1 Immobilization of anti-EpCAM on the PMMA Micro-Channel

4.1.1 Protocol for Surface Modification of PMMA Micro-Channel

A functionalized PMMA micro-channel was designed to trap the rare breast cancer cells from peripheral blood flowing through the micro-channel. Breast cancer cells over-express EpCAM on their surfaces, and will be caught by the strong binding affinity between the antibody and antigen, after anti-EpCAM antibodies are immobilized on the polymer micro-channel walls by the chemical surface modification of PMMA.

Figure 4-1 shows Poly (methyl methacrylate). The transition temperature (Tg) of PMMA is around 105°C. There are two reasons why PMMA was used as the material for the micro-channel. The first, was that the functional group, methyl ester, has chemistry modification potential; the second, was that PMMA is extensively used as a resist in lithographic applications in MEMS, and micro-fluidic devices.

![Poly(methyl methacrylate)](image)

Figure 4-1 Poly(methyl methacrylate)
The surface modification protocols composed three steps. First, UV radiation illuminated the PMMA surface to get carboxyl group-modified PMMA. Then Ethylenediamine, (H₃NCH₂CH₂NH₂) was added to yield amine-functionalized PMMA. Finally, the antibodies were attached, and sufficient reaction time to generate tethered antibodies on the PMMA micro-channel was allowed. This antibody-functionalized PMMA was prepared for targeting breast cancer cells. The protocol is listed schematically as follows,

\[
\text{PMMA} \xrightarrow{\text{UV}} \text{COOH-Modified PMMA} \xrightarrow{\text{NH₂-NH₂}} \text{NH₂-Modified PMMA} \xrightarrow{\text{Anti-EpCAM}} \text{Anti-EpCAM Modified PMMA}
\]

4.1.2 UV Modification of Micro-Channel

Commercial PMMA substrates as thick as 3 mm were obtained from Atofina (Philadelphia, PA).

1. Clean Channel;

2. Expose the micro-channel to UV;

3. Align the coversheets on the micro-channel and drilled holes on the coversheets;

In the first step, the PMMA micro-channel was placed into the sonicator for 20 minutes to get the particles and dust removed, and then the microchip was rinsed with IPA, and DI-H₂O respectively. Lastly, dry blow the chip with a stream of nitrogen.

In UV radiation step, the prepared microchip was placed on the platform of UV light station, with the front side of hot embossed micro-channel facing the UV light for 20
minutes. The distance between the UV light source and platform is 3 cm. Take the PMMA micro-channel away from UV light, and wash once with IPA and DI-H2O individually.

For aligning coversheets on the micro-channel, two pieces of PMMA coversheet as thin as 0.5mm were prepared. On one piece of coversheet, two holes were aligned and drilled according to the landscape of PMMA micro-channel reservoirs. This coversheet is put on the front side of micro-channel, and the other coversheet is put on the backside of the micro-channel. Stack the micro-channel with the two PMMA coversheets together in the middle of two pieces of square glass, and use binder clips to clamp the glasses on four sides to reinforce and level. These two pieces of coversheets design has less objective distance (0.5 mm) to reach micro-channel than that (3 mm) of the one piece coversheet design, hence showing is shown better image in microscopy than one piece.

The UV exposure step was extremely sensitive. Too long an exposure time or too small a distance between the UV source, and the substrate would destroy the PMMA micro-channel structure.

X-ray Photoelectron Spectroscopy (XPS) was used to confirm that the functional carboxyl group (COOH) was tethered to the PMMA after UV exposure. This was performed by Dr. McCarley’s group at LSU Chem. Dept.

4.1.3 Thermal Bonding

The prepared micro-channels were heated in the thermal bonding oven displayed in Figure 4-2. The holding temperature was set at 105°C, the glass transition temperature for PMMA, and the holding time was ten minutes. The temperature drops to 50°C eventually.
After the micro-channel was taken out of the oven, the coversheets and the micro-channel substrate were bonded together with the micro-channel geometry intact.

### 4.1.4 Preparation of NH$_2$-Modified PMMA Surfaces

The reaction between COOH-modified PMMA and H$_2$NCH$_2$CH$_2$NH$_2$ is shown in Figure 4-3.

![Chemical Reaction Diagram](image)

Figure 4-3 The reaction between COOH-modified PMMA and H$_2$NCH$_2$CH$_2$NH$_2$
The procedure is listed as follows,

1. Prepare amine functionalization solution;
2. Draw solution into micro-channel by vacuum;
3. Shake and incubate for 60 minutes;
4. Repeat step 3, and nitrogen dry;

After thermal bonding, the PMMA channels were enclosed by the coversheet, except that the two holes at the ends were open to the air for adding the chemical reagents. The Amine function solution was prepared by adding 0.0065 g EDC, and 10 µl Ethylenediamine expressed as H₂NCH₂CH₂NH₂ to 1 ml PBS (PH=7.4). Here, EDC is developed as a carboxyl (COOH) activating agent for amide bonding with primary amines.

Figure 4-4  Experiment of Set up for Chemical Surface Modification of PMMA Micro-Channels
In the second step, the solution was dropped to one end of fluid reservoir. Vacuum the other end of reservoir with the use of the pump to suck the amine function solution into the micro-channel as followed experimental demonstration in Figure 4-4.

During incubation step, shake the microchip for a while for freshly prepared amine-modified solution to contact the inner surfaces of the micro-channel adequately and keep the solution in the channels for 60 minutes. The second round solution was added and remained in the channels for 30 minutes. At the end, dry it under a stream of nitrogen.

4.1.5 Preparation of anti EpCAM-Modified PMMA Surfaces

The primary antibody, anti-EpCAM, was obtained from Oncogene (San Diego, CA). This monoclonal antibody recognizes and reacts with the human EpCAM antigen. The antibodies are formed from the clone VU-1D9 derived by immunizing mice with NCI-H69 small cell lung carcinoma cells. The isotype is mouse IgG.

The reaction between NH₂-modified PMMA and anti-EpCAM is displayed in Figure 4-5,

\[
\begin{align*}
\text{NH}_2 \text{PMMA} & + \text{Anti-EpCAM} \rightarrow \text{NH}_2 \text{PMMA} \text{anti-EpCAM} \\
\end{align*}
\]

Figure 4-5 The reaction between NH₂-modified PMMA and anti-EpCAM
The procedure of immobilization of Anti-EpCAM was listed as follows,

1. Prepare the primary anti-EpCAM antibody solution;
2. Flow the antibody solution through the micro-channel;
3. Incubate the antibodies in the channel overnight;
4. Rinse the micro-channel with PBS, and dry it with nitrogen.

5 µl of anti-EpCAM supplied at 1µg/µl in phosphate buffered saline (PBS), and 0.0008g EDC, were added to 100 µl PBS. The final concentration of antibody is 50µg/ml. The same flow system shown in Figure 4-4 was used to flow antibody through the PMMA micro-channels.

Leave the antibodies in the channel overnight at 5°C, and then dry the channels under a stream of nitrogen. Lastly, rinse the micro-channel with phosphate buffer solution (PBS). The antibody-modified PMMA micro-channel is prepared.

4.1.6 Visualization of Anti-EpCAM-Modified PMMA Micro-Channel

To test if anti-EpCAM could be immobilized on the PMMA micro-channel using this surface modification protocol, an antibody labeled with fluorochrome was used. If an antibody functionalized micro-channel is obtained, the fluorescence along the inner channel wall could be demonstrated with the fluorescence microscopy. Direct and indirect bondings were compared to determine which method generated better antibody immobilization.

For the indirect method, a secondary antibody, Fluorescein (FITC)-Donkey Anti-Mouse IgG, provided by Jackson ImmunoResearch (West Grove, PA) was used to react with
the primary antibody. Figure 4-6 demonstrates the immobilization of anti-EpCAM by indirect labeling.

This process is accomplished by adding one more step after the anti-EpCAM modification PMMA micro-channel. Draw 4 µl of secondary antibody, FITC-Donkey Anti-Mouse IgG (1.4mg/ml) into 200µl PBS. The dilution ratio is 1:50. Remain this secondary antibody in the anti-EpCAM modified micro-channel for 2 hours at 5°C. Last, dry blow the channels with nitrogen and rinse it with PBS.

In Figure 4-6 the bright green along the micro-channel, showed that the anti-EpCAM is immobilized on the inner channel wall. The indirect staining increased the sensitivity of the staining because multiple fluorochrome reagents will bind to each primary antibody molecule.

Figure 4-6 Immobilization and Fluorescence Image of anti-EpCAM by Indirect Labeling
For direct labeling, anti-EpCAM labeled with FITC replaced pure Anti-EpCAM. Figure 4-7 shows the immobilization of anti-EpCAM-FITC by direct labeling and displays the fluorescence image of the anti-EpCAM immobilization using the direct method. A very weak green line was evident along the channel wall.

There are two reasons why indirect labeling showed better results than direct labeling. For direct staining, the concentration of anti-EpCAM labeled with FITC, 12.5mg/ml, was too low, and in the small volume of the micro-channel, there were not enough antibodies immobilized. Also, FITC also interacts with the amine groups, so it inhibited the effective reaction between the antibodies and amine group and decreased the coverage of the antibody along the channel.

Figure 4-7  Immobilization and Fluorescence Image of anti-EpCAM by Direct Labeling
4.2 Breast Cancer Cell Line MCF-7 Culture

4.2.1 Cell Line Description

MCF-7 from ATCC (Manassas, VA) was kindly provided from Dr. Truax from Veterinary School in LSU. This breast cancer cell line can be described in Table 4-1.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Homo sapiens (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>breast; mammary gland; adenocarcinoma</td>
</tr>
<tr>
<td>Morphology</td>
<td>epithelial</td>
</tr>
</tbody>
</table>

Cell lines were grown in Dulbecco’s Modified Eagle’s Medium with high glucose (DMEMHG) containing 1.5 g/L sodium bicarbonate (NaHCO₃), 15 mM HEPES buffer, and 10% fetal bovine serum (FBS). In cell culture medium, HEPES is an organic buffer used to maintain physiological pH in CO₂ incubator. For the experiments, MCF-7 cells were suspended in the culture medium. The cell sizes ranged from 15 µm ~30 µm. Figure 4-8 shows a single MCF-7 cell under optical microscopy.

Figure 4-8 Single MCF-7 Cell under Optical Microscopy
4.2.2 **Cell Counting Using a Hemacytometer**

A hemacytometer was used to determine the MCF-7 cell concentration. It is an etched glass chamber with sides that will hold a coverslip exactly 1 mm above the chamber floor. The process of cell counting is listed as follows:

1. Disseminate cells very well until all the clumps are broken up;
2. Using a sterile Eppendorf pipette, draw a 100 µl cell sample into a micro centrifuge tube;
3. Put 100 µl trypan blue (0.4%) from Sigma into the tube with cells to differentiate the dead cells (darker blue) and healthy cells (brighter blue) shown in Figure 4-9.
4. Mix 20~25 times with Eppendorf pipette and load onto hemacytometer.
5. Count four corner squares on the top and bottom of the chamber.

From the cell counter, there were 165 cells counted in 4 squares.

![Figure 4-9 Counting MCF-7 Cells with a Hemacytometer](image-url)
Cell Concentration (Cells/ml)

\[ \text{Cell counted} \times \text{depth of hemacytometer (0.1 mm depth)} \times \text{dilution factor} \times 10^3 / \text{No. of square counted} \]

\[ = \frac{165 \times 10 \times 2 \times 10^3}{4} = 8.25 \times 10^5 \text{cells/ml.} \]

In the total 2.5 ml cell sample volume, there were \( 2 \times 10^6 \) cells.

The viability was defined as the concentration of all live cells among the whole cells. Dead cells have protein and enzymes, so they may react with stain, or fade the dye. The viability in our sample cells was recorded generally as 80%, which is very good in standard.

4.2.3 Medical Handling of the Human Cell Line and Biosafety

Appropriate safety procedures should be used when handling all cell lines, especially the human cell line. Protective gloves and lab coats were always used and a full mask was worn when handling the cells.

The waste was disposed of properly. The unfinished MCF-7 cell line was put into a big plastic ware container with heavy-duty soap, such as 7×cleaning solution from Linbro or Clorox detergent. They were left in the sink for more than 10 minutes until the harsh detergent killed the cells, and then discarded in the sink.

Disposable plastic tubes were used and discarded in biohazard bags. These were high temperature sterilized at a special facility on campus. Needles or sharps materials were put into a puncture resistant container.
4.3 Detection Design by Multi-Fluorescence Technique

To validate the capture of the rare breast cancer cells, three fluorescence markers, each identified by a separate color, were used to reliably identify the cancer cells. DAPI was the nuclei dye, which stained the nuclei of cells. White blood cells were stained with PE-CD45, which showed in orange under fluorescence microscopy. The cancer cells were defined by DAPI\(^+\) (blue), and the FITC-cell membrane linker\(^+\) (green). White blood cells, which will interfere in the detection of the cancer cells, were identified by DAPI\(^+\) (blue), CD45\(^+\) (red), and the FITC-cell membrane linker\(^+\) (green). Red blood cells do not have nucleus, so DAPI (blue) cannot stain them. Single color controls and unstained (autofluorescence control) cell samples were also run initially. In Table 4-2, cancer cell and white blood cells were compared with the use of three fluorochromes.

Figure 4-10 shows the schematically how the different types of labeled cells would be observed under fluorescence microscopy.

The Microphot fluorescence microscope from Nikon had an Epi-illumination mercury-arc light source. The microscope contained a trinocular observation head that was coupled to a charge-coupled device (CCD). The broadband source was able to excite many different fluorochromes. The Mercury-arc light source was a broadband illumination source, and produces a blue-green light that is rich in ultraviolet and near-infrared light region. Figure 4-11 shows the Mercury-arc lamp emission spectrum. Excitation and emission filter cubes for FITC and DAPI are B2 and UV-2, respectively.

The excitation-emission wavelengths for DAPI, PE and FITC are listed in Table 4-3.
Table 4-2  Multi-Fluorescence Comparison between Breast Cancer Cells and White Blood Cells

<table>
<thead>
<tr>
<th>Fluorescence Cell Type</th>
<th>DAPI (Blue)</th>
<th>PE-CD45 Antibody (Orange)</th>
<th>FITC-Cell Membrane Linker (Green)</th>
<th>Fluorescence Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC (Cancer Cell)</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>Blue in nuclei, and green halo outlining cell membrane</td>
</tr>
<tr>
<td>WBC (White Blood Cell)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Orange, Blue in nuclei, and green halo</td>
</tr>
</tbody>
</table>

Figure 4-10 Multi-Fluorescence Technique Used in Breast Cancer Detection in Micro-Channel

Table 4-3  Three fluorochrome Markers in the Detection Design

<table>
<thead>
<tr>
<th>Fluorescence Color</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Fluorescence color</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI (Phycoerythrin) PE</td>
<td>480-565</td>
<td>578</td>
<td>Orange-red</td>
</tr>
<tr>
<td>FITC</td>
<td>490</td>
<td>525</td>
<td>Green</td>
</tr>
</tbody>
</table>

4.3.1 DAPI Staining

DAPI nucleic acid stain was obtained from Molecular Probes (Eugene, OR). It is a popular dye for use in multicolor fluorescent techniques. DAPI stains only the nucleas of the cell, with no cytoplasmic labeling. Its blue fluorescence stands out in vivid contrast to other fluorescence. DAPI can be excited with the following radiation sources: xenon, Hg-arc lamp and UV laser. Figure 4-12 displays its excitation-emission spectrum. Ex/Em is 358/461 nm.
The procedure of labeling cells with DAPI is listed,

1. Dilute the DAPI stock solution (14.3mM) to 300nM with PBS.
2. Add 300 \( \mu \)l of this dilute DAPI staining solution to the cultured cells.
3. Incubate for 1-5 minutes.

A single MCF-7 cell nucleus stained with DAPI is displayed in Figure 4-13.

**4.3.2 Direct Immunofluorescence Assay with the CD45-PE**

Anti-CD45 conjugated with PE was purchased from BD Bioscience Pharmingen (San Diego, CA). It is an anti-human monoclonal antibody. It reacts with leukocyte common antigen (LCA) present on all human leukocytes including lymphocytes, monocytes, granulocytes, eosinophils and thymocytes. The CD45 PE will definitely stain human white blood cells but they should not stain the breast cancer tumor cell line.

The excitation-emission profile for PE is displayed in Figure 4-14.
Figure 4-14 Excitation-Emission Spectra for (Phycoerythrin) PE (BD Bioscience, Palo Alto, California)

The cells staining protocol with CD45-PE is proceeded as follows,

1. Start by adding 40 µl of anti-CD45 PE to two million cultured cells and incubate for 30 minutes probably on ice in the dark. (Ratio of antibody to number of cells: 20µl /10⁶ cells). A tube of proper volume should be used for staining process. A bigger tube will be used for more cells.
2. Wash the cell suspension with 1X Phosphate Buffer Solution (PBS) after the 30 minutes incubation period.
3. After the 30 minutes incubation period add the dual stained anti-CD45 PE / DAPI cell suspension to the micro-channel with syringe pump and view it immediately with the fluorescent microscope.
4. Fine tune the assay with the right temperatures, times, etc.

4.3.3 FITC-Cell Membrane Linker Labeling

It was possible that DAPI debris or cell debris was present in the micro-channel. To ensure that the cells were intact and not ruptured or torn apart, lipid labeling was used to double identify the breast cancer cells.
A green fluorescent cell linker kit was obtained from Sigma (St. Louis, MO). It included PKH67 dye stock ($1 \times 10^{-3}$ M in ethanol) and diluent C. This dye was used for cell membrane labeling. Diluent C was provided to maintain cell viability while maximizing staining efficiency. The Excitation / Emission is 490/502 nm.

The protocol of labeling the cells with FITC-cell membrane linker was listed as follows,

1. Centrifuge the cells at 400×g (1000 rpm) for 5 minutes into the loose pellet.
2. After centrifuging the cells, decant supernatant and let the pellet stay on the bottom of the conical tube.
3. Draw 10 µl of PKH 67 dye into the propylene tube, and dilute to $5 \times 10^{-6}$ molar with 2 ml Diluent C.
4. Rapidly add the 2 ml of staining solution to the cells and immediately mix the sample by gentle inverting the tube. Rapid and homogeneous mixing is the key for labeling due to the instantaneous staining.
5. Incubate at 25°C for 4 minutes and constantly invert the tube gently to assure mixing well between the cells and PKH67 dye.
6. Add 2ml of serum to stop the staining reaction.
7. Dilute the serum-stopped sample with 10ml of tissue culture medium

Figure 4-15  PKH 67 Excitation and Emission Spectra (Sigma, St. Louis, MO)
8. Centrifuge the samples at 400×g (1000 rpm) for 10 minutes. Decant supernatant fluid and resuspend the cells with 2ml tissue culture medium.

4.4 Flow System Experiment Set up

After an anti-EpCAM functionalized micro-channel was prepared, the cultured cells were labeled with DAPI, anti-CD45-PE, and FITC cell membrane linker. A Programmable Pump 22, from Harvard Apparatus syringe pump, shown in Figure 4-17, was used to generate a constant flow velocity. There were two input parameters, the syringe diameter and volume flow rate. The syringe diameters varied for different companies, and had different minimum and maximum flow rates.

Table 4-4 lists the minimum and maximum diameters available for Becton Dickinson plastic syringes of different volume.

![Figure 4-16 Cell Clusters Stained with DAPI and FITC](image)
Table 4-4 Becton Dickinson Plastic Syringe Diameters, Minimum & Maximum Flow Rates

<table>
<thead>
<tr>
<th>Volume Size (cc)</th>
<th>Diameter (mm)</th>
<th>Minimum Flow Rate</th>
<th>Maximum Flow Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.78</td>
<td>0.49 µl/min</td>
<td>805 µl/min</td>
</tr>
<tr>
<td>3</td>
<td>8.66</td>
<td>0.011 ml/h</td>
<td>181.4 ml/h</td>
</tr>
<tr>
<td>5</td>
<td>12.06</td>
<td>0.019 ml/h</td>
<td>317.0 ml/h</td>
</tr>
<tr>
<td>10</td>
<td>14.50</td>
<td>0.028 ml/h</td>
<td>461.0 ml/h</td>
</tr>
<tr>
<td>20</td>
<td>19.13</td>
<td>0.050 ml/h</td>
<td>821.0 ml/h</td>
</tr>
<tr>
<td>30</td>
<td>21.70</td>
<td>0.074 ml/h</td>
<td>1208.0 ml/h</td>
</tr>
<tr>
<td>50/60</td>
<td>26.70</td>
<td>0.002 ml/min</td>
<td>28.4 ml/min</td>
</tr>
</tbody>
</table>

The inlet of the micro-channel and syringe output tip were connected using assembly components from Upchurch (Oak Harbor, WA), including tubing, luer adapters, a short headless nut, and a ferrule. The same outer diameter for all those components was required to get a tight connection, which was the key issue for cell line transport. The syringe was used to pump the cell-loaded solution through the tubing, and the micro-channels, which were placed under the fluorescence platform was shown in Figure 4-18.
4.5 Micro-Channel Generation and Cancer Cell Capture Evolution

4.5.1 Cell Capture in 1st Micro-Channel Generation

A mold insert and hot embossing micro-fabrication were used to produce PMMA micro-channels (50 µm (width) × 100 µm (height) × 5 cm (length)). This nickel mold insert was fabricated at the Center for Advance Microstructures and Devices (CAMD) and the hot embossing was done in the Chemistry Department. In the final PMMA micro-channel, two holes were drilled at the each end of long channel to serve as inlet and outlet respectively.

Figure 4-19 Mold Insert Image of Micro-Channel (50µm×100µm×5cm)
Two microchips were prepared. One was PMMA micro-channel without surface modification and the other was a PMMA micro-channel with anti-EpCAM immobilized.

Figure 4-20 shows a round MCF cell in the non-functionalized channel instead of being attached to the walls. Cells retained their round shapes, and there were no deformation of the cells in the micro-channel.

Only three MCF-7 cells were captured at three different locations in micro-channel with the width of 50 µm (Figure 4-21). There were around a quarter of million cells flowed into the channel. These assured the breast cancer cells could be captured with the use of anti-EpCAM modified micro-channel, but was not optimized to get high cell capture efficiency. After cells initially contacted the wall, they flattened and elongated, and became shaped like half an ellipse. The cell contact area radius was approximately 12 µm, which was also used in the models of dissociation force and velocity determination.
Figure 4-21  Micro-Channel with Anti-EpCAM Immobilization on Walls
(50µm×100µm×5cm)

Figure 4-22 Skipped Cells in Outlet Reservoir in 50 µm Width Micro-Channel
Cells that were not captured on the micro-channel wall and passed through the outlet reservoir (Figure 4-22). In a micro-channel larger than the cells, cells tended to flow in the central part of the channel and leave a cell-free layer along the channel wall. Cells had less chance to contact the channel wall. Therefore, in order to get high target cell capture efficiency, a narrow and deep micro-channel seemed to have the tendency to improve the capture efficiency.

### 4.5.2 Cells Capture in 2nd Micro-Channel Generation

When the micro-channel width was reduced, the cells had a much higher probability of encountering the micro-channel wall, rolling along the channel wall, and eventually adhering to the channel by the strong affinity between anti-EpCAM and EpCAM. However, if the channel is too narrow, the cells would block at the entrance. Austin reported that white blood cells stopped at the entrance of a channel instead of deforming into it (Austin, 1997). When a micro-channel’s width was the same magnitude as the cells, it would increase the surface contact area between the cells and channel wall. It was reasonable to increase the aspect ratio to further increase the surface contact area, and improve the cell capture efficiency. This idea tended out to be the correct guess. Once the cells got trapped on the channel, the bond strength was very strong. The flow velocity of 2mm/sec was much lower than the critical dissociation velocity predicted by the model. The captured cells remained anchored to the channel wall and would not strip away under the continuous flow.

In Figure 4-24, a number of captured MCF cells are shown. Figure 4-25 demonstrates a global view of cell capture in the narrower micro-channel ((20µm×70µm×10cm) at a flow velocity of 2 mm/sec. Every where in the channel, the straight part, the curved channel,
the edge of the outlet reservoir, the cancer cells were anchored to the channel wall. From that, reasonable coverage of anti-EpCAM antibody coated on the PMMA surface was assumed. Compared with Figure 4-22 taken in the reservoir location of the 50 µm micro-channel, the reservoir in Figure 4-25 showed MCF-7 cells anchored only at the edge of outlet reservoir where the anti-EpCAM was immobilized. There were no skipped cancer cells staying in the reservoir. That displayed the high cell capture efficiency. In the picture of “Curved Channel Location”, the outer edge of the channel had more cancer cells captured due to the higher encounter rate compared with inner one. This phenomenon inspired consideration of the serpentine micro-channels in the future geometry for high capture cell efficiency and small footprint.

The cell capture efficiency can be evaluated approximately by method of cell counting. The cell concentrations of the initial cells suspension and final cells solution can be evaluated with the use of a hemacytometer. The numbers of cells at those two stages are the products of cell concentration and cell suspension volume. Therefore, the capture efficiency is determined by the ratio of the number of cells staying in the micro-channel to the initial number of cells.

Figure 4-23 Mold Insert Image of Micro-Channel (20 µm × 70 µm × 10 cm)
Figure 4-24 Micro-Channel with anti-EpCAM Immobilization on Walls (20 $\mu$m $\times$ 70 $\mu$m $\times$ 10 cm)

Figure 4-25 Breast Cancer Cells Capture in Micro-Channel (20$\mu$m$\times$70$\mu$m$\times$10cm) at the Flow Velocity of 2mm/sec
4.5.3 3rd Micro-Channel Generation

The cell-sorting micro-device should have high capture efficiency, quick processing times, and high throughput, so a parallel-multi micro-channel has been designed.

Figure 4-26 Mold Insert Image of Micro-Channel (50 µm × 120 µm × 5 cm)
Chapter 5       Summary and Conclusions

5.1 Summary of the Research Study

Breast cancer, the most common cancer in women, represents the second leading cause of death in women in the United States. Early cancer detection is looked upon as an effective strategy to reduce the burden of this disease. A popular early breast cancer detection technique, screening mammography, cannot eliminate death from breast cancer because only highly calcified tumors that are ~1 mm in size can be detected and misses subsequent metastasis formation in other tissues. Also, mammogram screening brings about inconclusive results, which require patients to undergo other invasive and expensive follow-up diagnostic procedures, such as the surgical biopsy, Fine Needle Aspiration (FNA), or Core Needle Biopsy (CNB) to determine if the tumors are malignant or benign.

At the early stage of tumorigenesis, tumor cells shed into peripheral circulating blood, which in principle makes it possible to detect cancer cells in the blood stream before the primary tumor is large enough to be detected by standard screening techniques. In human blood, the only epithelial cells are tumor cells, so the EpCAM antigen over-expressed by breast cancer cells will be a precise precursor to detect breast cancer cells at the early stage of tumor growth and metastasis.

The scale of the instrument available for diagnosis was reduced by using biochips and creating a Biological Micro-Electro-Mechanical Systems (Bio-MEMS) device to detect rare breast cancer cells in patients’ blood.
Antibody-antigen binding was used to detect and trap the target breast cancer cells from solution by designing a micro-device coated with anti-EpCAM antibodies.

5.2 Summary of Results and Conclusions

PMMA was chosen as the micro-device material due to its surface chemistry modification potential. Immobilization of anti-EpCAM antibodies was successfully done using a simple three-step protocol, introducing UV radiation, NH₂-modification of PMMA, and antibody attachment to the PMMA. Immunofluorescent staining was used to test whether antibodies were immobilized or not. Direct staining using anti-EpCAM conjugated with FITC and indirect staining, with the use of pure anti-EpCAM, and a secondary antibody labeled with FITC were compared for evaluating immobilization performance. The indirect staining was adopted and recognized as the best way due to the large number of antibodies coated along the micro-channel increasing the target cell capture efficiency. Compared with the direct method, the indirect method did not obstruct the reaction between the antibodies and the NH₂ group from fluorescein, and increased the density coverage of antibodies along the channel.

Cancer cell detection in the transparent micro-channel was based on a multi-fluorescence technique. Three fluorochromes, FITC (green), CD45-PE (red-orange), and DAPI (blue) were selected to differentiate the cancer cells from normal blood cells. Under fluorescence microscopy, breast cancer cells were identified by nuclei blue (DAPI positive), and green (FITC-cell membrane linker positive) lipid layers. White blood cells were identified by orange (CD45-PE positive), as well as having blue nuclei (DAPI positive) and green (FITC-cell membrane linker positive) lipid layers. With these three fluorochromes,
whether in the static case, with the target cancer cells anchored to the PMMA micro-channel or in the motile case with cancer cells rolling along the micro-channel, then binding to the surface and spreading in response to binding could be visualized under fluorescence microscopy.

In simulations, Hammer’s model of the effect of flow velocity on receptor-ligand binding was adapted. In antibody-antigen binding under the flow field in the micro-channel, there were two competing mechanisms involved. With the increase in the flow velocity of the cancer cells, they encountered the anti-EpCAM-modified micro-channel more often, which increased the probability of generating an encounter complex. On the other hand, when the flow velocity increased, the encounter duration time decreased dramatically. Given insufficient encounter duration times, would the encounter complex would not form and ultimately lead to cell binding.

In this non-dimensional model, the Peclet number (Pe) was introduced as a ratio of flow velocity, and 2-D convection-diffusion was used to obtain the relationship between the encounter rate and Pe. The probability of antibody-antigen binding was introduced to explain the relationship between the encounter duration time and Pe. Combining those two factors, encounter rate and encounter duration time, an optimal velocity, at which the maximum numbers of Anti-EpCAM and EpCAM binding was determined to be 2mm/sec. This model could be applied in any specific receptor-ligand biosystem to get the optimal velocity.

To calculate the binding strength between the captured cancer cells and PMMA micro-channel, the Dembo binding affinity model and Bell kinetic model were used. Dembo speculated that the mechanical strength of adhesion varied with the logarithm of the binding
affinity of receptor/ligand bonds. Given the parameters, anti-EpCAM/EpCAM binding affinity, extent of bond stretch to reach critical force, the critical binding force for a single bond was determined. In the Bell kinetic model, at equilibrium, the critical force to break down one EpCAM-antiEpCAM bond was developed and the same result obtained.

For contact area evaluation, the Cozens-Roberts model, which assumes that cell is a rigid and non-deformable sphere, yielded a surface contact area of 3.5 µm². When the enlarged contact area due to the cell spreading response to binding was considered, the actual contact area was approximatly as 452 µm². The estimated dissociation force integrated over the contact area after spreading was 0.48 dynes and the corresponding dissociation velocity was 10.6 m/s. The bond strength between the anti-EpCAM and EpCAM was very large once the cell spread, so it should be extremely difficult to uproot the cells from the surface.

Three generations of micro-devices were used for cell capture. The first was a channel of width 50 µm × height 100 µm × length 5cm. Cells were pumped through both a non-functionalized channel and an anti-EpCAM modified micro-channel. In the non-functionalized channel, round MCF cells remained in the middle of the channel, and were not captured; however, in the treated channel, low numbers of cells were captured on the channel wall and most passed through to the outlet reservoir. Once the cells attached to the surface, they became flattened and elongated. The cell capture assured us that the anti-EpCAM modified micro-channels would work, but the geometry had to be optimized to improve the cell capture probability.
To reduce the processing time and increase the high throughput, the 17 parallel micro-channels (50 µm × 120 µm × 5 cm) were set up for further flow experiment.

Therefore, in order to implement the separation of breast cancer cells from blood using the antibodies immobilized on the micro-channel wall, cells must be forced to contact the channel wall. Narrow and deep channels had been tested showing a good geometry because cells had more chances to contact the wall instead of staying in the middle part of the channel in the wider channel.

Cell spreading on the antibodies-modified surface largely increase the bond force, therefore, larger flow velocity were required to wash the targeted cells off the surface. Also, in order to achieve flow the sample solution through the micro-channel in less than 10 minutes, high throughput is necessary. Parallel multiplied micro-channels or annular geometries would be considered as potential channel geometries.

The antibodies immobilization protocol can be applied for different designed geometries to coat the surface with a layer of antibodies and the cells detection with the use of fluorescence technology can also be applied to different designs.

5.3 Areas of Future Research

The selectivity of the micro-device is not determined yet. A mixture of white blood cells, red blood cells and MCF-7 cells will be used as the sample to test if the anti-EpCAM modified micro-channel will only recognize and collect the target cancer cells as expected.

Cells may be run suspended in saline or other solution to improve capture rate. Removing the plasma from the blood through centrifugation and using normal saline would result in a less viscous suspension, so that the cells could be captured at faster rate. Altering
the salt concentration or lowering the temperature can sometimes increase the avidity of the antibody to enable the capture of cells in a faster flow. Experiments would need to be designed to evaluate these changes. A key issue is the improvement of the coverage of the antibodies along the micro-channel. To fully make use of the functionalized micro-channel, we may use the micro-pump assembled to this cell sorting micro-device to generate the repeated cycles.

To reduce the processing time and increase the capture efficiency as well as the high throughput, various geometries of micro-devices will be designed and compared. Annular (Figure 5-1), serpentine (Figure 5-2), spiral (Figure 5-3), micro-posts and other geometries of the micro-devices will be laid out and incorporated into the microchip using a laser writing machine. In addition to the narrow and deep channels, other geometries, such as serpentine or annular channels utilizing the inertia forces to obtain more cells contact on the wall will be experimented. A computational model should be developed to compare the performance of cell capture with different geometries of micro-devices.

Figure 5-1 Annular Micro-Device Geometry

Figure 5-2 Serpentine Micro-Channel Geometry
The future micro-devices would be coated with Molecular Imprinted Polymers (MIPs) monolayer instead of expensive antibody layer to catch the target cells. These MIPs are functionalized polymers, which can recognize and selectively bind guest EpCAM molecules because the shape and functionality of the guest has been "imprinted" during the polymerization. MIP is a promising new separation tool that has the potential of replacing antibodies in separations that demand high affinity and selectivity (Sellergren, 1997). Also, antibody-modified micro-device had to be stored in the dark and cold place. However, this future product, MIP coated microchip without any storage requirement would be more commercially popular as an early cancer detection device. The binding mechanism between MIP and molecules should be applied to get the dissociation force and the optimal velocity which guarantee the maximum bindings.

Also, based on the different types of cells having different electroosmotic properties, the cell sorting experiment with the use of the electro-kinetic flow other than the pressure driven flow would be carried out later.
References


Appendix 1: Non Specific Force

Van der Waals Force, $F_v$

$$F_v = -\frac{A_h R_v}{6} \left( \frac{1}{h^2} - \frac{2}{(h + \chi)^2} + \frac{1}{(h + 2\chi)^2} \right)$$

where $A_h$ is the Hamaker constant and $\chi$ is the constant on the order of $h$.

Electrostatic Force, $F_e$, For $h > 2L_c$,

$$F_e = 2\pi R_v \left( \frac{\rho^2 e^{-\kappa h}}{2\varepsilon \kappa^3} (e^\kappa - 1)^2 \right)$$

For $h < 2L_c$

$$F_e = 2\pi R_v \left( \frac{\rho^2}{2\varepsilon \kappa^3} (4L_c - 2h) + \frac{\rho^2}{2\varepsilon \kappa^3} e^{-\kappa h} (1 - 2e^{-\kappa L_c}) + \frac{\rho^2 e^{\kappa h}}{2\varepsilon \kappa^3} e^{-2\kappa L_c} \right)$$

where $\rho$ is the charge density of cell coats, $L_c$ is the thickness of macromolecular layer on each surface, and $\kappa$ is the reciprocal Debye-Huckel length.

Steric Stabilization Force, $F_s$.

For $h > 2L_c$

$$F_s = 0$$

For $h < 2L_c$

$$F_s = 2\pi R_v \frac{\lambda_{ss}(2L_c - h)}{L_c^2}$$

where $\lambda_{ss}$ is a phenomenological constant.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>value</th>
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</thead>
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<tr>
<td>$R_c$</td>
<td>Cell radius</td>
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<tr>
<td>$A_h$</td>
<td>Hamaker constant</td>
<td>$5.0 \times 10^{-21}$ J</td>
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<tr>
<td>$\chi$</td>
<td>Surface thickness</td>
<td>70Å</td>
</tr>
<tr>
<td>$L_c$</td>
<td>Cell coat thickness</td>
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</tr>
<tr>
<td>$1/\kappa$</td>
<td>Debye-Huckel length</td>
<td>8Å</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Charge density</td>
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</tr>
<tr>
<td>$\varepsilon$</td>
<td>Dielectric constant</td>
<td>$7.08 \times 10^{-19}$ C$^2/(\text{dyn-cm}^2)$</td>
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<tr>
<td>$\lambda_{ss}$</td>
<td>Steric constant</td>
<td>$2.5 \times 10^6$ dyn</td>
</tr>
</tbody>
</table>
Appendix 2: Comparisons of Different Types of Applied Forces

Assumptions:

\[
\sin \theta \sim \theta, \quad \cos \theta = 1 - 2 \sin^2 \theta \sim 1 - \theta^2 / 2, \quad \cos \phi \sim \pi / 2 - \phi = \alpha, \quad \sin \phi \sim 1 - \alpha^2 / 2
\]

\[
\frac{\tau}{aF} = \frac{\sin \theta \sin \phi - \cos \theta \cos \phi}{\cos \phi} \approx \frac{\theta (1 - \frac{\alpha^2}{2}) - (1 - \frac{\theta^2}{2}) \alpha}{\alpha} \approx \frac{(\theta - \alpha) - \frac{\alpha^2 \theta}{2} + \frac{\theta^2}{2} \alpha}{\alpha} \approx \frac{\theta - \alpha}{\alpha}
\]

Rearranging the above equation gives,

\[
\theta = \alpha \left( 1 + \frac{\tau}{aF} \right)
\]

The geometry of this model also shows,

\[
L \sin \phi = h + a(1 - \cos \theta)
\]

Using the same assumption we made before, \(\sin \phi \sim 1 - \alpha^2 / 2\) and \(\cos \theta \sim 1 - \theta^2 / 2\), the above equation can be simplified as,

\[
L (1 - \frac{\alpha^2}{2}) = h + \frac{a \theta^2}{2}
\]

With the approximation \(a >> L\), substituting \(\theta\) into the above equation gives,

\[
\alpha = \sqrt{\frac{2(L - h)}{L + a \left( 1 + \frac{\tau}{aF} \right)^2}} \approx \frac{1}{\left( 1 + \frac{\tau}{aF} \right)^2} \sqrt{\frac{2(L - h)}{a}}
\]

Applying the definition of \(\alpha\) given as \(\alpha = \cos \phi\), the relationship between shear force and normal force is expressed in term of \(\alpha\) as,
\[ F = T \cos \phi \cong \alpha T \cong \frac{T}{1 + \frac{\tau}{aF}} \sqrt{\frac{2(L - h)}{a}} \]

Therefore, from the above derivation based on the mechanical force balance and geometry assumptions, the relationship between the shear force (F) and the tension of the bond (T) is obtained as shown in the above equation. Hence \( \alpha \), the ratio between F and T, is the determinant value to compare the shear force (tangential force) required for remove the attached cell from the surface to the normal force required for the detachment.

When fluid flows through the micro-channel, the shear force is exerted on the cell body, and no torque exists (\( \tau = 0 \)).

\[ F = T \sqrt{\frac{2(L - h)}{a}} \]
Appendix 3: 3-D Velocity Distribution

The 3-D velocity distribution in the rectangular section: \(-a \leq y \leq a, -b \leq z \leq b:\)

\[
\begin{align*}
    u(y, z) &= \frac{16a^2}{\mu \pi^3} \left(-\frac{dP}{dx}\right) \sum_{i=1,3,5,...}^{\infty} (-1)^{(i-1)/2} \left[ 1 - \frac{\cosh(i \pi y / 2a)}{\cosh(i \pi b / 2a)} \right] \times \frac{\cos(i \pi y / 2a)}{i^3} \\
    Q &= \frac{4ba^3}{3\mu} \left(-\frac{dP}{dx}\right) \left[ 1 - \frac{192a}{\pi^5 b} \sum_{i=1,3,5,...}^{\infty} \frac{\tanh(i \pi b / 2a)}{i^5} \right]
\end{align*}
\]

The figure below showed the 3-D velocity profile of blood in micro-channel with the width and depth of 15 µm at the flow velocity of 2 mm/sec.
Vita

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