PHOTO-INITIATED CROSS-LINKED POLYACRYLAMIDE GELS FOR MICRO-DEVICE ELECTROPHORESIS

A Thesis

by

SHILPA AGRAWAL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Chemical Engineering

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Approved as to style and content by:

Victor M. Ugaz (Chair of Committee)	Mahmoud El-Halwagi (Member)
Zivko Nikolov (Member)	K. R. Hall (Head of Department)

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ABSTRACT

Photo-Initiated Cross-Linked Polyacrylamide Gels for Micro-Device Electrophoresis. (May 2005)

Shilpa Agrawal, B.Tech., National Institute of Technology Rourkela

Chair of Advisory Committee: Dr. Victor Ugaz

Photo-polymerized cross-linked polyacrylamide gels are becoming increasingly important for use in micro-fabricated DNA electrophoresis systems because they allow a concentrated sieving matrix to be precisely positioned at any location within a complex micro-channel network. The rate of photo-initiation in the free radical gel polymerization reaction, however, can exert a strong influence on the resulting gel structure. Experimental data on separation resolution of single stranded DNA (ssDNA) in photo-initiated polyacrylamide gels is very sparse. In this study, we investigate the performance of ssDNA electrophoresis in an ALF Express automated DNA sequencer using various photo-initiation chemistries. Cross-linked polyacrylamide gels with concentrations ranging from 6 to 12 %T were prepared using riboflavin, methylene blue, irgacure 651 and ReproGel (AP Biotech) photo-initiators. Separation resolution is compared with that attained in cross-linked polyacrylamide gels prepared using conventional chemical initiators (e.g. ammonium persulfate/ Tetramethylethylenediamine) in order to determine the polymerization conditions necessary for optimum performance.

DEDICATION

To my parents, Sunita and Amarnath Agrawal and my sisters Charu and Shweta

for all their love, encouragement and support

and my friend and philosopher J.N. Swamy.

ACKNOWLEDGMENTS

I would like to acknowledge the efforts made by everyone who helped in making this research a success. I would like to express my sincere gratitude to my advisor, Dr. Victor Ugaz, for giving me the opportunity to pursue this project and for all his guidance. I am also grateful to Dr. El-Halwagi for his continuous encouragement and for agreeing to serve on my committee. I would like to thank Dr. Zivko Nikolov not only for serving on my committee but also for his indispensable advice. Finally this work would not have been completed without all the help and facilities provided by my group members. I thank my group members Roger, Xiaojia, Rene, Arjun and Faisal for all their support and encouragement. Finally I would like to extend my special thanks to Department of Chemical Engineering for giving me this wonderful opportunity.

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CHAPTER I

INTRODUCTION

The ability to miniaturize a chemical analysis process enables faster analysis time, less waste of costly reagents, easier manipulations and the prospect of massive parallelization. Hence, numerous molecular biological and biochemical procedures are being re-examined in the hopes of miniaturizing the scale [1]. Such miniaturization not only improves current applications, but has potential to lead to a number of new and novel applications.

Different kinds of micro total analysis systems (μTAS) have been presented in literature [1,2]. Among these systems, a technology that promises to lead the next revolution in chemical analysis is microchip electrophoresis. The advantages of having such a system are : (a) high efficiency (b) high throughput (c) easy to operate and (d) relatively small consumption of samples and reagents. Given these advantages, there has been a significant interest from different fields in biomedical engineering (DNA sequencing, genomics, and proteomics), pharmacy (drug recovery, combinatorial libraries) and analytical chemistry and the like [1].

This thesis follows the style and format of *Electrophoresis*.

Over the years a number of studies have been presented on microchip electrophoresis [1, 2, 3, 4]. These studies include the use of capillary (CE) as well as slab gel electrophoresis (SGE) for microchip applications. Significant advantages have been identified for SGE over CE, at the typical length scales encountered for such applications [5]. Conventionally, polymer gels for SGE have been made using chemical polymerization techniques. In past few years, there has been considerable interest in using photo-polymerization as a viable technique for such a process. Photopolymerization scores over chemical polymerization in terms of time consumed and uniformity of the end product. Under the presence of a well chosen photo-initiator and uniform irradiation of UV light, photo-polymerization of polyacrylamide gels can be affected in 10-15 minutes for a slab of dimensions 31.5 cm x 17.0 cm x 0.03 cm. However, a comprehensive study on the role of photo-initiators in such a process is absent. This is critical to the suitable choice of photo-initiators for a given application. The current research is focused on this specific aspect of slab gel electrophoresis.

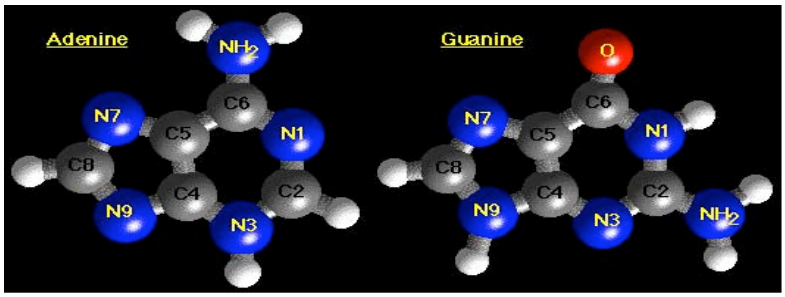
The overall objective is to use different photo-initiators in varying concentrations for the formation of different gels and obtain the separation performance based on the intensity data. The variation in performance is then used to obtain comparisons within photo-initiators to suggest the appropriate combinations. Processing costs and run time are also considered in order to determine the polymerization conditions necessary for optimum performance.

Structure of DNA

DNA is simply a polymer composed of monomeric units called nucleotides that store the genetic information important for all living organisms. A nucleotide consists of a 5-carbon sugar (deoxyribose), a base containing nitrogen attached to the sugar and a phosphate group. The sugar and phosphate groups perform a structural role whereas the bases carry genetic information. There are four different types of nucleotides found in DNA, differing only in the nitrogenous base: adenine (A), guanine (G), cytosine (C) and thymine (T). Adenines and guanines are the purines (Fig. 1a) while cytosine and thymine are the pyrimidines (Fig. 1b). Purines are the larger of the 2 types of bases found in DNA.

The structure of double stranded DNA is a right handed helix with 10 nucleotide pairs per helical turn. Each strand is connected to a complementary strand by hydrogen bonding (non covalent). A and T are connected by 2 hydrogen bonds whereas C and G are connected by 3 bonds as shown in Fig 2. This DNA sequence of a gene encodes information used to synthesize proteins necessary for cellular function. The ability to compare DNA sequences from a healthy population with those from individuals affected by disease can help to identify genetic changes that may have given rise to the disease. Consequently, DNA analysis offers a powerful tool for developing improved diagnostic techniques and more effective therapeutic interventions, such as new drugs and medical tests.

(a)



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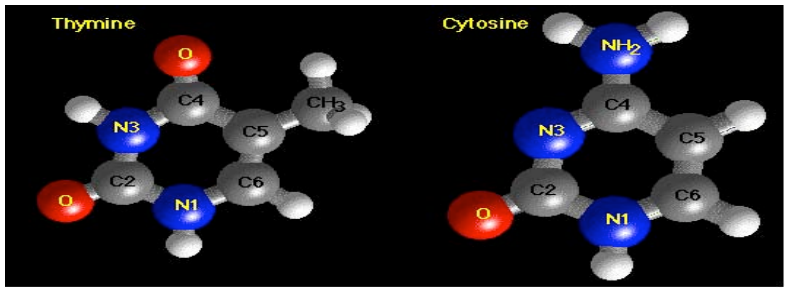


Figure 1. Chemical structure of bases that make up DNA. (a) Adenine and Guanine (b) Thymine and Cytosine [6].

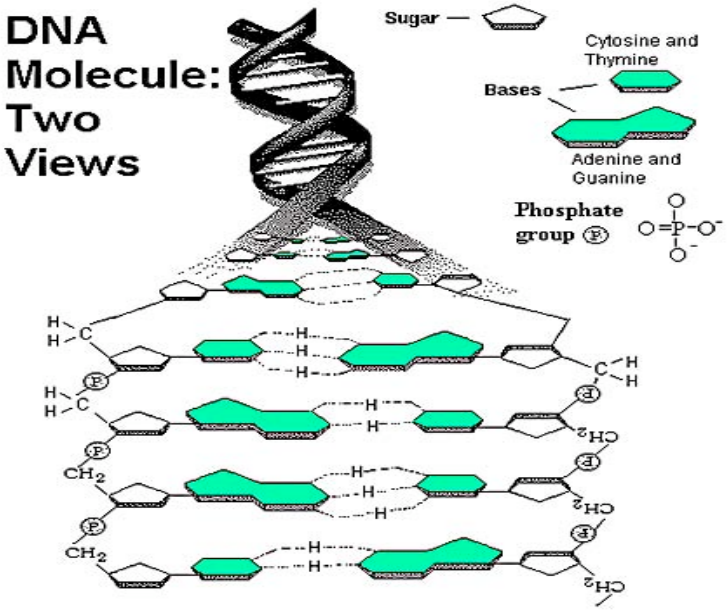


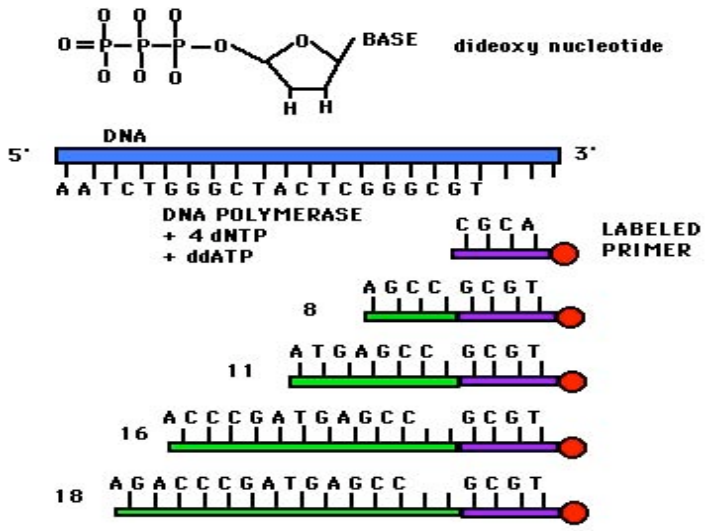
Figure 2. Double stranded DNA helix showing hydrogen bonding between the bases [7].

DNA Sequencing

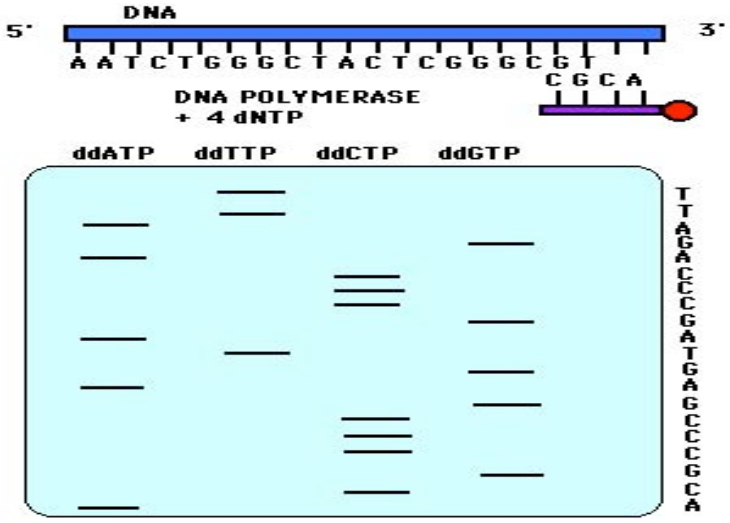
DNA Sequencing is the determination of the precise order of nucleotides in a sample of DNA. Two primary techniques are currently used: the Sanger method (also known as chain termination) and the chemical degradation method of Maxim & Gilbert. Of these, the Sanger method is most frequently used. It is based on the incorporation of chain terminating nucleotides (called dideoxy-nucleotide triphosphate (ddNTP)) during DNA replication. The ddNTP's contain a hydrogen atom in place of an OH group. Thus when a ddNTP is incorporated during the DNA replication process it is unable to form a phosphodiester bond and results in chain termination (Fig. 3a). The process is repeated using thermocycling. The concentration of ddNTP's in the sequencing reaction is around 1% of the concentration of deoxy-nucleotide triphosphate (dNTP) such that any position in the new DNA strand is likely to be the point of dideoxy termination and thus a complete distribution of chains terminating at each site is produced. The DNA fragments produced during the replication process can be detected using fluorophores on performing electrophoresis (Fig. 3b).

Electrophoresis Principles

Electrophoresis is a process by which charged molecules (DNA, proteins) can be separated under the influence of an electric field. (*Electro* refers to the energy of electricity and *Phoresis* means "to carry across") Thus electrophoresis is a process in which an electric field is used to transport molecules through a sieving medium. The process is effective because many biomolecules (amino acids, peptides, nucleic acids, and proteins) have ionisable groups and thus exist as charged species. At a given pH,



(a)



(b)

Figure 3. Sanger sequencing reaction. (a) The DDATP gets incorporated at various positions thus giving DNA fragments of different lengths. (b) Bands obtained when 2D gel electrophoresis is performed [8].

depending on the nature of their charge, they migrate towards either the cathode or the anode under the influence of an electric field. The frictional force exerted by the matrix gel acts as a sieve thereby separating the molecules by size [9]. Consequently the rate of migration through the gel depends on the strength of the electric field, gel pore size, the shape and size of molecules and the ionic strength and temperature of the buffer in which the molecules are moving [10]. For DNA in free solution, the frictional and electrostatic forces are both proportional to the size of the DNA fragment so electrophoretic mobility is independent of size [11]. As a result, sieving gel is required to introduce size dependence into the frictional component of the net force. The gel also inhibits convection currents that might arise due to heating and thus provides a stable matrix for retention of the sample after electrophoresis.

Electrophoresis is one of the major bottlenecks in development of DNA analysis assays because of the time associated with this process. It typically takes several hours to achieve the single base resolution required to extract sequence information. This is because of the large distance to be traveled by the DNA fragments. Micro fabricated gel-on-a-chip devices have the potential to perform the same analysis in only a few minutes because the migration distance gets reduced. This would make the analysis faster and cheaper because one can use smaller samples and reduce the amount of chemicals needed for the test. Such miniaturization necessitates development of improved gel media capable of achieving high separation resolution over short distances.

Theoretical Background

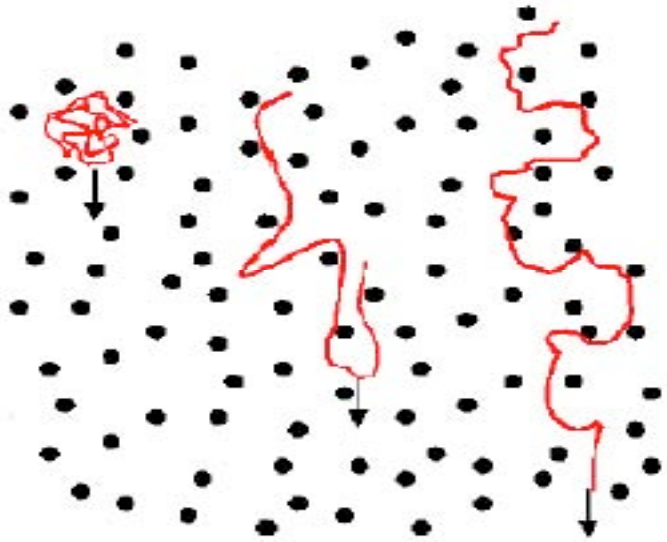
Mobility

Mobility of ssDNA fragments through a gel can be divided into two regimes- Ogston and Reptation regime. Ogston regime refers to the regime where filtering occurs when the size of the molecule is smaller than the average pore size of the sieving matrix (Fig. 4a). It is based on the following assumptions- that the molecule has a rigid spherical shape, field intensity is small so that molecule can go backtrack if it reaches a dead end, mobility is proportional to the fraction of pores available to the migrating molecule and finally that the gel is a random distribution of gel fibers [9]. Smaller molecules migrate quickly because they have a larger fraction of the available gel pores given by the following equation

$$\log \mu = \log \mu_0 - \pi L\left(r + R_g\right)^2 T \qquad (1)$$

where μ is the electrophoretic mobility of the analyte in the matrix, μ_0 is the free solution mobility, L is gel polymer length per unit volume, r is the radius polymer, R_g is the radius of the migrating molecule and T is total gel concentration. But these assumptions do not necessarily hold for migration of DNA fragments in a gel. This is because gels cannot be treated as random array of fibers and DNA molecule is neither rigid nor spherical. Thus the model is valid only if the assumptions hold true and usually this happens for molecules between 50-300 bases [9].

When the size of DNA molecule is larger than the average pore size of the gel it stretches and changes shape as a result of their viscoelastic character to move through the gel pores. This includes the reptation regime (Fig. 4b) in which the DNA fragment



(a) (b) (c)

Figure 4. Representation of the different mechanisms of DNA migration. (a) Ogston sieving (b) Reptation without orientation. (c) Reptation with orientation (alignment in electric field) [9].

adopts an extended coil conformation and moves head first in a snake like fashion through the tight tubes formed by the gel pores surrounding the DNA chain. In the reptation with orientation regime DNA gets oriented in the direction of the field and all resolution is lost (Fig. 4c). Lumpkin developed an expression [12] that explains this process

$$\mu = \frac{k}{N} + bE^2 \qquad (2)$$

where N is the DNA molecular weight, K is a constant, b is a function of the mesh size as well as charge and length of the migrating solute and E is the electric field strength.

At low field strengths the first term in equation (2) dominates whereas at high field strengths the second term in the equation dominates.

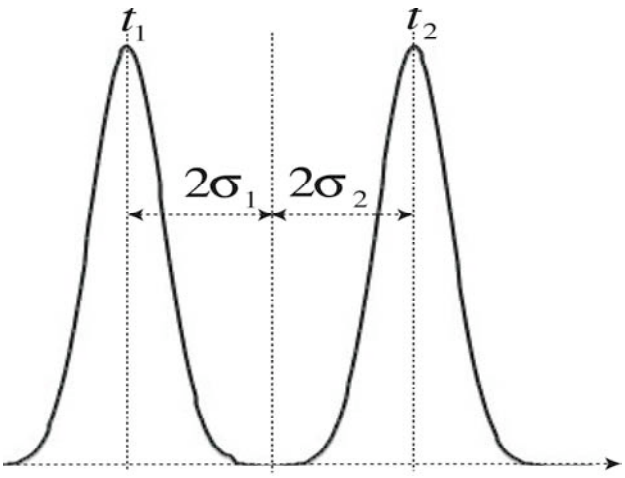
Resolution

Analysis is performed using electrophoresis, whereby fragments of different lengths move through a sieving medium with different speeds. The migration speeds are recorded yielding a pattern of of distinct peaks versus time. The ability to distinguish different peaks can be expressed in terms of a parameter called separation resolution,

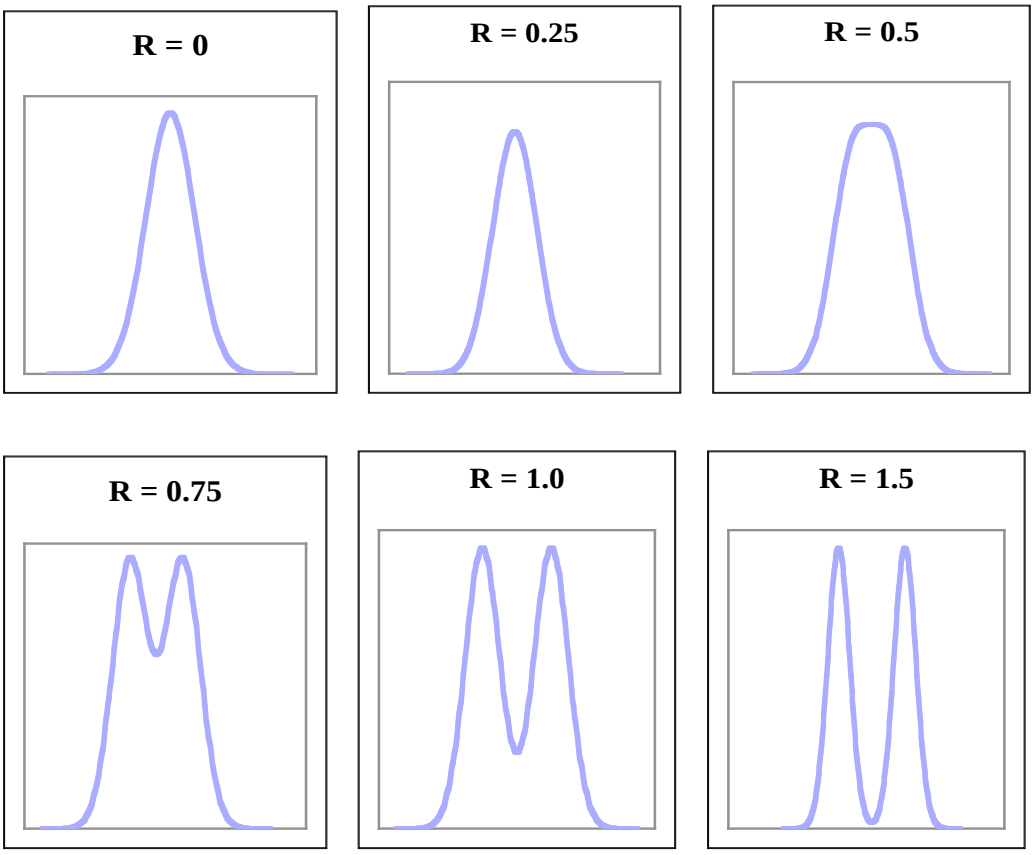
$$R = \frac{t_2 - t_1}{2(\sigma_1 + \sigma_2)} \tag{3}$$

where σ is the standard deviation of the peak and t_2-t_1 is the distance between the peaks.

A value of R=1.5 signifies two completely separated peaks (Fig. 5a) whereas values less than 1.5 are an indication of overlap between peaks (Fig. 5b). For miniaturized systems, it is desirable to obtain the highest possible resolution in the smallest migration distance.



(a)



(b)

Figure 5. Separation resolution (R) (a) Definition of R (b) Different R values.

Comparison between Slab Gel and Capillary Electrophoresis

Electrophoretic separations can be performed in either slab gels or capillaries. Capillary electrophoresis (Fig. 6) is normally used in high throughput applications while slab gels (Fig. 7) are often used when fewer samples need to be run. The most commonly used types of matrices for this purpose are agarose and polyacrylamide. Agarose matrices are typically used for larger DNA because they generally incorporate characterstic pore sizes equal to or greater than 200 nm and higher [13]. Polyacrylamide gels were first introduced by Raymond and Weintraub [14]. These gels have pore sizes ranging from approximately 1 nm to 20 nm and are very efficient in DNA sequencing and protein separations [13]. Although capillary technology does not necessarily offer significant improvement in separation resolution, it offers other advantages including the ability to easily replace the separation medium between electrophoresis runs. In slab gel electrophoresis (SGE) the old gel has to be removed and a freshly prepared polymer solution cast between the plates, which is a tedious job while in capillary electrophoresis, (CE) the freshly prepared polymer is pumped into the capillaries replacing the old material without dissembling the instrument. Secondly, very dense arrays of capillaries can be built for large scale sequencers allowing hundreds of samples to be run simultaneously. On the other hand slab gel sequencers use laser beam to scan across the slab sequentially monitoring fluorescence from each lane. As the number of channels increases the duty cycle decreases and signal to noise ratio becomes reduced [15]. Hence capillaries are useful for high throughput sequencing and genotyping applications [16].

Slab gels are still of interest because they offer improved separation effectively in shorter lengths at lower electric fields which are useful properties in miniaturized systems. A major drawback of slab gel electrophoresis is the creation of Joule heat when electric current passes through the gel and buffer solution. Electrophoretic mobility increases by about 2% for every 1°C rise in temperature [5]. This can cause a non-uniform temperature distribution in the gel such that analytes at edges run more slowly than those in the center. These temperature gradients can also cause band broadening, and in either case cause damage to the gel or DNA sample [5]. The limiting values of voltage gradients to prevent joule heating are 5V/cm for agarose, 8V/cm for polyacrylamide gels [5]. Nevertheless, slab gels are still used because they provide a very high resolution separation, are transparent to UV light, are electrically neutral having no charged groups and finally DNA samples isolated from polyacrylamide gels are very pure [5].

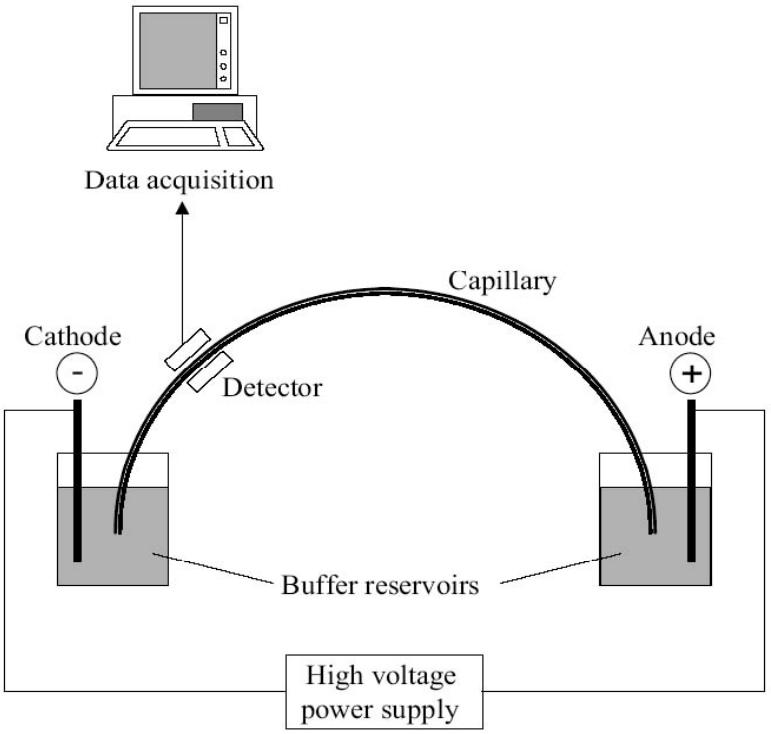


Figure 6. Capillary electrophoresis instrument.

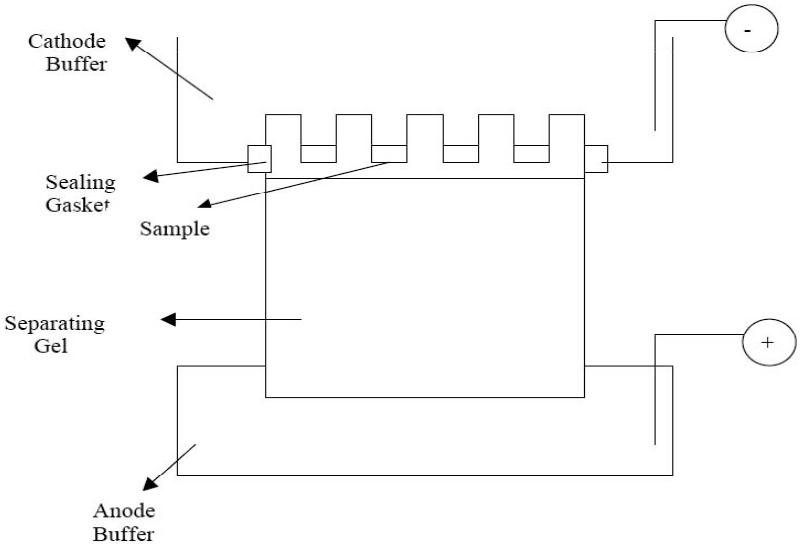


Figure 7. Slab gel electrophoresis.

Selecting the Right Gel

Gel electrophoretic separation of molecules depends upon the pore size relative to the size of migrating molecules. In order to produce a gel matrix with the appropriate pore size distribution, proper choice of monomer, cross linker and initiator is required. Agarose gels are formed from a highly purified polysaccharide derived from agar. In the powdered form, agarose is made up of loose random coils of polysaccharide. On mixing it with water, melting and cooling it these coils adopt a helical conformation. The molecules participate in forming double helical structure resulting in gel formation with the spaces between them acting as pores. Table 1 gives the effective range of separation for various concentration of agarose.

Table 1. Separation range of agarose gels [17]

Agarose (%)	Effective Range of Separation (base pairs)
0.3	5,000-60,000
0.6	1,000-20,000
0.7	800-10,000
0.9	500-7,000
1.2	400-6,000
1.5	200-4,000
2.0	100-3,000
3.0	40-1500

Polyacrylamide gels on the other hand are formed by chemical cross-linking of individual monomeric units. Pore size can be controlled, by varying the concentration of relative amounts of monomer and cross-linker (Table 2). The pore sizes are much more

uniform and can be effectively used to separate very small DNA fragments. The major disadvantage associated with polyacrylamide gels is that they are tedious to prepare.

Table 2. Separation range of polyacrylamide gels [17]

Acrylamide (%)	Effective Range of Separation (nucleotides)
3.5	100-1000
5.0	80-500
8.0	60-400
12.0	40-200
20.0	10-100

The polymerization reaction can be initiated either chemically or photo-chemically. Chemical polymerization typically uses ammonium persulfate as the initiator peroxide and the quaternary amine, N, N',-tetramethylethylenediamine (TEMED) as the catalyst. It can take up to 1.5-2 hours to completely polymerize. In the case of photochemical polymerization, riboflavin and long-wave ultraviolet (UV) light can be used as initiators, with TEMED as the catalyst. The gel matrix consists of an aqueous solution of acrylamide monomer, bis-acrylamide (N, N'-methylenebisacrylamide) cross-linker (Fig. 8) and the initiators- ammonium persulfate and TEMED or riboflavin and TEMED. Polymerization of this mixture produces a three dimensional network where pore size can be varied over a wide range. For DNA sequencing applications average pore sizes of 5-10 nm are required. Polyacrylamide gel concentrations are typically expressed in terms of %T and %C as follows.

$$\%T = \frac{acrylamide(g) + bisacrylamide(g)}{100mL} \tag{4}$$

$$\%C = \frac{bisacrylamide(g) \times 100}{acrylamide(g) + bisacrylamide(g)} \tag{5}$$

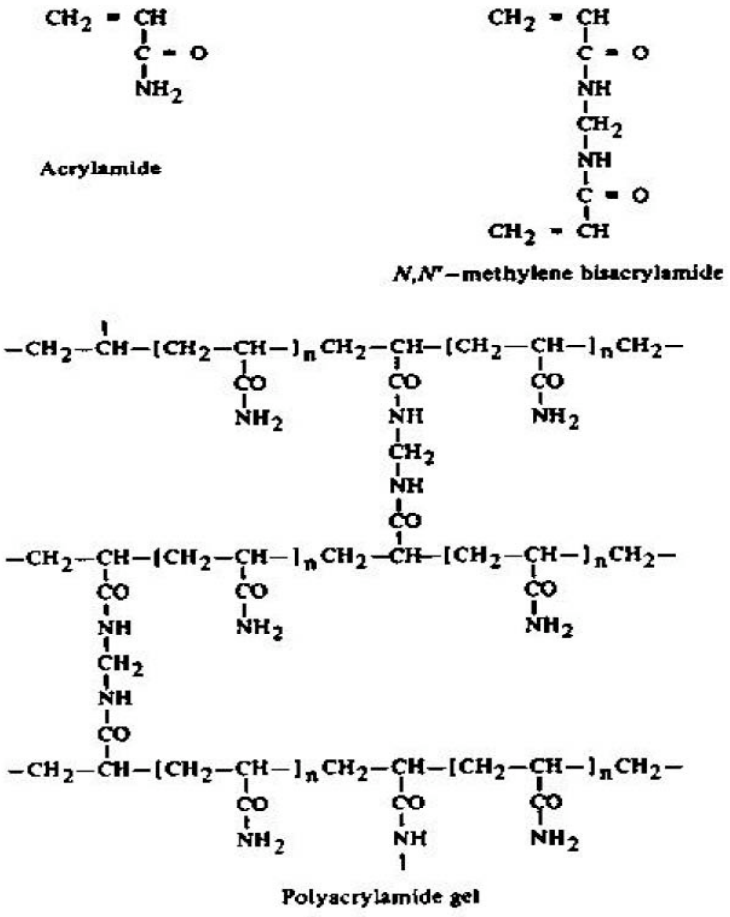


Figure 8. Chemical structure of monomer, cross-linker and polyacrylamide gel [18].

The type and concentration of initiators used strongly influence properties of the resulting gel as well as the rate of polymerization. If the concentration of initiator is

increased it results in a reduction in the average polymer chain length that is accompanied by a rise in gel turbidity and decrease in gel elasticity [18]. On the other hand, reducing the initiator concentration produces gels with longer polymer chains, lower turbidity and greater elasticity. However, reducing the initiator concentration also causes slower polymerization, which is not desirable as dissolved oxygen in the monomer solution can begin to inhibit the reaction resulting in porous or mechanically weak gels.

The mechanism for photo-polymerization takes place via free radical polymerization reaction. These are chain polymerizations where each polymer molecule grows by adding a monomer to a free radical radical active centre. The process can be divided into three distinct steps-initiation, propagation and termination. During initiation free radical active center is created. This occurs by formation of free radical active center from an initiator and then by adding these free radicals to a molecule of monomer. These free radicals are generated by photolysis i.e the action of UV light. One of the advantages of this is that the formation of free radicals begins as soon as light is incident and ceases when the light source is removed. Propagation involves growth of polymer chain by addition of monomer to the active center. Finally termination takes place by joining of two growing chains to form a single polymer molecule.

Polyacrylamide gels used in slab gel electrophoresis applications are formed by chemical cross-linking of monomeric units. Cross-linking implies the formation of permanent junctions between different molecules i.e a chemical reaction between a monomer on one chain and a cross-linker on a neighboring chain. The monomeric units

are linked together with the aid of free radicals generated by the initiators. In the case of UV cross-linking the photo-initiator creates the free radicals through absorption of photons in the UV range. The denaturant also plays an important role. Urea is generally used for this purpose at concentrations of around 7 or 8 molar to prevent folding of the DNA molecules and formation of secondary structures which would alter their mobility. The gel is also run at higher temperatures (around 55°C) to prevent secondary structure formation.

Microchip Applications

The chief disadvantage of the use of polyacrylamide gels in high throughput applications is the necessity to cast a gel every time a new run is performed. Photo initiated gels speed up the process by replacing chemical initiators with UV activated ones, allowing the polymerization time to be reduced to 10-15 minutes. They also alleviate difficulties associated with loading externally polymerized matrices into micron sized capillary channels because the monomer solution for the UV gels has approximately the same viscosity as water and spontaneously wicks into a micro channel by capillary action [19]. Another advantage of UV initiated polymerization is that the reaction can be isolated within a specific location inside a micro fabricated device by discriminately illuminating the channel or only where the gel needs to be positioned. This ability to precisely position the gel within a well defined flat interface allows more precise sample injection and compaction [19]. It is also possible to control the UV initiated polymerization process by varying the intensity of incident light.

Research Objective

Considerable effort has gone into fabrication of microchip electrophoresis systems for DNA sequencing because they are portable and inexpensive. A conventional laboratory set up, involves sample preparation process that incorporates DNA extraction, purification and chemical reactions (e.g. PCR). In areas like environmental microbiology, there is a requirement to analyze unique ecosystems in extreme environments such as deep sea or under-glacial lakes. In such cases regular lab-scale techniques are not practical. Further, the utility of such systems demands time and man-power which are expensive [2]. Thus, there is a need for automation whereby, sample preparation, reaction and analysis can take place in a single system. Unfortunately chip design, optimization and integration of components are still challenging.

Photo-polymerized cross-linked polyacrylamide gels offer a number of advantages for use in micro fabricated DNA electrophoresis systems because they allow a concentrated sieving matrix to be precisely positioned at any location within a complex micro channel network. The rate of photo-initiation in the free radical gel polymerization reaction can exert a strong influence on the resulting gel structure [20]. Consequently there is a need to compare the effects of various photo-initiators on separation resolution. This thesis investigates the performance of single stranded DNA electrophoresis in an automated slab gel DNA sequencer among gels cast by various photo initiation chemistries. Cross-linked polyacrylamide gels with concentrations ranging from 6 to 12%T have been prepared using riboflavin, Methylene blue, Irgacure 651 and ReproGel photo-initiators.

CHAPTER II

EXPERIMENTAL METHODS

Materials

Acrylamide, N, N-methylbisacrylamide (Bis), Ammonium Persulfate, Riboflavin 5'Monophosphate, urea, 10X TBE buffer were purchased from Bio-Rad Labs. Reprogel High Resolution A (24%T) and B solution, Reprogel Long Read A (21%T) and B solution were from Amersham Biosciences Corp, Long Ranger (50%) was from Cambrex Bio Sciences, Methylene blue was obtained from Fluka and Irgacure 651 was obtained from Ciba Speciality Chemicals Corp. All the reagents were used as received.

Gel Preparation

All electrophoresis experiments were performed using an ALF Express II (Amersham Biosciences Corp.) DNA sequencer (Fig. 9). This instrument is designed for automated detection of fluorescently labeled DNA fragments and consists of a gel cassette incorporating 0.3mm spacers and combs coupled with a 633 nm laser (Fig. 10). Cassette, spacers and combs were cleaned using alconox and DI (de-ionized) water. Alconox was prepared by dissolving 40g of alconox in 500 mL DI water. Cleaning is very important because all the dust particles have to be removed for the laser to penetrate through the spacers and illuminate the entire gel. Next the gel cassette was treated with a diluted solution of PlusOne Bind Silane in order to impart mechanical strength to the loaded wells. It was prepared by mixing 1000μL of Bind Silane solution (30μL of

PlusOne Bind Silane in 10 mL ethanol) with 250μL of acetic acid. Finally the gel plates were assembled and the comb was inserted.

The gels were prepared using various combinations of monomer, cross-linker and initiator. A detailed description of the process is given in Appendix A. In order to prepare 6%T, 9%T and 12%T Long Ranger gels one part of diluted Long Ranger gel solution (monomer and cross-linker) was mixed with two parts of Reprogel High Resolution photo-initiator (solution 'B'). For preparing 8%T High Resolution and 7%T Long Read gels one part of the solution 'A' of the respective gels was mixed with two parts of photo-initiator (solution 'B').

(a) (b)

Figure 9. ALF express DNA sequencer. This instrument consists of a temperature controlled gel electrophoresis unit. Fluorescently labeled DNA samples are illuminated using a laser and the resulting fluorescence is recorded by an array of photo detectors and displayed on the attached computer. Up to 40 samples can be run simultaneously. (a) lid is closed and the instrument is running. (b) lid is open showing the gel cassette.

Gels were also prepared using monomer and cross-linker in powdered form. 6%T, 5%C gels were prepared by mixing 0.06g of Bis with 1.14g of acrylamide and 9%T, 5%C gels were prepared by mixing 0.09g of Bis with 1.71g of acrylamide. Next 7.2g of urea was added with DI water to a volume of 16L. Finally 10X TBE was added until the solution reached a final volume of 20 mL.

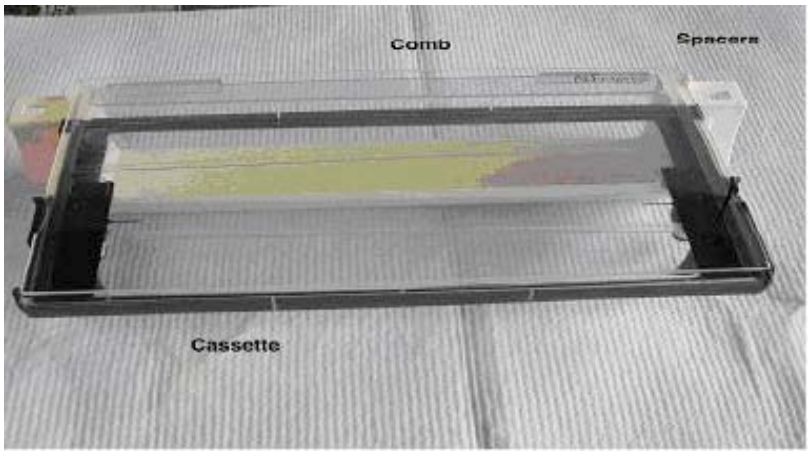


Figure 10. Gel cassette. The assembled gel cassette consists of 2 spacers and a comb having 0.3 mm thickness. The plates are held together by gel clips.

Gels were cast using two polymerization chemistries. In case of photo-polymerization, riboflavin, methylene blue and irgacure 651 were investigated. For High Resolution, Long Ranger and Long Read gels the following amounts of photo-initiator were used.

Riboflavin-3 concentrations of riboflavin were used-0.0005%, 0.001% and 0.002%. 0.0005% initiator solution was prepared by mixing 0.25mg of Riboflavin in 50ml DI water. Similarly 0.001% and 0.002% were prepared by adding 0.5mg in 50 ml DI water and 1mg in 50ml DI water respectively. The solution was then placed on a magnetic stirrer for a few minutes until the initiator was completely dissolved. The solution obtained was yellow in color, and the color deepened on increasing the concentration of riboflavin.

Methylene Blue-A 2mM methylene blue (MB) solution was prepared by adding 32mg of MB in 50mL of DI water. 1mL of this diluted mixture was then added to 20mL of the gel mixture along with 1ml of of 20mM Sodium Toluene Sulfinate (reducer) and 1mL of1mM diphenyliodonium chloride (oxidizer) [21, 22]. MB is sometimes used because it produces polyacrylamide gels with greater elastic properties than persulfate activated gels [18].

Igacure 651(alpha, alpha-dimethoxy-alpha-phenylacetophenone)-A 2% Irgacure solution was prepared by dissolving 100mg of irgacure powder in 5mL ethanol. 15μL, 30μL and 60μL aliquots of this solution were then added to 20mL of the gel mixture.

In the case of Long Ranger and Reprogel the photo-initiator solutions described above were added directly added to the monomer cross-linker mixture. For gels prepared using acrylamide and Bis in powder form, 30μL of 10% ammonium persulfate (APS) solution was added along with the above mentioned photo-initiators. Next the gel was carefully injected between the plates in order to avoid trapping air bubbles. If air bubbles were present, they were removed using a plastic ‘bubble catcher” strip. After loading the

gel the entire gel cassette was exposed to UV illumination for approximately 12 minutes using a Repro Set UV illuminator (4*40W low pressure mercury lamps emitting UV-A) (Fig. 11). After polymerization was completed combs were removed and the cassette loaded in the ALF instrument.

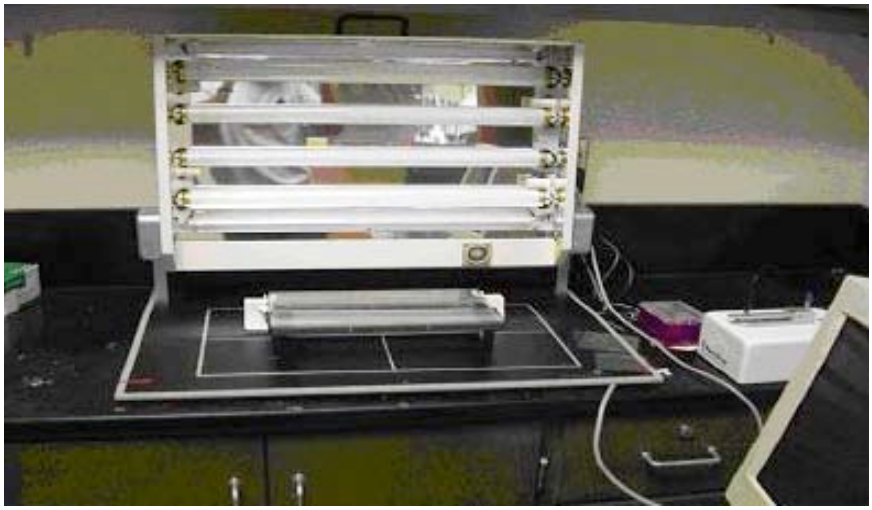


Figure 11. Repro Set UV illuminator. Repro Set UV illuminator has 4*40W low pressure mercury lamps emitting UV light. It has 4 fluorescence lamps that have 99% of their light content in the UV region (315-400 nm).

A series of chemically polymerized gels were also prepared by adding 30μL of 10% APS and 20μL of Tetramethylethylenediamine (TEMED) to the various mixtures described earlier. The mixture was then injected into the gel cassette, checked for bubbles and left for 60-90 minutes to polymerize, after which the combs were removed and the cassette was loaded in the ALF instrument. Running buffer was prepared by adding 100 mL of 10X TBE to 1900 mL of DI water to obtain 0.5X final concentration.

The buffer solution was poured in the upper and lower reservoirs, and the temperature was allowed to reach 55°C. Prior to sample loading the wells were flushed with running buffer in order to remove the unpolymerized monomer residues.

Preparation of DNA Sample

DNA samples were prepared by mixing 2μL of Cy 5.0 Map Marker single stranded DNA ladder (BioVentures,Inc) with 4μL of tracking dye and 8μL of formamide in 200μL thin walled tubes. The excitation wavelength for Cy 5.0 is 649 nm and the emission wavelength is 670 nm. The Map Marker ladder contains bands of 70, 80, 90, 100, 120, 140, 160, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380 and 400 bases in length. The tracking dye contains 25mg/mL blue dextran of an average molecular weight of 2,000,000 dissolved in 25mM EDTA. The DNA samples were mixed thoroughly and placed in a preheated dry bath incubator (Fisher Scientific) where a temperature of around 95°C was maintained for 5 minutes in order to fully denature the fragments.

After denaturing, the DNA samples were removed and placed on ice at 4°C prior to loading in the wells. 7μL of this sample mixture was loaded in each lane.

Electrophoresis experiments were allowed to run from 3-20 hours depending upon the gel composition. The field strength was maintained at 30V/cm and current at 60 mA. After the run was completed the cassette was removed and dismantled carefully prior to cleaning for the next run.

Data Analysis

After the electrophoresis run was completed fluorescence intensity versus run time data were obtained from the ALF Express II file format (.flx). This data was converted to text file format using ImageMagick software. Then this converted file was used for various calculations like separation resolution, mobility and pore size estimation using Matlab codes.

Separation Resolution

Separation Resolution was calculated using equation (3) where σ is the standard deviation of the peak and t_2-t_1 is the time between the peaks. σ was calculated by fitting each peak to a Gaussian profile.

Mobility

Mobility calculations were performed by recording the migration time for each peak and using it to calculate the migration velocity. Mobility was then calculated by dividing the migration velocity (v=L/t) by the electric field strength (E=V/L) as shown in the following equation.

$$\mu = \frac{L^2}{Vt} \tag{6}$$

where μ is the mobility, E is the electric field, V is voltage, L is the separation length, v is migration velocity and t is the migration time.

Absorbance Spectra

The absorbance spectra were measured using Bio-Rad Smart Spectrometer. In this case the gel was cast in cuvettes and after UV polymerization scanned for a definite wavelength.

CHAPTER III

RESULTS

We have done a systematic study of the resolution and mobility of ssDNA in cross-linked polyacrylamide gels with varying concentration of photo-initiators under a fixed set of operating conditions (E=30V/cm, T=55°C, separation length=8.5 cm).

Separation Resolution

Fig. 12 shows the separation resolution versus base number for 400 base pair ssDNA fragments in gels with varying concentration of photo-initiators. The following can be concluded from the graphs.

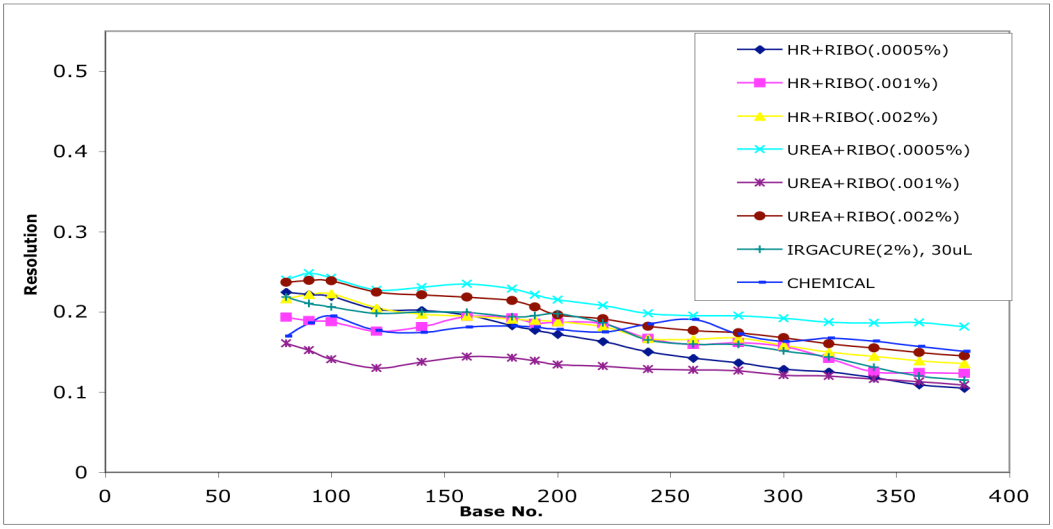
From Fig. 12(c, d, e, f, g) we can see that Irgacure 651 does not give a good resolution. One of the reasons might be that the run time is very short compared to other photo-initiators, possibly due to poor cross-linking that results in larger pore size and hence inefficient separation. Even increasing the amount and concentration from 2% to 10% does not help, instead it further lowers the resolution (Fig. 12f).

Solution B (Reprogel Long Read and High Resolution initiator) does not give high values of resolution (Fig. 12a, b) and in all the cases urea is better than solution B. This might be because the average pore size with solution B is larger in comparison to urea with riboflavin as initiator.

Separation runs could not be performed with Methylene Blue because it gives an opaque gel that is not transparent to red laser illumination (Red laser used has a

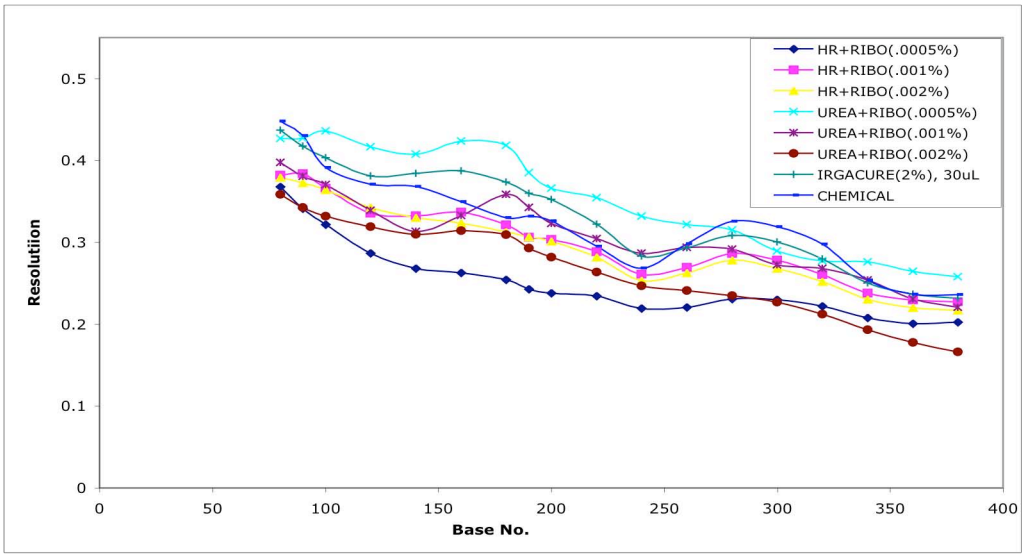
wavelength of 633 nm whereas blue gel obtained has a wavelength of around 475 nm). Hence the transmittance is too low for detection of DNA fragments.

Gel (9%T, 5%C) gave high values of separation resolution in nearly all the cases (Fig. 12g), but the resolution curves obtained were not smooth. This might be due to the decrease in transmittance due to increase in gel concentration. Morever, the average pore size obtained was very small and the run time long s compared to other runs, which might have resulted in higher resolution.

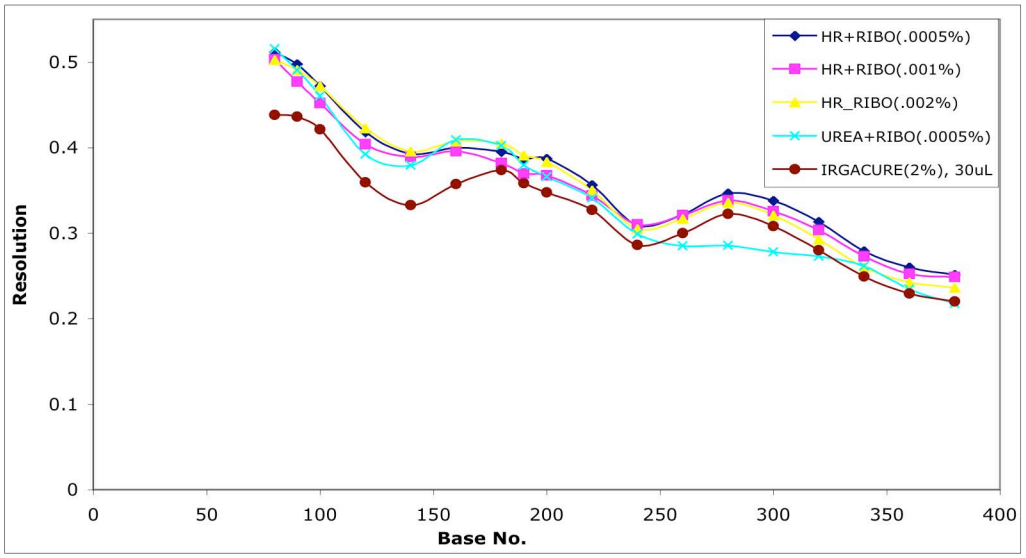


(a)

Figure 12. Separation resolution. (a) Long Ranger (6%) (b) Long Ranger (9%) (c) Long Ranger (12%) (d) High Resolution (8%) (e) Long Read (7%) (f) Gel Powder (6%T, 5%C) (g) Gel Powder (9%T, 5%C) obtained for the gels with varying concentration of photo-initiators.

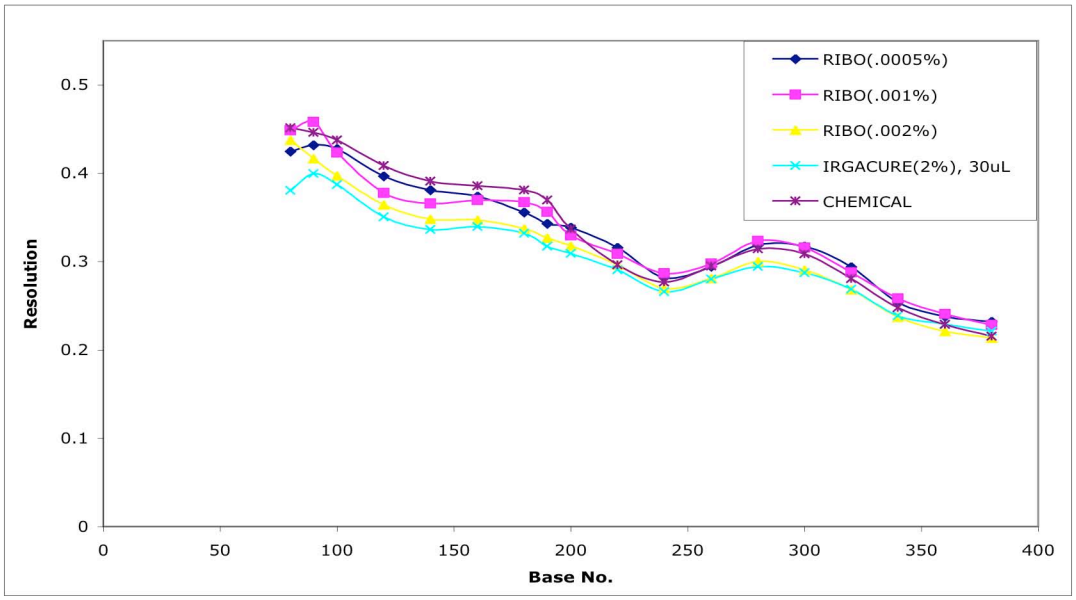


(b)

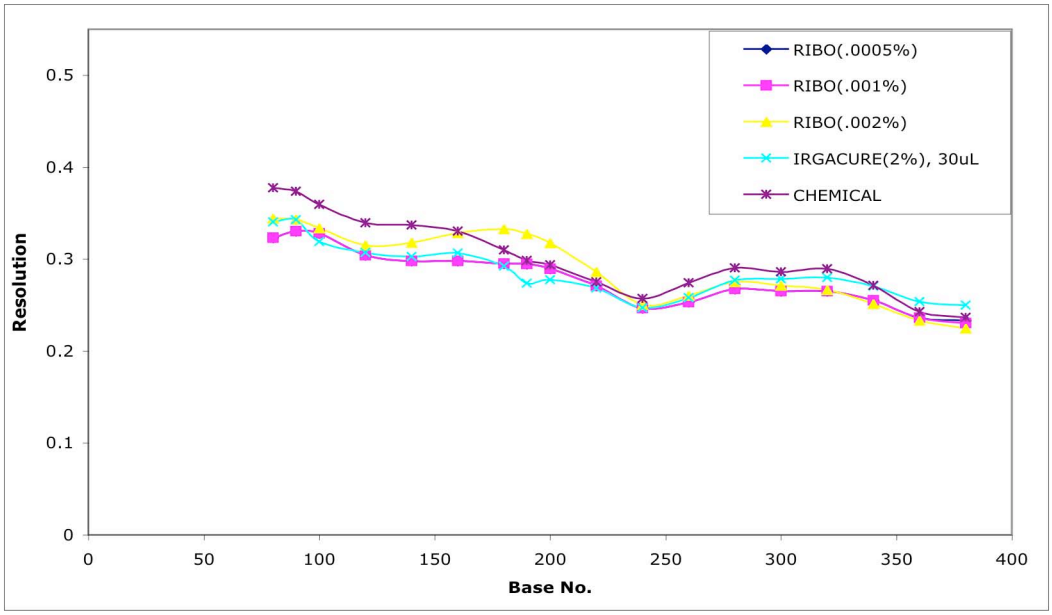


(c)

Figure 12. Continued

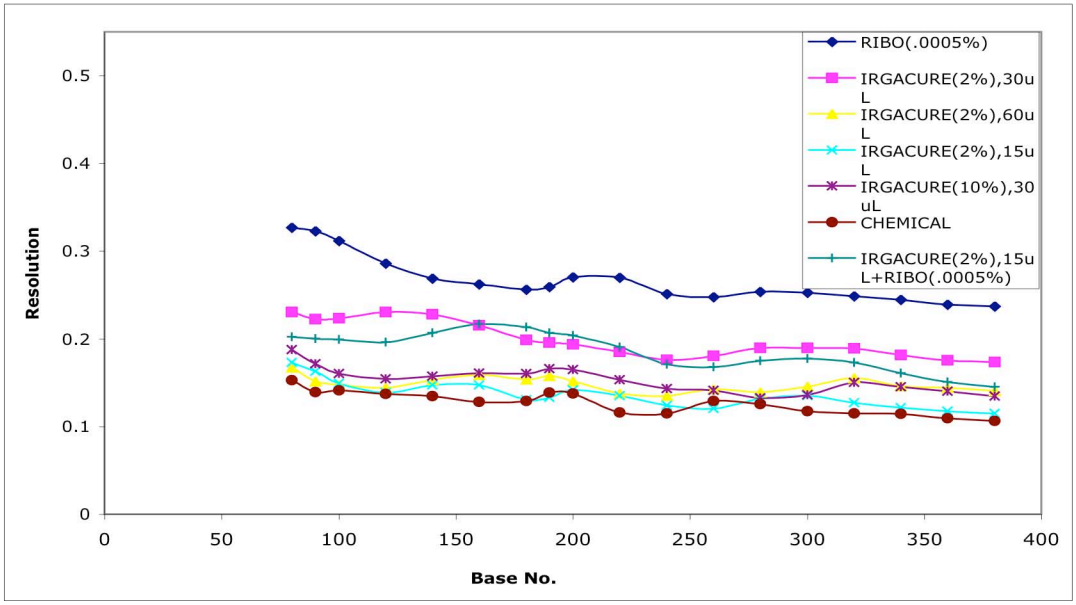


(d)

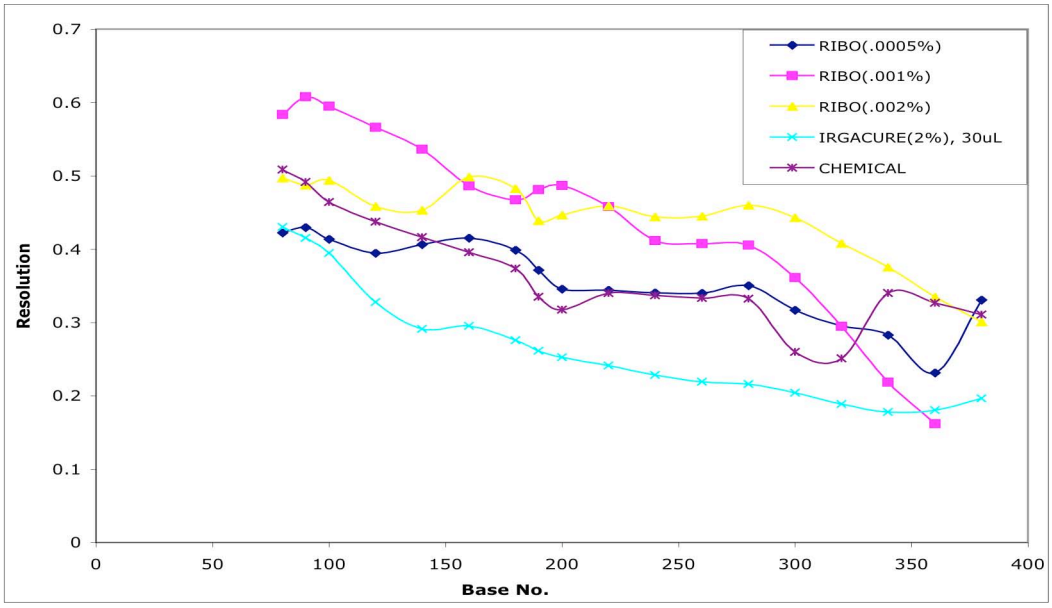


(e)

Figure 12. Continued



(f)



(g)

Figure 12. Continued

Mobility

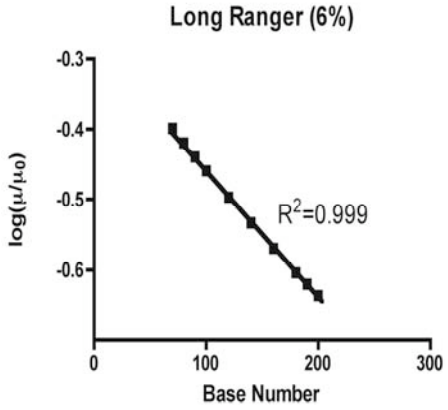
The mobility of ssDNA fragments was measured for various gels at different concentrations. The data obtained from these was used to estimate pore sizes for each of the gel concentrations and compared with those obtained in the literature under a similar set of conditions.

Ogston Regime

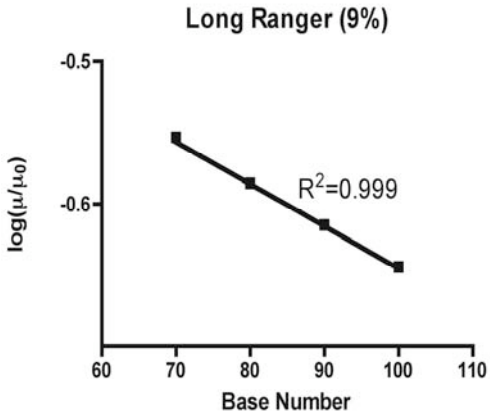
Small fragments of DNA with radius of gyration (R_g) less than the gel pore size denoted by 'a' belongs to Ogston regime. The original concept was put forward by Rodbard and Chrambach [23, 24]. It assumes DNA molecule to be a rigid spherical particle migrating through a network of rigid cylindrical fibers. The radius of gyration has been calculated using the Kratky-Porod equation where the contour length L_D=Mb (M=the number of monomers of size b; b=.43 nm for ssDNA).

$$R_g^2 = \frac{pL_D}{3}\left[1 - 3\left(\frac{p}{L_D}\right) + 6\left(\frac{p}{L_D}\right)^2 - 6\left(\frac{p}{L_D}\right)^3 \times (1 - e^{-L_D/p})\right] \qquad (7)$$

This model predicts an exponential dependence of mobility on base number. Thus a plot of log (μ/μ_0) versus base number at a given gel concentration should be linear. A .0005% concentration of riboflavin has been used while plotting the graphs. From Fig. 13 we see that the above relationship holds for following ranges-Long Ranger 6%, 9%, 12%-200, 100, 100 bases, High Resolution 8%-100 bases, Long Read 7%-100 bases, powdered gel 6%, 9%-160, 100 bases. Here the cut off value for Ogston regime was taken at the point where the R^2 value for the linear fit drops below 0.999.

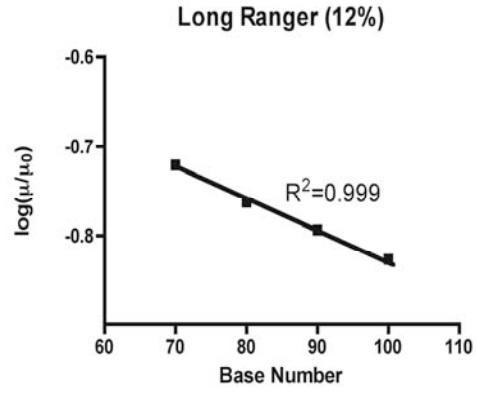


(a)

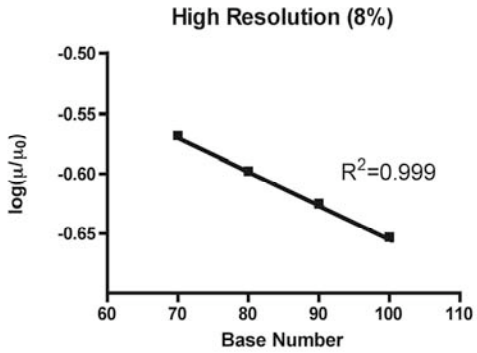


(b)

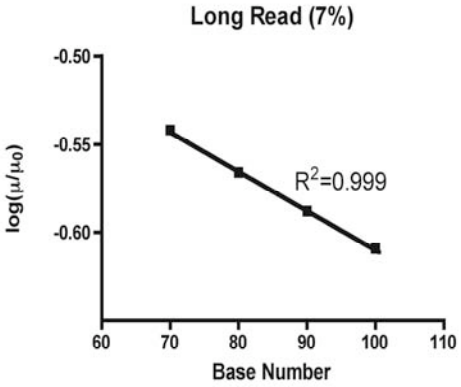
Figure 13. Ogston plots. (a) Long Ranger (6%) (b) Long Ranger (9%) (c) Long Ranger (12%) (d) High Resolution (8%) (e) Long Read (7%) (f) Gel Powder (6%T, 5%C) (g) Gel Powder (9%T, 5%C).



(c)

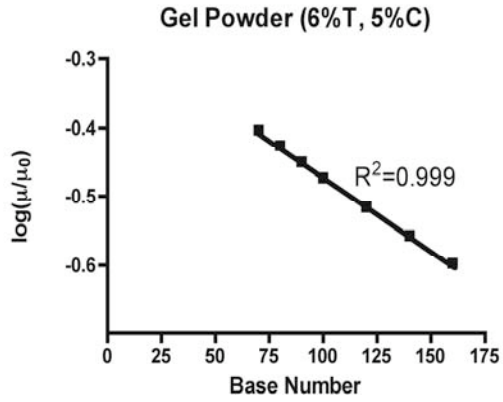


(d)

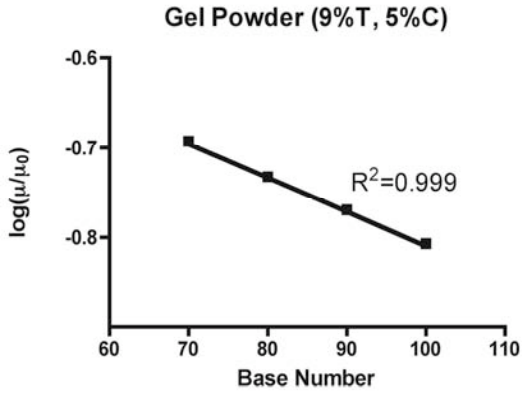


(e)

Figure 13. Continued



(f)



(g)

Figure 13. Continued

The free solution mobility of ssDNA fragments can also be calculated using this model. The Ogston model gives a linear relation between mobility versus gel concentration on a semi-log plot and the intercept gives the free solution mobility (μ_o) [24]. This graph is often called Ferguson plot. Now using the limiting values for base numbers obtained from Fig. 13 a series of linear fits in the Ferguson plot was obtained

(Fig. 14) and it was found that the value of μ_o converges to 3.1+ .06 x 10^{-4} cm^2/Vs for Long Ranger with riboflavin (.0005%) as initiator. These values obtained are in good agreement with those obtained in literature under similar conditions. (Brahmasandra et. al: μ_o=2.8 x10^{-4} cm^2 /Vs [25], Pluen et. al.: μ_o=3.3 x 10^{-4} cm^2 /Vs [26]).

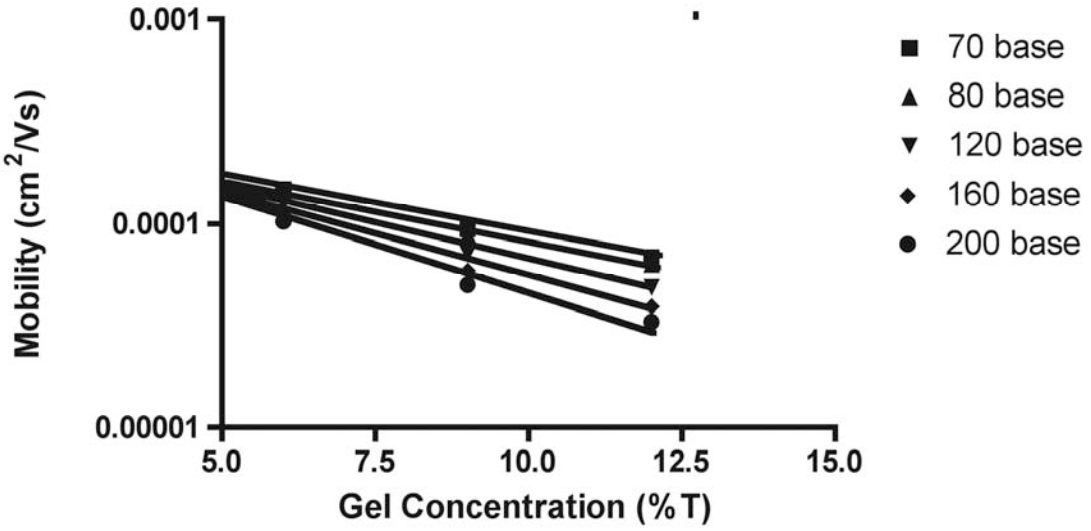
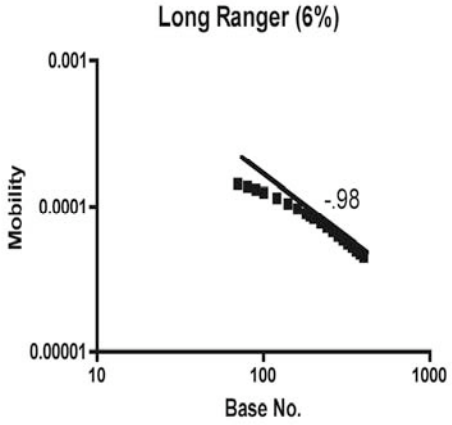


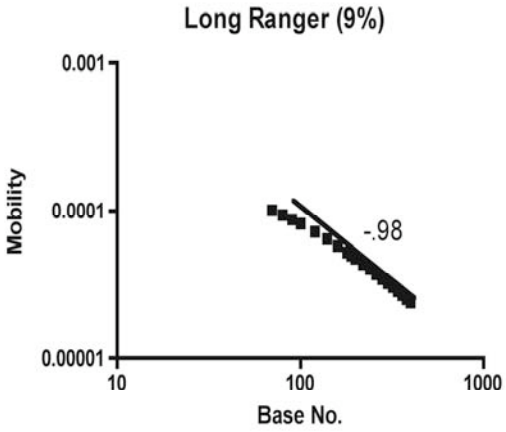
Figure 14. Ferguson plot for Long Ranger.

Reptation Regime

This regime is used to describe DNA fragments with radius of gyration greater than the average pore size of the gel. In this case the migrating molecule adopts an extended configuration and moves in a snake like fashion head first through the gel pores. In this case we have plotted mobility versus base number on a log log plot (Fig.15). From the plot we can make the observation that the mobility varies inversely with the base number and hence it is in accordance with the reptation theory.

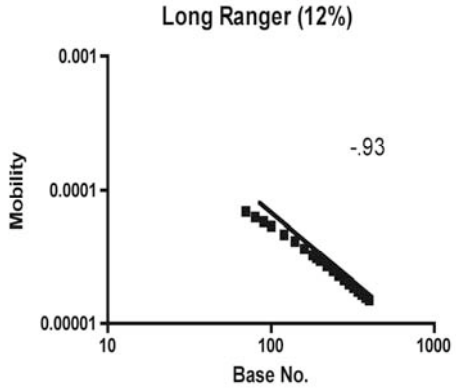


(a)

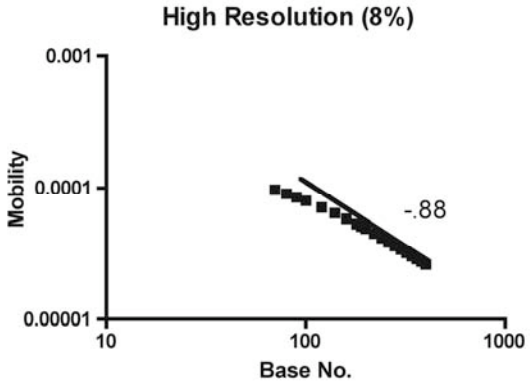


(b)

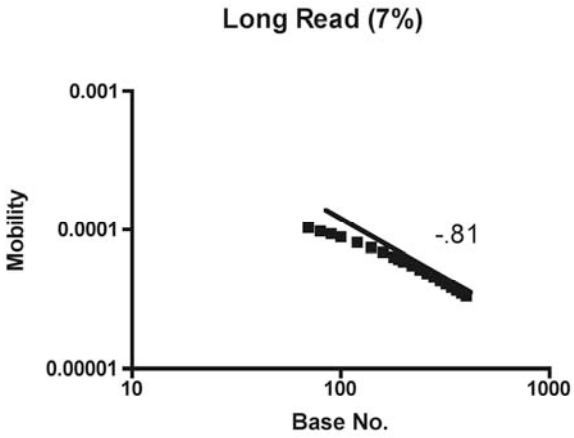
Figure 15. Reptation plots. (a) Long Ranger (6%) (b) Long Ranger (9%) (c) Long Ranger (12%) (d) High Resolution (8%) (e) Long Read (7%) (f) Gel Powder (6%T, 5%C) (g) Gel Powder (9%T, 5%C).



(c)

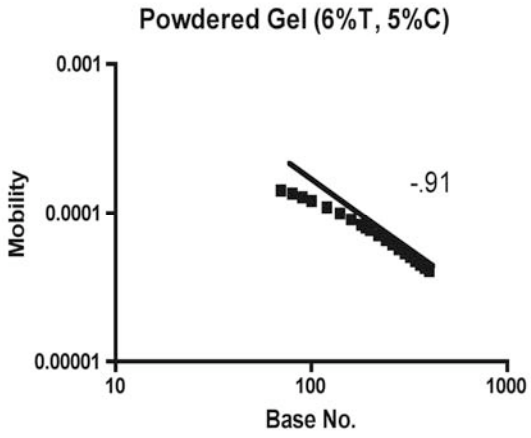


(d)

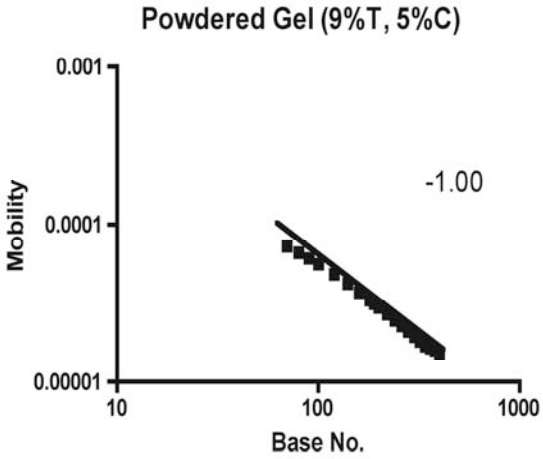


(e)

Figure 15. Continued



(f)



(g)

Figure 15. Continued

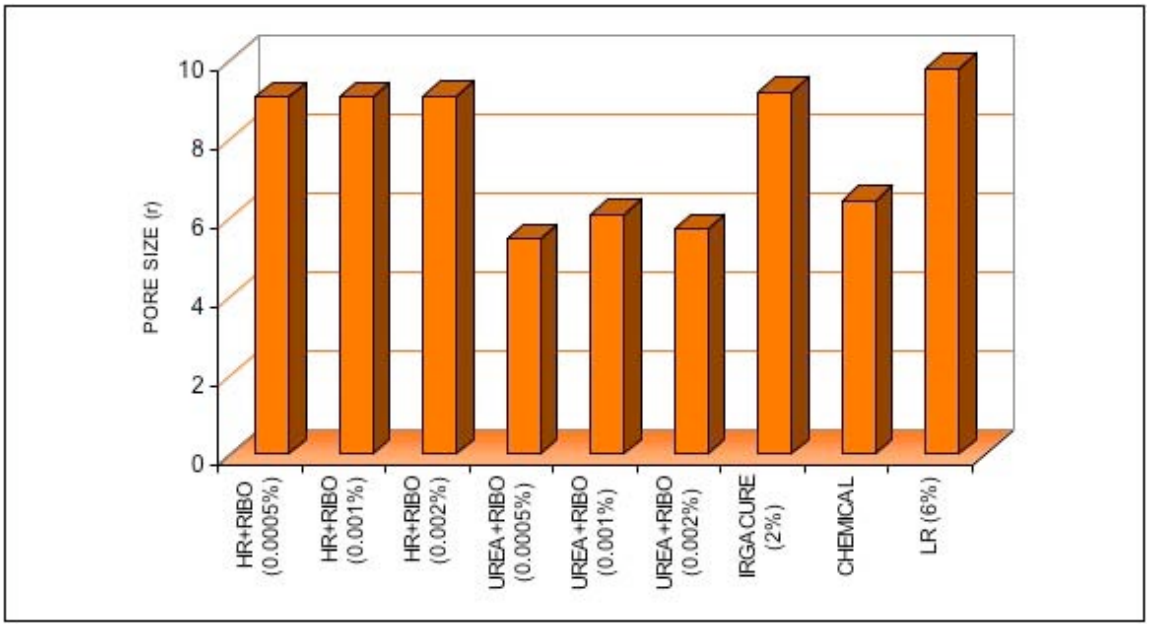
Pore Size

Pore size has been calculated using a formulation proposed by Mercier and Slater. It uses the following relationship to fit the mobility data

$$\sqrt{\frac{\mu_o - \mu}{\mu}} \cong \frac{R + r}{\xi} \tag{8}$$

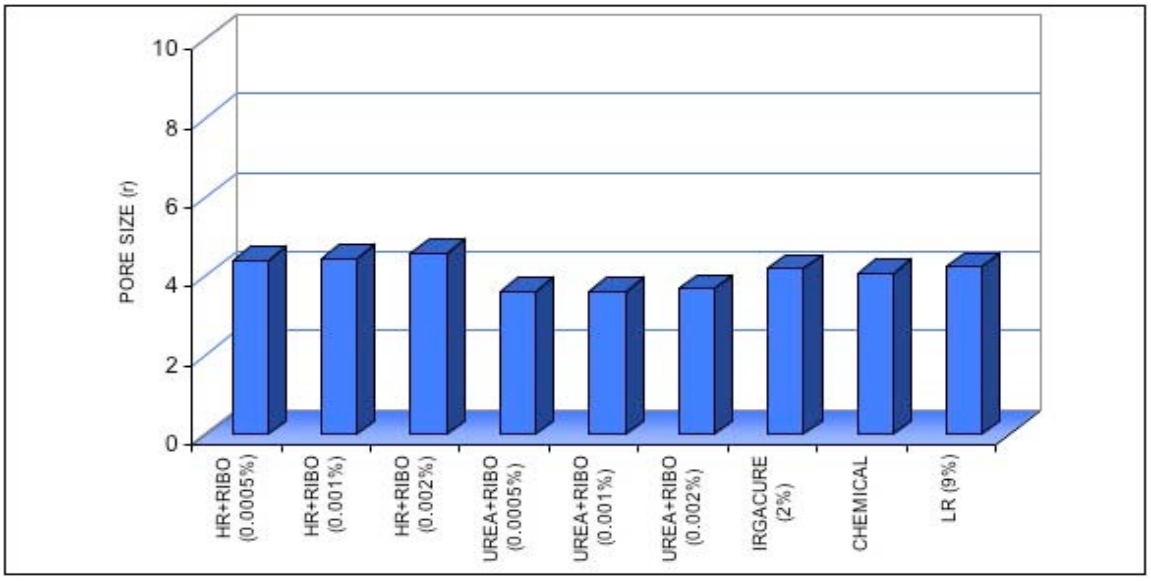
where μ_0 is free solution mobility, μ is observed mobility, R is radius of gyration, ξ is pore size and r is effective fiber size. Plot of L.H.S versus R gives a straight line with slope $1/\xi$ and intercept of R/ξ (Appendix A). Though this equation is strictly valid for Ogston regime it has been shown to be valid even in the Reptation regime [23]. Fig. 16 shows bar graphs of pore sizes for different gels with varying concentration of photo initiators.

From Fig. 16a we can say that the pore size for Long Ranger 6% varies between 7.5±1.5 nm. As the gel concentration increases the pore size gets reduced. For Long Ranger 9%, 12% it varies between 3.9±.3 nm and 2.6±.4 nm (Fig. 16b, c). Pore size for High Resolution and Long Read varies as 3.85±.15 nm and 3.2±1 nm (Fig. 16c, d). The powdered gel 6%T, 9%T has pore size varying between 5.9±1.7 nm and 2.9±.9 nm (Fig. 15f, g).

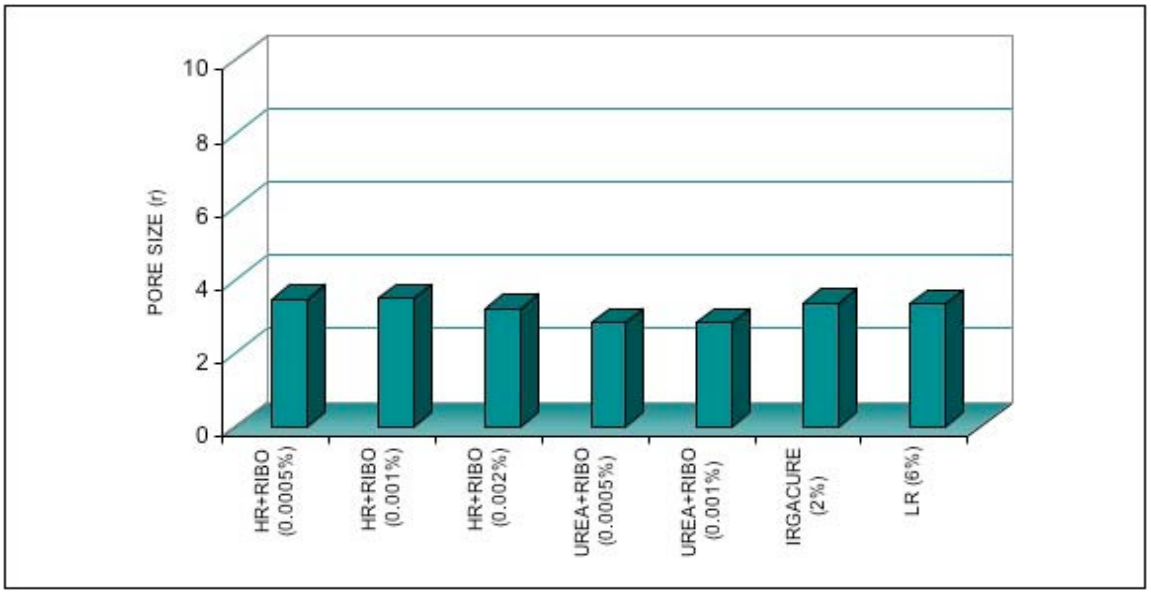


(a)

Figure 16. Pore sizes of various gels using different photo-initiators. (a) Long Ranger (6%) (b) Long Ranger (9%) (c) Long Ranger (12%) (d) High Resolution (8%) (e) Long Read (7%) (f) Gel Powder (6%T, 5%C) (g) Gel Powder (9%T, 5%C).

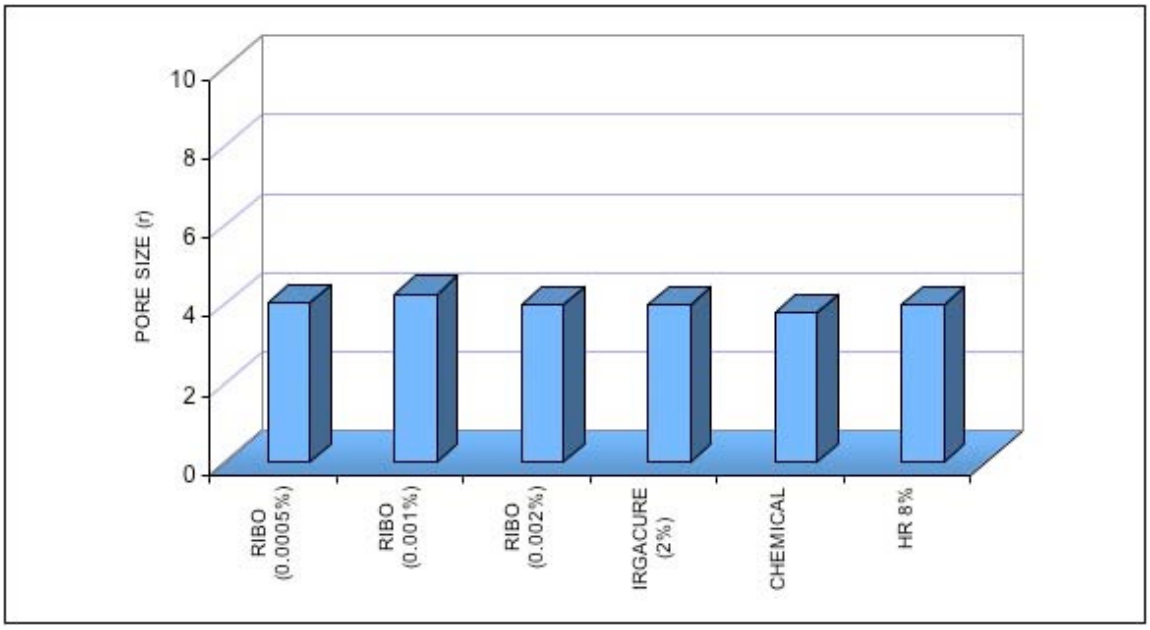


(b)

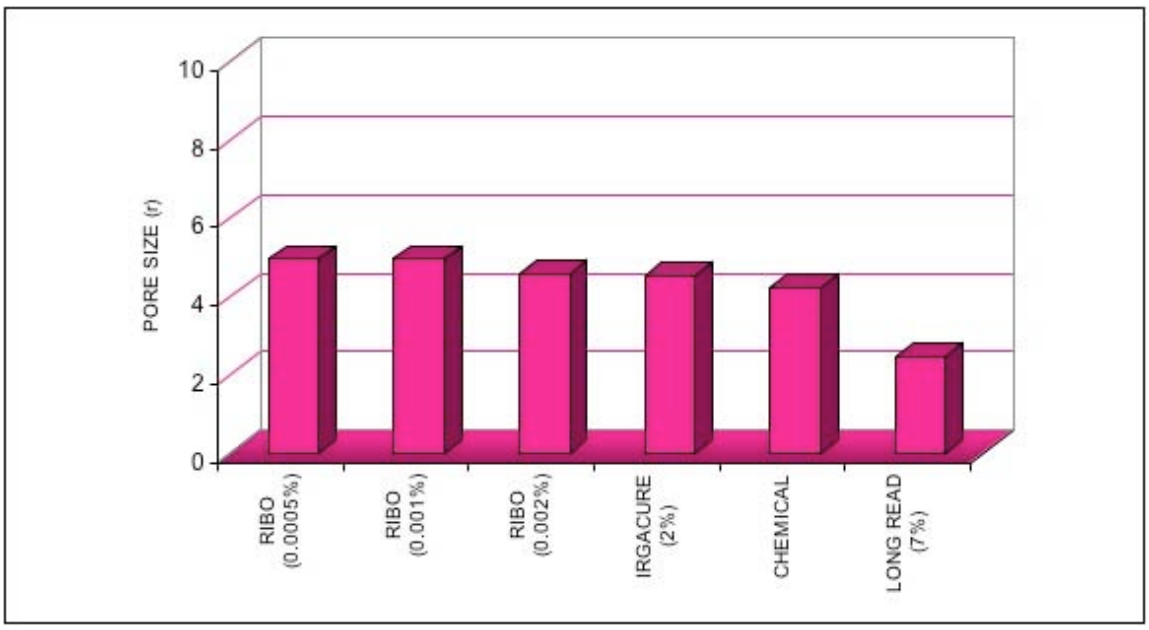


(c)

Figure 16. Continued

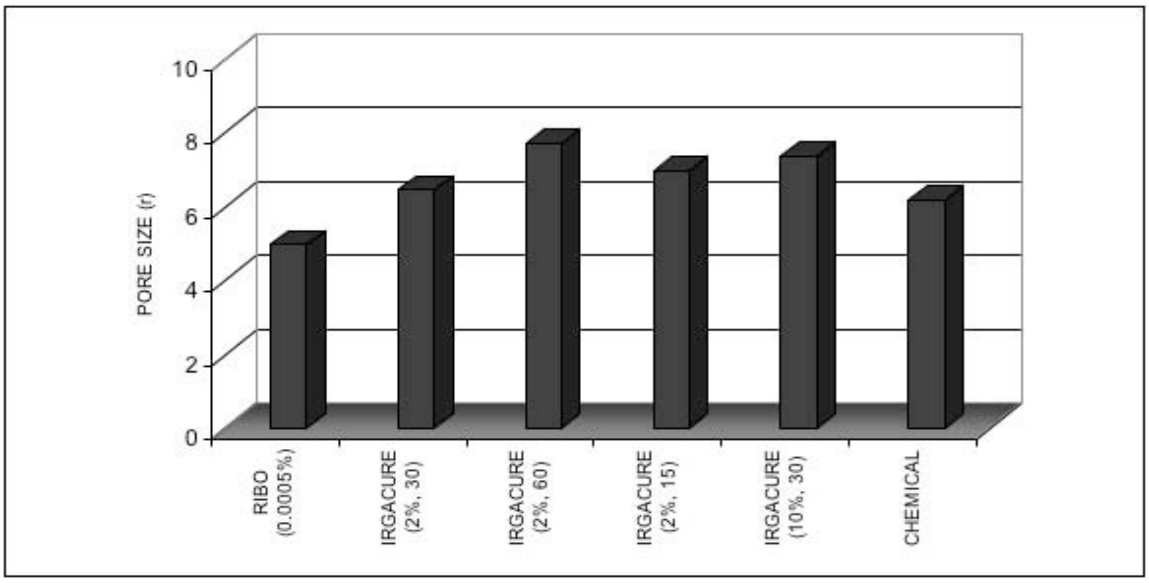


(d)

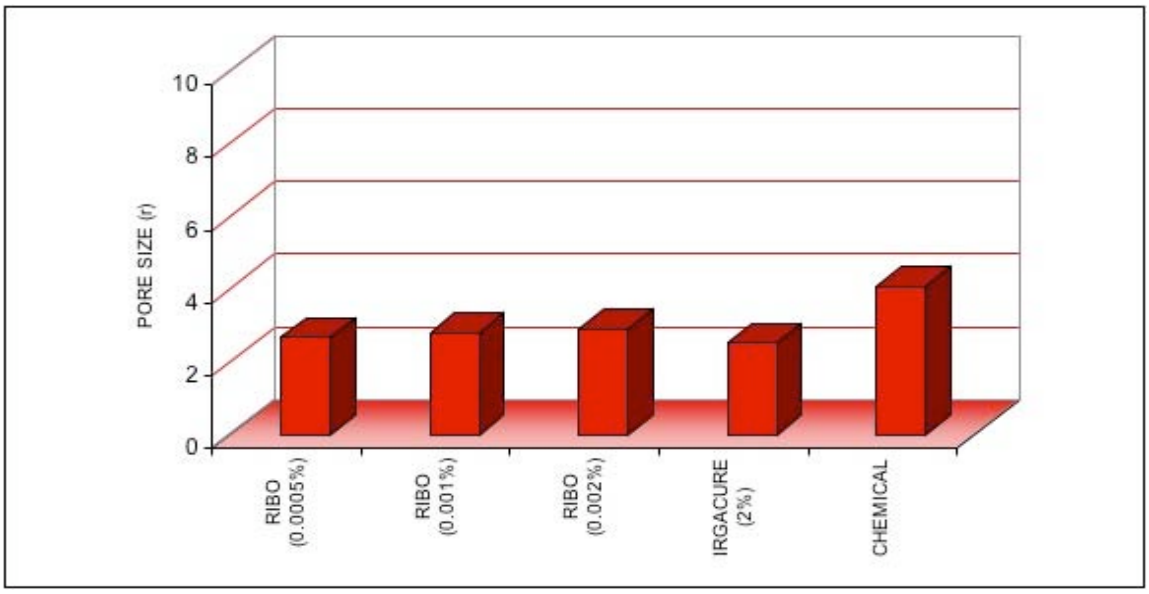


(e)

Figure 16. Continued



(f)



(g)

Figure 16. Continued

Run Time

Table 3 has been prepared for comparing the time taken by the various gels to obtain single stranded resolution. From the data in Table 4 it can be seen that Irgacure 651 gels require much less run time at low concentrations, though the resolution obtained is not good. This might be due to poor degree of cross-linking between the monomeric units resulting in large pore sizes and thus ineffective separation. It is also seen that as the gel concentration increases, the run time also increases. This holds true for Long Ranger, High Resolution, Long Read and powdered gel. In addition chemically polymerized runs take a longer time in comparison to other runs. This is because of the large time involved during polymerization, which is around 1-1.5 hours for chemical and only 10-15 minutes for photo-polymerized runs.

Cost Estimation

A rough cost estimation was performed for each of the gels. Only the cost of the reagents was taken into account. It was seen that powdered gels are cheaper than the ready made gels available in the market and the cost for performing chemical polymerization is slightly higher as compared to using photo-initiators. It is also seen that as the concentration of the gel increases the cost also increases. Table 4 shows the cost involved for performing various runs.

Table 3. Cost estimation for various gels ($ per run).

	Riboflavin				Urea+ Riboflavin			High Resolution+Riboflavin			Chemical
	Regular	.0005	.001	.002	.0005	.001	.002	.0005	.001	.002	
Repro High Resolution(HR)	7.4	7.4	7.4	7.4							7.47
LongRead	7.4	7.4	7.4	7.4							7.4
Gel powder (6%T,5%C)		3.88									3.94
Gel Powder (9%T,5%C)		4.13	4.13	4.13							4.19
Long Ranger-6%					5.01	5.01	5.02	7.46	7.46	7.46	7.53
Long Ranger-9%					5.84	5.84	5.84	8.28	8.28	8.28	8.35
Long Ranger-12%					6.66			9.1	9.1	9.1	9.17

Table 4. Run Time in minutes.

		Riboflavin			Urea+Riboflavin			High Resolution+Riboflavin			Chemical	Irgacure
	Regular	.0005	.001	.002	.0005	.001	.002	.005	.001	.002		
ReproHigh Resolution	175	200	175	200							240	200
Long Read	160	150	150	160							235	150
Gel Powder (6%T,5%C)		125									140	80
Gel Powder (9%T,5%C)		300	300	300							360	300
Long Ranger-6%					100	90	100	62	70	70	140	75
Long Ranger-9%					200	150	190	175	175	150	220	180
Long Ranger-12%					270			250	250	260	320	270

CHAPTER IV

CONCLUSIONS

Summary

Thus from the resolution calculations it is observed that:

1) Irgacure 651 does not give a good resolution. One of the reasons might be the run time is very short as compared to other photoinitiators, possibly due to poor crosslinking that results in larger pore size and hence inefficient separation.
2) Solution B (Reprogel Long Read and High Resolution crosslinker) does not give high values of resolution.
3) Riboflavin gives better resolution in the range .0005%-.002%.
4) In all the cases urea is better than Reprogel High Resolution B.
5) Separation runs could not be performed with Methylene Blue because it gives an opaque gel that is not transparent to red laser illumination.
6) It was also seen that gel (9%T, 5%C) gave high values of separation resolution in nearly all the cases. But the resolution curves obtained are not so smooth. This might be due to the decrease in transmittance due to increase in gel concentration.

From the mobility calculations the cut off values for the Ogston regime for various gels were Long Ranger 6%, 9%, 12%-380, 260, 240 bases, High Resolution 8%-320 bases, Long Read 7%-340 bases, powdered gel 6%, 9%-400, 240 bases. Here the cut off value for Ogston regime was taken at the point where the R^2 value for the linear fit

below 0.99. Ferguson plot gave a value of μ_o as $3.1 \pm .06 \times 10^{-4}$ cm^2/Vs for Long Ranger with riboflavin (.0005%) as initiator.

Future Work

Application of photo-initiator to polymerization is rather limited and deserves further exploration. It is seen that different photo-initiators in varying concentration result in changes of single stranded resolution under the same operating conditions. Morever temperature, field strength, buffer composition and degassing reagents also affect the separation performance. The influence of these parameters coupled with the various photo-initiators will be required to elucidate the effects on DNA separation that will help to design matrices suitable for microchip electrophoresis, which might impart a high resolution during electrophoresis.

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APPENDIX A

PROCEDURE FOR RUNNING ALF EXPRESS II DNA SEQUENCER

To run a sequencing process:

1. Take 100ml of 10X TBE and dilute it to 2000ml (0.5X).
2. Stir the TBE solution to mix it well.
3. Dilute acetic acid (glacial) to 10% in a 10ml tube. Remember to label the tube with date and chemical name.
4. Clean the sequencer plate

   First wash it with regular tap water and then rinse it with DI water. Finally, use kimwipe to clean it with ethanol and leave for drying in air. Note that only alconox can be used and no other commercial detergents. Use of a sink pad can reduce the probability of breaking the plate.
5. Mix 1000 microliters of bind silane and 250 microliters of acetic acid in an eppendorf tube.
6. Apply bind silane onto the bottom plate to fix the comb.

   Use kimwipe to spread it.
7. Put on the gel plate clips. The side with rubber is up.
8. Insert the comb between the top and bottom plates.
9. Gel casting

   Check the desk with a bubble level. Make sure it does not tilt. Mix portion A and B, and then pour the mixture into the bottom channel on the plate. Tap the plate

gently so that the monomer can be evenly distributed between the top and bottom plates. The wells of the comb must be completely filled with the gel monomer. Avoid bubbles in the gel. Leave unused gel monomers in the UV machine and turn on the lamp to initiate gel polymerization for 10 minutes. After that, remove the unused portions. Note that the gel monomer is highly toxic.

10. DNA and dye attachment

    Mix 20 microliters of DNA sample and 20 microliters of CY5 dye in a thin walled eppendorf tube. Then heat the mixture in the PCR machine to 95°C and maintain it for 5 minutes. After heating, cool the mixture in an ice bath.

11. Pour buffer solution into the lower reservoir in the sequencer to the marked level (about 1000ml).

12. Place the gel plate

    Make sure that the buffer solution is in touch with the gel.

13. Pour the buffer solution into the upper reservoir.

    Make the black line on the plate aligned with the white one on the machine.

14. Insert the electrodes (black to black and red to red).

15. Start the sequencer from the ALF program.

    Go to the “file” menu and select the “start sequencer”. Open a new casebook and then hit the “preset” button. Keep the laser beam in the middle of the sensors and then check the transmittance reading. It is required to be over 70%. Realign the laser beam if the transmittance is too low.

16. Sample loading.

Wait until the system reaches the operating temperature (about 55°C). Before loading, flush each lane with the buffer solution to wash out unwanted particles. Use loading tips to load samples and remember to change tips between lanes.

17. Press the “start” button in the program window.

    Check the logbook file if the machine is shutdown unexpectedly. Note that the gel integrity starts to degenerate in 24 hours.

To unload a finished process:

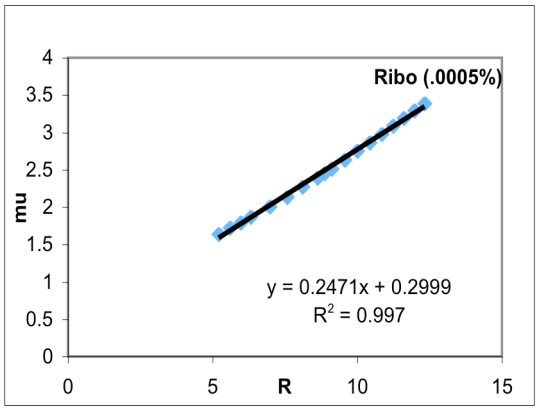
1. Remove the 2 electrodes (black and red) and wash them with DI water.
2. Remove buffer pipes.
3. Remove the thermoplates from the sequencer.
4. Take off all plate clips and rinse them with DI water.
5. Wait until the plates become lukewarm and wet them with floating water.
6. Push outward gently to remove the cover plate.
7. Peel off the spacers, rinse them with DI water and put them back in the storage box.
8. Use a brush to scrub the gel under running water and then rinse with DI water. Note that no detergent is needed here.

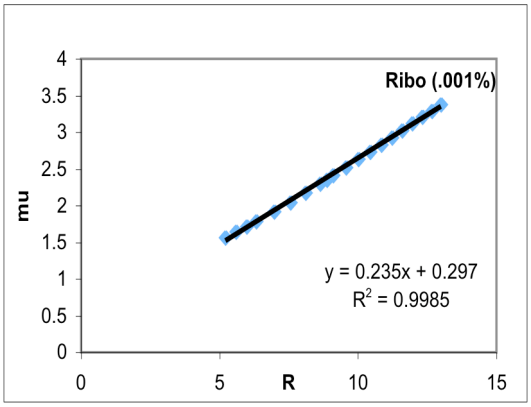
Wash the bottom plate in the same way.

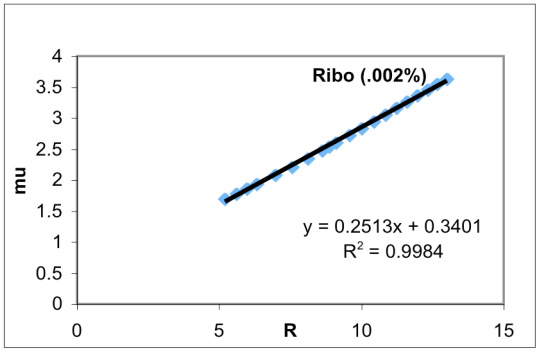
APPENDIX B

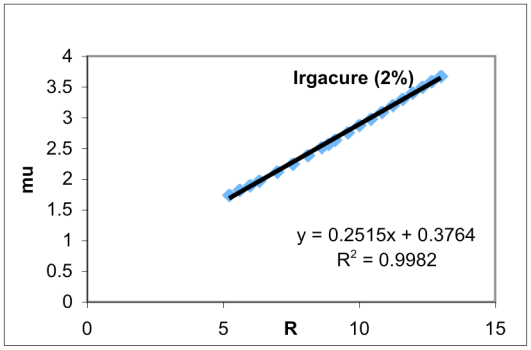
MERCIER SLATER FITS TO MOBILITY DATA FOR ALL GEL SAMPLES STUDIED

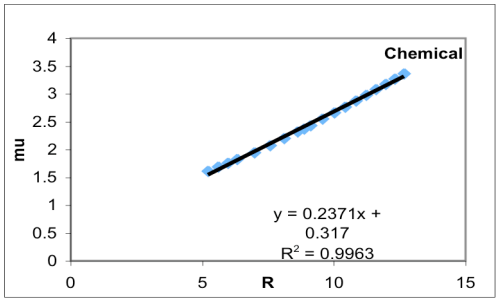
High Resolution (8%)



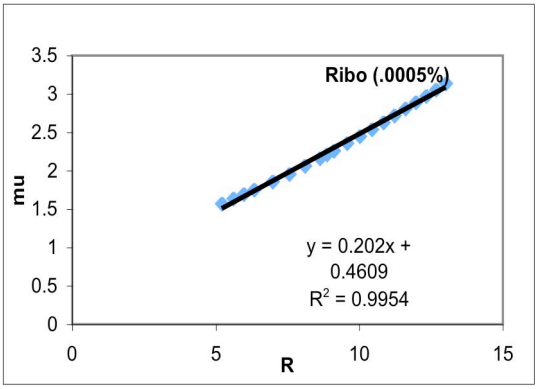


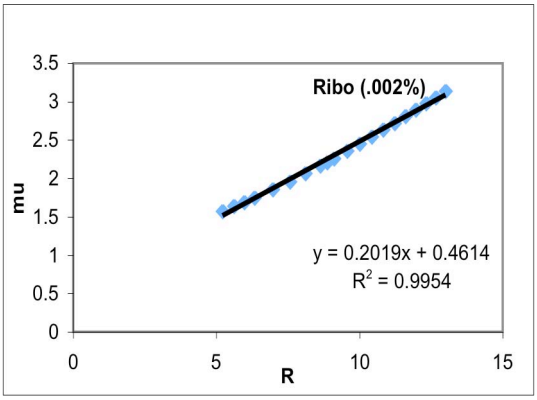


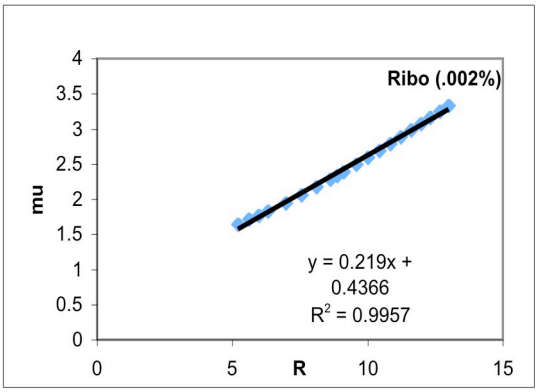


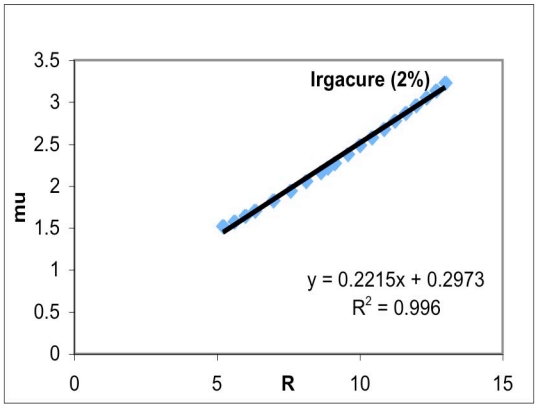


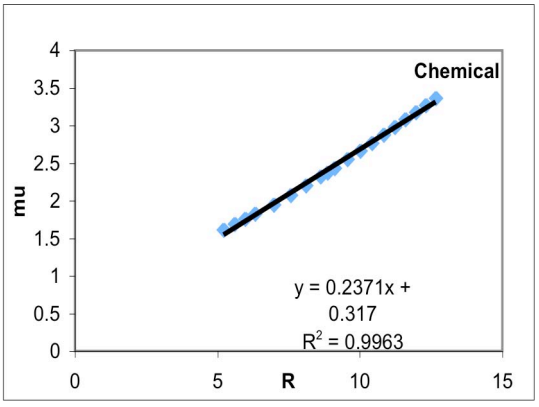
Long Read (7%)


Ribo (.0005%)
y = 0.202x + 0.4609
R² = 0.9954
mu
R

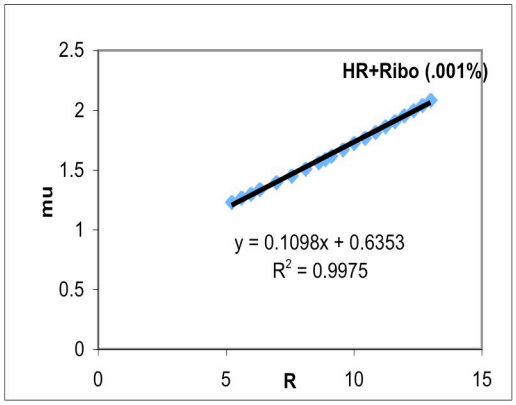

Ribo (.002%)
y = 0.2019x + 0.4614
R² = 0.9954
mu
R

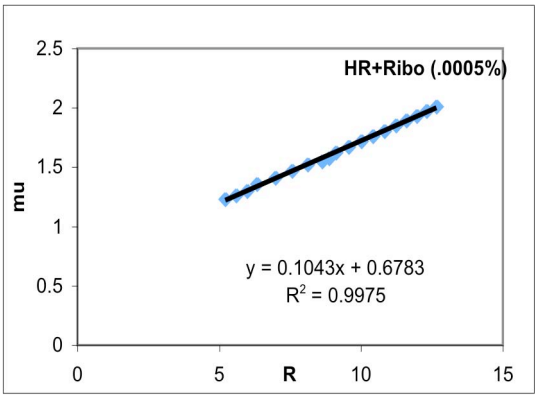

Ribo (.002%)
y = 0.219x + 0.4366
R² = 0.9957
mu
R

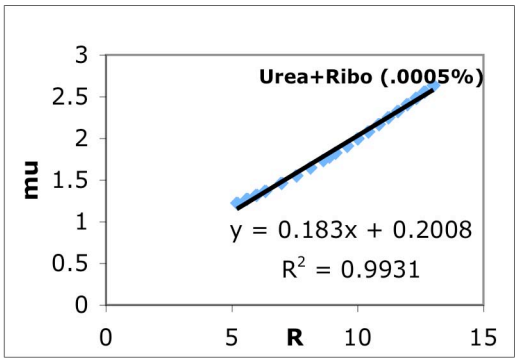

Irgacure (2%)
y = 0.2215x + 0.2973
R² = 0.996
mu
R

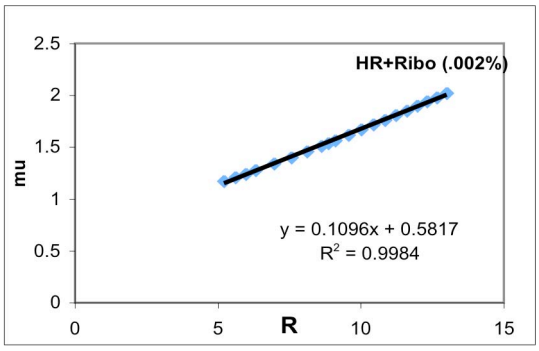

Chemical
y = 0.2371x + 0.317
R² = 0.9963
mu
R

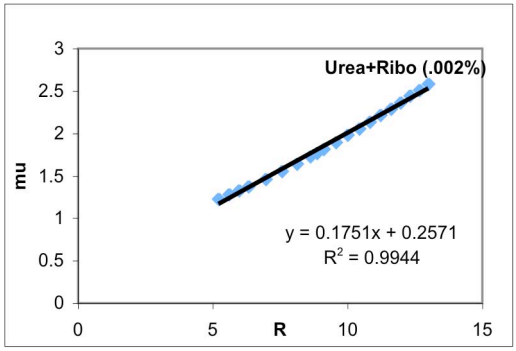
Long Ranger (6%)

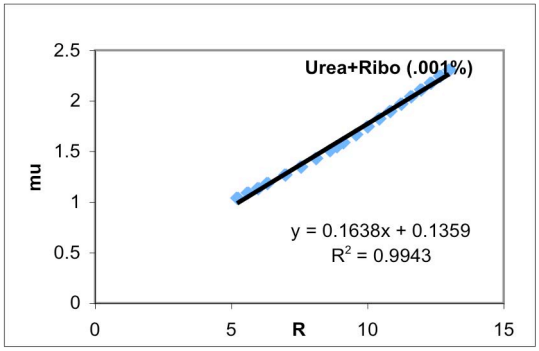


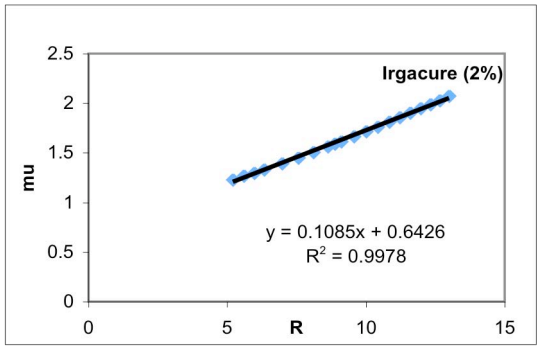


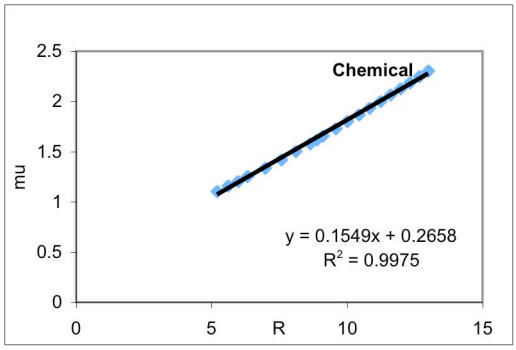




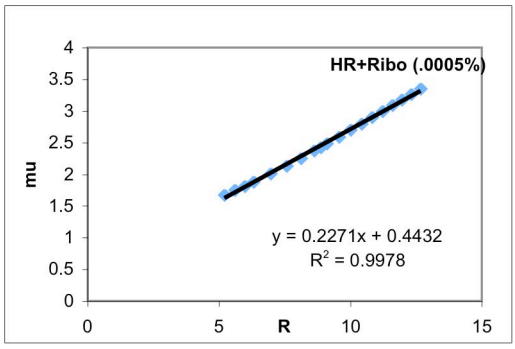


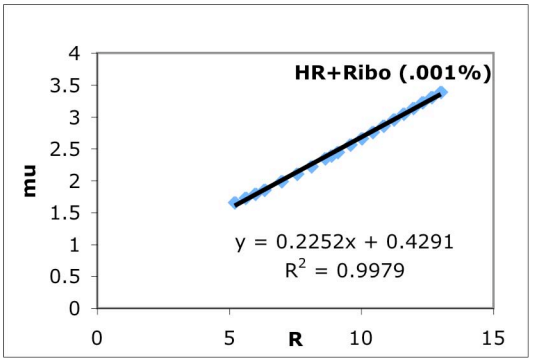


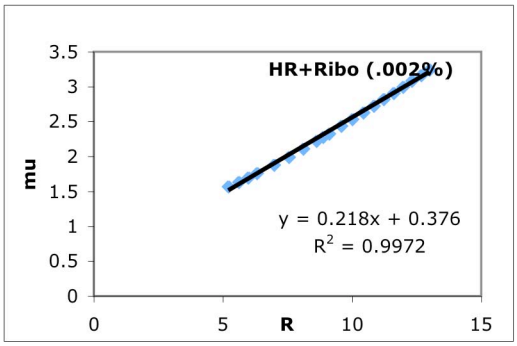


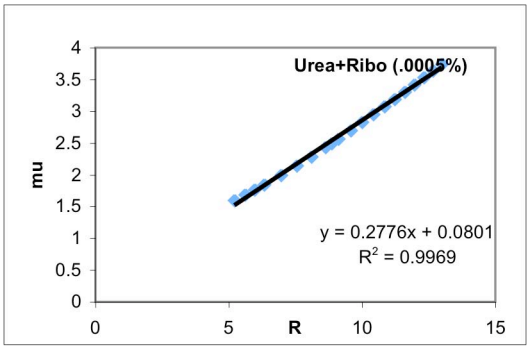


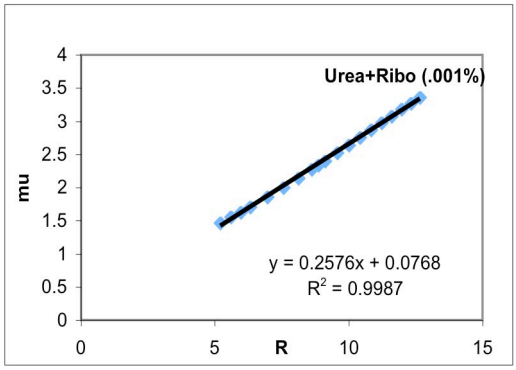
Long Ranger (9%)

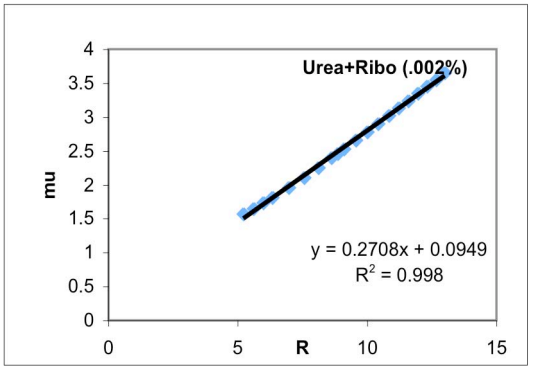


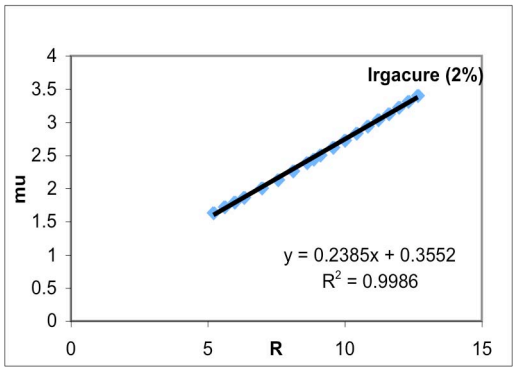


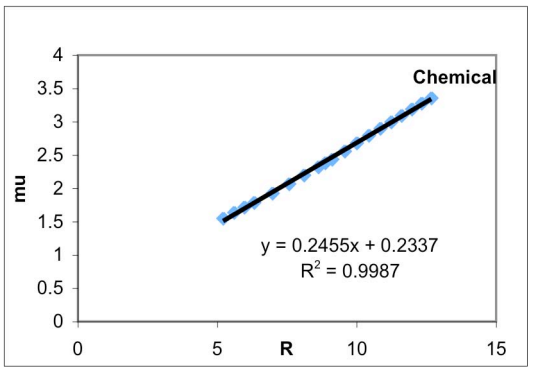




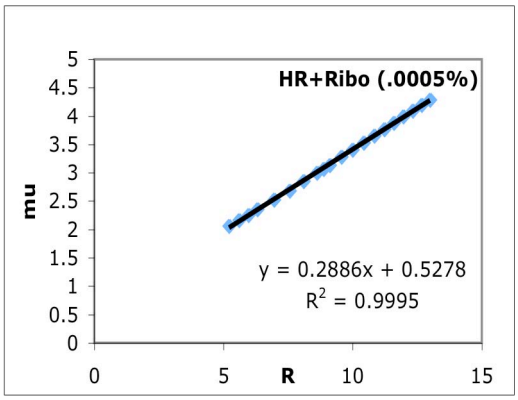


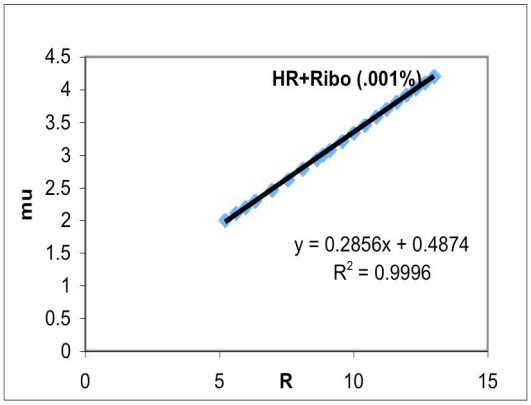


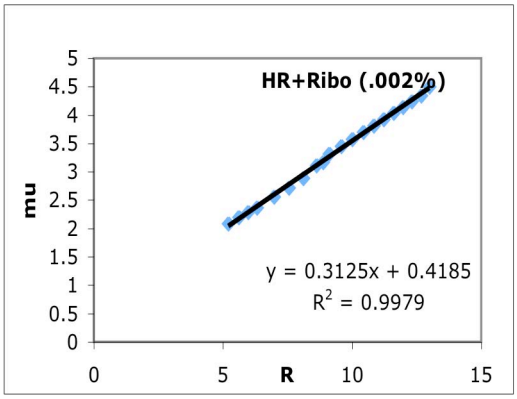


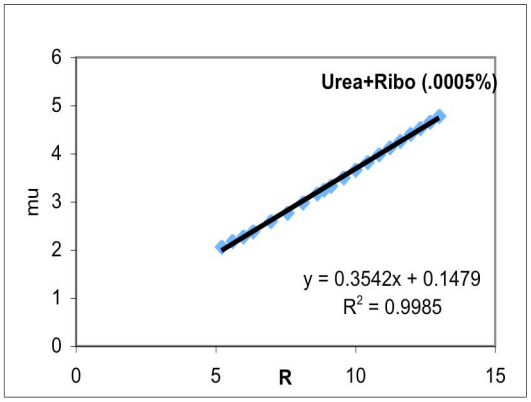


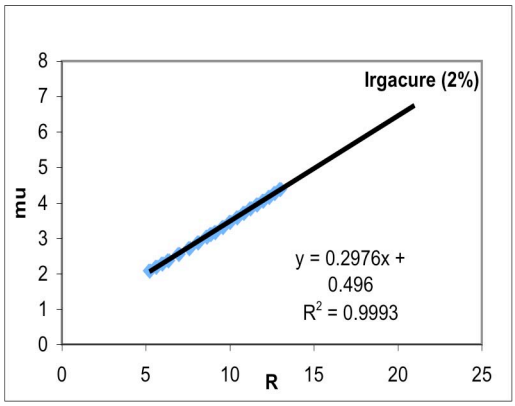
Long Ranger (12%)



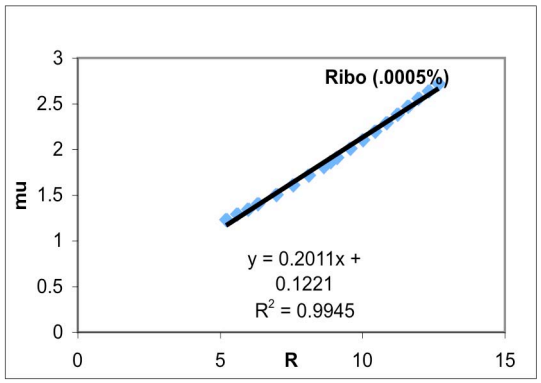


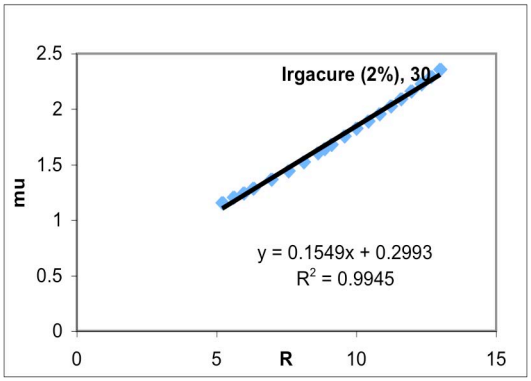


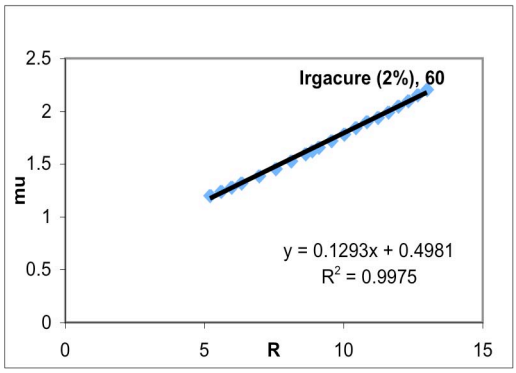


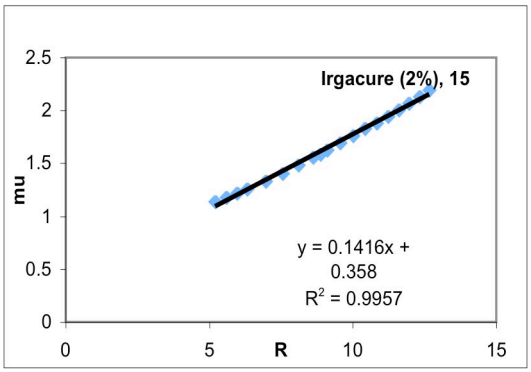


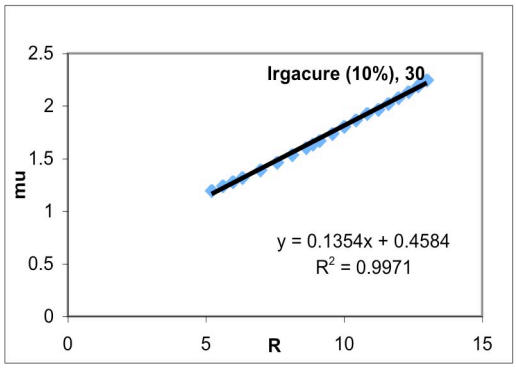
Gel (6%T, 5%C)

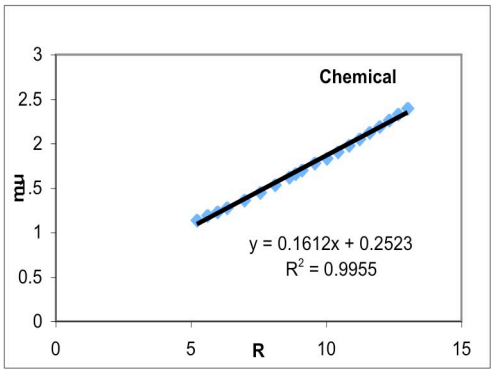

Ribo (.0005%)
y = 0.2011x + 0.1221
R² = 0.9945
mu
R

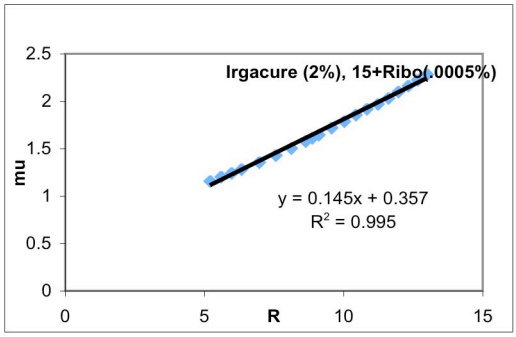

Irgacure (2%), 30
y = 0.1549x + 0.2993
R² = 0.9945
mu
R


Irgacure (2%), 60
y = 0.1293x + 0.4981
R² = 0.9975
mu
R

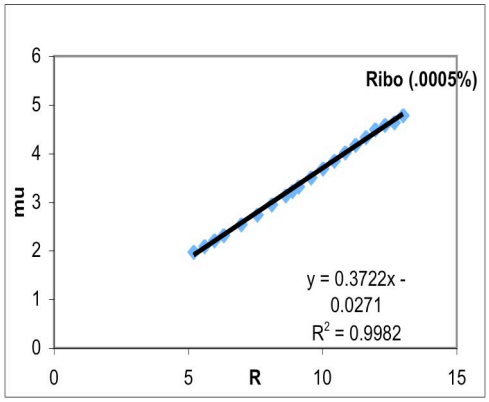

Irgacure (2%), 15
y = 0.1416x + 0.358
R² = 0.9957
mu
R

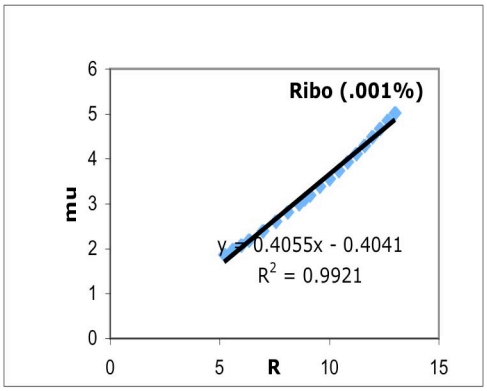

Irgacure (10%), 30
y = 0.1354x + 0.4584
R² = 0.9971
mu
R

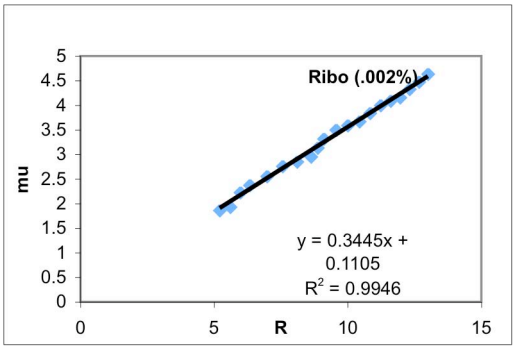

Chemical
y = 0.1612x + 0.2523
R² = 0.9955
mu
R

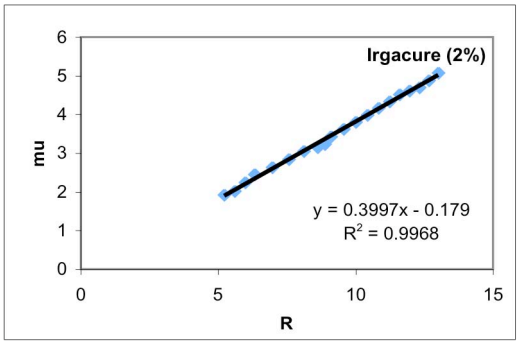

Irgacure (2%), 15+Ribo(.0005%)
y = 0.145x + 0.357
R² = 0.995
mu
R

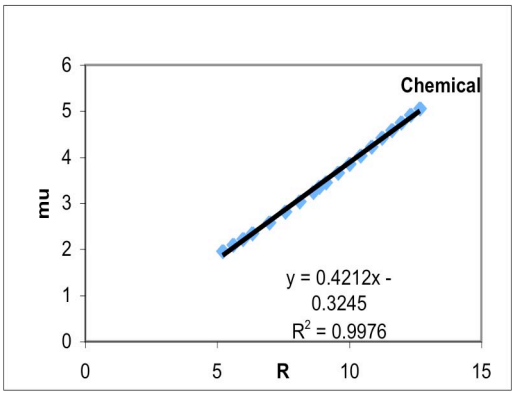
Gel (9%T, 5%C)


Ribo (.0005%)
mu
R
y = 0.3722x - 0.0271
R² = 0.9982


Ribo (.001%)
mu
R
y = 0.4055x - 0.4041
R² = 0.9921


Ribo (.002%)
mu
R
y = 0.3445x + 0.1105
R² = 0.9946


Irgacure (2%)
mu
R
y = 0.3997x - 0.179
R² = 0.9968


Chemical
mu
R
y = 0.4212x - 0.3245
R² = 0.9976

VITA

Shilpa Agrawal was born in Rourkela, India on December 22, 1981, to Sunita and A.N.Agrawal. After completing her schooling from Carmel School in Rourkela she joined Ispat English Medium School. She then pursued her chemical engineering at National Institute of Technology, Rourkela from 1999 to 2003 receiving her B.Tech degree in 2003. In Fall 2003 she entered Texas A&M University to pursue her graduate studies in chemical engineering.

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