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I am submitting herewith a thesis written by Abitha Sundararajan entitled “Synthesis and Analysis of Anionic Hydrogel Scaffolds for Endothelial Cell Tissue Engineering”. I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Engineering Science.

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Synthesis and Analysis of
Anionic Hydrogel Scaffolds for Endothelial Cell Tissue
Engineering

A Thesis
Presented for the
Master of Science
Degree
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Abitha Sundararajan

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DEDICATION

I dedicate my master's thesis to my parents who have always supported me in accomplishing what I wanted and encouraging me. If not for their encouragement and support I would not have been here for pursuing my higher studies.

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Abstract

Acidic hydrogels are cross-linked polymer networks that contain neutral and negatively charged monomers. The degree of negative charge on these soft materials plays a critical role in their biomedical applications. In particular, their use as a scaffold for cardiovascular applications depends on their charged and swollen states. The amount of negative charge, however, is a complicated function of the fabrication conditions, monomeric dissociation constants, ionic strengths and pH of the surround bath solution. This study, therefore, considered four representative acidic hydrogels and their physical properties and interactions with endothelial cells. The acidic monomers methacrylic acid (MAAc) and sulfoethylmethacrylate (SEMA) were polymerized with the neutral monomer 2-hydroxyethylmethacrylate (HEMA) or polyethylene glycol dimethacrylate (PEG) to produce acidic hydrogels with a range of charges and physical properties. The acidic monomers MAAc and SEMA represent weak and strong acidic monomer, respectively. Swelling measurements showed a dependence on both the degree of charge and the properties of the neutral monomer. Diffusion potentials measured were found to be in consistent with the theoretical predictions. Endothelial cells were inoculated on these acidic hydrogels and an algorithm using MATLAB was developed for calculating the cell covered area. Highly charged hydrogels showed greater affinity for cell adhesion while hydrogels with low charge densities showed little or no affinity for cell adhesion.

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Abbreviations

APS	Ammonium Per Sulfate
BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
HEMA	Hydroxyethyl methacrylate
MAAc	Methacrylic acid
PEG	Poly ethylene glycol
PPAEC	Porcine Pulmonary Artery Endothelial Cells
PBS	Phosphate Buffered Saline
SEMA	Sulfo ethyl methacrylate
SMBS	Sodium Meta Bi Sulfate

Nomenclature

C_{A-}	Concentration of dissociated acidic groups
C_{AH}	Concentration of undissociated acidic groups
C_b	Total concentration of all positive monovalent ions in the external bath
C_{bh+}	Hydrogel ion concentration
C_{H+}	Hydrogen ion concentration
C_i	Ion concentrations of the i^{th} ion in the salt solution
$\bar{C}_i$	Ion concentrations of the i^{th} ion in the hydrogel
C_{m0}	Concentration in the reference state
C_{mobile}	Total mobile ion concentration within the hydrogel
C_s	Bath salt concentration
$C_s^{'}$	Concentrations of the solution in left- hand side
$C_s^{''}$	Concentrations of the solution in right-hand sides
C_x	Fixed charge density
$\bar{C}_+^{'}$	Cation concentrations at the left-hand side of the charged membrane
$\bar{C}_+^{''}$	Cation concentrations at the right-hand sides of the charged membrane
D_0	Diameter in the reference state
e	Unit charge
F	Faraday constant
F_b	Bath free energy
F_g	Hydrogel free energy
F_{el}	Elastic free energy
F_M	Free energy of mixing
F_N	Mobile particle free energy
$F_{polyion}$	Polyion contributions of the hydrogel Helmholtz free energy
J_i	Flux across the membrane of the i^{th} ion
K_a	Acid dissociation constant
k_B	Boltzmann's constant

N_{bi}	Total number of i^{th} bath ion
N_{f-}	Total number of negative fixed charges
N_{gi}	Total number of the i^{th} mobile particle within the hydrogel
N_x	Average number of monomers between cross-links
R	Gas constant
r	Cation-to-anion mobility ratio in the membrane
V_g	Hydrogel volume
V_0	Hydrogel volume in the reference state
T	Absolute temperature
z_i	Valence of the i^{th} ion
z_{pi}	Valence of the polyion charge
z_x	Valence of fixed charge groups
μ_i	Electrochemical potentials of the i^{th} ion in the salt solution
$\bar{\mu}_i$	Electrochemical potentials of the i^{th} ion in the polyelectrolyte hydrogel
γ_i	Ion activity coefficients of the i^{th} ion in the salt solution
$\bar{\gamma}_i$	Ion activity coefficients of the i^{th} ion in the hydrogel
$\gamma_{\pm}$	Mean activity coefficient of the electrolyte
ϕ_0	Polymer volume fraction in the reference state
ϕ	Polymer volume fraction
ϕ_{self}	Self-consistent potential created by the mobile ions and polyions
ϕ	Electrical potentials in the salt solution
$\bar{\phi}$	Electrical potentials in the charged membrane
$\Delta\phi$	Membrane potential
$\Delta\phi_{Don}$	Donnan potential
$\Delta\phi_{diff}$	Diffusion potential
v_{site}	Lattice site volume
v_0	Solvent particle volume
χ	Flory interaction parameter
$\bar{\omega}_+$	Cation mobility in the membrane
$\bar{\omega}_-$	Anion mobility in the membrane

Part I: INTRODUCTION

I. Introduction

A. Basics of Hydrogels

Hydrogels also called the ‘smart gels’ have been of great interest to mankind for the past few decades because of its properties and numerous applications. They are insoluble polymer network chain that can swell in aqueous environment. The properties of hydrogels that make them very special and important for bioengineering are its tissue like structure or properties, responsive swelling, degradability and in situ formability. Few places where hydrogels are used are as sensors, separation membranes, drug delivery systems, hygienic bandages, artificial skins systems, in pharmaceutical industries and contact lens. [1, 2]

Hydrogels can be categorized based on their cross-linker as covalent and physical. Physical gels are formed by noncovalent cross-links and they can be further divided into hydrophilic and hydrophobic. Polyethylene glycol (PEG) based gels are hydrophilic. Polypropylene oxide based ones are hydrophobic. Ionic/Polyelectrolyte and Polyampholyte is another type of classification, based on the type of the monomer the hydrogel contains. Acidic Hydrogel is the one that contains a negative monomer; the one that contains positive monomer is the basic hydrogel. Polyampholytes can be classified as neutral, contains both the types of monomer. [2-5]

There are a number of ways to prepare hydrogels, which are discussed in the next chapter. Physiological parameters such as change in temperature, pH, ionic strength, pressure, electromagnetic events, charge density and solvent quality causes the hydrogel

to swell or deswell. These make them suitable for sensors and drug delivery system. They can be used to prevent tissue-tissue adhesion after an operation, prevent thrombosis and restenosis in vessels after vascular injury, etc. [1, 6, 7]

B. Functions and Properties Endothelial Cells

The cell is the fundamental structural and functional unit of all living organisms. Endothelial cell is the important cell found in the inside lining of blood vessels, lymph vessels, and the heart. They are a thin layer of cells lining the interior surface of the blood vessels, heart to the smallest capillary. The endothelial cell layer controls the flow of material into the blood stream. They must maintain an open vessel lumen by growing as a single cell layer adherent to the basement membrane of the vessel wall. This makes these cells very important and an interesting field for research for both the engineers and biologists. These cells are deeply involved in many physiologic and pathologic processes. [8]

The functions of the endothelial cells are very similar but there exists a considerable physiological difference between those in the capillaries and in large arteries and veins. Other than the physiologic difference, the morphologies of these cells also differ remarkably between small and large vessels. In the small vessels, the cells wrap around to form hollow cylindrical tubes through which the blood flows compared to the classic monolayer - flat sheets of tightly packed polygonal cells in the large vessels. The most important property of this cell is that, they don't grow in layers; they always attach themselves firmly to the underlying membrane. [9, 10]

It has also been found that capillaries also differ from organ to organ. For example, in the brain and the retina, the capillaries take part in the maintaining the blood brain barrier. Here, there are no breaks in the vessel walls and continuous intercellular tight junctions connect the cells. Similar growth properties are found in muscle, lung and adipose tissue. Spleen, liver and bone marrow have discontinuous capillaries. Adjacent endothelial cells have gaps. Another type of capillary, called fenestrated, has intracellular gaps within the cytoplasm of individual cells. Intestinal villi and endocrine glands have this capillary. [11, 12]

Cell culture of capillary endothelial cells have been of great interest because of its various functions. Bovine aortic endothelial cells (BAEC), porcine pulmonary artery endothelial cells (PPAEC), cell from human umbilical vein, endothelial cells of rats and some more. BAEC, PPAEC and human umbilical vein are the most commonly used ones in the research for the past few decades. There are number of techniques that are available to isolate endothelial cells and grow them in the laboratories. Endothelial cell culture techniques of artery and venous has had rapid progress, but only for the past two or three years there has been successful long term culture of capillary endothelial cells. [11]

II. Literature Review

Research on hydrogels has been done for decades. Swelling experiments on hydrogels are very useful in finding its thermodynamic and mechanical properties. Number of experiments can be conducted on hydrogels to study its swelling properties.

In 1979 M. Ilasky, K. Dusek, J. Vacik and J. Kopecek published a paper about the deformational, swelling and potentiometric behavior of HEMA-MAAc gels. The hydrogels were prepared by co-polymerization of 2-hydroxyethyl methacrylate and methacrylic acid. 3 wt% ethylene dimethacrylate and azobisisobutyronitrile were the crosslinker and initiator respectively. The results they obtained indicated that with increase in the degrees of neutralization, the degree of swelling increases with a decrease in the modulus. The gels with high MAAc content have minimum dependency between modulus and degrees of neutralization. When they compared their results with the modified theory of rubber elasticity, it was found that decrease in the modulus at lower concentration is controlled by the degree of swelling. At high degrees of neutralization, both the degree of swelling and electrostatic interactions contribute to the modulus. The results also indicate that the interaction parameter and the volume fraction of polymer in the gel do not depend on the degrees of neutralization, which is similar to other hydrophilic hydrogels. [13]

A paper published in 1980 by Vlastimil Kudela, Jirf Vacik and Jindrich Kopecek, was about the effects of cross-linker, concentration of the dissociable groups and pH on the membranes concentration potentials, electrical conductivity and swelling of the membranes, of copolymers of 2-hydroxyethyl methacrylate – sulfooxyethyl methacrylate (SSEM). Cross-linker used was ethyldimethacrylate. Hydrogels prepared by free radical polymerization was initiated by azo-bis-isobutyronitrile and UV radiation. Swelling of gels with 2% cross-linker was quite high in 0.1M of potassium chloride. With increasing SSEM but same amount of cross-linker, the degree of swelling was slightly

lower compared to gels with high content of SSEM and the cross-linker. This implies that the concentration of cross-linker is important in swelling characteristics. Theoretical model for electrochemical properties was developed using Teorell-Meyer-Sevier theory. Gels that are strongly acidic and hydrophilic showed variations from the theoretical model expected for the hydrophobic gels. However, their model for hydrophilic gels with high permselectivity and electrical conductivity with a virtually theoretical electrochemical behavior and with a small dependence on pH was obtained when the ionogenic component and the degree of cross-linking was combined. [14]

In 1984 J.Ricka and T.Tanaka investigated the quantitative consistency of Donnan theory of swelling for weakly charged ionic gels. Swelling experiments as a function of ionic composition of the solvent was done on a polyacrylamide -acrylic acid copolymer gels. They were able to predict quantitatively the ionic solvent compositions at which the swelling of gels be maximum. The study was valid for gels that did not contain multivalent salts. [15]

Later in 1988, Jim H. Kou, Gordon L. Amidon and Ping I. Lee published a paper on pH-dependent swelling and solute diffusion characteristics of hydroxyethyl – methacrylate gels. The gels were prepared by free radical polymerization, with tetraethylene glycol dimethacrylate (TEGDMA), as cross-linker and 1% of 2, 2''-azobisisobutyronitrile. The concentration of cross-linker varied with gels. HEMA-MAAc gels swelling were highly dependent on the pH and the MAAC content. Swelling was high at high pH, where the carboxylic groups are ionized. With the non-ionized gel, the swelling did not depend on TEGDMA; concentration was ranging from 0.5% to 3%. The

result was in supportive of the existence of secondary interactions that shield the effect of covalent cross-links. Diffusion characteristics were studied with phenylpropalamine (PPA), at various pH. Measurements indicated that Yasuda's free volume theory was in agreement with it. There was an exponential increase. [16]

The phase behavior of hydroxyethyl methacrylate – methacrylic acid gels were examined as function of relative charge composition, solvent quality and bath salt concentrations by Anthony English, Toyochi Tanaka and Elazer R. Edelman. Sodium Chloride and Calcium Chloride were the salts used for the study. These gels showed continuous and discontinuous swelling transitions as a function of bath salt concentration (sodium chloride). The various experiments indicated that it is necessary to consider both equilibrium and non-equilibrium thermodynamics in the characterization of polyelectrolytes for use. The polyelectrolyte gels, N-isopropylacrylamide, acrylic acid and acrylamide were studied for analyzing its equilibrium and non-equilibrium swelling transitions. The experiments were carried out by changing the hydrogel and bath composition systematically. The study revealed that the theory and the experimental results were in qualitative agreement. [17]

Belma ISIK, from Hacettepe University, Turkey, studied the acrylamide – Hydroxyethyl methacrylate (HEMA) gels for its swelling properties in 1998. The polymer contained PEG 4000 to increase its microporosity. The gels diffusional behavior in water was also studied with the equilibrium swelling values. The diffusional characteristics were a non-fickian type. Swelling of the gels decreased with the increase

in total monomer, HEMA and cross-linker concentrations, while it increased with increase in the PEG 4000 content. [18]

In 2001 swelling of superabsorbent acrylamide/sodium acrylate hydrogels prepared using multifunctional crosslinkers have been studied by Erdener Karadag and Dursun Saraydin. Cross-linkers such as trimethylolpropane triacrylate, ethylene glycol dimethacrylate (EGDMA), 1,4-butanediol dimethacrylate and N,N'-methylenebisacrylamide were used. The research involved the study of the influence of cross-linkers and the relative content of sodium acrylate on swelling, initial swelling rate, swelling rate constant, swelling co-efficient and diffusional behavior of water in the hydrogel. Results indicate that acrylamide – sodium acrylate hydrogels showed greater swelling in water compared to acrylamide hydrogels and the water intake of hydrogels followed non-fickian type diffusion. [19]

In order to study the equilibrium swelling and kinetics of pH sensitive ionic hydrogels, Sudipto K. De, N.R. Aluru, synthesized poly-hydroxyethyl methacrylate-acrylic acid hydrogels in the year 2002. Copolymers of hydroxyethyl methacrylate - acrylic acid gels were prepared by photo polymerization, using 1% of a diacrylate as the cross-linker. Equilibrium and kinetic models were developed and compared with the experimental results. The models were accurate in predicting the swelling processes. In their model they describe the swelling process as chemoelectromechanical model and the kinetics of swelling is limited by diffusion. Results indicate that increasing fixed charge or lowering the ionic strength of the solution, swelling can be improved. Their study also

revealed that Young Modulus changed greatly with the pH of the solution while Poisson's ratio does not. [20]

In the paper published by P.B. van Wachem, A.H. Hogt, T. Beugeling, J. Feigen, A. Bantjes, J.P. Detmers and W.G. van Aken in 1987, growth properties of human endothelial (HEC) cells on copolymers of hydroxyethyl methacrylate – methyl methacrylate, copolymers of hydroxyethyl methacrylate (HEMA) or methyl methacrylate (MMA) and 15 mol% methacrylic acid (MAAc) or 15% trimethylaminoethyl methacrylate (TMAEMA-Cl). Human endothelial cells were seeded on the hydrogels in the presence of CMS (serum containing culture medium) or in the presence of CM (complete medium). The cells were counted using a haemocytometer. The HEC did not adhere to pure HEMA, more hydrophobic and hydrophilic copolymers. Maximum adhesion was shown on moderately wettable copolymer 25HEMA/75MMA with a contact angle of 39°. All those mentioned above were in the presence of CMS. The adhesion may be due to the fact that adsorption of serum proteins on the gel surfaces influenced the HEC adhesion. Fibronectin present in the serum is known to promote cell adhesion. Vitronectin may also influence cell adhesion. High growth of HEC was also seen on 85MMA/15MMA, 85MMA/15TMAEMA, and 85HEMA/15TMAEMA-Cl. In the absence of the serum, the same growth rate was not found on the hydrogels. This indicates that the serum is necessary in adhesion of the HEC to the hydrogels. An interesting result obtained was that in the absence of serum, HEC adhered onto the positively charged gels very strongly, as removing them using trypsin was impossible. Complete cell spreading on the MMA, a positively charged gel was observed, which may

be due to the electrostatic interactions. The same was not found with negatively charged gels. [21]

Burmeister JS, McKinney VZ, Reichert WM and Truskey GA in 1999, examined the effect of substrate hydrophobicity on cell-substrate contact area and the affinity between adsorbed fibronectin (Fn) and its receptor. Copolymer of ethyl methacrylate (EMA) and hydroxyethyl methacrylate (HEMA) were used for the study. The gels were made to absorb Fn, the protein necessary for cell adhesion. Bovine aortic endothelial cells (BAEC) were plated for 2 h in serum-free medium onto the gels. The topography of the basal surface as a function of distance from the substrate was examined using total internal reflection fluorescence microscopy (TIRFM). The total area covered by the cells on the gels was also studied. Maximum adhesion and contact area was found on the gels with no HEMA in it and minimum adhesion was found on gel with 100% HEMA. The affinity between Fn and its receptor and the bond strength was determined. The affinity and force per bond decreased with increasing in HEMA. These results indicate that differences in the strength of endothelial cell adhesion to polymers are influenced by the conformation of the adsorbed adhesion proteins. [22]

III. Motivation

Lewis was the first person to observe the outgrowth of endothelial cells from cultured fragments of chick embryo liver in the 1920s. Some years later he came to the conclusion that endothelial cells were unrelated to either parenchymal or connective tissue cells, which holds true till today. Until the early 1970s, endothelial cells could only

be studied at a distance; progress in endothelial cell research was very slow. Culturing endothelial cells became very easy with the development from the 70s resulting in a great deal of research. As stated earlier, these cells are very important to the human body. [11]

Development of artificial organs such as blood vessels, etc, involves these cells. Therefore knowledge of endothelial cells in depth is essential. Aortic endothelial cells grow quickly on unconditioned plastic tissue culture dishes and non-conditioned medium containing only serum. This explains why growing aortic endothelial cells in laboratories for research purposes have become easy and popular. Studying the growth of these cells and the rate at which they grow are the most important part of the research. Haemocytometer is the most commonly used counter to count the number of endothelial cells. Properties of endothelial were given earlier; it stated that morphology of these cells change with the layer they grow on. This is the basic idea behind artificial organs involving endothelial cells is - growing them on different polymers or hydrogels. [23]

Fabrication of hydrogels for various applications and studies is not a new area of research. There is no limit to the different type of hydrogels that can be made according to the required property. Properties and applications of hydrogels were given in the beginning of the chapter. As said before, hydrogels are useful in drug delivery systems, etc. For such applications, the swelling properties of the gels are the deciding factor. Diffusion characteristics of the hydrogels are the determining factor for. Hydroxyethyl methacrylate gels are the most commonly made gels, because it is biodegradable and has properties that are very useful in many fields.

This thesis involves the study of HEMA based gels and PEG based gels for its swelling and diffusion characteristics. Four acidic gels have been studied; they are HEMA-SEMA, HEMA-MAAc, PEG-SEMA and PEG-MAAc. The other part of the research involves the study of porcine pulmonary endothelial cells. Growth rate of these cells on naked Petri dishes and on the acidic gels that were mentioned were studied. The traditional method of counting the number of endothelial cells using haemocytometer was followed in this study. Image Processing techniques were used to count the number of cells and to calculate the area they have covered on the hydrogels. A MATLAB algorithm was developed to calculate the area the cells have covered on the hydrogels. Morphology of the cells grown on different hydrogels was also examined to determine the area of applications of these gels.

IV. Thesis Outline

Thermodynamic characteristics such as the swelling and diffusion potential of acidic hydrogels were studied and compared to the theoretical model. Endothelial cells were grown on naked Petri dish and on various hydrogels that were made, their growth rate and morphology was studied and distinguished. The results obtained were very close to the expected values. A detailed explanation of the algorithm developed to count the number of endothelial cells and to calculate the area covered, the advantages of the same are also given.

Part 2 of this thesis gives the different methods of synthesizing ionic hydrogels. The theory of swelling and diffusion potentials of acidic hydrogels is given in detail.

Methods and preparation of the hydrogels are also given. Various experiments were done with these hydrogels for examining their mechanical properties. All the results that were obtained are shown in graphs and each graph is discussed in detail. Diffusion potential measurements results are compared to the expected or theoretical graphs. A C code was developed to simulate the theoretical model, which is added at the end of the chapter. Part 3 is dedicated to the studies that were done on endothelial cells. The image processing techniques used are explained. Methods of cell culture and staining are also added. The results obtained for each gel are compared and discussed. The algorithms used are given at the end of the chapter. The last chapter is completely dedicated to the conclusions of all the previous chapters and a concise conclusion of the thesis.

**Part II: ACIDIC HYDROGEL THERMODYNAMIC
CHARACTERIZATION**

Abstract

Acidic hydrogels consists of negative monomeric species that are polymerized and cross-linked into a three dimensional network. A series of (logarithmically spaced charges) gels of hydroxyethyl methacrylate (HEMA) and polyethylene glycol (PEG) with sulfoethyl methacrylate (SEMA) and methacrylic acid (MAAc) were prepared. The swelling of these gels in deionised water, different bath concentrations of Sodium Chloride and the diffusion potential measurements were studied. Results indicate that HEMA –SEMA gels showed greater swelling characteristics compared to HEMA-MAAc gels. Swelling increases when the concentration of the monomer increases, whereas the swelling of HEMA-MAAc gels occurred only with very high concentrations of the monomer. As the concentration of HEMA and PEG decreases, the range of the diffusion potential for various concentrations of NaCl solution increases which, corresponds to the amount of swelling of the gels.

I. Introduction

Polyelectrolyte or Ionic hydrogels can be divided into two different types namely, Anionic and Cationic hydrogels. Cationic hydrogels are those with positive charge and Anionic hydrogels are the one with negative charge. Ionic gels are composed of a solid and a liquid phase. The solid phase consists of a cross-linked polymer network with acidic or basic groups attached to it the polymer chains. When the gel is immersed in a solvent, the chains in the network become solvated. Due to the cross-links the chains do not completely mix with the solvent. This is because the cross-links provide an elastic restoring force that counters the expansion of the network. A hydrogel is a combination

of a polymer network and a internal solution that surround the chains. All the ionizable groups, acidic or basic do not become fully protonated/deprotonated. [1, 24, 25]

HEMA and PEG based gels are biocompatible and can be polymerized easily. HEMA is one of the most commonly used hydrogel and it was first applied for making contact lenses in the 1950s. These hydrogels can be prepared in various shapes and forms and they can swell up to a 1000 times more than their size and shrink back to its original size without any changes. The mechanical properties of these gels are very important for their applications. [26] Four different acidic hydrogels were made by this method; they are HEMA-SEMA, HEMA-MAAc, PEG-SEMA and PEG-MAAc. These hydrogels are polyelectrolyte hydrogels. They can also be classified as pH-dependent hydrogels. They can be prepared by a number of methods, some of which that are relevant to this study are discussed in this section. [27-32]

Co-polymerization of an ionic monomer with a neutral monomer or homopolymerization of ionic monomers, are the methods mostly used to synthesize hydrogels. Co-polymerization or cross-linking method is a reaction of a co-monomer with a multifunctional co-monomer which acts as a cross-linker in the presence of an initiator and a solvent. Phase separation occurs in co-polymerized hydrogels, if the amount of water present is greater than the water content corresponding to the equilibrium swelling. This results in a heterogeneous hydrogel, which consists of domains of highly cross-linked micro gels, which are connected by loosely cross-linked chains. This is known as microsineresis. The resultant hydrogel is opaque. These hydrogels are interesting in the study of drug-delivery systems. [30]

Another method is cross-linking linear polymers by irradiation or by chemical compounds. Free radical polymerization and step or condensation polymerization are two of the commonly used techniques in the preparation of homopolymerized hydrogels. Most of the hydrogels are polymerized using free radical polymerization. This reaction can be carried out in bulk, in solution or in suspension. The reaction is initiated by adding a small amount of a free radical generator, also called initiator. For this study, hydrogels were prepared by free radical polymerization and the free radical generator used was Ammonium Per Sulphate (APS). [33]

A number of initiators can be used, such as azo-compounds, peroxides and redox initiators. Most commonly used cross-linkers are ethylene glycol dimethacrylate, N,N'-methylene bisacrylamide and divinyl benzene. Gamma-irradiation can also be used to initiate the polymerization reaction. Residue free hydrogels can be produced by this method compared to the chemical initiation method. [33]

Monomers used for synthesizing the polyelectrolyte hydrogels contain a ionizable group, a group that can be hydrolyzed or a group that can undergo a substitution reaction after polymerization is completed. The resulting hydrogels can be weak basic or acidic hydrogels or strong basic or acidic hydrogels. Weak ionic hydrogels may contain primary or secondary substituted amines or carboxylic acids. Strong polyelectrolyte hydrogels may contain sulfonic acids or quaternary ammonium salts.

A. Swelling/ Deswelling Phenomena

The important property of the hydrogel which is swelling, occurs due to the presence of hydrophilic groups such as $-\text{OH}$, $-\text{CONH}-$, $-\text{CONH}_2$, $-\text{COOH}$ and $-\text{SO}_3\text{H}$.

Neutral hydrogels swell due to the contribution from the water-polymer thermodynamic mixing to the overall free energy and also from elastic polymer contribution. Along with these contributions, ionic interactions between charged polymer and free ions cause swelling in ionic hydrogels. Due to the ionization of the mentioned ionizable groups in the monomers, the hydrogel is more hydrophilic and thus leads to a high water intake. An increase in the ionic content in the polymer, results in increase in the hydrophilicity leading to higher equilibrium and faster swelling. [34] [35-38]

For an acidic hydrogel, hydrogen ion from the basic solution combines with the hydroxide ion to form water. Charge compensation occurs due the cations that enter the gel with the hydroxide ion. The increase in cation concentration gives rise to an osmotic pressure that will cause the gel to swell or deswell. Ionic gels swell due to the nature of the polymer network to mix with the solution. Typically, the osmotic pressure is much greater than the mixing force. Equilibrium of ionic gels occurs when the elastic restoring force of the network balances the osmotic pressure. [35, 39, 40]

II. Theory of Hydrogels

The system here is a one with constant temperature, T and volume, V , subdivided into a hydrogel phase of volume V_g and a very large bath of volume V_b . The total number of each mobile ion and solvent species, N_i , is fixed and distributed between the hydrogel and bath phases. Helmholtz free energy is the free energy to be considered with the defined temperature, total system volume and mobile particle numbers. The considered free energy is in turn a function of the mobile solvent and ion species distribution in the hydrogel and bath phases. [41] In the limit that the bath ion numbers and volume become

very large the ion chemical potentials become fixed by the corresponding bath ion concentrations. Also considering the solvent interaction parameter, a temperature dependent variable, the bath ion concentrations and solvent quality become the independent control variables. For a given solvent quality and bath ion concentrations the equations of constraint that arise from the balance of chemical potentials and local charge electroneutrality define the Helmholtz free energy as a function of the hydrogel volume. Therefore, the hydrogel volume becomes the state variable in this system and the equations of constraint implicitly define an equilibrium surface as a function of solvent quality and bath ion concentration. By considering the entire manifold of locally stable and unstable solutions for each value of solvent quality and bath salt concentration the complete thermodynamic system is more fully appreciated. [17, 36, 42-44]

A. Free Energy Model

The total system Helmholtz free energy consists of contributions from both the hydrogel and the surrounding bath such that

$$F(V_g; V_b, \chi(T), N_{g0}, \dots, N_{b0}, \dots) = F_g + F_b \quad (1)$$

where F_g - the hydrogel free energy and

F_b - the bath free energy.

It is assumed that the hydrogel Helmholtz free energy consists of four additive components,

$$F_g = F_M + F_{el} + F_N + F_{polyion} \quad (2)$$

where, F_M gives the mixing,

F_{el} the elastic,

F_N the mobile particle and

F_{polyion} the polyion contributions of the hydrogel Helmholtz free energy.

The Helmholtz free energy of mixing between monomer and solvent molecule, based on Flory – Huggins lattice theory is

$$F_M = k_B * T * V_g / v_{\text{site}} \quad (3)$$

where, V_g is the hydrogel volume,

v_{site} the lattice site volume,

ϕ the polymer volume fraction,

χ the Flory interaction parameter,

k_B is Boltzmann's constant and

T the absolute temperature.

The elastic contribution to the Helmholtz free energy is

$$F_{\text{el}} = (3 * k_B * T / 2) (V_g * \phi / N_x * v_{\text{site}}) [(\phi_0 / \phi)^{2/3} - 1 - 1/3 \ln(\phi_0 / \phi)] \quad (4)$$

where, N_x is the average number of monomers between cross-links. [17]

The term $(V_g * \phi / N_x * v_{\text{site}})$ is the effective number of chains in the network while

(ϕ_0 / ϕ) is the swelling ratio. Hydrogel volumes and polymer volume fractions are related through

$$V_g / V_0 = \phi_0 / \phi = C_{m0} / C_m = (D / D_0)^{2/3} \quad (5)$$

where, V_0 , ϕ_0 , C_{m0} and D_0 are the hydrogel volume, polymer volume fraction, concentration and diameter in the reference state respectively. Mobile particle

contribution to the Helmholtz free energy using the entropy of an ideal solution and an electrostatic component due to charges in the self-consistent potential created by the polymer charges and mobile ions is given by,

$$F_N = k_B * T \sum_i N_{gi} \{ \ln(N_{gi}/V_g) - 1 \} + \sum_i z_i * e * N_{gi} * \phi_{self} \quad (6)$$

where, N_{gi} is the total number of the i th mobile particle within the hydrogel

z_i is the valence of the i^{th} ion,

e is the unit charge and

ϕ_{self} is the self-consistent potential created by the mobile ions and polyions.

Debye-Huckel corrections and contributions that arise from other forms of charge fluctuations were ignored.

The polyion free energy is given by,

$$F_{polyion} = z_{pi} * e * N_f * \phi_{self} \quad (7)$$

where, z_{pi} is the valence of the polyion charge and

N_f is the total number of ionized polyion charges.

When the ion distributions are uniform the inclusion of the mobile ions at low bath ionic strengths eliminates the electrostatic contribution to the total free energy. This occurs at low ionic strengths where the electrostatic screening length is long compared to the polyion or polymer chain separation distance. [45]

The bath contribution to the Helmholtz free energy can also be defined using an ideal solution approximation

$$F_b = k_B * T \sum_i N_{bi} \{ \ln(N_{bi}/V_b) - 1 \}, \quad (8)$$

where, N_{bi} is the total number of i th bath ion.

Corrections due to the fluctuations are ignored.

B. Equilibrium Equations of Constant

For fixed volume, temperature and particle numbers the Helmholtz free energy is an extremum in equilibrium. Hence in equilibrium

$$dF = \sum_i [(\partial F_g / \partial N_{gi})_{T,V,\dots,N_i,\dots} - (\partial F_b / \partial N_{bi})_{T,V,\dots,N_i,\dots}] dN_{gi} = 0 \quad (9)$$

here it is assumed that $dN_{gi} = -dN_{bi}$

Since,

$$\mu_i = F / N_i$$

this implies a balance of chemical potentials in all phases. The equations that arise from the balance of chemicals implicitly define a surface of equilibrium solutions as a function of bath salt concentration and solvent quality.

1. Solvent equilibrium constraints, balance of solvent chemical potentials

As $V_g \approx v_0 N_{g0}$, the first equilibrium equation of constraint can be written as

$$\Pi_{\text{swelling}} = (\mu_{g0} - \mu_{b0}) / v_0 = 0 \quad (11)$$

v_0 is the solvent particle volume.

v_0 is the solvent molar volume.

The swelling equilibrium condition obtained by equating hydrogel and bath solvent chemical potentials is

$$-1/v_{\text{site}} [\ln(1-\phi) + \phi + \chi\phi^2] + \phi_0/N_x v_{\text{site}} [1/2(\phi/\phi_0) - (\phi/\phi_0)^{1/3}] + C_{\text{mobile}} - 2C_b = 0, \quad (12)$$

Molar volume of the lattice sites is given by $v_{\text{site}} = N_A v_{\text{site}}$

ϕ – polymer volume fraction,

χ – Flory interaction parameter,

N_x – number of monomers between cross-links,

ϕ_0 – polymer volume fraction in the reference state,

C_{mobile} – total mobile ion concentration within the hydrogel

C_b – total concentration of all positive monovalent ions in the external bath,

$$C_b = C_s + C_{\text{bh}^+}$$

C_s – bath salt concentration

C_{bh^+} – hydrogel ion concentration.

2. Ionic equilibrium, balance of ion chemical potentials

With the assumption that the potential background within the hydrogel is uniform, balance of ion chemical potentials, leads to the Donnan equilibrium relations. Donnan equilibrium relations with the constraint of local charge neutrality gives,

$$C_{\text{mobile}} = 2\sqrt{(N_{\text{f}^-}/2N_A V_g)^2 + C_b^2} \quad (13)$$

N_{f^-} – total number of negative fixed charges

$$C_{\text{mobile}} = \sum_i N_{\text{gi}}/N_A V_g \quad (14)$$

Excluded volume effect is assumed to be negligible.

3. Dissociation equilibrium balance of bound and unbound chemical potentials

We know, acid dissociation is given by

$$K_a = C_{A-} * C_{H+} / C_{AH} \quad (15)$$

K_a – acid dissociation constant

C_{A-} – concentration of dissociated acidic groups

C_{H+} – hydrogen ion concentration

C_{AH} – concentration of undissociated acidic groups.

In the case of monovalent ions, local charge electroneutrality and Donnan equilibrium gives a third order polynomial equation in terms of the known bath ion concentration and the unknown internal hydrogen ion concentration,

$$C_{H+} + K_a C_{H+}^2 - (C_{bH+}^2 + C_A K_a C_{bH+} / C_b) C_{H+} - K_a C_{bH+}^2 = 0, \quad (16)$$

Effects due to electric field and charge inhomogeneities are being ignored because the above equation is based on Donnan equilibrium condensation.

Newton's and bisection method can be used to obtain analytical solutions to the equilibrium swelling for the above equation. All other ion distributions follow from the known bath ion concentrations and the Donnan equilibrium conditions. Osmotic pressure and free energy components can then be determined knowing the ion distributions. Iterating the above procedure, the equilibrium swelling can be determined by minimizing the difference between hydrogel and bath osmotic pressures. For a given bath salt concentration and solvent quality, all solutions and their stability can be determined.

C. Principle of Diffusion

Ions move freely in solutions, in order to maintain equilibrium, therefore, between different regions in a simple solution of electrolytes, potential cannot be measured. However, if NaCl solutions of different ionic strengths are brought together, a Diffusion

Potential or Liquid Junction Potential results immediately for a short period, because of the differences in the mobilities of the ions. Na^+ and Cl^- diffuse as separate units. Na^+ moves more slowly than Cl^- and the resulting separation of the opposite charged ions establishes a potential at the boundary (junction) between the two solutions, the positive side in this case being the solution with higher concentration. Difference in mobility can be attributed to the shell of hydration of each type of ion. Each ion carries an associated “cloud” of water molecules that are attracted by the charged state of the ion. In sodium (Na^+) this “cloud” is larger than that found in chloride (Cl^-). Thus the (Cl^-) on the left side will rise faster than (Na^+) and this voltage difference (or potential) is the Diffusion Potential. Ultimately this potential dissipates as equilibrium is attained.

When a semi-permeable membrane is used to separate solutions of different concentrations, a membrane potential is obtained, which is a combination of Donnan potential resulting due to the accumulation of either positive or negative charges over the surface of the membrane, the diffusion potential due to the diffusion of charges from higher concentration to lower concentration and also the charge of the semi-permeable membrane, as the concentration gradient created across the membrane, the charge at the pore walls can play a significant role on the ions flow in the membrane. [46]

D. Donnan Potential

There exists an electrical potential difference between two salt solutions of different concentration separated by an ion-exchange membrane in the absence of any current flowing through the membrane. The difference in concentration between the salt solutions will set up a diffusional force driving the sodium chloride from the higher

concentration into the lower concentration through the hydrogel membrane. The chloride ions have higher diffusivity rate than sodium ions. Consequently, excess negative electrical charges will accumulate on the low concentration solution side of the membrane, while excess positive electrical charges will accumulate on the high concentration side because of the positively charged sodium cations that are left behind. This charge separation will induce an electrical potential difference that will drive the electro-migration of the chloride ions in the direction opposite to that of the diffusion. The overall result will be that the net movement of the chloride ions to the lower concentration will slow down and eventually stop, when the two opposing forces are equal and the two opposing fluxes are equal. This equilibrium condition is called the Donnan equilibrium and the electrical potential difference across the membrane is the "Donnan potential". The Donnan potential, which depends on the membrane charge density, plays a very important role in the selectivity of the membrane with respect to the valance of the ions. [43, 47, 48]

The electrochemical potentials of the i^{th} ion in the salt solution and in the hydrogel are given by μ_i and $\bar{\mu}_i$, respectively:

$$\mu_i = \mu_i^0 + RT \ln \gamma_i C_i + z_i F \phi, \quad i=+,- \quad (17)$$

$$\bar{\mu}_i = \bar{\mu}_i^0 + RT \ln \bar{\gamma}_i \bar{C}_i + z_i F \bar{\phi}, \quad i=+,- \quad (18)$$

where μ_i and $\bar{\mu}_i$ are the standard electrochemical potentials, z_i is the valence of the ion, R is the gas constant, T is the absolute temperature, γ_i and $\bar{\gamma}_i$ are the ion activity coefficients of the i^{th} ion in salt solution and in the hydrogel, C_i and $\bar{C}_i$ are the ion concentrations, F is the Faraday constant while ϕ and $\bar{\phi}$ are the electrical potentials; the bar denotes the values in the charged membrane.

At the interface between the membrane and the external electrolyte solution, the electrochemical potentials in the two phases are equilibrated:

$$\mu_i = \bar{\mu}_i, \quad i = +, - \quad (19)$$

The Donnan potential, $\Delta\phi_{Don}$ between the membrane surface and the electrolyte solution is given as

$$\Delta\phi_{Don} = \bar{\phi} - \phi = -\frac{RT}{z_i F} \ln \frac{\bar{\gamma}_i \bar{C}_i}{k_i \gamma_i C_i}, \quad i = +, - \quad (20)$$

where k_i is the distribution coefficient of the i^{th} ion, defined as

$$\bar{\mu}_i^0 - \mu_i^0 = -RT \ln k_i, \quad i = +, - \quad (21)$$

Under the condition, $z_+ = -z_-$, the relationship between the concentrations of ions in the membrane and in the external solution can be obtained:

$$\bar{C}_+ \bar{C}_- = \frac{\gamma_{\pm}^2 C_s^2}{Q^2} \quad (22)$$

where C_s is the bath salt concentration in the external solution equated as $(C_+ C_-)^{1/2}$

$\gamma_{\pm}$ is the mean activity coefficient of the electrolyte equated as $(\gamma_+ \gamma_-)^{1/2}$

Q can be expressed as

$$Q = \left(\frac{\bar{\gamma}_+ \bar{\gamma}_-}{k_+ k_-} \right)^{1/2} \quad (23)$$

On the other hand, the condition of electro neutrality in the membrane is given by

$$\sum_{i=+,-} z_i \bar{C}_i + z_x C_x = 0 \quad (24)$$

where z_x is the valence of fixed charge groups, and C_x is the fixed charge density.

$$\bar{C}_+ = \sqrt{\left(\frac{z_x C_x}{2z_+} \right)^2 + \left(\frac{\gamma_{\pm} C_s}{Q} \right)^2} - \frac{z_x C_x}{2z_+} \quad (25)$$

$$\overline{C_-} = \sqrt{\left(\frac{z_x C_x}{2z_+}\right)^2 + \left(\frac{\gamma_{\pm} C_s}{Q}\right)^2} + \frac{z_x C_x}{2z_+} \quad (26)$$

The most important point in equations (25) and (26) is that the mean activity coefficient of electrolytes in the external, aqueous, organic solution, $\gamma_{\pm}$ is multiplied to the electrolyte concentration, C_s . The effect of mean activity coefficient of the electrolyte is usually neglected in aqueous solution. Donnan potential at the left- and right-hand sides of the interface between the membrane and the surrounding solution are given by inserting Equation (25) into equation (20). If it is assumed that the activity coefficients of ions are not the function of the ion concentrations in the membrane, the total Donnan potential through the membrane can be expressed by the sum of the Donnan potentials at the left- and right-hand sides for an aqueous, organic solution system cited above:

$$\Delta\phi_{Don(l \rightarrow r)} = -\frac{RT}{z_+ F} \ln \left[\frac{\gamma_+'' C_s'' \overline{C_+}'}{\gamma_+' C_s' \overline{C_+}''} \right] \quad (27)$$

where C_s' and C_s'' are the concentrations of the solution in left- and right-hand sides, and $\overline{C_+}'$ and $\overline{C_+}''$ are the cation concentrations at the left- and right-hand sides of the charged membrane, which are given by equations (25) and (26) respectively. The value of γ_+''/γ_+' cannot be obtained separately, thus we approach it by $\gamma_{\pm}''/\gamma_{\pm}'$, to avoid errors. Therefore the above equation becomes

$$\Delta\phi_{Don(l \rightarrow r)} = -\frac{RT}{z_+ F} \ln \left[\frac{\gamma_{\pm}'' C_s'' \overline{C_+}'}{\gamma_{\pm}' C_s' \overline{C_+}''} \right] \quad (28)$$

E. Membrane Diffusion Potential

There is a potential developed due to the diffusion of ions from higher concentration to lower concentration, this voltage difference is called as diffusion potentials because they are generated by selective ion diffusion. The diffusion potential depends on the ionic mobility in the membrane and affects the transport of ions in the membrane. The voltage difference established between the two compartments is thus a direct result of the selective diffusion of ions across the membrane. The membrane potential becomes more negative as more cations diffuse from left to right, and the voltage in turn limits their outward diffusion. At some point the tendency of a cation to diffuse down its concentration gradient is balanced by its equal tendency to move in the opposite direction "down" the electrical gradient. This balance of electrical and diffusion tendencies creates an electrochemical equilibrium between the opposing chemical or concentration and electrical forces. (Consequently, diffusion potentials are also often called Nernst potentials). Over time, about the same number of cations diffuses into the outside compartment as diffuse in the opposite direction, and no additional membrane potential develops. The ionic flux across the membrane is given by the Nernst-Planck equation, which can be applied to the phase of the membrane

$$J_i = -\bar{\omega}_i RT \frac{d\bar{C}_i}{dx} - z_i F \bar{\omega}_i \bar{C}_i \frac{d\bar{\phi}}{dx}, \quad i=+,- \quad (29)$$

where $\bar{\omega}_i$ is the ionic mobility of the i^{th} ion in the membrane. The electro-neutrality condition in a membrane requires that

$$J_+(x) = J_-(x) \quad (30)$$

Differentiating hydrogel concentration terms used earlier,

$$\frac{d\overline{C_+}}{dx} = \frac{d\overline{C_-}}{dx} \quad (31)$$

Now after substitutions the diffusion potential can be expressed as

$$\Delta\phi_{diff} = \overline{\phi}^n - \overline{\phi}' = -\frac{RT}{z_+F} \frac{r-1}{r+1} \times \ln \left[\frac{(r+1)\overline{C_+}^n + (z_x/z_+)C_x}{(r+1)\overline{C_+}' + (z_x/z_+)C_x} \right] \quad (32)$$

where r is the cation-to-anion mobility ratio in the membrane defined as

$$r = \frac{\overline{\omega_+}}{\overline{\omega_-}} \quad (33)$$

where $\overline{\omega_+}$ and $\overline{\omega_-}$ are the cation and anion mobility in the membrane, respectively.

F. Membrane Potential

The membrane potential $\Delta\phi$ is the sum of the Donnan potential and the membrane diffusion potentials, which are given by equations (28) and (32), respectively

$$\Delta\phi = \Delta\phi_{Don(l \rightarrow r)} + \Delta\phi_{diff} \quad (34)$$

Thus equation (34) gives the total membrane potential of the hydrogel.

III. Methods and Materials

A. Procedure for Making Acidic Hydrogels

The hydrogels were prepared by the free radical polymerization of monomers. All the chemicals were used as bought. The total monomer concentration used, after dilution was 1.54M to avoid phase separations. The reaction temperature was 25°C. Ethylene glycol dimethacrylate (EGDMA) was the cross-linker used. Ammonium Per Sulphate (APS) and Sodium Meta Bi Sulfate (SMBS) were used to initiate and speed up the

polymerization reaction respectively. All the materials used in the preparation of the hydrogels were washed thoroughly with de-ionized water before use. The stock solutions of the monomers were prepared as given below.

Preparation of 450mL of 1.54M HEMA: 89.002mL of HEMA, 180mL of ethylene glycol (EG) and 1749 μ L of EGDMA were added. The beaker was filled with de-ionized water upto 450mL.

Preparation of 450mL of 1.54M PEGDMA: 90.187gm of PEGDMA, 180mL of (EG) and 1749 μ L of EGDMA were added. The beaker was filled with de-ionized water upto 450mL.

Preparation of 50mL of 1.54M of SEMA: 3.08gm of NaOH, 12.041mL of SEMA, 20mL of (EG) and 194 μ L of EGDMA were added. The beaker was filled with de-ionized water upto 50mL.

Preparation of 50mL of 1.54M of MAAC: 6.597mL of MAAC, 20mL of (EG) and 194 μ L of EGDMA were added. The beaker was filled with de-ionized water upto 50mL. 4 wt% of APS was prepared. 0.4gm of APS (1.76mM) was added to every 10mL of de-ionized water to prepare the same. 15 wt% of SMBS was prepared. 1.5gm of SMBS (7.6mM) was added to every 10mL of de-ionized water to prepare the same. 20.2mM concentration of EGDMA was used. For the preparation of 50mL of EGDMA solution, 194 μ L of EGDMA was added.

All these solutions were mixed thoroughly using a magnetic stirrer. While mixing SEMA, the beaker was placed in a beaker with ice, as the reaction is exothermic. The hydrogels were prepared in the form of sheets and cylinders of various sizes. Hydrogels with different charges were prepared according to the Table I. For example, to make

Table I
Hydrogel Sample Preparation

Sample	Charge(mM)	HEMA/ PEGDMA (mL)	SEMA/ MAAc (mL)
0	0	10.00	0.00
1	1.54	9.99	0.010
2	2.74	9.982	0.018
3	4.87	9.968	0.032
4	8.66	9.944	0.056
5	15.40	9.900	0.100
6	27.39	9.822	0.178
7	48.70	9.684	0.316
8	86.60	9.438	0.562
9	154.00	9.000	1.000
10	273.86	8.222	1.778
11	486.99	6.838	3.162
12	866.01	4.377	5.623
13	1540.00	0.000	10.000

1.54mM of HEMA-SEMA, 9.99mL of HEMA and 0.010mL of SEMA was added in a test tube. 100 μ L of APS and SMBS were added to each test tube to initiate and speed up the reaction as mentioned earlier.

For making sheets of hydrogels, glass slides of two different sizes, one slightly smaller than the other was washed and dried, and a Teflon sheet in the size of the small glass slide was kept between the slides, with a small opening to pour the solution. Micro-pipettes of various sizes were added in each test tube to obtain hydrogels in the form of cylinders for swelling experiments.

B. Diffusion Potential Measurements

The gels from the glass slides were taken and kept in a dish containing de-ionized water. The gels were washed with deionised water everyday and the gels were then placed in 154mM of NaCl Solution. In order to conduct diffusion measurements on the hydrogels, these gels were cut into small circular gels, so it can fit in the membrane test chamber.

The equipment used for this experiment to measure the steady state potential is the EVC-4000 Multi-Channel Voltage/Current Clamp instrument. A four-channel Voltage/Current clamp apparatus, the EVC4000 employs the voltage clamp technique to monitor membrane permeability as a function of membrane voltage or applied chemicals. Each module, with its companion preamplifier, can operate independently in one of three different modes: Voltage Clamp, Current Clamp, or Open Circuit Potential measurement.

The hydrogel was placed in the chamber. Two voltage electrodes, each on either side of where the membrane were inserted into the appropriate ports of the chamber. Two

current electrodes were also connected at the ports located at each distal end of their respective chambers. The '1' electrodes, i.e., V1 and I1 were connected on the same chamber half; similarly the '2' electrodes, V2 and I2 on the other half. The concentration of the NaCl solution on the left chamber was changed, whereas the concentration of the NaCl solution on the right chamber remained at 154mM. The ionic strength of the salt solution was changed from 1.54mM to 4.87mM, then to 15.4mM and so on till 1540mM. The membrane needs to be at equilibrium while starting the experiment on each hydrogel. Therefore the solution on the left chamber was 154mM that is the same as the right chamber. The solution from both the chamber was allowed to flow and the voltage was adjusted to zero on the preamplifier. After this initial adjustment, the concentration on the left chamber was varied as given earlier and the voltage was measured.

C. Swelling Measurements

Small pieces of gels were taken from the 100 μ L micropipettes and washed in deionised water continuously for 5 days. Diameter of the gels was found after washing it with de-ionized water. The gel diameter was found by placing it under the microscope and National Vision Assistant software was used to find the length. A Zeiss 1,25x/ 0,035 lens was used for the measurement. Later the gels were washed with different concentrations of Sodium Chloride. The salt was used as it was bought, from Sigma-Aldrich. The concentration of the bath was made to vary from 1.54 μ M, 4.87 μ M, 15.4 μ M until 1540mM. The gels were washed for a day in each concentration before the diameter was measured.

IV. Results and Discussions

A. Swelling Ratio

Figure I shows the swelling of HEMA-SEMA gels after it was washed with Deionised water for a few days. The x-axis is the charge concentration of hydrogels and the y-axis is the ratio of the measured diameter of the gel to the standard. These gels show a steady increase in swelling as the concentration of the monomer SEMA increases. Swelling can be seen from the gels with charge 15.4mM. There is a steady increase in the swelling thereafter. The gel that has swollen the most is the one made of pure SEMA, which is HEMA-SEMA 1540mM.

Figure II gives the swelling/deswelling of HEMA-SEMA gels in various bath concentrations as a function of the charge of the gels. Bath salt concentration is given on the x-axis and ratio of the diameter of the gels in the y-axis. The HEMA-SEMA gels, with charge from 0mM till 8.66mM, do not show any swelling/deswelling for various ionic strengths. Only the gels with the charge 15.4mM and higher show swelling transitions. This is so because the osmotic pressure within the gel is higher as the charge increases. Each gel has swollen slightly more than the lower charged gel for the same ionic strength. Gels with charges from 0mM to 86.6mM have collapsed to the same size for the salt concentration 1540mM. All the gels with charge greater than 86.6mM have collapsed to a great extent, but not to the same size. The size of the gel with the maximum charge, HEMA-SEMA 1540mM has been the largest for all the salt concentrations. The osmotic pressure for this gel has been the highest for all ionic strengths and therefore the gel has remained larger than the other gels for corresponding ionic strengths.

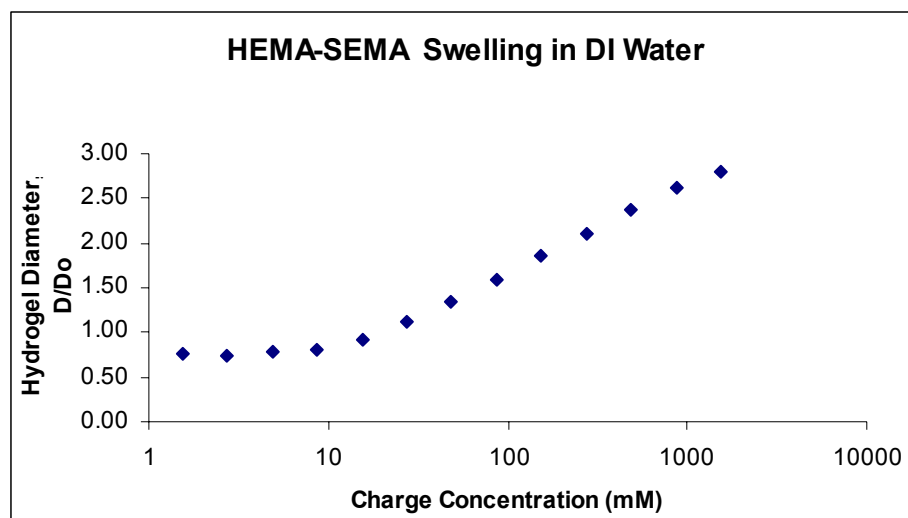


Figure I. Swelling of HEMA-SEMA in DI water

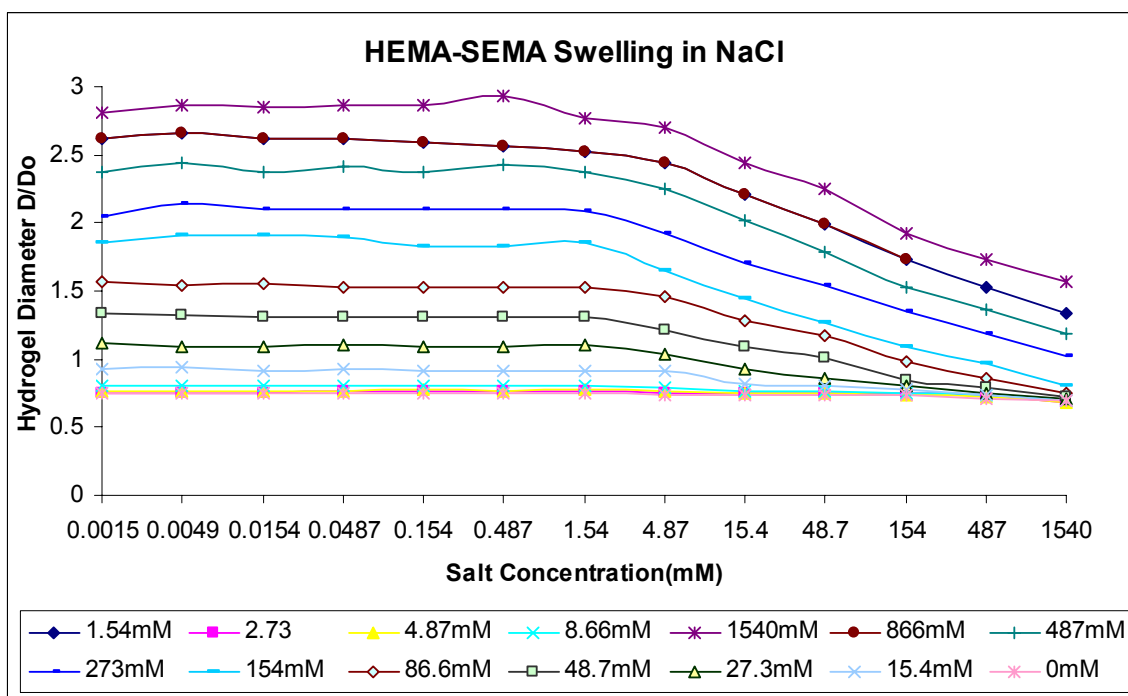


Figure II. Swelling of HEMA-SEMA in different concentrations of NaCl

Diameters of HEMA-MAAc gels after washing them in deionised water is given in figure III. The graph is a straight line expect for pure MAAc. These gels remain collapsed in deionised water, except for pure MAAc, which shows a high degree of swelling.

Figure IV shows the equilibrium swelling diameters of HEMA-MAAc hydrogels, measured as a function of bath sodium chloride concentration. At low ionic strengths the hydrogen ion is the only available counter ion to maintain charge neutrality. As a result, hydrogen ion binding produces a collapse transition in pure MAAc hydrogel at low ionic strengths. As the bath salt concentration increases from 1.54 μ M to 1.54mM, it leads to an exchange of hydrogen ions for sodium ions. Therefore, the internal pH increases, hydrogen ions dissociate from the acidic MAAc monomers and the internal osmotic pressure rises. When the bath concentration increases from 1.54mM to 1.54M, polyelectrostatic shielding and a reduction in the osmotic pressure difference between the hydrogel and the surrounding bath causes the gels to deswell. [17, 26]HEMA-SEMA 0mM that is the pure HEMA, remains collapsed as the concentration of the bath increases, whereas the pure MAAc gel shows a continuous swelling and deswelling. The gels with large amount of MAAc content shows swelling or deswelling compared to gels with very less MAAc content. Pure MAAc and HEMA-MAAc 866mM shows maximum swelling at 1.54 μ M. Gels with charge from 866mM down to 273mM remain collapsed till 4.87 μ M and exhibit swelling only from 1.54 μ M. These gels do not show a steady swelling or deswelling, but they all collapse at 1.54M. All the other gels from 154mM till 1.54mM do not have a considerable amount of swelling or deswelling. [17, 27, 49, 50]

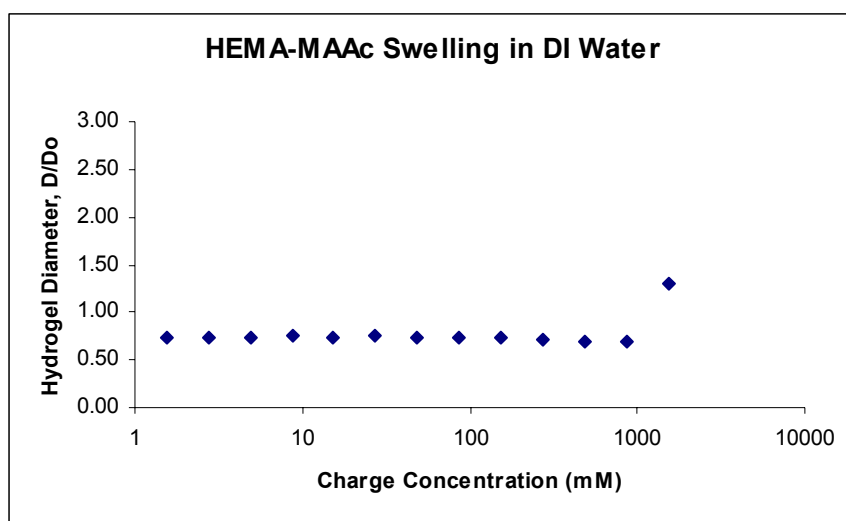


Figure III. Swelling of HEMA-MAAc in DI water

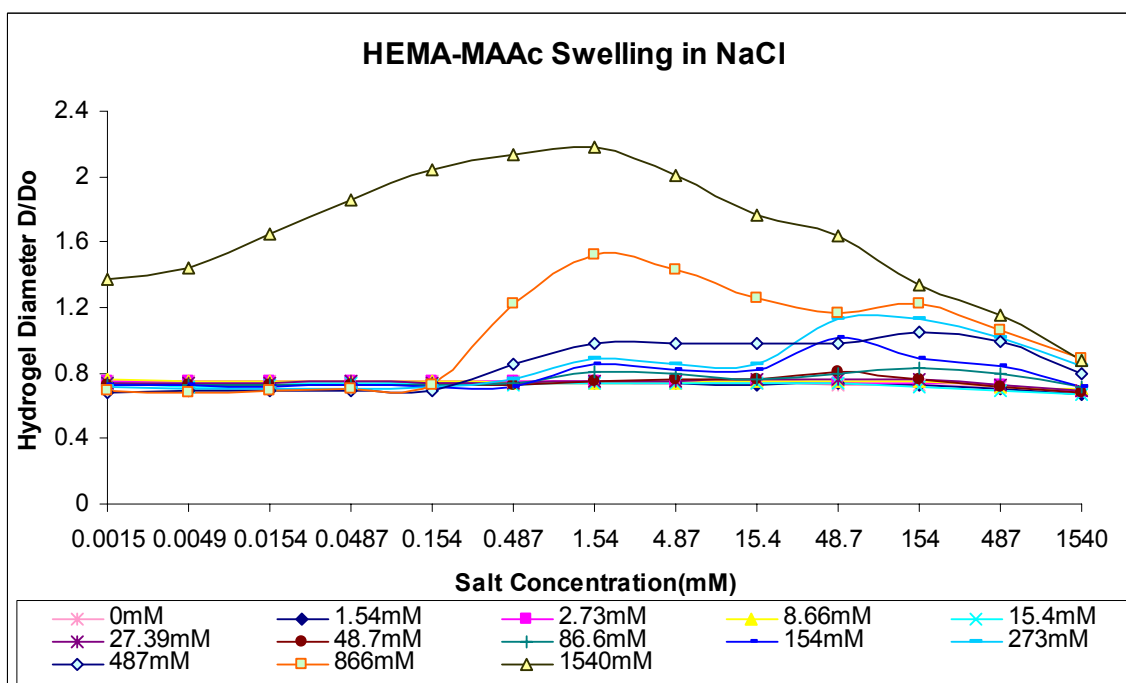


Figure IV. Swelling of HEMA-MAAc in various concentrations of NaCl

Swelling measurements of PEG-MAAc hydrogels, after being washed in deionised water, are given in figure V. As seen, the graph is a straight line. Only the pure MAAc gel shows a slightly higher swelling compared to the rest of the gels. Figure VI shows the equilibrium swelling diameters of PEG-MAAc hydrogels, measured as a function of bath sodium chloride concentration. All the gels remain collapsed for all the concentrations of the bath, except the pure MAAc, which shows a continuous swelling transition about which it has already been discussed.

Given in the figures VII and VIII are the swelling data obtained for PEG-SEMA in deionised and different bath concentration of sodium chloride respectively. A small amount of swelling in deionised water can be seen only from the gels with charge 273mM. The gels with charge lower than that do not show any difference in their diameters. The diameters of the gels with charge from 0mM to 154mM are very much the same as the PEG-MAAc gels with the same charge.

Swelling in different bath concentrations, are a little similar to the PEG-MAAc gels. Gel with charge 866mM show, swelling in low ionic strengths. It begins to deswell at 15.4mM concentration of the bath and completely collapses to the same size as the lower charged at concentration 1.54M. PEG-MAAc 487mm, has swollen slightly higher than the gel with charge 273mM, but lesser than 866mM. It begins to collapse at the ionic strength 48.7mM and reaches the same size as the other lower charged gels at the ionic strength 1.54M. Hydrogel with charge 273mM shows a similar swelling transition as the gel with charge 487mM. Hydrogels, with charge lower than 273mM remains collapsed for all the ionic strengths.

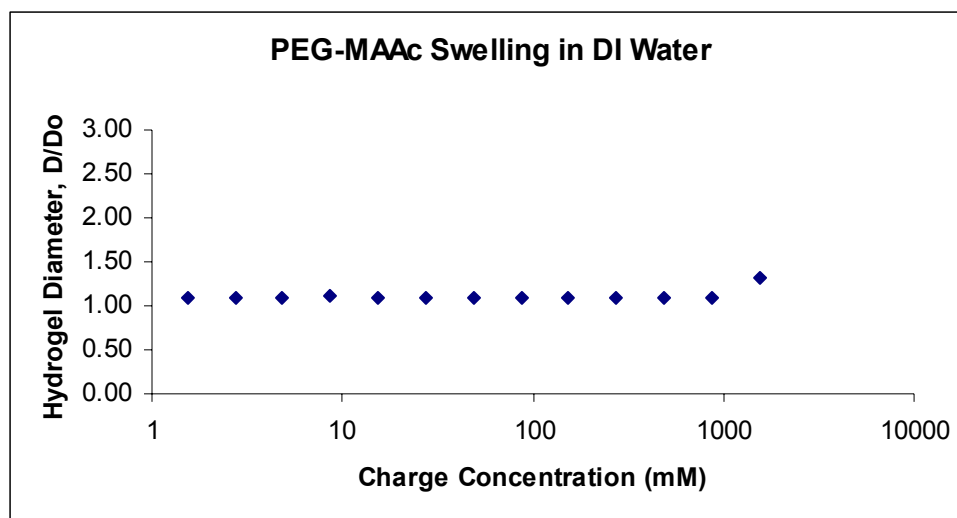


Figure V. Swelling of PEG-MAAc in DI water

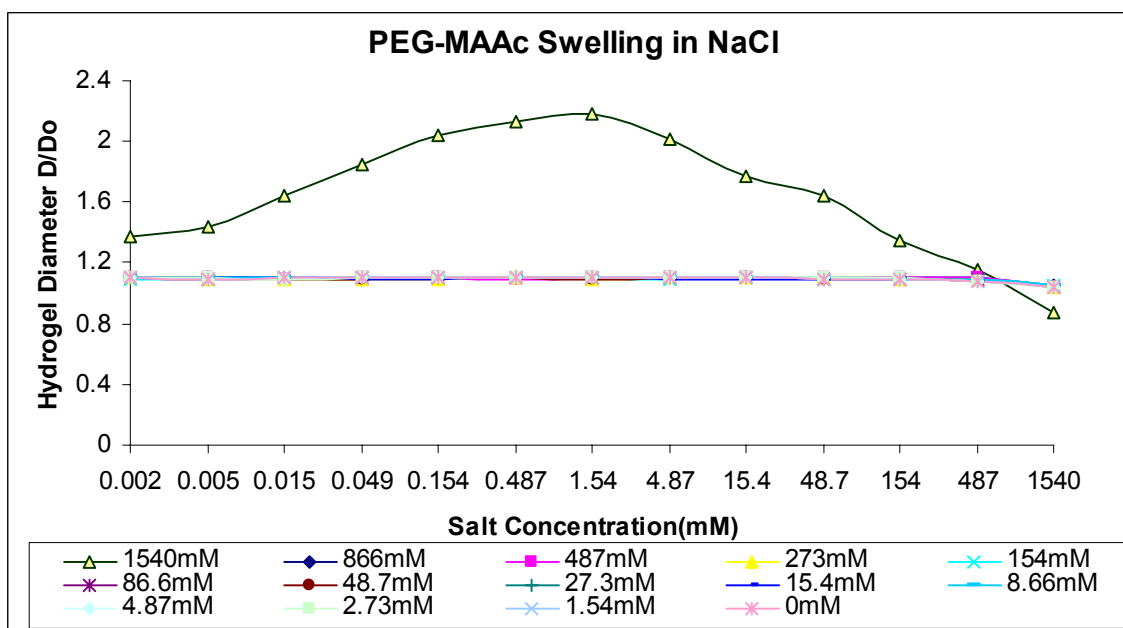
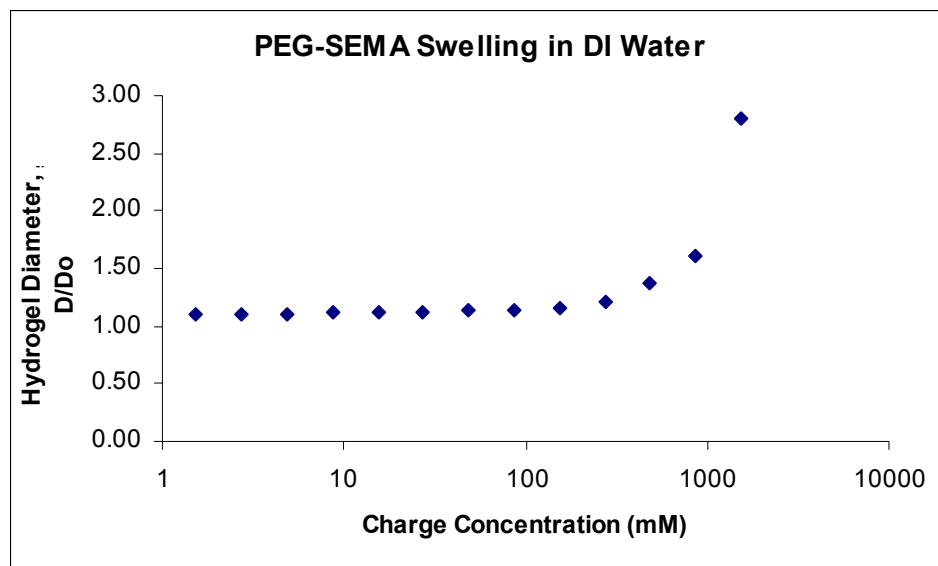


Figure VI. Swelling of PEG-MAAc in various concentrations of NaCl



FigureVII. Swelling of PEG-SEMA in DI water

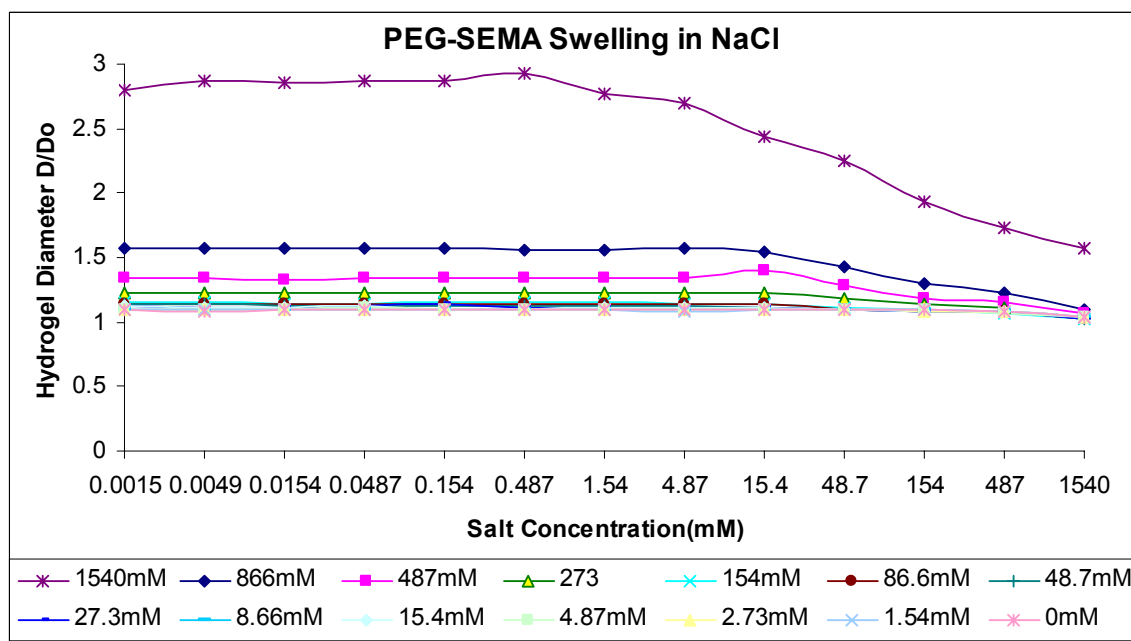


Figure VIII. Swelling of PEG-SEMA in various concentrations of NaCl

B. Diffusion Potential Measurements

Figure IX gives the expected diffusion potential values and was simulated using C. The figure gives the potentials of acidic and the basic hydrogels. The acidic hydrogels are the ones with a negative charge and are given in blue line. The potential keeps decreasing as the charge of the hydrogel decreases. For the pure hydrogel the line is almost straight, showing that there is negligible charge in it. Figure X gives the diffusion potential measurements for PEG-MAAc obtained experimentally. The concentration ranges over 0mM to 1540mM. The graph obtained is similar to the expected graph. The gel with the highest charge has the maximum potential at lowest ionic strength and crosses the zero line at 154mM and has negative potential for ionic strengths more than 154mM. The potential measured for pure MAAc at 1.54mM ionic strength was 40mV, the maximum for the PEG-MAAc gels. The potential gradually decreased and reached a value of -5mV for ionic strength 1540mM.

Steady state ion potentials measured for the PEG-SEMA gels are shown in figure XI. The results match with the expected values. The maximum positive potential is shown by the hydrogel with charge 866mM. Theoretically, the gel with the maximum charge should show the maximum potential, but here the gel with the maximum charge do not exhibit that, gels with lower charge have higher potential. This can be explained with the swelling graph. Figure VIII shows the swelling of PEG-SEMA gels at various concentrations of sodium chloride. PEG-SEMA 1540 has swollen to a very high extent compared to the other lower charged gels. The next highest charged gel 866mM has swollen a very small amount followed by 487mM and the rest of the gels have remained

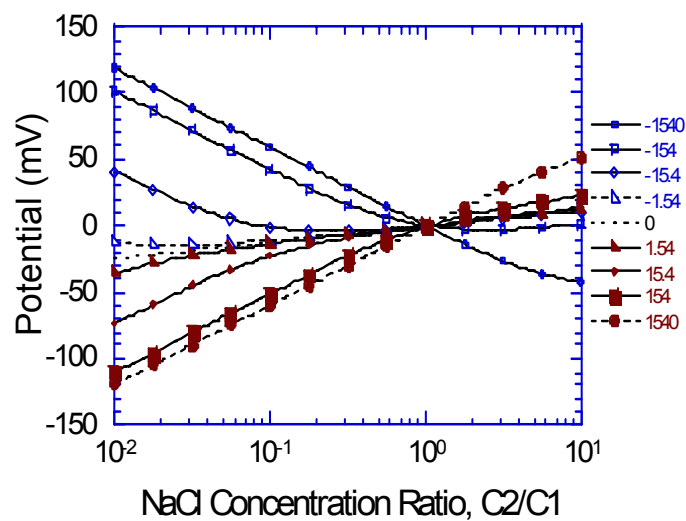


Figure IX. Theoretical diffusion potential values for acidic hydrogels.

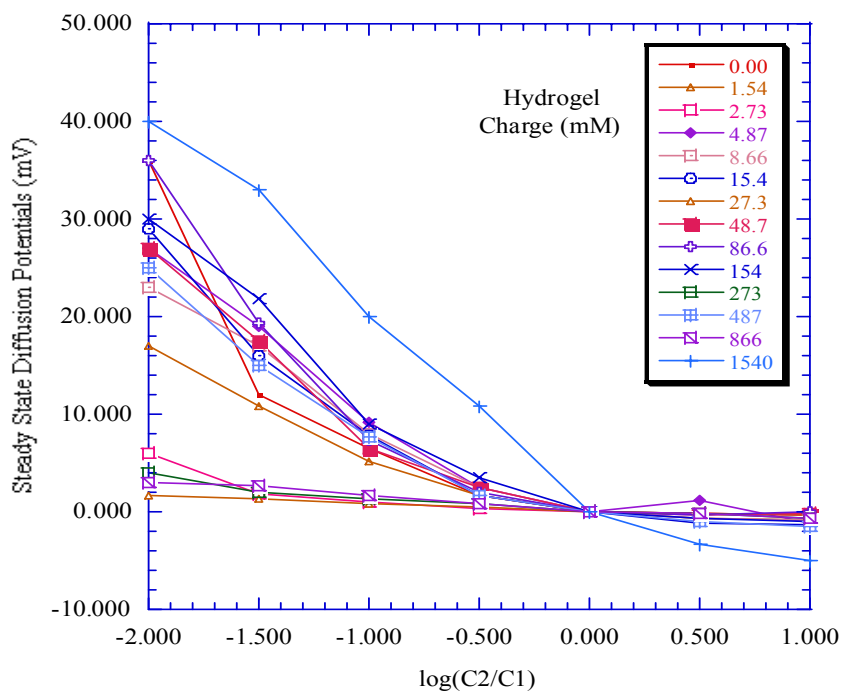


Figure X. Experimental diffusion potential measurements of PEG-MAAc.

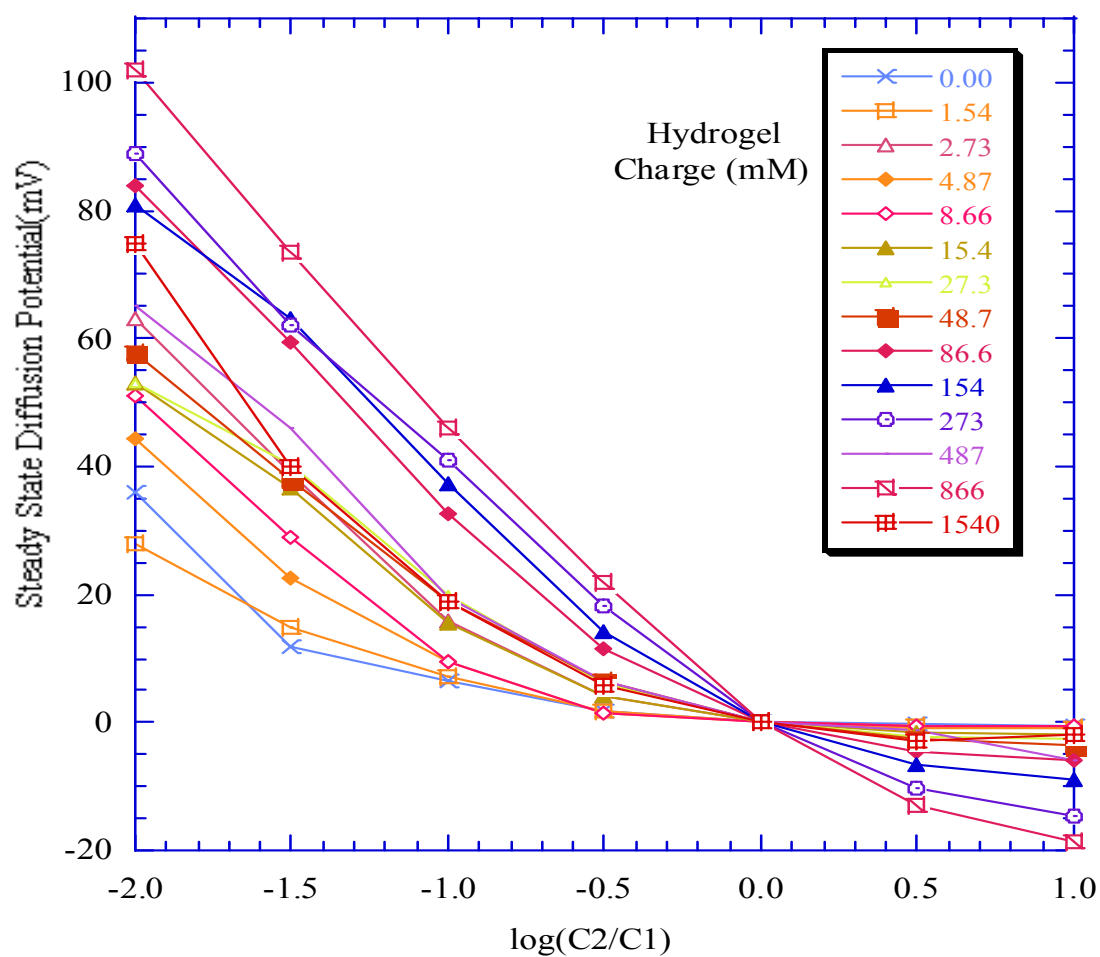


Figure XI. Experimental diffusion potential measurements of PEG-SEMA

collapsed throughout. As the swelling increases, the area of the gel increases, though the charge within it remains constant. Therefore, charge per unit area will decrease, resulting in a decrease in potential. Comparing the swelling and the steady state potential figures, the gels have shown a gradual decrease in the potential.

Diffusion Potentials measured for HEMA-SEMA gels are given in figure XII. The maximum voltage measured was 90mV for the gel with charge 15.4mM. Pure SEMA and the gels with charge lower than that till 86.6mM show a steady decrease in potential, though pure SEMA's maximum potential is not as high as the gel HEMA-SEMA 15.4mM. They follow the same pattern as shown in figure IX. Figure XIII is a plot of the diffusion potential measured across the HEMA-MAAc hydrogels. The maximum potential that was measured for the whole set of gels is 97mV for the gel with the charge 274mM. The gel with the highest charge do not show a high potential difference across it, which can be explained by the swelling of the same gel in the different concentration of NaCl solution. The gel MAAc 1540mM had shown maximum swelling throughout. All the gels with the charge from 274mM and below that show a steady decrease in the potential measured across it.

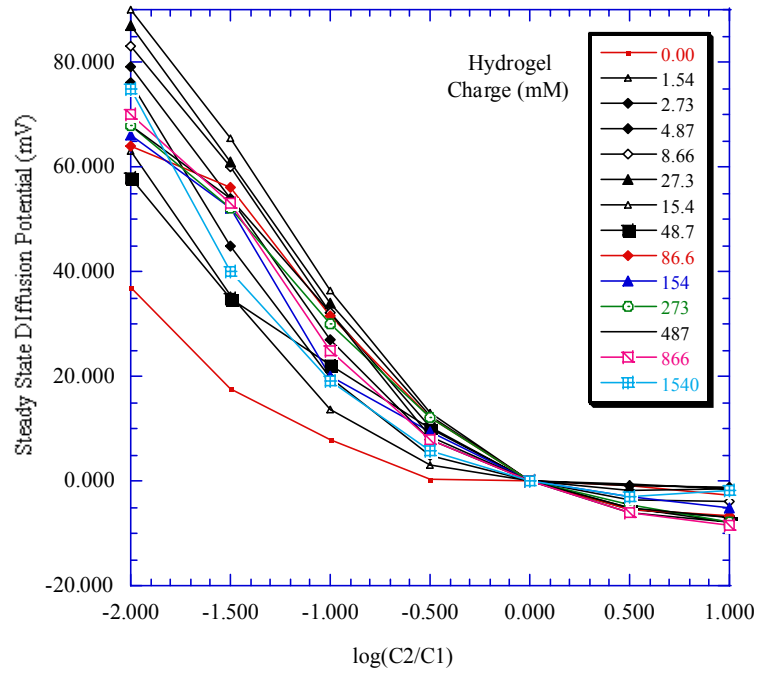


Figure XII. Experimental diffusion potential measurements of HEMA-SEMA

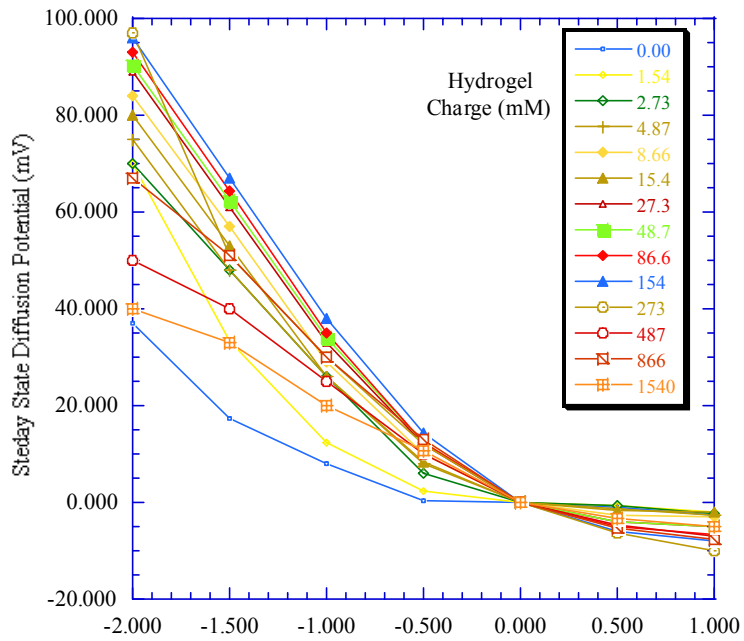


Figure XIII. Experimental diffusion potential measurements of HEMA-MAAc

Part III: ENDOTHELIAL CELLS ON ACIDIC HYDROGELS

Abstract

Acidic hydrogels may and may not support cell adhesion to it, depending on its charge. Endothelial cells were grown on some of the acidic hydrogels and their affinity for these cells was studied. It was found that pure SEMA, pure MAAc, PEG-SEMA 866mM, HEMA-MAAc 866mM and HEMA-SEMA 866mM hydrogels showed good affinity for cell adhesion. At the end of 96 hours almost whole of the gel was covered with the cells and had formed a similar pattern. Pure HEMA and lower charged gels that whose charge were close to 0mM did not let any cells to adhere to them. Very few cells were sticking onto the gels and were not healthy. Image processing techniques were used to calculate the amount of area covered by the cells and compared.

I. Introduction

Growing endothelial cells in laboratories has not been a difficult task for the past few decades. These cells are grown in the research labs for various studies; most common is to monitor their growth factor. These can be grown on different surfaces with different medium to study their characteristics. Counting these cells is difficult because it's hard to differentiate them from the background and the smooth muscle cells that may grow along with them. As mentioned before, using a haemocytometer is the traditional method to count cells. Counting using the meter requires lot of care and is time consuming. Other than the growth rate, the shape of the cells is also important, whose importance has been mentioned in the first chapter. In order to find out the shape and size of the cells, just looking at the cells through a microscope is not enough. Images need to be taken for studying the shape and size of the cells. For this purpose a CCD camera, attached to the

microscope was used. The images were captured using the Zeiss lens and Vision Assistant 6.0 software of National Instruments. A minimum of four images was taken for each dish. The cells were monitored for 96 hrs. Pictures were taken after 4 hrs and then after every 24 hrs. When the images were captured it was made sure that all parts of the dish were covered. More than one image was taken because the result from just one image cannot be taken as the true result.

PPAEC were grown on naked Petri dish to monitor their growth rate. Later, these cells were also grown on the various hydrogels that were made, which were mentioned in the previous chapters. MATLAB algorithms were developed to study the growth rate of the cells. The morphology of the cells on the various hydrogels was observed. The algorithm that was developed is explained in detail in this chapter and few methods are also compared to predict the best method to count the number of cells and the area covered by them.

II. Methods and Materials

A. Endothelial Cell Culture Preparation

Endothelial cells were isolated from porcine pulmonary arteries obtained from a local abattoir. The endothelial cells were then cultivated in an incubator at 37 °C and 5% CO₂. The cell culture media consisted of M199 (GibcoBRL) and 10% fetal bovine serum (Hyclone) supplemented with vitamins (Sigma), glutamine (GibcoBRL), penicillin, streptomycin (GibcoBRL), and amino acids (Sigma). The cells were passaged for further use.

B. Cell Inoculation on Acidic Hydrogel

Acidic hydrogels were made according to the method described in the previous chapter. The gels that were used for the cell culture were those from the glass slides. The gels were cored out using a 9. These gels were sterilized. The gels were allowed to soak in 2mL of M199 overnight, so the gels will absorb the protein necessary for the cells to adhere to them and grow. Cells that were passaged were taken for the experiment. The media in which the cells grow was removed and 10mL of trypsin was added to the dish, so the cells that are sticking onto the dish will come loose. After few the dish was washed thoroughly with the trypsin in it, to remove the endothelial cells sticking onto the dish. Trypsin containing the cells were then poured into a test tube and centrifuged for 6mins, so the cells form a pellet at the bottom of the test tube. Trypsin was removed from the test tube and 10mL of M199 was added to it. The pellet was broken using a pipette and 2mL of this media containing the endothelial cells was added to each gel. The gels were then placed in the incubator at 37 °C and 5% CO₂, the environment suitable for endothelial cell growth. Images were taken after 4hrs and every 24hrs thereafter. Phase inverted microscope was used for this purpose. A CCD camera was interfaced with the computer for capturing digital images. National Instruments software, Vision Assistant 7.0 was used to capture and store the images. Four different parts of the gel was photographed for the study.

C. Staining

The media was removed, the gel was then washed with approximately 1ml of 1x PBS thrice and then 500μL of formalin was added. After 10 minutes formalin was removed

and the gels were washed again with 1xPBS. 500 μ L of Tritun was added and 10 minutes later it was removed. The gels were washed with 1x PBS three times and 500 μ L of BSA (Bovine Serum Albumin) was added. An hour later the gel was washed with 1x PBS thrice and 500 μ L of Texas Red was added to it. The gels were washed with 1x PBS after 60 minutes. Approximately 1mL of 1x PBS was added and the dish was covered with aluminum foil and stored in the incubator for obtaining images.

III. Image Processing Techniques

Images can be a 1-bit, 2-bit, 4-bit until 16-bit image. Grayscale and color images are two basic types of images. A grayscale or a pseudo-color image is composed of one plane, whereas a true-color image contains three planes – the three planes are for the red component, blue component and the green component. A grayscale image is composed of a single plane of pixels. Each pixel is assigned a number between 0 and 255, the grayscale values. All the images that were taken for the research were 8-bit grayscale images. The image shown is of the endothelial cells grown on a naked Petri dish taken after 96 hours. The cells are small and have a cobbled stone structure. Certain part of the image contain white dots, they are the dead cells that are floating in the medium. As can be seen from the image, counting the number of cells is a difficult task. Doing them manually with the knowledge of each cell's boundary will be bound to error and also time consuming. Developing codes to count the cells, calculate the area covered by them, dividing the cells according to their boundary for further study and analysis are easy to achieve with knowledge of image processing. [51-53]

The image obtained needs to be enhanced, that is, it needs to be processed so the resultant image is suitable for the study. The first step is to find the histogram of the image. The histogram of an image is very important as it gives the number of pixels on each gray level. Figure XV gives the histogram of the image given in figure XIV. This is essential for segmentation purposes. The image should be filtered and then segmented to find the area covered by the cells. As can be seen from the image the difference between the cells and the background is very less. Their grey levels are very close which makes the study difficult. [54-56]

Need for a filter

A digital image as mentioned earlier will contain noise that needs to be removed. The noise added to the image will be of different types depending on how the image was obtained. Few cases of noise addition are, during electronic transmission of the digital image, when the image has been scanned from a photograph, wherein the scanner might have scratches, etc and when an image has been captured in the digital format using a CCD camera in which the process itself introduces noise to the acquired image. There are a number of filters that can be used according to the noise and its source. Here, for the images of the endothelial cells that have been obtained through a CCD camera Gaussian filter can be used. This filter may not be very effective in removing the noise completely; a couple of methods have been used to predict the best method or steps that are needed.

For the image given in figure XIV, Gaussian low pass filter has been used to the Fourier Transform of the image. Figure XVI is the image after low pass Gaussian filter has been used on the image in figure XIV, to filter the noise. This process has darkened the foreground and made the image easier to process, but still the output is not perfect for

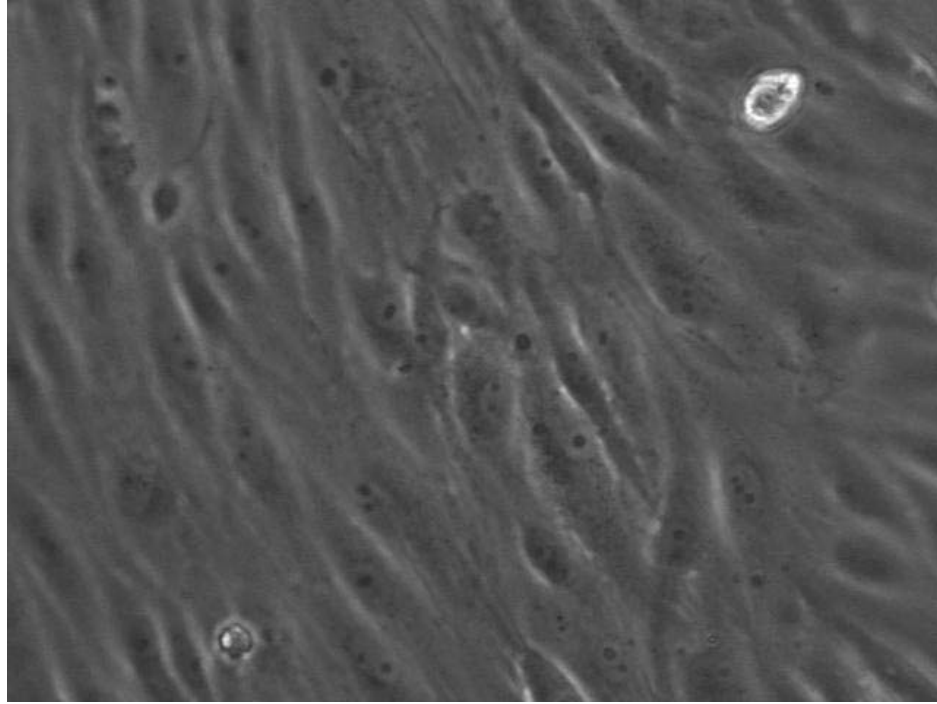


Figure XIV. Endothelial cells grown on PEG-SEMA 866nm. Image taken after 96 hours.

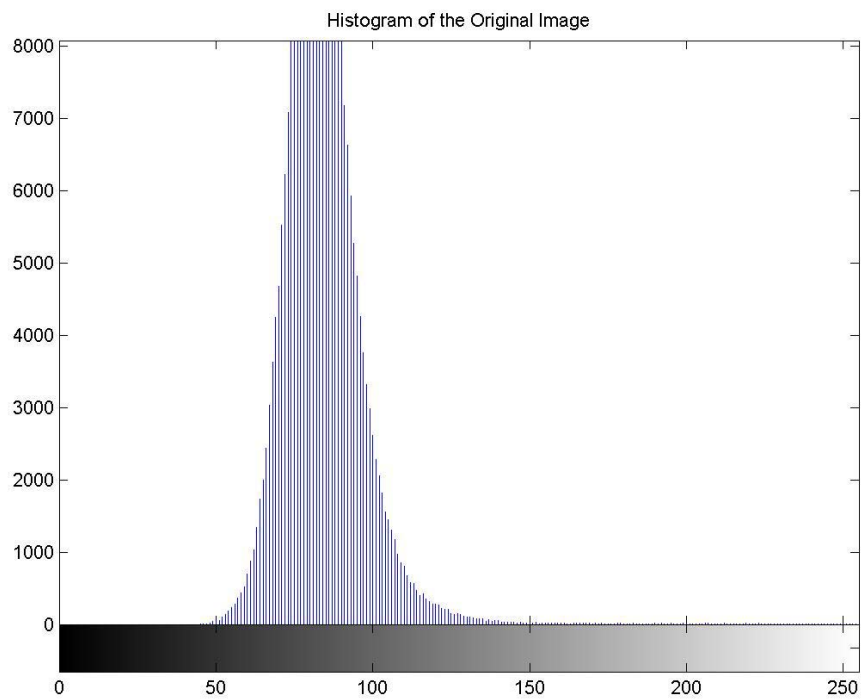


Figure XV. Histogram of the image given in figure XIV.

Image with Gaussian Filter

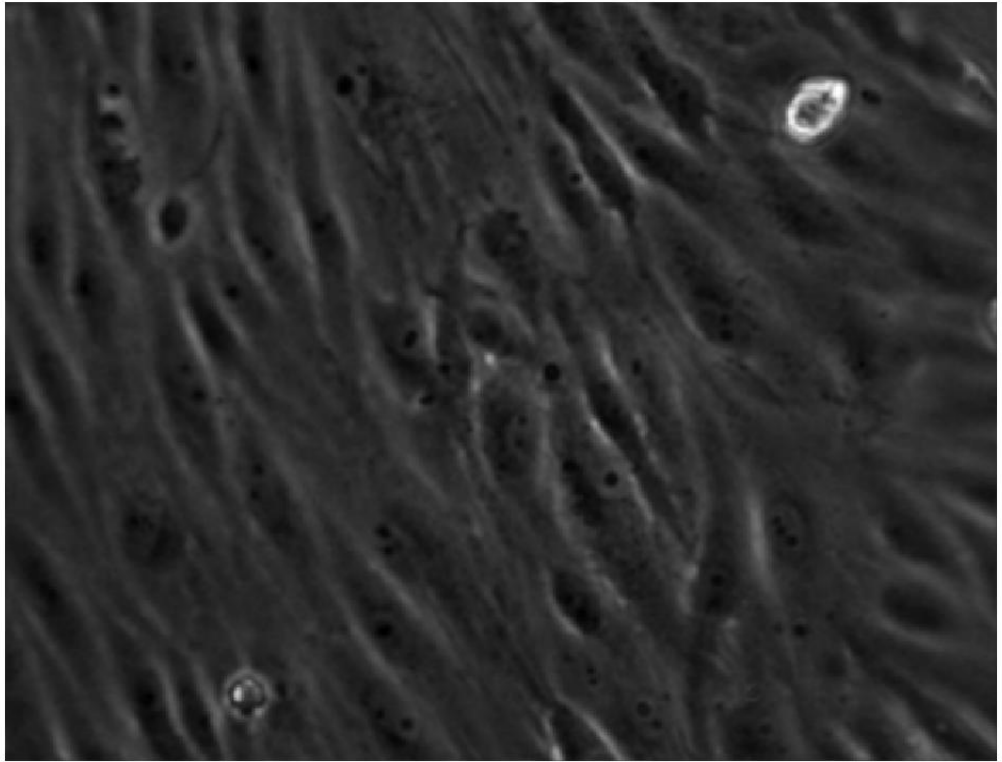


Figure XVI. Image after filtering using low pass Gaussian filter.

calculating the area covered without any errors. Therefore image enhancement was done on the image given in figure XIV.[57]

Enhancing an image is process by which the noise in the image is removed, the areas of interest are made brighter or in other words, the brightness has been adjusted to make the image clearer. Figure XVII is enhanced image of figure XIV. This image is clearer than the original and better than the image filtered using low pass Gaussian filter. In the analysis of the cells in images it is essential to distinguish between the objects of interest and "the rest" or the background. The techniques that are used to find the objects of interest are referred to as segmentation techniques - segmenting the foreground from background. Most common techniques for segmentation are thresholding and edge finding. As there is no universally applicable segmentation technique that will work for all images and no segmentation technique is perfect, the best that can be built is given in this chapter. [58]

Image after thresholding is a binary image and there is more than one way to threshold an image. Few of them are fixed threshold, triangle algorithm, isodata algorithm and histogram based threshold. Histogram based thresholding is used for this algorithm. This method was chosen as all the images contain the same grey level and the cells and the background have almost the same grey level values. Before the image was converted into a binary one, morphological operations such as opening and subtracting the image were done on the image to remove small lines and dots that are not part of the required data and also to fill few places where there are holes. The segmented image is given in figure XVIII. It can be seen that the area where the cells are in greyish or white.

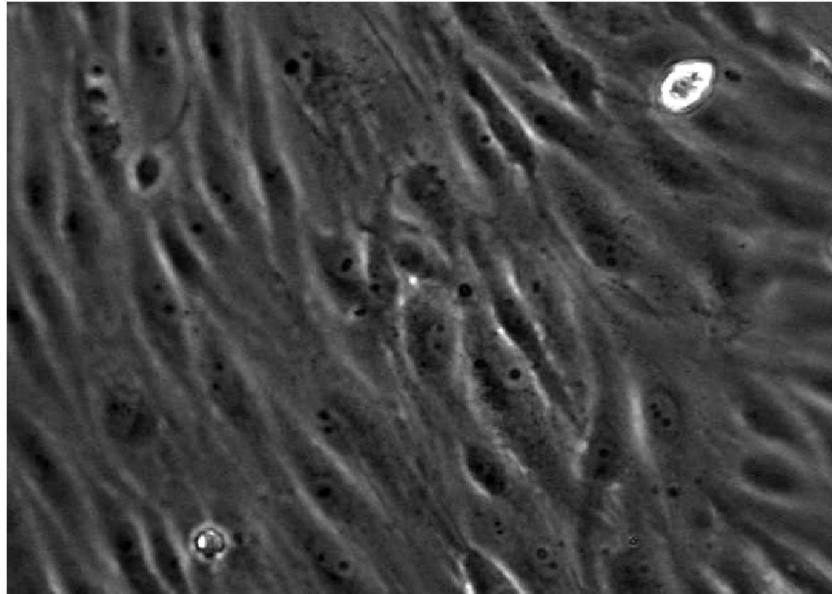


Figure XVII. Enhanced image of figure XIV.

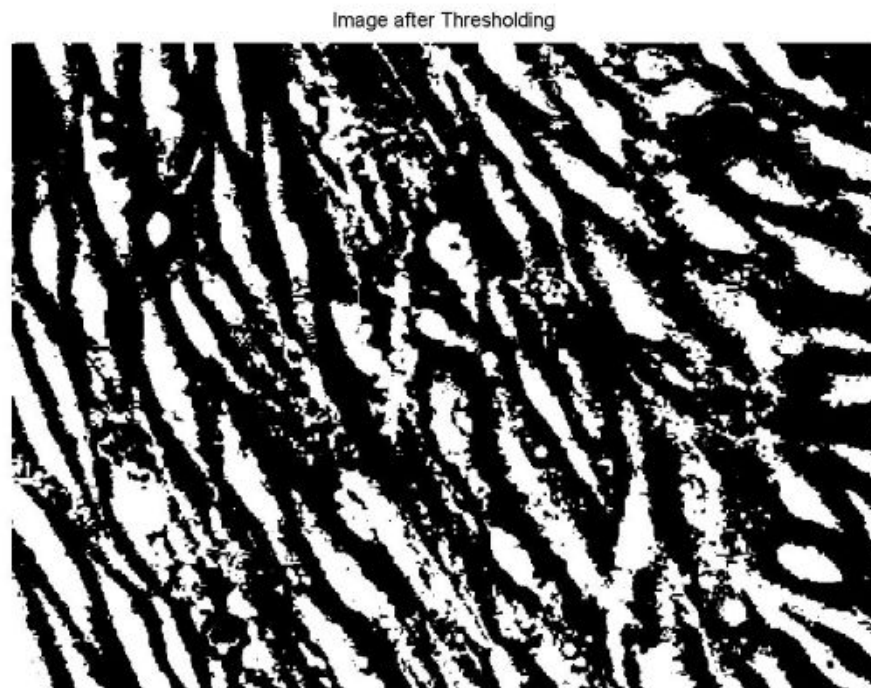


Figure XVIII. Segmented Image.

The numbers of pixels that are above certain grey level were counted and the percentage of these levels in the image was calculated. As there were four images taken at different parts of the dish, an average of these four images was taken and a graph was drawn to show the growth rate. [59]

For the same image given in figure XIV, a LabVIEW program was created to calculate the number of cells in the image. The steps used were similar to the above given steps. To the original image a smoothing filter was used. The image obtained after filtering using the smoothing filter is given in figure XIX. The image that was filtered was further filtered using Convolution highlighting filter, the image obtained is given in figure XX. The image that was filtered twice was converted to a binary image by giving a threshold. This process is also called as segmentation, as mentioned before. The thresholded image is given in figure XXI. [59] The image given in figure XXI was inverted, that is the pixels in white were made black and vice versa.

Morphological operations such as closing and filling holes were performed. 3*3 kernels were used for the purpose. By these two steps, tiny holes were filled and the inner contour of particles was smoothened out. Particle filter function was used to remove the blocks that are less than a specified area, as they are unwanted noise. Image after the particle filter function was applied is given in figure XXII. The number of cells that are in the image were then counted by specifying the minimum and maximum area of the particle to be counted that is the cell area in this case. The number of cells that were present in the image was 1058. The VI used is given in appendix C.

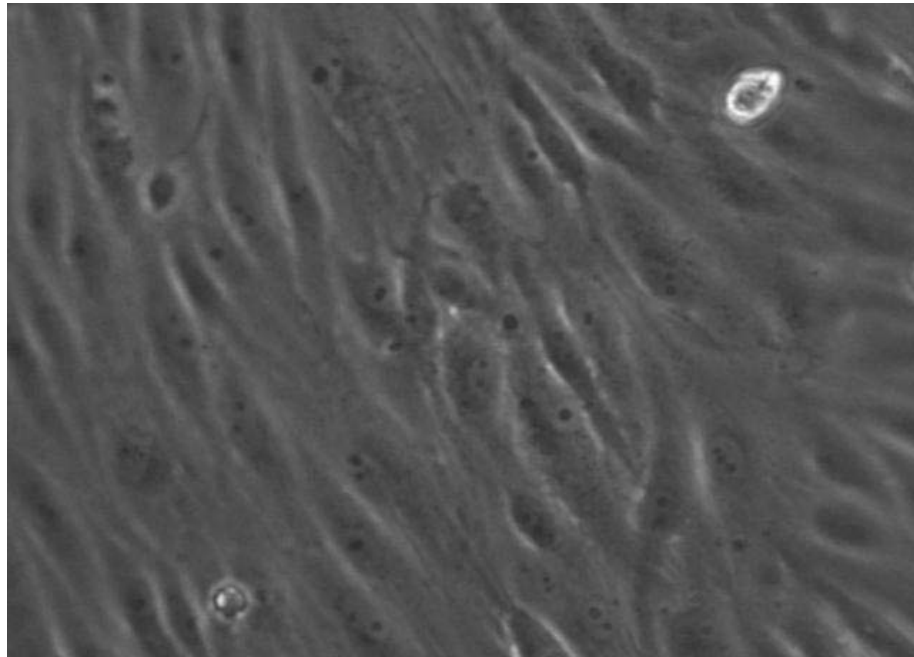


Figure XIX. Gaussian Smoothing Filter applied to the image in figure XIV.

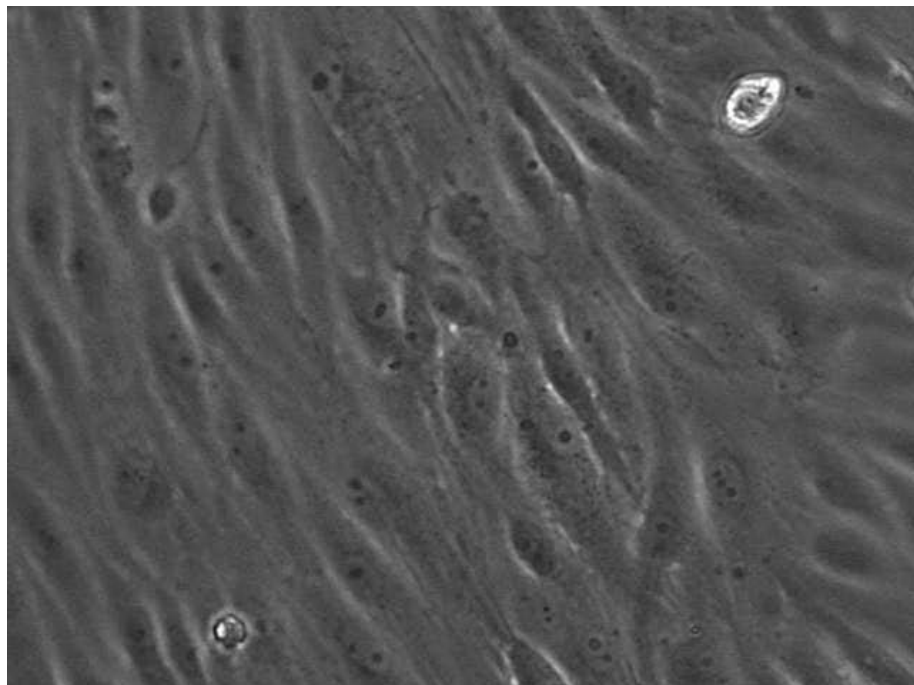


Figure XX. Convolution Highlighted Filter applied on image given in figure XIX.



Figure XXI. Thresholded image of figure XIX.

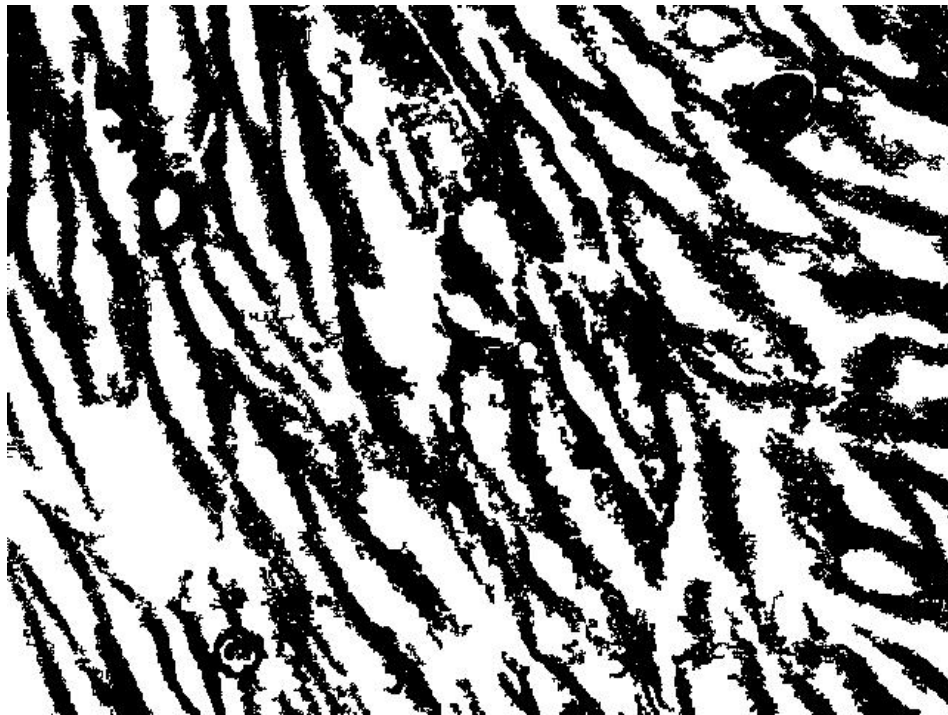


Figure XXII. Morphological operations and particle filter applied to the image given in figure XXI.

IV. Results and Discussion

From figure XXIII it can be seen that the hydrogel with the maximum charge has more affinity for endothelial cells, than the hydrogels with the least charge. After 4 hours of seeding the percentage of cells that grew on the PEG-SEMA 1540mM gel was close to 2%. In 24 hours the percentage rose by 10 times. The growth was steady from then and by the end of 96 hours all parts of the gel was completely covered by endothelial grown in a particular pattern. The cells started to form a pattern after 48 hours. The cells grow in thin, long tubular forms. There is no gap between the adjacent cells. Similar growth pattern is found on all the gels. The next highly charged gel, PEG-SEMA 866mM showed similar affinity for the endothelial cells. Initially the growth rate was high compared to the pure SEMA; however at the end of 96 hours the area covered was a few percentages lower than the pure SEMA. The amount of cells that grew on the PEG-SEMA 154mM was comparatively really less. The maximum area covered was 70% at the end of 96 hours, though the growth rate till 72 hours was very similar to the higher charged gels. PEG-SEMA 1.54mM showed least affinity for the growth of cells on it. The number of cells that grew on it or the area covered in percentage remained very close to zero, indicating that this gel is not suitable for growing endothelial cells on them.

The growth pattern of endothelial cells on the HEMA-SEMA hydrogels is given in the graph XXIV. The growth pattern is similar to the one seen in graph XXIII. Endothelial cells are able to stick onto HEMA-SEMA 866mM hydrogel, similar to pure SEMA. The initial growth rate was the highest for this gel, but at the end of 96hours, the

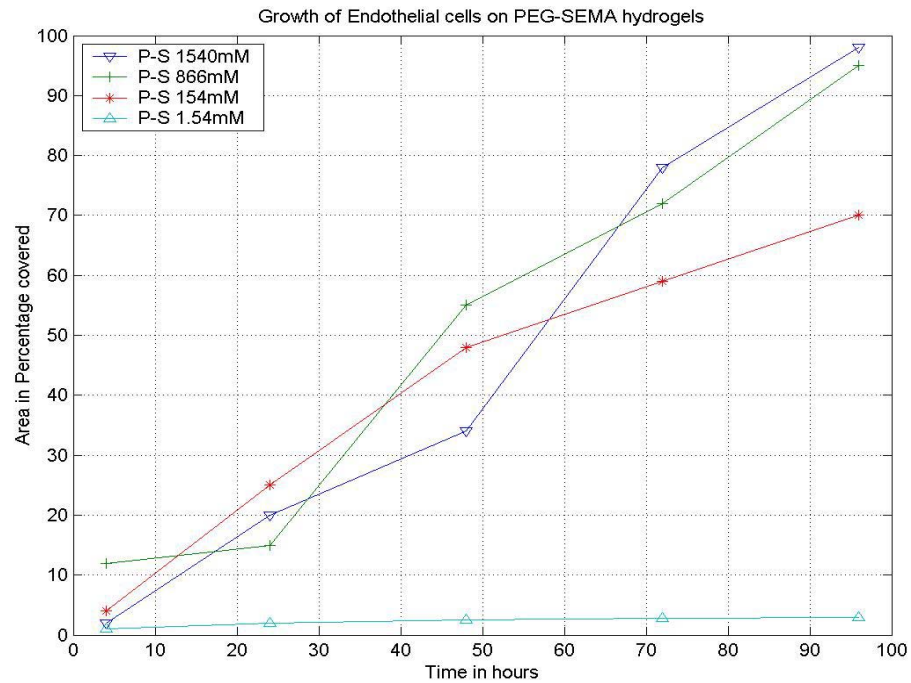
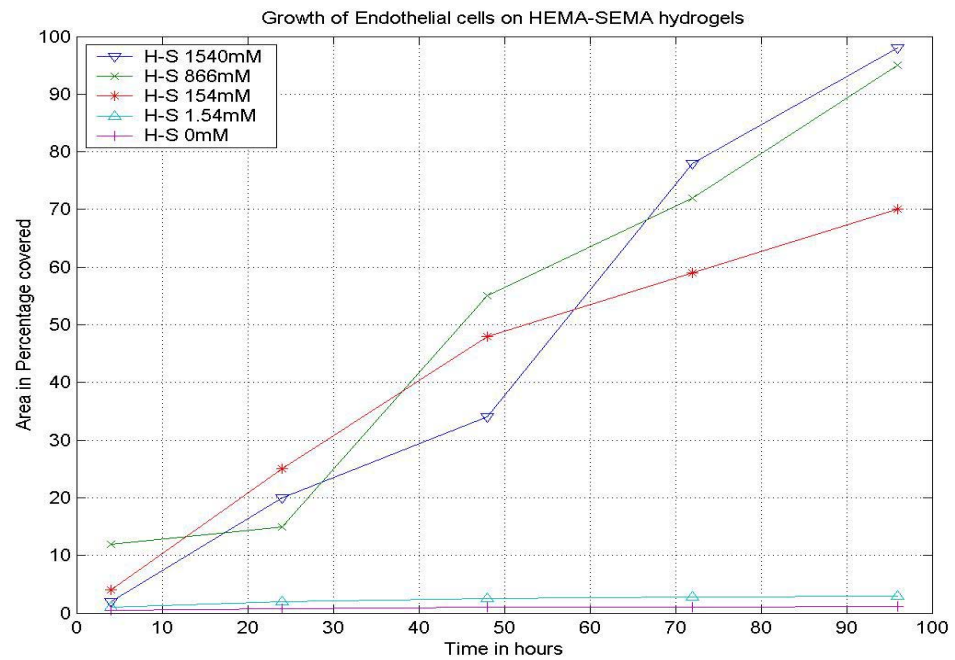


Figure XXIII. Growth rate of endothelial cells on PEG-SEMA hydrogels with charges 1540mM, 866mm, 154mM and 1.54mM.

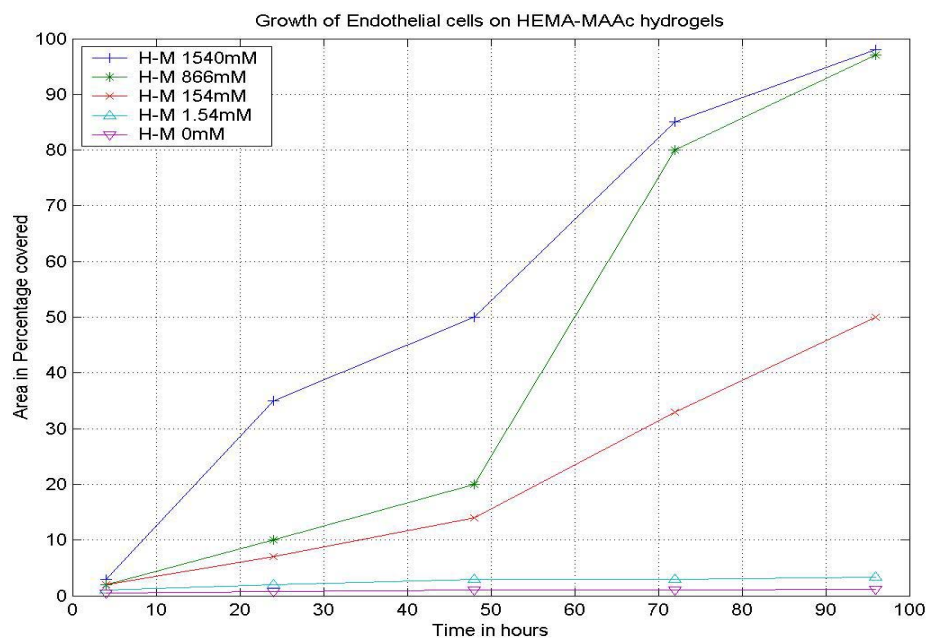


Graph XXIV. Growth rate of endothelial cells on HEMA-SEMA hydrogels with charges 1540mM, 866mm, 154mM, 1.54mM and 0mM.

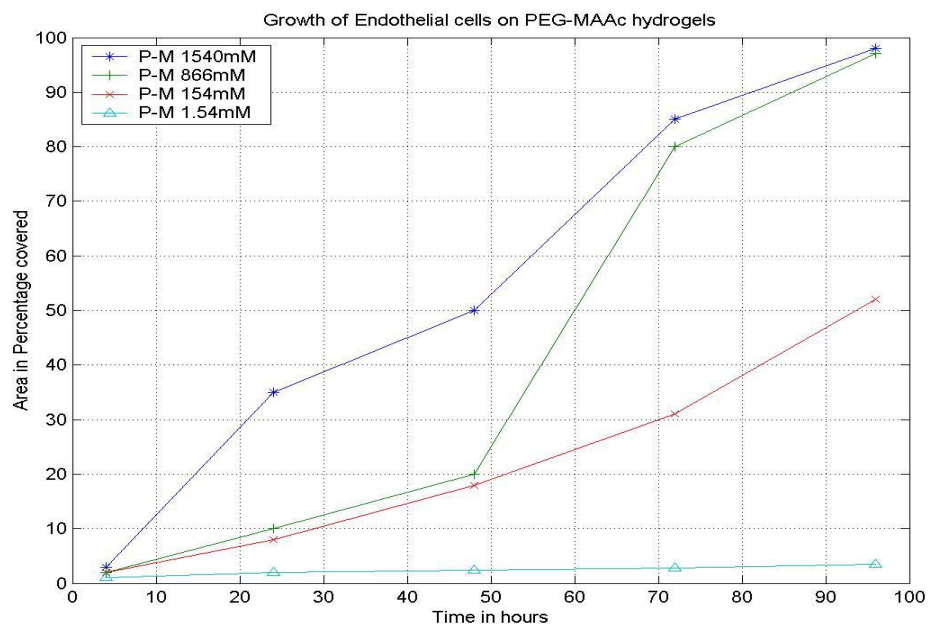
area covered by the cells was slightly less than pure SEMA. HEMA-SEMA 154mM shows a moderate affinity for cell adhesion. Though, initially the cell adhesion was very much like the gels with higher charge, after 48 hours the cells did not grow at the same rate. The maximum area covered was just close to 70%. HEMA-SEMA 1.54mM and pure HEMA shows the least affinity for cell growth. This is in acceptance with the earlier work done by by P.B.van Wachem, A.H. Hogt, T. Beugeling, J. Feigen, A. Bantjes, J.P. Detmers and W.G. van Aken in 1987 and also by Burmeister JS, McKinney VZ, Reichert WM and Truskey GA in 1999.

Graph XXV gives the growth pattern on HEMA-MAAc hydrogels. Pure MAAc shows the maximum adhesion or affinity for the growth of the cells, followed by HEMA-MAAc 866mM. These gels show similar characteristics like the other higher charged gels. The shape of the gels on these gels was also similar to the rest. HEMA-MAAc 154mM shows very little affinity for the cells. The maximum growth on this gel is lower than that on HEMA-SEMA 154mM and PEG-SEMA 154mM. As seen before, least adhesion was shown on the lower charged gels. HEMA-MAAc 1.54mM and pure HEMA has no cells growing on them. The few cells that grew on the gels with very low charge did not have a specific shape. There were one or two cells spread out on the surface and at few places they were aligned in a manner similar to that seen on a naked petri dish.

The hydrogel, PEG-MAAc 1.54mM in graph XXVI also shows the same characteristics of the other gels with charge 1.54mM and the pure HEMA. The number of cells that grew on them was almost close to zero and the number remained close to it even at the end of 96 hours.



Graph XXV. Growth rate of endothelial cells on HEMA-MAAc hydrogels with charges 1540mM, 866mM, 154mM, 1.54mM and 0mM.



Graph XXVI. Growth rate of endothelial cells on PEG-MAAc hydrogels with charges 1540mM and 1.540mM.

The results can be summarized as, the gels with high charge in them like the pure SEMA, pure MAAC, HEMA-SEMA 866mM, HEMA-MAAC 866mM, PEG-SEMA 866mM and PEG-MAAC 866mM has maximum affinity for the endothelial cells to grow on them. The gels with the higher HEMA or PEG content show the least affinity for the growth of cells on them. The pattern of growth is the same on the gels. The cells grow in a long spindle shape without any gap between the adjacent ones and in a single layer. After 48 hours the cells begin to show a pattern in their growth.

Part IV: CONCLUSIONS

I. Introduction

The aim of this Masters thesis was to synthesize and find the mechanical properties of the acidic hydrogels, and also to grow endothelial cells on them and come up with a robust algorithm to find the area covered by the cells and to count the number of cells. Four different acidic hydrogels were made and their swelling and diffusion properties were studied, which are given in part 2. A robust algorithm to count the number of cells and also to find the area covered by the cells grown on the acidic hydrogels and also on naked Petri dish was developed using MATLAB. Few methods were developed to find the area covered and compared to find the best method. Part 1 of thesis gives the basics of hydrogels, its properties and applications. Functions and importance of the endothelial cells in the human body are also discussed in the chapter. The work done so far on acidic hydrogels based on HEMA, PEG, SEMA and MAAC and were reviewed and mentioned. The same study was done on endothelial cells grown on acidic hydrogels. This chapter gives a good introduction to many of the work done by J.Ricka and T.Tanaka on the swelling theory, effect of cross-linker on HEMA based gels by Vlastimil Kudela, Jirf Vacik and Jindrich Kopecek and many more. It can be seen that many work has been done on HEMA based hydrogels because of its properties that makes it very important and interesting. Extensive work is being done on various types of endothelial cells for developing artificial organs and various applications. For all the work on endothelial cells counting the number of cells and finding their growth rate is the first step and the most important step. The research done on these areas has also been mentioned in this chapter.

II. Part Conclusions

Part 2 of the thesis deals with the fabrication of hydrogels, their swelling theory, diffusion principle, swelling and diffusion potential results. Introduction part of chapter 2 explains the various methods by which hydrogels can be made and swelling/deswelling phenomena. Helmholtz free energy model for the swelling of the gels is given in detail. Diffusion principle and the potentials that contribute to the diffusion potential of the acidic hydrogels are also explained in depth. The procedure that was followed in making of the four different acidic hydrogels are given and the experimental methods to find the swelling and diffusion potentials. Figure I and II gives the swelling of HEMA-SEMA in DI water and in various concentrations of NaCl solutions respectively. HEMA-SEMA hydrogels showed continuous swelling in DI water, Most of the gels showed swelling/deswelling when washed with NaCl solutions and all the gels collapsed when they were washed with 1540mM of sodium chloride solution.

Figure III and IV gives the swelling of HEMA-MAAc in DI water and NaCl solutions with different ionic strengths. On the contrary to HEMA-SEMA gels, HEMA-MAAc gels did not show any swelling or deswelling, they remained in the collapsed state except for pure MAAc in DI water. In sodium chloride solutions, only pure MAAc shows swelling transitions throughout, other gels showed swelling at some point and most remained inert to the solution. It can be concluded that high amount of HEMA in any gel is more inert to any change in the surroundings. Swelling of PEG-MAAc in DI water and NaCl solutions are given in the figures V and VI. The experimental results for PEG-SEMA are given in figures VII and VIII. Figures show that PEG-MAAc gels have remained in the collapsed state except for pure MAAc. Most of PEG-SEMA hydrogels

also remained inert to any bath surroundings. PEG-SEMA 866mM has swollen to a small extent, but the difference in size between this and pure SEMA is vast. It can be inferred that high content of PEG in gels, makes the gel inert, just like high content of HEMA in a gel. Presence of MAAC in gels also does not support swelling except for presence of SEMA. All the gels that contain a lot of SEMA showed swelling or deswelling when washed with DI water and sodium chloride solutions.

Diffusion measurements were done on all the gels, and the results were plotted and checked with the theoretical graph that was obtained using C. Theoretical graph obtained for the basic and acidic hydrogels is given in figure IX. The lines in blue are the values for acidic hydrogels. The figures X, XI, XII and XIII are the plots of the diffusion potentials obtained for PEG-MAAC, PEG-SEMA, HEMA-SEMA and HEMA-MAAC gels. The results obtained are similar to the theoretical values generated. The results obtained have been discussed in detail in the second chapter, The aim to study the mechanical properties of the acidic hydrogels was achieved.

Part 3 of the thesis is completely dedicated to image processing. The experimental procedure followed for the endothelial cell culture preparation and inoculation on the hydrogels are given in detail. A robust algorithm was developed to count the number of cells and also to calculate the area covered by the cells on the gels. The algorithm was explained step by step and few methods were compared. Figure XIV is the original image taken 96 hours the cells were inoculated on PEG-SEMA 866mM hydrogel. Histogram of the image is given in figure XV. The image was filtered using a low pass Gaussian filter; the filtered image is given in figure XVI. Though this image is clear and better than the original image, it is still not a good one to calculate the area covered. Better techniques of

enhancement and morphological operations can be applied to improve the image even further. The figure XVII is the enhanced image of the original image given in figure XIV. The final step is to separate the background and the area of interest. This was done using a segmentation technique – thresholding the image. Figure XVIII gives the binary image that was obtained after segmentation.

Higher charged and lower charged gel were selected for inoculation with endothelial cells. A number of images were taken at regular intervals. Using the algorithm developed the area covered was calculated and they are given in the figures XXIII, XXIV, XXV and XXVI. Figure XXIII gives the result for PEG-SEMA gels. Figure XXIV gives the results for the HEMA-SEMA hydrogels. Results of HEMA-MAAc gels are in figure XXV. Finally, the figure XXVI gives the results obtained for PEG-MAAc gels. The results obtained have been discussed in the chapter. It can be concluded that gels with high SEMA and MAAc acid content can be used for inoculating endothelial cells on them for various applications. Gels with a lot of HEMA and PEG in them cannot be used for growing these cells on them, but these gels have shown very good resistance to the surroundings. They are very inert and are biodegradable too. They can be used where gels with high resistance are needed. The algorithm that was developed has been given in the appendix at the end of the chapter. The main program and the subroutine that is built have been given.

III. Thesis Conclusions

The master's thesis aim was to investigate the thermodynamic characteristics of acidic hydrogels, to find the gels affinity for the growth of endothelial cells on them and also to develop a robust algorithm to calculate the area covered by the cells. PEG-SEMA, HEMA-SEMA, PEG-MAAc and HEMA-MAAc hydrogels were made by free radical polymerization. Thirteen different charged hydrogels were made in each type. Thermodynamic characteristics of these gels, such as swelling properties and diffusion potential characteristics were found experimentally and compared to the theoretical values generated. The program to generate the theoretical graph was coded in C. The results obtained were very close to the expected values. Theory of the hydrogel swelling, diffusion characteristics and a detailed description of the various methods of preparation of hydrogels has been given in the part 2 of the thesis. A step by step description of the procedure followed in the making of the hydrogels has been given. Cell culture on the acidic hydrogels was done and it was very successful in terms of the results obtained. The results obtained were consistent with previous work. MATLAB was used for developing the code to calculate the area. The software was extremely useful for the purpose. Image processing fundamentals were clearly understood and a detailed explanation of the techniques used has been given and few methods have been compared.

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APPENDIX

Appendix A

Acidic Hydrogel

The set of particle numbers in the hydrogel phase are:

$$\{N_i\} = \{N_{A^-}, N_{AH}, N_{H^+}, N_{OH^-}, N_{Na^+}, N_{Cl^-}\}$$

and corresponding set of concentrations

$$\{C_i\} = \{C_{A^-}, C_{AH}, C_{H^+}, C_{OH^-}, C_{Na^+}, C_{Cl^-}\}$$

The set of particles in the bath phase are:

$$\{N_{ibath}\} = \{N_{H^+bath}, N_{OH^-bath}, N_{Na^+bath}, N_{Cl^-bath}\}$$

$$\{C_{ibath}\} = \{C_{H^+bath}, C_{OH^-bath}, C_{Na^+bath}, C_{Cl^-bath}\}$$

Electro neutrality in the bath can be written as

$$C_{H^+bath} + C_{Na^+bath} - C_{Cl^-bath} - C_{OH^-bath} = 0$$

or

$$C_{H^+bath} + C_{Na^+bath} - C_{Cl^-bath} - \frac{K_w}{C_{H^+bath}} = 0$$

Charge electro neutrality for the acidic hydrogel phase

$$C_{H^+} - C_{A^-} + C_{Na^+} - C_{Cl^-} - C_{OH^-} = 0$$

Acid dissociation equilibrium is equivalent to

$$K_a = \frac{C_{A^-} C_{H^+}}{C_{AH}},$$

where K_a is the acid dissociation constant.

The total concentration of acidic monomers is the sum of undissociated, C_{AH} , and dissociated, C_{A^-} , groups

$$C_{Ao} = C_{AH} + C_{A^-}$$

Writing the dissociated acidic monomer concentration using the dissociation equilibrium equation and the definition of the total acidic monomer concentration gives

$$K_a = \frac{C_{A^-} C_{H^+}}{C_{Ao} - C_{A^-}}$$

$$K_a (C_{Ao} - C_{A^-}) = C_{A^-} C_{H^+}$$

$$K_a C_{Ao} = C_{A^-} (C_{H^+} + K_a)$$

$$C_{A^-} = \frac{C_{Ao} K_a}{(C_{H^+} + K_a)}$$

Using the Donnan relations we can write the internal chloride and hydroxide ion concentrations in terms of the know bath concentrations

$$\frac{C_{Na^+}}{C_{Na^+ bath}} = \frac{C_{H^+}}{C_{H^+ bath}} \quad \text{or} \quad C_{Na^+} = C_{Na^+ bath} \frac{C_{H^+}}{C_{H^+ bath}}$$

Similarly,

$$\frac{C_{Cl^-}}{C_{Cl^- bath}} = \frac{C_{H^+ bath}}{C_{H^+}} \quad \text{or} \quad C_{Cl^-} = C_{Cl^- bath} \frac{C_{H^+ bath}}{C_{H^+}}$$

and

$$\frac{C_{OH^-}}{C_{OH^-bath}} = \frac{C_{H^+bath}}{C_{H^+}} \quad \text{or} \quad C_{OH^-} = \frac{K_w}{C_{H^+}}$$

Combining the above relations gives

$$C_{H^+} - C_{A^-} + C_{Na^+} - C_{Cl^-} - C_{OH^-} = 0$$

$$C_{H^+} - \frac{C_{Ao}K_a}{(C_{H^+} + K_a)} + C_{Na^+bath} \frac{C_{H^+}}{C_{H^+bath}} - C_{Cl^-bath} \frac{C_{H^+bath}}{C_{H^+}} - \frac{K_w}{C_{H^+}} = 0$$

$$(C_{H^+})^2 - \frac{C_{Ao}K_a}{(C_{H^+} + K_a)} C_{H^+} + C_{Na^+bath} \frac{(C_{H^+})^2}{C_{H^+bath}} - C_{Cl^-bath} C_{H^+bath} - K_w = 0$$

$$\left\{ 1 + \frac{C_{Na^+bath}}{C_{H^+bath}} \right\} (C_{H^+})^2 - \frac{C_{Ao}K_a}{(C_{H^+} + K_a)} C_{H^+} - C_{Cl^-bath} C_{H^+bath} - K_w = 0$$

$$\left\{ 1 + \frac{C_{Na^+bath}}{C_{H^+bath}} \right\} (C_{H^+})^2 - \frac{C_{Ao}K_a}{(C_{H^+} + K_a)} C_{H^+} - C_{H^+bath} (C_{Cl^-bath} + C_{OH^-bath}) = 0$$

$$\left\{ 1 + \frac{C_{Na^+bath}}{C_{H^+bath}} \right\} (C_{H^+})^2 - \frac{C_{Ao}K_a}{(C_{H^+} + K_a)} C_{H^+} - (C_{H^+bath})^2 \left(1 + \frac{C_{Na^+bath}}{C_{H^+bath}} \right) = 0$$

$$(C_{H^+})^2 - \frac{1}{(C_{H^+} + K_a)} \left(\frac{C_{Ao} K_a}{1 + C_{Na^+ bath} / C_{H^+ bath}} \right) C_{H^+} - (C_{H^+ bath})^2 = 0$$

$$(C_{H^+})^3 + K_a (C_{H^+})^2 - \left\{ \frac{C_{Ao} K_a}{(1 + C_{Na^+ bath} / C_{H^+ bath})} + (C_{H^+ bath})^2 \right\} C_{H^+} - K_a (C_{H^+ bath})^2 = 0$$

For numerical root finding the first derivative is needed, i.e.,

$$3(C_{H^+})^2 + 2K_a C_{H^+} - \left\{ \frac{C_{Ao} K_a}{(1 + C_{Na^+ bath} / C_{H^+ bath})} + (C_{H^+ bath})^2 \right\} = 0$$

If only HCl is added without any NaCl the above expression reduces to

$$(C_{H^+})^3 + K_a (C_{H^+})^2 - \{C_{Ao} K_a + (C_{H^+ bath})^2\} C_{H^+} - K_a (C_{H^+ bath})^2 = 0$$

If the acid remains completely dissociated over the range of bath hydrogen ion concentrations, i.e. $K_a \rightarrow \infty$ we have

$$(C_{H^+})^2 - \left(\frac{C_{Ao}}{1 + C_{Na^+ bath} / C_{H^+ bath}} \right) C_{H^+} - (C_{H^+ bath})^2 = 0$$

$$\left(\frac{C_{H^+}}{C_{H^+ bath}} \right)^2 - \left(\frac{C_{Ao}}{C_{H^+ bath} + C_{Na^+ bath}} \right) \frac{C_{H^+}}{C_{H^+ bath}} - 1 = 0$$

$$\frac{C_{H^+}}{C_{H^+ bath}} = \frac{\frac{C_{Ao}}{C_{H^+ bath} + C_{Na^+ bath}} \pm \sqrt{\left(\frac{C_{Ao}}{C_{H^+ bath} + C_{Na^+ bath}} \right)^2 + 4}}{2}$$

Taking the positive root

$$\frac{C_{H^+}}{C_{H^+ bath}} = \sqrt{\left(\frac{C_{Ao}}{2(C_{H^+ bath} + C_{Na^+ bath})}\right)^2 + 1} + \frac{C_{Ao}}{2(C_{H^+ bath} + C_{Na^+ bath})}$$

Let

$$C' = (C_{H^+ bath} + C_{Na^+ bath}) = (C_{Cl^- bath} + C_{OH^- bath})$$

Then

$$\frac{C_{H^+}}{C_{H^+ bath}} = \sqrt{1 + \left(\frac{C_A}{2C'}\right)^2} + \frac{C_{Ao}}{2C'}$$

$$\frac{C_{H^+}}{C_{H^+ bath}} = \sqrt{1 + \left(\frac{\rho / F}{2C'}\right)^2} + \frac{\rho / F}{2C'}$$

The Donnan potential follows from the relation

$$\Phi_D = \frac{RT}{zF} \ln \left(\frac{C_{H^+}}{C_{H^+ bath}} \right)$$

$$\Phi_D = \frac{RT}{zF} \ln \left(\sqrt{1 + \left(\frac{\rho / F}{2C'}\right)^2} + \frac{\rho / F}{2C'} \right)$$

Appendix B

Program to calculate area of the cells grown on acidic hydrogels

```
z = 1;
I = imread('P-S 866 4hrs-1.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

x = 1; z = z+1;
I = imread('P-S 866 4hrs-2.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 4hrs-3.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 4hrs-4.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

f(x) = (a(1)+a(2)+a(3)+a(4))/4;

x = x+1; z = 1;
I = imread('P-S 866 24hrs-1.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 24hrs-2.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 24hrs-3.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 24hrs-4.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);
```

```

z = z+1;
I = imread('P-S 866 24hrs-5.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

f(x) = (a(1)+a(2)+a(3)+a(4)+a(5))/5;

x = x+1; z = 1;
I = imread('P-S 866 48hrs-1.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 48hrs-2.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 48hrs-3.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 48hrs-4.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 48hrs-5.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

f(x) = (a(1)+a(2)+a(3)+a(4)+a(5))/5;

x = x+1; z = 1;
I = imread('P-S 866 96hrs-1.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 96hrs-2.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

```

```

z = z+1;
I = imread('P-S 866 96hrs-3.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 96hrs-4.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

z = z+1;
I = imread('P-S 866 96hrs-5.jpg');
[41] = size(I);
a(z) = subpegsema(I, r, m);

f(x) = (a(1)+a(2)+a(3)+a(4)+a(5))/5;

```

%To plot the % of area covered by the cells with respect to time.

```

t = [4 24 48 96];
figure(6),plot(t,f);grid on
title('P-S 866mM');
xlabel('Time in hours');
ylabel('Area in Percentage covered');

```

Subroutine for the program to calculate the area covered by the cells on acidic hydrogels

```

function az = subpegsema(I, r, m)
figure(1),imshow(I);
title('Original Image, P-S 866mM');
J = imadjust(I,[0.2 0.7],[]);
figure(2), imshow(J)
figure(3),imhist(J);
title('Histogram of the Original Image');
B = ones(15);
TI = imopen(I,B);
FI = imsubtract(J,TI);

% To threshold the image to get a binary image.
for i = 1:480
    for j = 1:640
        if FI(i,j) >= 57
            FI(i,j) = 0;
        else

```

```

        FI(i,j) = 256;
    end
end
end

% To calculate the number of pixels of a particular value.
c = 0;
for i = 1:480
    for j = 1:640
        if FI(i,j) == 256
            c = c+1;
        end
    end
end
end

figure(4), imshow(FI);
title('Image after Thresholding');

b = r*m;
az = c*100/b;

```

Program that uses Gaussian Low Pass filter to enhance the image

% Gaussian Filter used on Cell grown on gels to find the area covered by the cells.

```

I = imread('P-S 866 96hrs-2.jpg');
[m n] = size(I);
figure(1), imshow(I)
title('Original Image')
figure(2), imhist(I);
title('Histogram of the Original Image');

h = low_pass_filter('gaussian',m,n,1);
[a b] = size(h);
fh = fftshift(fft2(h));
fi = fftshift(fft2(I));
g = fi.*fh;
gh = abs(ifft2(ifftshift(g)));
figure(3), imshow((gh),[])
title('Image with Gaussian Filter');

for i = 1:m
    for j = 1:n

```

```

        if I(i,j) <= 170
            I(i,j) = 0;
        end
    end
end
end

% To calculate the percentage of area covered by the cell.
a = 0;
for i = 1:m
    for j = 1:n
        if I(i,j) < 170
            a = a+1;
        end
    end
end
end

v = m*n;
az = a*100/v;

figure(4),imshow(I);
title('Image after Thresholding');

```

Subroutines for Gaussian Low Pass Filter Design:

```

function [U, V] = freq_mat(M, N)
%Computes meshgrid frequency matrices.

u = 0:(M - 1);
v = 0:(N - 1);

% Compute the indices for use in meshgrid.
idx = find(u > M/2);
u(idx) = u(idx) - M;
idy = find(v > N/2);
v(idy) = v(idy) - N;

% Compute the meshgrid arrays.
[V, U] = meshgrid(v, u);

function [H, D] = low_pass_filter(type, M, N, D0, n)
%LOW_PASS_FILTER Computes frequency domain lowpass filters.
% H = LOW_PASS_FILTER(TYPE, M, N, D0, n) creates the transfer function of
% a lowpass filter, H, of the specified TYPE and size (M-by-N). To

```

```

% view the filter as an image or mesh plot, it should be centered
% using H = fftshift(H).
%
% Valid values for TYPE, D0, and n are:
%
% 'ideal' Ideal lowpass filter with cutoff frequency D0. n need
%         not be supplied. D0 must be positive.
%
% 'btw'   Butterworth lowpass filter of order n, and cutoff
%         D0. The default value for n is 1.0. D0 must be
%         positive.
%
% 'gaussian' Gaussian lowpass filter with cutoff (standard
%            deviation) D0. n need not be supplied. D0 must be
%            positive.

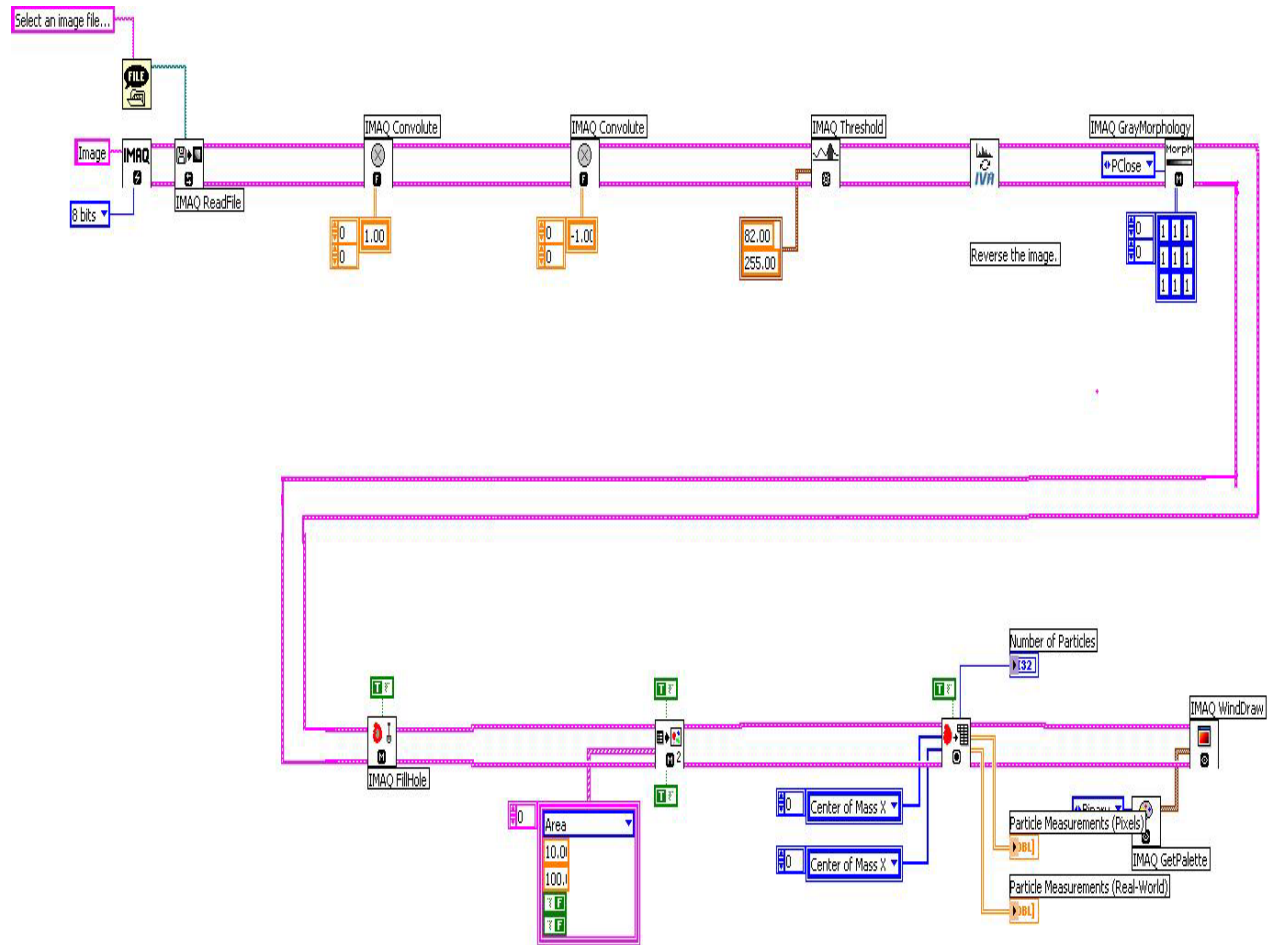
% computing the required distances.
[U, V] = freq_mat(M, N);

% Compute the distances D(U, V).
D = sqrt(U.^2 + V.^2);

% Begin filter computations.
switch type
case 'ideal'
    H = double(D <= D0);
case 'btw'
    if nargin == 4
        n = 1;
    end
    H = 1./(1 + (D./D0).^(2*n));
case 'gaussian'
    H = exp(-(D.^2)./(2*(D0^2)));
otherwise
    error('Unknown filter type.')
end

```

Appendix C



LabVIEW VI for counting the number of cells.

Vita:

Abitha Sundararajan was born on June 12th 1981 in India. She obtained her Bachelor of Engineering in Instrumentation and Control from the University of Madras, India in 2002. She wanted to continue her education in Biomedical Engineering, therefore she came to the University of Tennessee, Knoxville in Fall 2003 for her Masters degree. Abitha is currently looking for a position as a biomedical engineer.