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Reversible Phospholipid Nanogels: An Alternative Matrix for Tunable High Resolution DNA Sieving with Enhanced Separation Efficiency and Accurate Extended Range Sizing by Capillary Gel Electrophoresis

Brandon C. Durney

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Reversible Phospholipid Nanogels: An Alternative Matrix for Tunable High Resolution DNA Sieving with Enhanced Separation Efficiency and Accurate Extended Range Sizing by Capillary Gel Electrophoresis

By: Brandon C. Durney

Dissertation submitted to The Eberly College of Arts and Sciences at West Virginia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

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Reversible Phospholipid Nanogels: An Alternative Matrix for Tunable High Resolution DNA Sieving with Enhanced Separation Efficiency and Accurate Extended Range Sizing by Capillary Gel Electrophoresis

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Abstract:

In an aqueous solution the phospholipids dimyristoyl-*sn*-glycero-3-phosphocholine (DHPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DMPC) self-assemble to form thermo-responsive non-Newtonian fluids (i.e., pseudo-gels) in which small temperature changes of 5-6 °C decrease viscosity dramatically. This characteristic is useful for sieving-based electrophoretic separations (e.g., of DNA), as the high viscosity of linear sieving additives, such as linear polyacrylamide or polyethylene oxide, hinders the introduction and replacement of the sieving agent in microscale channels. While a practical advantage of utilizing phospholipid pseudo-gels for sieving is the ease with which they are introduced into the separation capillary, the separation performance of the material for DNA analyses is exceptional. Capillary electrophoresis separations of DNA are achieved with separation efficiencies ranging from 400,000 to 7,000,000 theoretical plates in a 25 micrometer inner diameter fused silica capillary. Assessment of the phospholipid nanogel with a Ferguson plot yields an apparent pore size of ~31 nm. Under isothermal conditions, Ogston sieving is achieved for DNA fragments smaller than 500 base pairs, whereas reptation-based transport occurs for DNA fragments larger than 500 base pairs. Single base resolution of short tandem repeats relevant to human identification is

accomplished with 30 minute separations using traditional capillary electrophoresis instrumentation. Applications that do not require single base resolution are completed with faster separation times. This is demonstrated for a multiplex assay of biallelic single nucleotide polymorphisms relevant to warfarin sensitivity. The thermo-responsive pseudo-gel preparation described here provides a new innovation to sieving based capillary separations. Specific DMPC-DHPC medium is developed to effectively separate and size DNA fragments up to 1,500 base pairs by decreasing the total lipid concentration to 2.5%. A 2.5% phospholipid nanogel generates a resolution of 1% of the DNA fragment size up to 1,500 base pairs. This separation additive is used to evaluate size markers ranging between 200 and 1,500 base pairs in order to distinguish invasive strains of *Streptococcus pyogenes* and *Aspergillus* species by harnessing differences in gene sequences of collagen-like proteins in these organisms. For the first time, a reversible stacking gel is integrated in a capillary sieving separation by utilizing the thermally-responsive viscosity of these self-assembled phospholipid preparations. A discontinuous matrix is created that is composed of a cartridge of highly viscous phospholipid assimilated into a separation matrix of low viscosity. DNA sample stacking is facilitated with longer injection times without sacrificing separation efficiency.

Reversible Phospholipid Nanogels: An Alternative Matrix for Tunable High Resolution DNA Sieving with Enhanced Separation Efficiency and Accurate Extended Range Sizing by Capillary Gel Electrophoresis

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List of Symbols and Abbreviations

1. CE : capillary electrophoresis
2. i.d. : inner diameter
3. DNA : deoxyribonucleic acids
4. LOC : Lab-on-a-chip
5. CGE : capillary gel electrophoresis
6. bp : base pairs
7. dsDNA : double stranded DNA
8. PCR : polymerase chain reaction
9. dNTPs : deoxynucleotide triphosphates
10. E_{ph} : electrophoretic mobility
11. EOF : Electroosmotic flow
12. R_s : chromatographic resolution
13. LPA : linear polyacrylamide
14. PDMA : polydimethylacrylamide
15. HEC : hydroxyethylcellulose
16. PVP : polyvinylpyrrolidone
17. cP : centipoise
18. STRs : short tandem repeats
19. POP™ : performance optimized polymer
20. MLVA : multi-locus variable number analysis
21. VNTRs : variable number tandem repeats
22. SSCP : single strand conformation polymorphism

23. Da : Dalton
24. PHEA : polyhydroxyethylacrylamide
25. PEG : polyethylene glycol
26. PVA : polyvinyl alcohol
27. ELFSE : end-labeled free-solution electrophoresis
28. SNPs : single nucleotide polymorphisms
29. CODIS : FBI Combined DNA Index System
30. FAM : 6-carboxyfluorescein
31. DMPC : dimyristoyl-*sn*-glycero-3-phosphocholine
32. DHPC : 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine
33. PEO : polyethylene oxide
34. HPC : hydroxypropyl cellulose
35. MeOH : methanol
36. MOPS : 3 - (N-morpholino) - propane sulfonic acid
37. NaOH : sodium hydroxide
38. diH₂O : deionized water
39. LIF : laser induced fluorescence
40. o.d. : outer diameter
41. UV-vis : UV visible absorbance
42. Pa : Pascals
43. R_g : Radius of gyration
44. K_r : retardation coefficient
45. ξ : apparent pore size

- 46. L_{tot} : total length
- 47. L_{eff} : effective length
- 48. E_{app} : electric field strength
- 49. A : ampere
- 50. V_{app} : applied voltage
- 51. RFU : relative fluorescence units
- 52. EtBr : ethidium bromide
- 53. K_d : dissociation constant
- 54. HPMC : hydroxypropylmethyl cellulose

**Reversible Phospholipid Nanogels: An Alternative
Matrix for Tunable High Resolution DNA Sieving with
Enhanced Separation Efficiency and Accurate Extended
Range Sizing by Capillary Gel Electrophoresis**

By: Brandon C. Durney

Chapter 1

Capillary Electrophoresis Applied to DNA Analysis

Reproduced from [1]:

Durney, B.C., C.L. Crieffeld, and L.A. Holland, *Capillary Electrophoresis Applied to DNA and RNA: Determining and Harnessing Sequence, Modifications, or Structure to Advance Bioanalyses (2009-2014)*. Analytical and Bioanalytical Chemistry, submitted.

1.0 Introduction

Capillary electrophoresis (CE) separations are significant because they provide fast separations of limited sample volumes. Following reports of outstanding separation efficiencies of amines, amino acids, and peptides achieved using a 75 micrometer inner diameter (i.d.) glass capillary [2, 3], the technology was quickly adapted to the analysis of deoxyribonucleic acids (DNA) [4, 5]. The growth in the use of CE for DNA analyses is best illustrated by the number of annual journal publications, which is summarized in Figure 1-1. Critical innovations reported early in the method development [6-11] dramatically increased the applicability to sequence and size DNA fragments. Landmark applications include genome sequencing [12], forensic analysis of DNA with commercial systems [13, 14], and integrated lab-on-a-chip (LOC) platforms [15-18].

Figure 1-1: Trend for the Number of Publications on Capillary Electrophoresis and DNA Based on SciFinder® Database. Reproduced from [1].

As the technology matures, research driven advances have been transformed into heavily utilized applications, generating a large user-base focused on applying the methodology. Perhaps the best indicator of progress in the field of CE DNA separations is the translation of this technology into the teaching laboratory [19, 20]. CE separations of DNA have been integrated in teaching exercises in genomic identification of food with a commercial chip [19]. Despite the maturity of this technology innovative research and new applications are still reported.

This report summarizes the methodology and optimization of an alternative separation matrix composed of phospholipid additives for applications based on DNA analyses. The peer-reviewed publications reproduced in this dissertation are listed below:

Chapter 1:

Durney, B. C., Carihfield, C., Holland, L. A.; Capillary Electrophoresis Applied to DNA: Determining and Harnessing Sequence and Structure to Advance Bioanalyses (2009-2014); Analytical Bioanalytical Chemistry **(2015)** accepted.

Chapter 2:

Durney, B. C., Lounsbury, J. A., Poe, B. L., Landers, J. P., Holland, L. A.;
A Thermally Responsive Phospholipid Pseudogel: Tunable DNA Sieving
with Capillary Electrophoresis; *Analytical Chemistry* **(2013)** 85, 6617-6625.

Chapter 3:

Durney, B. C., Bachert, B. A., Sloane, H. S., Lukomski, S., Landers, J. P.,
Holland, L. A.; Reversible Phospholipid Nanogels for Deoxyribonucleic
Acid Fragment Size Determinations up to 1,500 Base Pairs and Integrated
Sample Stacking; *Analytica Chimica Acta* **(2015)** accepted.

Tuntevski, K., Durney, B. C., Snyder, A. K., LaSala, P. R., Nayak, A. P.,
Green, B. J., Beezhold, D. H., Rio, R. V. M., Holland, L. A., Lukomski, S.;
Aspergillus Collagen-Like Genes (*acI*): Identification, Sequence
Polymorphism, and Assessment for PCR-Based Pathogen Detection;
Applied and Environmental Microbiology **(2013)** 79, 24, 7882-7895.

This dissertation contains three chapters detailing a phospholipid capillary gel electrophoresis (CGE) method used in DNA analysis. Chapter 1, describes CE methodology as it applies to gel facilitated DNA separations, and the pivotal applications for how DNA can be harnessed for identifying humans and pathogens. The most prevalent gel matrices used for these DNA applications are

discussed in detail to emphasize the significance and innovation in the work discussed in the following chapters. Chapter 2, describes the use of phospholipid additives as an inexpensive alternative to commonly implemented sieving gels due to its optimal separation performance. The nanogel performance at isocratic and gradient temperature separations is also discussed. Chapter 3, outlines an approach for the use of a nanogel matrix for the identification of pathogens and has enhanced sizing ability for DNA fragments longer than 500 base pairs (bp). The use of a discontinuous nanogel matrix is also described resulting in the enhancement of DNA sample stacking upon electrokinetic injection prior to capillary nanogel electrophoresis separations of DNA fragments.

1.1 Background on DNA

1.1.1 Genomic DNA

Within eukaryotic cells, the nucleus stores DNA, which encodes for information necessary for life. Understanding the genome sequence is pivotal to studying protein function. Genetics are advanced by detailed knowledge of the genome. Except for reproductive cells (i.e., the egg and the sperm), every cell in the human body is diploid, containing copies of genes originating from each parent. This role of DNA in life is harnessed through bioanalyses to treat diseases,

develop new therapeutics, test for paternity, identify humans, or detect pathogens.

1.1.2 Fundamentals of DNA

DNA is a biopolymer that encodes for proteins that comprise a living organism.

The nucleotide monomers of DNA are comprised of a saccharide, or ribose sugar, a phosphate ester, and one of four nucleic acid monomers of similar molecular weight, which are adenine 135 amu, guanine 151 amu, cytosine 111 amu, or thymine 126 amu. Base pairing is stabilized by hydrogen bonds. The purine, adenine, pairs with the pyrimidine, thymine, resulting in two hydrogen bonds. The other purine, guanine, pairs with the second pyrimidine, cytosine, generating three hydrogen bonds. The size of each paired base is similar as the combined mass of adenine-thymine or of guanine-cytosine are 261 and 262 amu, respectively. The nucleotides are polymerized in a single strand as a result of a phosphodiester bond at the 3' carbon of one ribose residue and the 5' carbon of a second ribose residue. The nucleotides within a single strand of DNA can pair with complementary nucleotides to form double stranded DNA according to Chargaff's rule (i.e., adenine pairs with thymine, and guanine pairs with cytosine). The double stranded DNA (dsDNA) adopts a double helix with the negatively charged phosphodiester on the exterior of the helix and the DNA bases forming hydrogen bonds within the interior. On each strand every monomer carries a single negative charge on the phosphate backbone. When mass correlates with

DNA size, the charge-to-size ratio is the same for DNA fragments regardless of length.

1.1.3 DNA Amplification – Polymerase Chain Reaction

The complementary pairing of adenine to thymine and guanine to cytosine enables DNA amplification through a process known as polymerase chain reaction (PCR), which was invented and then reported [21] shortly before CE separations were applied to DNA. The three phases of a PCR cycle are (1) melting (or denaturing) the DNA, (2) annealing the primers to the template, and (3) polymerization of the nucleotides to form the new copy. The reaction requires the presence of DNA primers, DNA polymerase, and free deoxynucleotide triphosphates (dNTPs). In the first cycle of PCR, the original template is melted to separate the complementary strands of DNA at approximately 95°C. The temperature is reduced to allow primers to anneal to a specific site on each strand of the template by complementary base recognition. The temperature is then increased to 72°C to enable DNA polymerase to ligate the nucleotides that base pair to the template. During this phase, the complementary strand is formed. The three steps of PCR are repeated by cycling the temperature. Exponential replication of the template sequence occurs at a rate of approximately 2^n , where n is the number of PCR thermal cycles. With the ability to amplify DNA fragments with high fidelity, separation and identification of these fragments can be accomplished using CE.

1.2 Separation

CE is a high throughput separation method commonly employed for DNA analysis due to rapid analysis times and small sample volumes. Free-zone and gel-facilitated sieving are the most commonly reported modes implemented for DNA analyses, and are summarized in Table 1-1.

Table 1-1: Capillary/Microchip Electrophoresis Mechanisms
--

Free solution / electrophoretic mobility

Mechanism:

Electrophoretic mobility (i.e. charge-to-size ratio of fragment)

Attributes

- + Simple to implement
- Poor separation of similar DNA charge-to-size ratio

Application:

DNA aptamer with affinity for protein

Ogston sieving: sieving through a gel with pores $\geq$ DNA Radius of Gyration

Mechanism:

Electromigration through pores; size determines probability of impeded motion

Attributes

- + Relationship between length and migration linear
- + Sizing ± 1 base
- Limited to fragments $< 1,000$ nucleotides

Application:

Sizing for human and pathogen identification, or disease markers
Accurate sequencing to support parallel sequencing methods

Reptation: sieving through a gel with pores $\leq$ DNA Radius of Gyration

Mechanism:

Electromigration of DNA through pores requires deformation; nonlinear correlation between size and time to traverse gel

Attributes

- + Suited for fragments $> 1,000$ nucleotides
- Size precision poor

Application:

Coarse sizing/fingerprinting, comet assays

Table Reproduced from [1].

The various modes of CE, which are depicted in Figure 1-2, are used depending upon the specific DNA application being investigated. These modes of separation will be discussed in greater detail in the sections that follow.

Figure 1-2: Mechanisms of DNA Separations in Capillary Electrophoresis

1-2 A. Electrophoretic mobility

1-2 B. Ogston sieving

1-2 C. Reptation

Figure 1-2: A depiction of capillary electrophoresis separation mechanisms for DNA analysis. **(A)** Free zone CE, DNA migrates based on electrophoretic mobility. DNA molecules of all lengths have similar migration times. **(B)** In Ogston sieving, small DNA fragments migrate faster than large. **(C)** Reptation occurs when DNA must take a more linear conformation to migrate through pores created by the gel separation matrix. Reproduced from [1].

1.2.1 Capillary Electrophoresis

Free-zone CE is the most fundamental mode of separation (see Fig 1-2A) and is based on the sum of the electrophoretic mobility (E_{ph}) of the analyte and the electroosmotic flow (EOF), when it is present. The charge-to-size ratio of an analyte determines the E_{ph} . The charged analyte is attracted to the electrode of opposite charge. Anions move towards the anode, while cations move toward the cathode. The charge of the analyte dictates the attractive force driving the electrophoretic transport and the size of the molecule affects the frictional drag. EOF is caused by the negatively charged surface of glass or fused silica used for the separation. Cations accumulate at the negative surface and in the presence of an electric field migrate toward the cathode, creating a bulk fluid flow. Under normal polarity, the direction of fluid flow is from the anodic reservoir toward the cathodic reservoir. Injection occurs at the anode and detection occurs at the cathode. Reversed polarity separations generate an EOF directed from the cathodic reservoir to the anodic reservoir. EOF is typically stronger than E_{ph} , and separations performed using reversed polarity are limited to negatively charged analytes only. In reversed polarity separations, the EOF is frequently suppressed, usually through chemically modifying or dynamically coating the surface.

Free-zone CE is easily implemented since the only requirement is a buffer and an understanding of the charge-to-size ratios of the analytes. Molecules with significantly different electrophoretic mobilities are easily separated. However, as the charge-to-size ratio of two molecules becomes more similar, the E_{ph} of each molecule becomes more difficult to distinguish. Unfortunately, free-zone CE methods for DNA separations are limited because of the similar charge-to-size ratio of fragments of different length. To circumvent this problem gels are incorporated in CE separations to sieve DNA fragments based on size.

1.2.2 Gel – facilitated sieving

Gel-facilitated sieving is one of the most commonly used methods for sequencing DNA in parallel analyses and sizing. EOF is suppressed by coating the surface of the capillary, and then the capillary is filled with sieving gel. Ogston sieving and reptation are the mechanisms of DNA transport through the gel. In Ogston sieving shown in Figure 1-2B, DNA behaves as an incompressible sphere. The separation, which occurs with suppressed EOF under reversed polarity, is based on the ability of DNA to pass unobstructed through the pores formed by the gel matrix. Smaller fragments of DNA migrate faster than longer fragments, and a linear relationship is observed between fragment size and migration time. Both sequencing and sizing are conducted within the Ogston regime. Reptation, which is depicted in Figure 1-2C, occurs when a DNA molecule is too large to pass freely through the pores of the gel and must deform or unfold to fit through

the matrix. With this mode of sieving, the relationship between migration time and DNA fragment size is non-linear and peak resolution is worse, which makes sizing difficult. The fragment size at which the separation transitions from Ogston sieving to reptation can be approximated with a DNA size ladder and determined experimentally. A variety of gel matrices for DNA separations have been reviewed [22-28].

1.2.3 Characteristics of DNA sieving gels

Prevalent matrices used for sequencing or sizing DNA fragments are summarized in Table 1-2. The factors that determine which separation matrix is utilized for a specific application are included in the table.

Table 1-2: Gels used for DNA sequencing and sizing applications

Linear polyacrylamide (LPA)

- + Low cost
- + 2-3 base resolution up to 1,000+ nucleotides in 1 to 2 hours
- High viscosity (e.g. ~260,000 cP at 2%)
- Not self-coating

Polydimethyl-acrylamide (PDMA)

- + Self-coating
- High cost

POP-4™ for sizing applications

- + 1 base resolution up to 250, 2 base up to 350 nucleotides in 30 minutes
- + Low viscosity (e.g. <400 cP at 4%)

POP-7™ for sequencing applications

- + 2-3 base resolution up to 950 nucleotides in 2 hours
- + Low viscosity (e.g. ~400 cP at 6.5%)

Hydroxy-ethyl cellulose (HEC) + Polyvinylpyrrolidone (PVP)

R = H or CH₂CH₂OH
(HEC)

(PVP)

HEC + PVP for microfluidic sizing applications

- + Self-coating
- + Low cost
- + Low viscosity (e.g. ~400 cP at 3.5%)
- + 1 base resolution up to 200, 2-3 base up to 400 nucleotides in 10 minutes

HEC

- + Low cost
- + Low viscosity (e.g. ~3,000 cP at 4%)
- 2-3 base resolution up to 570 nucleotides in 70 minutes
- Batch variability, not synthesized
- Minimal self-coating ability

PVP

- + Self-coating
- + Low cost
- + Very low viscosity (e.g. ~15 cP at 4%)
- 2-3 base resolution up to 500 nucleotides in 90 minutes

Table Reproduced from [1].

The separation performance, which is the most critical figure of merit of a sieving gel, is measured by the chromatographic resolution (R_s) and the upper size limit for Ogston sieving that can be obtained in a given analysis time.

Chromatographic peak resolution (R_s), is defined as $R_s = (\Delta t)/W_{ave}$, where Δt is the difference in migration times of two adjacent peaks and W_{ave} is the average width of the peak at the base (estimated as 4σ) [29, 30]. Resolution is more often calculated using the width at half-height [31], which for a Gaussian peak is

estimated as 2.35σ . For Gaussian peaks this calculation generates the same value as obtained using the width of the base [29]; however, it is preferable because it obviates practical issues associated with a noisy baseline or overlapping peaks [32]. Peak resolution can also be described in terms of the minimum number of nucleotides that are distinguishable upon separation of two DNA fragments. This is calculated by dividing the difference in the number of bases for the two adjacent peaks by the calculated chromatographic resolution. DNA sequencing requires a chromatographic resolution equal to 0.59 (or 2 base resolution) [31, 32], although base calling can be achieved with lower resolution with sophisticated base calling methods [33]. DNA sizing applications require a separation matrix that can distinguish fragments with single-base resolution up to 300 bases [14]. For simplicity, the resolution that can be expected for different sieving matrices is reported in Table 1-2 in terms of the size in bases by which fragments can reliably be distinguished from one another.

In addition to separation performance, the cost, viscosity, and coating capability of a gel must also be considered. Both cost and availability play a role in the selection of a matrix for DNA applications. A gel that is easily synthesized or readily available at a low cost is preferred. The viscosity of the gel matrix is also critical because high pressure systems are required to introduce and remove viscous gels from capillaries. Such high pressures are incompatible with microfluidics. Coating the capillary or channel surface is necessary to suppress the EOF. The coating must be stable and the effect on EOF reproducible.

Linear polyacrylamide (LPA) and polydimethylacrylamide (PDMA) are widely reported from 2009-2014. The use of blended matrices comprised of hydroxyethyl cellulose (HEC) and polyvinylpyrrolidone (PVP) are especially useful in applications of microfluidics for human identification.

LPA is used in CGE techniques due to outstanding performance and low cost. LPA provides high resolution in short analysis time, with an upper size limit of 1,000 bases. Methods using LPA report an 80 minute separation, distinguishing fragments with 2-base resolution and an upper size limit approximately 1,000 bases [33, 34]. LPA can be synthesized from the acrylamide monomer, which costs as little as \$0.30 per gram [35] or can be purchased as the purified high molecular weight LPA at a cost of \$1.20 per gram [36]. The disadvantages of LPA are the high viscosity and inability to coat the surface of the capillary. LPA is among the most viscous matrix used in DNA analysis; however, under a sheer force of 1.32 s^{-1} , the viscosity of a 2% LPA gel drops to 27,000 centipoise (cP) [34]. A suppressed EOF is required, and different strategies for surface modification have been reported [37, 38], which are based on a previously reported method [39].

LPA matrices have been used for various applications. The synthesis and optimization of two LPA matrices for the CE separation of DNA fragments less than 70 bases was reported and applied to size PCR markers for wild type and

mutant gastric cancer tissues with a resolution below 5 bases [40]. A 5% LPA matrix was used in an integrated microfluidic lab on a chip platform for DNA extraction, amplification, separation, and detection from a crude biological sample, and a full profile of short tandem repeats (STRs) was obtained for a standard DNA template in a 40 minute analysis time [41]. Other microfluidic platforms utilizing LPA were employed for the analysis of *E. coli* [41-43], *Staphylococcus Aureus* [42, 44], *Salmonella typhimurium* [42], human respiratory viruses [45], *Alu* insertions used for gender and ethnicity determination [46, 47], *p53* gene mutations [48], and EndoV/DNA ligase mutations [49].

PDMA matrices overcome the two major limitations of LPA sieving gels: viscosity and coating ability. The most prevalently used PDMA matrix is performance optimized polymer 4 (POP-4™), which contains 4% polymer with 5% 2-pyrrolidinone and 8M urea [50]. Single-base resolution of DNA fragments up to 250 bases, and 2 base resolution up to 350 bases has been demonstrated within a 30 minute separation for forensic DNA applications [51]. Other POP™ matrices containing higher percentage polymer concentrations have been optimized for sequencing applications. Matrices of 6.5% PDMA have a viscosity between 75 cP and 1,200 cP depending on whether the low or high molecular weight polymer is used in the synthesis reaction [52]. The POP-7™ formulation offers 2 to 3 base resolution up to 950 bases in a 2 hour separation, but has a viscosity of 395 cP [53]. The low viscosity is an advantage of PDMA. Unlike LPA, PDMA can coat the surface, so other coating materials or modifications are

not required. The advantages of using a PDMA sieving matrix come at a cost, as it is the most expensive matrix available with POP-4™ (cat # 402838 or # 4363752) available at a cost of approximately \$60 per mL [54]. It is also expensive to synthesize a PDMA matrix using the dimethylacrylamide monomer, but has been shown to yield comparable separation performance to commercially available matrices of PDMA in capillary [55] and LPA in a microfluidic [56]. The material is heavily used in forensics applications. New integrated microfluidics have been applied to methylated DNA using PDMA sieving gel to identify whether a forensic sample source was tissue [57], body fluid [58], or semen [59] and has been utilized to analyze seminal stains as old as 56 years [60], as well as analyzing polymorphisms of STRs [61].

Matrices composed of PDMA are also employed for a variety of applications outside of forensics. Sizing DNA with new matrices [62] and microfluidic platforms developed for STR analysis [63, 64] are often compared to bench-top analyses achieved using PDMA matrices. Applications of PDMA matrices outside of forensics includes multi-locus variable number repeat analyses (MLVA) to genotype bacteria by harnessing differences in targeted gene sequences for regions in an organisms genome, these loci are known as variable number tandem repeats (VNTRs). Specific biomarkers were targeted for the identification of several bacterial species including *Shigella* spp. [65], *Streptococcus agalactiae* [66], *Staphylococcus aureus* [67], *Clostridium difficile* [68], *Listeria monocytogenes* [69], *Legionella pneumophila* [70], *Pseudomonas*

aeruginosa [71], and *Francisella noatunensis* [72], and study the association of specific point mutations with susceptibility to particular antimicrobial agents [73]. These matrices also have merit in the agricultural field having aided in methods for sizing biomarkers for the identification of 7 pathogenic species in bovine milk [74]. They have also been used in distinguishing genetically modified cotton and soybean [75], and single strand conformation polymorphism (SSCP) analysis for the identification of 7 infectious disease causing pathogens [76].

HEC, a polysaccharide based gel derived from cellulose is a low cost and low viscosity matrix; however, with this matrix the EOF is not eliminated but is only suppressed by 20% [77]. A drawback to utilizing HEC is polydispersity of the polymer chain because it is a naturally occurring polymer. HEC matrices cost approximately \$0.14 per gram [78] with low viscosities at dilute concentrations. An early application of HEC for DNA separations yielded 2 base resolution at an upper size limit of 570 bases [79]. A 2% HEC matrix using 97 kiloDalton (kDa) average molecular weight polymers was reported to have a viscosity of 5,000 cP [79]. However, the viscosity of HEC matrices can be adjusted so that it can be suited for separating DNA of different size ranges by varying the percentage of low and high molecular weight HEC in the preparation [80]. A lower molecular weight, 90 kDa HEC matrix was used for the identification of genetically modified maize with DNA markers less than 200 bases [81]. A blended HEC matrix consisting of 0.21% 27 kDa and 0.07% 1 MDa HEC with 0.12% 7 MDa LPA was used for the separation of DNA fragments ranging from 200 to 40,000 bases in

2 minutes in a glass microfluidic coated with polyhydroxyethylacrylamide (PHEA) [82]. The poor surface passivation by HEC can be overcome by blending other effective surface coating agents such as polyethylene glycol (PEG) [83], polyvinyl alcohol (PVA) [84], and PDMA [84].

PVP is a sieving matrix with mediocre separation performance, but excellent surface coating properties, low cost [85], and low viscosity, which can range from 3 to 27 cP [86]. PVP matrices have been reported to demonstrate the feasibility of using short capillaries [87], performing portable methods [88], and improving detection through base stacking and field gradients [89]. Although, this matrix is not widely used, a newly developed blended sieving matrix comprised of 20% PVP and 80% HEC [62] harnesses the complementary properties of each material. PVP is an excellent coating material, and HEC provides better separation performance. The viscosity of the mixture is lower than a matrix containing only HEC and has been used for more than 90 consecutive CE runs without deterioration in separation performance [90]. The matrix is mainly used in microfluidic platforms for DNA sizing for human identification STR analysis because it provides single base resolution up to 200 bases and 2-3 base resolution up to 400 bases in a 10 minute separation [63].

1.3 Pivotal Applications of Capillary Gel Electrophoresis for DNA Sieving

1.3.1 DNA Sequencing

CGE is best known for first generation DNA sequencing. The methodology involves Sanger sequencing, in which the DNA template to be sequenced is transformed into a base ladder as summarized in Figure 1-3. The terminating nucleotide of each fragment in the ladder corresponds to a specific fluorophore. The fragments are separated depending on the length by CGE. The fluorescence is monitored at each of four emission wavelengths to assign the terminal base corresponding to the fluorophore matching each of the four bases. CGE can sequence up to 1,000 bases in under 1 hour with high precision. Research advances in CGE Sanger sequencing culminated with the report of the Human genome [12]. With this goal achieved, CGE Sanger sequencing has become a more applied method.

Figure 1-3: Conceptual Depiction of Sanger Sequencing with Capillary Gel Electrophoresis

1-3 A) Dye labeled Terminator Chemistry for one base (e.g. T)

1-3 B) - D) Dye labeled Terminator Chemistry performed individually for each base

1-3 E) Pool Amplification Products

1-3 F) Separate with Capillary

Gel Electrophoresis

1-3 G. Record Observed Wavelength of Fluorescent Response and Convert to DNA Sequence

Figure 1-3: Fig. 4 is a description of capillary electrophoresis Sanger sequencing. Four separate PCR reactions are used to convert a DNA sequence into a ladder in which the terminal base is associated with a specific fluorophore. The reactions are then pooled and separated using capillary gel electrophoresis. The order that each wavelength of fluorescence is recorded corresponds to the sequence of the amplicon, which is the complement of the original template. Reproduced from [1].

1.3.1.1 End-labeled free-solution electrophoresis

End-labeled free-solution electrophoresis (ELFSE) relies upon free zone CE to separate DNA fragments that are covalently attached to a large molecule, such as synthetic peptoids [91] or proteins [92], often referred to as drag-tags. DNA mobility increases with the fragment size, so migration becomes inversely proportional to fragment length, meaning larger fragments migrate faster than smaller fragments, which is due to the increased frictional drag associated with the tag. By exploiting the balance of forces between the frictional drag caused by the tag (equal for all fragments) and the mobility stemming from the electronic driving force felt by the DNA under an applied voltage, DNA migration becomes charge dependent where larger fragments possess more negative charges and therefore migrate faster in free solution. A decrease in the polydispersity of the proteins used for drag-tags, decreases variation in charge and size distribution, which extends the sequencing read length [92]. Increase in the charge on the drag-tag increases wall-interactions, which increases band broadening [93, 94]. Micelle drag-tags have been utilized in commercial CE instruments and microfluidics to improve the readout time by optimizing the electroosmotic counter-flow [95]. Additional applications for drag-tag methods include hybridization assays with short single strand DNA targets [96] for the detection of single nucleotide polymorphisms (SNPs) [97] or to assess the formation of primer-dimers in multiplex PCR reactions [98].

1.3.1.2 Beyond *de novo* genome sequencing

Next generation sequencing strategies are now commercialized as cheaper and faster alternatives based on highly multiplexed analysis of short reads [99].

However, Sanger sequencing via CGE is still commonly used to correct for errors in assembling the sequence data, for example in long repeats of DNA polymers.

Thus, CGE is reported as an analytical technique used to assist in genome sequencing with next generation sequencing technology. CGE is used to improve quality control in next generation sequencing [100], or to quantify the DNA library [101]. Droplet microfluidics was used in conjunction with CGE to ensure that a suitable amount of DNA is generated by PCR without a bias in size distribution [102].

1.3.2 DNA Sizing

Beyond the use of CGE for DNA sequencing of the entire human genome, CGE continues to play a significant role in assigning STRs for human identification or detecting pathogen biomarkers. Repetitive sequences within the genome are harnessed to uniquely identify specific biomarkers for a number of applications relevant to pathogen detection, human disease, and especially human identification. DNA analyses are critical to forensic laboratories around the world. Human identification methods are based on the analysis of DNA sequences known as STRs, which contain 2 to 5 base repeats. For example, the short

tandem repeat Penta E has the recurring sequence AAAGA, and can vary among individuals from 5 to as many as 24 repeats. An individual will have two different sets of this repeat, one from each parent. Thirteen different STRs are used in the FBI Combined DNA Index System, also known as CODIS, for probability matching of a DNA sample to a specific individual. An allelic ladder containing all possible repeats present for each of the STR loci in a given STR analysis kit is used to discriminate and identify the markers for an individual. In a single forensic analysis, a minimum of sixteen markers are separated and sized. Four different fluorescent labels (e.g. FAM, JOE, TAMRA, ROX) are used to distinguish the STRs because some have similar length and overlap in separation space. Therefore, these overlapping lengths are resolved spectrally. The amplicons reflect the number of repeats at a specific loci, and the assignment of size is accomplished using an internal standard DNA ladder. These overlapping fragments are most commonly analyzed using a PDMA sieving gel matrix. The relationship between migration time and fragment size is linear, so the lengths of the amplicons is calculated using the migration times. Sizing is used for genotyping through the identification of markers that have specific lengths and DNA sieving gels are critical to these separations.

1.3.2.1 Chip-based forensics

Microfluidic systems for forensic analyses continue to advance. Newer device designs generate results from buccal cells in under 3 [103] or 4 [63] hours.

Sample volumes of complex samples of whole blood or semen were reduced to 1.2 and 1.5 μL , respectively [104]. A simple disposable chip fabricated in 10 minutes using a printer and polyester toner at a cost of \$0.15 was reported [105]. A more sophisticated plastic microchip was reported for integrated sample extraction, PCR amplification, and DNA separation that achieved single base resolution of buccal samples with only a 7-cm long separation channel [106]. The plastic device is cost-effective and was used over a 6 month period. In another report a microfluidic droplet generator was used for high throughput isolation of single cells prior to integrated extraction, amplification, and sizing [107]. This approach of isolating single cells circumvents issues of analyzing and interpreting data obtained with DNA from multiple donors. The total analysis time with the device, which was 22 hours including a required 10 hour cell lysis step, is equivalent to the time required for bench top analyses.

1.3.2.2 Self – assembled gels

Self-assembled gels with tunable selectivity are an attractive alternative matrix for DNA separation. The phospholipids dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) spontaneously form a thermally-reversible nanogel capable of high accuracy DNA sizing [108, 109]. The nanogel adopts a bilayer nanodisk morphology at 19°C, which corresponds to a low loading viscosity of 50 cP for a 20% solution [110]. When the temperature is increased to 30°C the phospholipids assume a

nanoribbon-like structure that forms a higher viscosity interconnected network. The DMPC-DHPC preparation is self-coating, effectively suppressing EOF by simply flushing the capillary with the phospholipid [111]. The phospholipid nanogel is roughly one-third the cost of the POP-4™ gel used for human STR analyses in commercial systems, and has been demonstrated with identical separation performance figures of merit [112-114]. Single base resolution up to 350 bases was demonstrated with a 10% phospholipid nanogel [108]. Nanogels diluted to 2.5% extend the range for accurate DNA sizing up to 1,500 base pairs [115]. The thermally-responsive viscosity can support sieving gradients [108] as well as stacking cartridges that pre-concentrate DNA upon injection and can be thermally erased prior to separation [115].

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Chapter 2

Theory and mathematical representation for capillary electrophoresis and DNA separations

2.0 Introduction

The use of CE for the separation of analytes is governed by basic mathematical principles. This chapter describes, in terms of mathematical models or theories, the use of CE methods for the separation of DNA fragments. Due to DNA carrying a nearly identical charge-to-size ratio independent of fragment length, the implementation of free zone CE for the analysis of DNA is limited; especially for samples containing multiple fragments. However, incorporating a gel sieving matrix into the capillary separation provides a sized dependent mobility for the DNA migrating through it, allowing for a separation of fragments that can also be explained through mathematical models and theories. A detailed description as to how the use of phospholipid additives for tunable high resolution DNA sieving fits into these theories is explained, and extended to how experimental variables can be manipulated to achieve a wanted separation performance.

2.1 Theory

2.1.1 Mathematical representation for capillary electrophoresis

Analyte species to be separated and detected using CE separations are governed by two terms. When a voltage is applied across a capillary, the analyte

molecules move as a result of their $E_{ph} (\mu)$, which can be explained as the pulling force felt by the molecule towards the electrode of opposite charge. The E_{ph} of a particular analyte is a function of its velocity (v) and the applied electric field (E_{app}) (see Equation 1).

$$\mu = \frac{v}{E_{app}} \quad \text{Equation 1}$$

A related expression, derived by Hückel [1], relates the molecules' E_{ph} to its charge (q) using the expression below (see Equation 2), which is where the commonly referred charge-to-size ratio (q/r) originates.

$$\mu = \frac{q}{(6\pi\eta r)} \quad \text{Equation 2}$$

As mentioned, the mobility of the species are charge dependent; therefore, cations migrate toward the cathode (+) and anions migrate toward the anode (-). A secondary force that plays into a molecules mobility in CE, is EOF. This phenomenon occurs due to the negatively charged surface of the glass separation channel. With buffer pH values above 4.5, the silanol groups on the inner surface of the capillary impart a negative surface charge. This charge

attracts positively charged ions contained in the background electrolyte (BGE) solution, creating an electric double layer. Under an applied voltage, this layer of cations migrate toward the cathode, resulting in a bulk fluid flow in this direction. The EOF is typically an order of magnitude greater than E_{ph} and the extent of its force is driven by the pH and ionic strength of the BGE. Operating at a pH below 4.5 can remove this effect, and many strategies exist for coating the capillary surface to eliminate its presence. For CE separations operating under normal polarity (NP) conditions, depicted in Figure 2-1 A, meaning the migration of analytes is driven from the anodic buffer reservoir to the cathodic buffer reservoir, positive and negative analyte species can be separated. The limitation of normal polarity CE (NP-CE) is its inability to separate neutral species, as any and all uncharged molecules will migrate with the EOF force. The order of migration, or elution, for analytes reaching the detector can be represented as a sum of two vectors (see Figure 2-1 B); one for the analyte species E_{ph} combined with the EOF force vector. A schematic representation for a normal polarity CE instrument setup is depicted in Figure 2-1 below.

Figure 2-1: Normal polarity capillary electrophoresis

Figure 2-1: (A) Instrumental setup for normal polarity capillary electrophoresis. (B) Vectors and vector sums for determining the order of analyte migration based on their q/r values. (C) An example electropherogram for CE separation.

The vector sum for EOF and neutral analytes are equal as they have no attraction towards either electrode, and only traverse the capillary length due to the bulk fluid flow carrying them. Cations reach the detector first obeying the charge-to-size (q/r) migration pattern, with smaller molecules with higher charge

coming before larger molecules containing the same charge. Switching the polarities between the two electrodes is known as reverse polarity CE (RP-CE). In this experimental setup (see Figure 2-2), the EOF is still a bulk fluid flow toward the cathode buffer reservoir and due to its direction and magnitude being greater than the E_{ph} for neutral and cationic analytes, these methods are limited to the analysis of anionic species only.

Figure 2-2: Reverse polarity capillary electrophoresis

Figure 2-2: Instrumental setup for reverse polarity capillary electrophoresis

Under these conditions, anions will migrate through the capillary but because they are fighting the EOF, separation times can be very long. To circumvent this, eliminating the presence of the EOF is typically accomplished to shorten the separation time. A good surface coating is important for eliminating EOF so analyte migration times are reproducible. Because DNA carries a negative charge, it can easily be separated under RP-CE conditions with suppressed EOF, as depicted in Figure 2-3. For DNA sequencing and sizing applications, a

gel matrix most commonly incorporated to act as a sieve, where small fragments migrate more rapidly than larger fragments.

Figure 2-3: Reverse polarity capillary electrophoresis for DNA separations

Figure 2-3: Reverse polarity capillary electrophoresis with suppressed EOF used for DNA sieving analyses.

A representative electropherogram depicting the sieving behavior of a standard DNA ladder containing fragments with lengths ranging from 50 to 1,000 bp through a gel matrix, is shown in Figure 2-3. Optimizing a matrix for DNA separations is generally driven by the size range of the fragments under analysis and the closeness of the fragment sizes contained in the sample. The CE method figure of merit that is closely monitored during matrix optimization for the separation of DNA fragments is resolution.

2.1.2 Resolution of DNA analytes

A general representation for the separation performance of a method can be explained in terms of chromatographic resolution (R_s). This variable is determined for the separation of two analyte species, with different sizes (N), in CE, through Equation 3, below.

$$R_s = \frac{1}{4(N_2 - N_1)} \left| \frac{\Delta\mu}{\mu_{ave}} \right| N_{pl}^{1/2} \quad \text{Equation 3}$$

Another way to quantitatively monitor the separation performance of a method is known as selectivity (S), and it can be calculated using Equation 4, seen below. Replacing the variables for analyte size (N), and electrophoretic mobility [E_{ph}] (μ), this expression can be simplified to an easier form, where the migration time of the analytes is implemented.

$$S_{1,2} = \frac{1}{N_2 - N_1} \left| \frac{\Delta\mu}{\mu_{ave}} \right| = 2 \left| \frac{t_{m1} - t_{m2}}{t_{m1} + t_{m2}} \right| \quad \text{Equation 4}$$

Defining the remainder of the terms, $\Delta\mu$ is the difference in electrophoretic mobility, μ_{ave} is the average mobility between the two analyte species, and N_{pl} is the number of theoretical plates which is calculated using Equation 5.

$$N_{pl} = 5.5 \left(\frac{t_m}{w_{1/2}} \right)^2 \quad \text{Equation 5}$$

As you can tell, migration time and peak width have the most pronounced effect on the number of theoretical plates. A general rule for CE is to maximize the number of theoretical plates by optimizing the separation conditions. Baseline resolution is obtained when the value for $R_s = 1.5$. This correlates to two analyte peaks being fully resolved from one another. As this value decreases, the two peaks overlap more and more until they can no longer be distinguished from one another.

For a DNA separation matrix, these figures of merit for performance are evaluated and optimized. The resolution between two DNA fragments can be calculated using the width at the base (w_b), see Equation 6 below.

$$Rs = \frac{2(t_{m(1)} - t_{m(2)})}{(w_{b(1)} + w_{b(2)})} = \frac{\Delta t}{w_{ave}} \quad \text{Equation 6}$$

Because many DNA applications require high resolution through the analysis of several fragments with minimal differences in length, there can often be complications with unresolved peaks and noisy baselines, making measuring the peak width at the base difficult. Therefore, the expression can be altered to include an easier measured value in these circumstances, the width at half height ($w_{1/2}$), which is sometimes referred to as the full width at half maximum (*fwhm*). Calculating resolution in this manner is accomplished using Equation 7, seen below.

$$Rs = \sqrt{2 \ln(2)} \frac{t_{m(2)} - t_{m(1)}}{w_{\frac{1}{2}(1)} + w_{\frac{1}{2}(2)}} \quad \text{Equation 7}$$

To further simplify the calculation, Equation 8 can also be used to calculate the resolution between two fragments.

$$Rs = 0.589 \left(\frac{\Delta t}{w_{\frac{1}{2}ave}} \right) \quad \text{Equation 8}$$

Most often, resolution values are calculated and reported using the previous two expressions. One final calculation is typically done in order to eliminate any misunderstanding in how a reported resolution value should be interpreted. As seen in Equation 9 below, divide the difference in fragment lengths (Δbp) for two adjacent peaks by the calculated chromatographic resolution (Rs). This yields an expression in bases or bp. Calculating the base (or bp) resolution for a given separation matrix is one way to elucidate the minimum number of bases or bp two fragments must differ by in order to effectively resolve them from one another under a particular set of experimental conditions. How manipulating experimental conditions and variables change this value directly points to how the optimum separation performance for a matrix is determined.

$$Rs_{bp} = \frac{\Delta bp}{0.589 \left(\frac{\Delta t}{w_{\frac{1}{2} ave}} \right)} \quad \text{Equation 9}$$

2.1.3 Modeling the separation matrix

Key parameters of concern for a DNA separation matrix in CGE are viscosity, coating ability, cost, and separation performance. Unlike slab gels, which are physical gels, dynamic solutions of entangled polymers are much easier to control in a capillary or microfluidic format. A matrix with a low viscosity is preferable due to easy manipulation and control during loading and flushing steps.

Polymers used for CGE DNA sieving are water-soluble and possess a few key characteristics making them amenable to the methodology including; low to moderate viscosity when used at concentrations above the entanglement threshold (c^*), high sieving ability, good surface coating ability to reproducibly suppress EOF, and chemical stability at a pH range of 7-9. The properties of concern displayed by an aqueous solution containing an entangled polymer, most commonly referred to as a gel, for sieving based separations can be modeled through a number of mathematical models and scaling theories. To better understand some of these relationships, the fundamental principle behind key variables will be explained within the view of this chapter, and values from referenced journal articles will be used in some examples.

First, to gain a general understanding of the physical properties and molecular interactions displayed by different concentrations of polymers in solution, the diagram in Figure 2-4 illustrates these different stages. At dilute concentrations and below, the polymer chains are isolated from one another and do not interact in solution, but as the concentration increases the chains begin to overlap and entangle. The concentration of polymer in solution at which individual chains begin to interact with one another is known as the overlap or entanglement threshold (c^*). This value is important to determining the pore size created by a polymer sieving matrix and is used in following calculations.

Figure 2-4: Physical properties and molecular interaction of different concentrations of polymer in solution

[polymer]			
water	ultra-dilute	dilute	semi-dilute
Liquid		Entangled Network	
		Gels	
		Transient Interactions	Covalent crosslinks
		Reversible	Permanent

Figure 2-4: Diagram for the different physical properties of polymers in solution with increasing concentration. For entangled networks, or gels, the type of molecular interactions that leads to their physical state.

The transition from a dilute to a semi-dilute polymer solution is generally marked by a large increase in matrix viscosity, at which point the solution of water-soluble polymers transiently interact and form a dynamic gel. Because the interactions between polymer chains are temporary and weak, the formation of a dynamic gel solution is entropically driven and stabilized primarily through hydrophobic and Van der Waals forces.

The most important parameter of a sieving matrix used for DNA or other biomolecule separation, is the pore size (ξ_b), as this commands the mechanism of transport to which the DNA will obey while travelling through it. The point at which a DNA molecule becomes too large to fit through the pores created by the gel matrix, is the upper limit for high resolution DNA sieving.

An entangled network solution is often characterized by the average pore size it forms at a given concentration. One of the most prevalently cited descriptions for modeling and experimentally testing a separation matrix composed of HEC is given by Grossman and Soane [2], where a $(c^*) = 0.4\%$ was found to form pores on the order of 265 Å. In this example, the c^* can be estimated using an expression for the number of segments in the polymer chain (N), see Equation 10, originally derived by de Gennes [3].

$$c^* \approx N^{-\frac{4}{5}} \quad \text{Equation 10}$$

But because polymer chains can differ in their respective values of N and MW, a second approach proposed by Viovy and Duke [4], uses the polymer radius of gyration ($R_{g(\text{polymer})}$) to estimate this value. In this approach, the expression below can be implemented (see Equation 11).

$$c^* = \frac{M_w/N_A}{\frac{4}{3}\pi R_{g(\text{polymer})}^3} = 1.5 [\eta]^{-1} \quad \text{Equation 11}$$

Therefore, for polymer solutions with concentrations above this critical value, a derived mathematical relationship for determining the pore size of the matrix also uses the polymer molecular weight (M_w) and radius of gyration ($R_{g(\text{polymer})}$); (see Equation 12).

$$\xi_b = 1.43 R_{g(\text{polymer})} \left(\frac{c}{c^*} \right)^{-(1+a)/3a} \quad \text{Equation 12}$$

The exponent term in this expression is a function of a polymers' three dimensional orientation at a given temperature and in a given solvent. This value

can be theoretically modeled to obtain a rough estimate for the pore size of a matrix. However, as seen in Equation 11 above, the viscosity of a polymer solution can also be used to calculate the average pore size it forms through measurable experimental means and a more precise value for the exponent term can be rationalized through a number of calculation, detailed in the following section.

Experimentally determining and plotting the log of the intrinsic viscosity $[\eta]$ as a function of the polymer volume fraction yields a plot whose inflection point provides a graphical representation for the point at which a polymer solution crosses from being dilute to semi-dilute. This increase in the slope from a constant value of 1 is marked with a viscosity increase because this is where individual chain segments begin to interact and entangle. To verify the estimated value obtained for (c^*) using Equation 10 above, it can be compared with the experimentally determined value from a $\log[\eta]/\log[c^*]$ plot.

The Kuhn–Mark–Houwink–Sakurada equation (see Equation 13 below) which describes the relationship between a matrix's intrinsic viscosity and its MW can be used to determine the average MW of the polymer chain in solution. The Mark-Houwink constants (K) and (α) vary for different polymers and are evaluated under a particular solvent system at different temperatures [5]. They can also be determined experimentally using standard polymer solutions of

known MWs, solvent, and temperature; by plotting the $\ln$ of the measured $[\eta]$ versus $\ln[M_w]$, and fitting the data with a linear regression line. The slope of this line is the value of α and the intercept is equal to $\ln K$ (see Equation 14).

$$\ln[\eta] = \ln[K] + \alpha \ln[M_w] \quad \text{Equation 14}$$

Switching to exponent orientation, the expression becomes that seen in Equation 15 below.

$$[\eta] = KM_w^\alpha \quad \text{Equation 15}$$

The exponent term, α , is the same constant seen in Equation 12, and is a function of the polymer geometry and relates the polymer chain's three-dimensional configuration for a given solvent system. Values of the term generally vary from 0.5 to 2.0 [5]. For α - values from 0.0–0.5, the polymer is envisioned as a rigid sphere contained in an ideal solvent system; values from 0.5–0.8 reflect a random coil configuration in an acceptable solvent; and values from 0.8–2.0 reflect the polymer shape as a stiff rigid chain or rod [5]. The fact that the intrinsic viscosity of a given polymer sample differs depending upon the solvent used provides an insight into the general shape of polymer molecules in

solution. In a good solvent, which shows a zero or negative heat of mixing with the polymer, the molecule is loosely extended, and the intrinsic viscosity is high.

Starting with Equation 16 (below) and plugging in measured values for the intrinsic viscosity (η) in units of mL/g, then 2.1×10^{23} for (Φ_c) which is a universal constant for a random-coil polymer in solution [2,6], a value for the root-mean-squared end-to-end distance (r^2) of the polymer chain can be obtained.

$$[\eta] = \frac{\Phi_c \langle r^2 \rangle^{3/2}}{M_w} \quad \text{Equation 16}$$

Using this value and the expression in Equation 17 (below) for an unperturbed chain [2,6], yields the polymer coils radius of gyration ($R_{g(\text{polymer})}$).

$$\langle R_{g(\text{polymer})}^2 \rangle = \frac{\langle r^2 \rangle}{6} \quad \text{Equation 17}$$

This value can be used in the expression below (see Equation 18), relating the polymer's radius of gyration to its segment length.

$$R_{g(\text{polymer})} = \alpha N^{0.6} \quad \text{Equation 18}$$

With this value for the polymer chain segment length (N), it is now possible to more accurately solve Equation 10, obtaining a better but still estimated average pore size for the matrix.

The importance of these variables is realized when optimizing a DNA separation matrix because the transition from linear Ogston sieving to non-linear reptation occurs at the relationship seen in Equation 19 (below) which was proposed by Slater and Noolandi [7,8].

$$R_{g(\text{DNA})} \cong 1.4 \xi \quad \text{Equation 19}$$

This relationship makes determining the average pore size of a matrix possible through a Ferguson study. A Ferguson plot shows the relationship between DNA mobility as a function of gel concentration. Estimating the radius of gyration for the DNA ($R_{g(\text{DNA})}$) fragments being separated using the relationship seen in Equation 20 below, and the assumptions that dsDNA has a persistence length of 45nm and a contour length of 0.34nm per bp [9].

$$R_{g(\text{DNA})}^2 = \frac{1}{3}pL \left(1 - \frac{p}{L} + \frac{p}{L} e^{-L/p} \right) \quad \text{Equation 20}$$

This is how the limit for Ogston sieving is determined for standard DNA separation matrices as well as the nanogel matrix detailed in the final section. This is generally the more accepted approach for determining a pore size of a matrix based on the size of DNA fragments that can easily and reproducibly migrate through it. The matrix used is generally indicative of the specific DNA application being investigated. This is where the significance of the phospholipid nanogel used in the publications supporting this dissertation comes to light. Because the matrix can easily be tuned to accommodate DNA fragments of varying size, it does not necessarily follow many of the assumptions made in the calculations.

2.1.4 Mobility of DNA through sieving matrices

To model the process of DNA traversing a gel matrix, it was important to first understand the network formed by the sieving media. The models for DNA migration relate the E_{ph} for DNA in free solution to its relative mobility through the gel matrix. The attractive force between the anode (+) and the DNA (-), causes the fragments to migrate through the separation channel to the detection module, and understanding how this process can change due to the DNA size, matrix

properties, temperature, and other controllable experimental variables is theorized through a number of relationships. This section will explain the basis behind the most utilized models and the limitations of each.

DNA is a polyelectrolyte, built from a sequence of nucleotide monomers. One way to model DNA is as a flexible cylinder with a diameter (d), contour length (L), and persistence length (l_p); which is a measure of the DNA chain rigidity. This introduces a new term known as the Kuhn length (l), which is estimated using Equation 21, seen below.

$$l = 2 l_p \quad \text{Equation 21}$$

For values of $L > l$, the random walk distance defined by the Kuhn length (l) and the average end-to-end chain distance (R_N) can be related to the radius of gyration for the DNA molecule ($R_{g(\text{DNA})}$) through the expression seen below (see Equation 22). Which can also be expressed using the number of Kuhn segments (N_K) through the relationship seen in Equation 23.

$$R_N = \sqrt{6} R_{g(\text{DNA})} = l \sqrt{N_K} = \sqrt{(l L)} \quad \text{Equation 22}$$

$$N_K = L/l \quad \text{Equation 23}$$

Tying this relationship to the excluded volume interaction model, in order to account for the repulsive forces between two DNA segments, is characterized by the excluded volume parameter (v_F), which serves as a scaling estimate for this observation and is seen in Equation 24 below.

$$v_F \approx l^2 d \quad \text{Equation 24}$$

By this means an end-to-end distance for a DNA fragment can be represented and scaling laws permit estimations for any chain size.

During capillary electrophoretic separations of DNA under an external electric field, the DNA polyelectrolytes are subjected to a number of balanced forces that govern its' velocity (v) travelling through the separation channel which is represented through Equation 25 below.

$$F_{external} - \xi(v - \mu_0 E_{app}) = 0 \quad \text{Equation 25}$$

The main contributors affecting this process are; the force created by the applied voltage (E_{app}), the hydrodynamic friction between the DNA and surrounding fluid (ξ_K), and the steric effects exerted by the gel sieving matrix. One way to present this relationship is outlined in Equation 26 below.

$$v = \frac{F_{electro}}{F_{drag}} = \frac{q_{eff} E_{app}}{\xi_K} \quad \text{Equation 26}$$

As described by Equation 27 below, the mobility of DNA through a sieving matrix is a function of its mobility in free solution (μ_0), the concentration of the gel (c), and the retardation coefficient (K_R).

$$\log[\mu] = \log[\mu_0] + K_R c \quad \text{Equation 27}$$

Adjusting the previous equation to an exponent orientation, the expression can also be presented in the form seen in Equation 28 below.

$$\left(\frac{\mu}{\mu_0}\right) = e^{[-K_R c]} \quad \text{Equation 28}$$

The retardation coefficient (K_R) for a particular sized DNA fragment is proportional to its length or number of bases or bp (see Equation 29), as larger fragments have bigger R_g . This variable is calculated for DNA using the Porod-Kratky stiff-chain model, which assumes a persistence length (L) of 45 nm and contour length (l) of 0.34 nm per bp. This relationship is shown in Equation 29, below.

$$K_R \propto (R_g + r)^2 \quad \text{Equation 29}$$

With this expression, the effective sieving limit for high resolution DNA separations can be determined. Mathematical representations for the modes of migration that DNA of differing sizes obey, with an assumed cut off limit for this value, makes modeling this process possible. A general equation relating DNA mobility to size as a function of gel concentration is shown below (see Equation 30).

$$\mu = \mu_0 e^{[-K_R C (R_g + r)^2]} \quad \text{Equation 30}$$

As the following section will explain, these models are loosely based on the correlation between the DNA size and the matrix pore size, as the migration of DNA fragments varies as a function of this relationship.

2.1.4.1 Sieving Mechanisms

The migration of DNA through sieving media has been theorized and studied mainly because it is a model polyelectrolyte but also because of its importance to understanding the human genome. As explained in previous sections, the mobility and migration pattern for DNA of a particular size is indicative of the pore formed by the matrix. These mechanisms of migration are important to specific applications of DNA analysis. The sieving mechanisms of concern are linear Ogston and non-linear reptation. As depicted in the Figure 2-5 below (Adapted from reference [10], the mechanism to which the DNA molecules obey is directly proportional to the pore size created by the matrix.

Figure 2-5: Sieving mechanism of DNA

Figure 2-5: Displays the relationship between DNA size and matrix pore size. Concept for Figure Adapted from reference [10].

2.1.4.1.1 Ogston model

Ogston sieving is important to accurately size DNA fragments. Sieving occurring within this domain is linear therefore making calculating the size of unknown DNA fragments possible. The assumptions for this model are that the pore size created by the matrix is much larger than the size of the DNA molecules passing through it as unobstructed spherical objects. Equation 31 below shows the

relationship for an Ogston model electrophoretic mobility of a DNA molecule being equal to its free solution mobility multiplied by the probability (P) that it will encounter a pore large enough to fit through.

$$\mu = \mu_0 P (\xi \geq R_g) \quad \text{Equation 31}$$

The probability term is estimated using the Ogston model through the relationship seen in Equation 32 below.

$$P (\xi \geq R_g) = e^{[-\pi n l (R_g + r)^2]} \quad \text{Equation 32}$$

Which predicts the fraction of pores large enough to accommodate a DNA fragment with a determined radius of gyration where n is the average number of polymer strands per unit volume, l is the average length of the polymer strands, and r is the thickness of the polymer strands. Another assumption to this model is that the product of ($n \cdot l$) is proportional to the concentration of the gel matrix. Thus, making it possible to use the expression in Equation 33 below to also predict the pore size distribution of a matrix.

$$P(\xi \geq R_g) = e^{[-K_{RC}(R_g + r)^2]} \quad \text{Equation 33}$$

Which ties together with the previous section where the DNA fragments retardation coefficient was used to determine its mobility through the matrix. When r is much smaller than R_g , the plot of $\log(\mu)$ versus concentration yields a straight line with a slope proportional to R_g^2 (see Figure 2-5), also referred to as a Ferguson plot.

The limitation of the Ogston model comes to light when the DNA molecular size is larger than the average pore size for the sieving matrix. According to theory, this fragment of DNA should lose mobility and become trapped in the matrix because it cannot fit through the pore. In practice, this observation is not seen, as the DNA molecule can deform its shape in order to squeeze through the pore and continue migrating under the applied electric field. This process is known as reptation and will be discussed in the following section.

2.1.4.1.2 Reptation model

The relationship between a DNA fragments electrophoretic mobility and molecular size travelling under reptation can be estimated using the expression seen below in Equation 34. In this model, the mobility is inversely proportional to the length of the fragment.

$$\mu \sim \frac{N}{N^2} = \frac{1}{N} \quad \text{Equation 34}$$

When large DNA molecules are subjected to high electric field strengths, they align with the field and become elongated and stretch through the pore matrix. This phenomenon is known as biased reptation and separation efficiency suffers drastically making it very difficult to separate similar sized DNA fragments. The mobility of DNA during biased reptation can be estimated using Equation 35, seen below.

$$\mu \approx K \left(\frac{1}{N} + bE^2 \right) \quad \text{Equation 35}$$

Due to the poor resolution obtained during biased reptation, it is typically avoided and serves no advancement to currently implemented DNA analysis techniques where the majority of DNA markers are small and can be easily separated within the Ogston regime.

2.1.4.1.3 Other relevant models pertaining to DNA separation

A diagram relating the previously mentioned DNA separation mechanism as a result of matrix concentration and DNA size is shown below in Figure 2-6. An additional mechanism theorized by Barron et al (1994) [11], discusses the phenomenon when the polymer concentration is below c^* and the DNA mobility is hindered through collisions with polymer molecules in solution. This relationship is difficult to model because the above models are only accepted when the polymer concentration is above c^* .

Figure 2-6: Diagram for the separation mechanism in relation to gel concentration and DNA size

Figure 2-6: A representation for the sieving mechanism for DNA transport through different MW polymer solutions of varying concentrations which dictate the overlap concentration (c^*) and the average pore size of the matrix (ξ_b). At a maximum DNA size alignment occurs and resolution drops. Concept for Figure adapted from Reference [12].

2.2 Phospholipid Nanogels

The lipid molecules used to create the nanogel matrix are amphiphilic, meaning they possess both hydrophobic and hydrophilic regions. They differ only in the length of their aliphatic chain. Both lipid molecules have zwitterionic phosphocholine head groups which orient themselves facing the water

molecules, and the aliphatic carbon chain tails align towards the interior, forming a bilayer disk or bicelle. The thickness of the disk is governed by the thickness of the DMPC bilayer which is estimated to be around 5 nm [13]. However, techniques and reported values for this bilayer thickness vary and range from 7 to 9 nm for molar ratios of [DMPC]/[DHPC] of 1.5 at 25 °C to 30°C [14], and up to 10 nm using theoretical modeling [15]. Thus, making modeling the morphologies of the nanogel matrix difficult. While the circumference of the disk is attributed to the amount of DHPC in solution. At a temperature of 19°C, the isotropic nature of the lipid preparations allows for free tumbling nanodisks in solution, resulting in a low viscosity state. Upon a temperature increase, these materials re-assemble to form higher order morphologies that can be rationalized by the marked increase in matrix viscosity. At a temperature of 29-30°C, a viscosity maximum is reached, which for a nanogel with [DMPC]/[DHPC]=2.5 at 10% hydration shows a 370-fold increase over the low viscosity state obtained at 19°C [16]. This phenomenon occurs in response to the temperature change and is explained through a morphology shift from disks to ribbons that at high enough concentrations, begin to interact and entangle with one another. This dynamic ribbon entanglement is what causes the viscosity increase and formation of a gel, which allows for separations by sieving. When disks become ribbons, the DMPC centers combine to form an elongated bilayer and the DHPC molecules remain protecting the edges as they did in the disk morphology. An analogy for how this process occurs would be seen during cell division (one parent cell splitting to become two daughter cells) in reverse (two cells becoming one). These

processes are alike because the lipids composing the cell membrane are very similar to the lipid molecules used to form the sieving matrix, making it very biocompatible. The length and entanglement of these ribbons is impacted by three main factors; which are, the composition or ratio of DMPC to DHPC, the concentration or hydration percent of lipid in aqueous buffer, and the temperature of the solution. How each of these factors is affecting the performance of the matrix for DNA separations is detailed in the following chapters. To briefly explain each of these variables, the composition is defined as the molar ratio of long-chain (DMPC) to short chain (DHPC) lipid. Previous optimization studies show a ratio of 2.5 is best for separations based on subtle differences in hydrodynamic size [17], providing evidence the material would be effective for high resolution sieving separations of DNA. Composition varies most significantly due to the amount of DHPC in solution because it protects the exposed hydrophobic edges of the DMPC bilayer (see Figure 2-7 A). Lesser amounts of DHPC lead to smaller circumference disks and shorter ribbons. Greater amounts of DHPC lead to larger circumference disks and longer ribbons (see Figure 2-7 B). However, it is important to note that when the ratio of DMPC to DHPC drops below 1.5 to 2 the material does not have the same gel-phase transition and stays much more fluid like, even at elevated temperatures. The concentration of lipids in solution is reported as a weight to volume percentage. This variable ties to the number of disks or ribbons present in solution for that particular matrix preparation. As noted in Figure 2-7 C, a more concentrated matrix will have very high entanglement and a higher viscosity, a less

concentrated matrix will have lower entanglement and a lower viscosity. The more concentrated matrix will form smaller pores and will separate smaller DNA more efficiently; whereas, the dilute matrix will form larger pores and will be able to accommodate the separation of a wide range of larger DNA fragments. This observation has been realized for other commonly employed DNA separation media including LPA, HEC, and other thermos-responsive matrices [18].

Temperature is the most important variable for nanogel separations because it dictates the morphology of the matrix. For this reason, the instrument and ambient air temperature are regulated. An ambient temperature of 19°C aids in keeping the matrix in its low viscosity state while being inserted into and being expelled out of the separation capillary. The instrument is programmed to regulate temperatures within 0.2°C of the programmed setting, and will cancel the analysis if the temperature reading increases or drops outside of this accepted range. The interaction between the DNA and nanogel matrix as a result of these three variables is detailed in the following chapters but is what allows the fine tunable nature of the matrix for the separation of a wider range of DNA fragments.

Figure 2-7: Phospholipid Nanogel Sieving Matrix Components

Figure 2-7: (A) Molecular structures of DMPC and DHPC. A representation of the bilayer structure adopted by the amphiphilic lipid molecules in aqueous solution. **(B)** The phospholipids self-assemble into different morphologies in response to temperature changes including disks and ribbons. (inset) The process for disk to ribbon morphology switch, ribbons entangle and viscosity of the matrix increases. The circumference of the disks and length of ribbons is proportional to the molar ratio of the two lipids composing the matrix. **(C)** The low viscosity disk orientation that extend into a ribbon geometry that entangles to a degree proportional to the concentration or hydration percentage of the lipids in solution and the temperature. These morphologies adopted by the phospholipids are temperature dependent and reversible. Adapted from reference [19].

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Chapter 3

A Thermo-responsive Phospholipid Pseudo-gel: Tunable DNA Sieving with Capillary Electrophoresis

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3.0 Introduction

Sieving-based microscale electrophoretic separations of DNA are prevalent for diverse applications including research on humans [2-8], pathogens [9, 10], and food [11]. These separations are performed using commercially available automated instruments [2, 3], as well as custom or commercially available microfluidics [12]. Aqueous solutions of linear polymers are used as sieving gels rather than covalently cross linked gels because of advantages in performance and implementation [13]. Capillary gel separations based on linear polymers, such as LPA [14, 15], PVP [16], polyethylene oxide (PEO) [17], or HEC [18, 19], are completed in approximately 30 minutes in a capillary and less than 2 minutes in a microchip [20] or short capillary [21]. The total analysis time can be reduced through parallelization [22, 23] or by integrating the analytical processing [4-7]. For any microchannel separation, the sieving material is introduced with pressure into a channel in which EOF is eliminated. The bulk EOF may be controlled with a covalently modified surface; however, it is preferable to eliminate this flow by using an additive that both sieves and dynamically modifies the surface. Migration time reproducibility, carryover, and channel plugging are concerns, which can be alleviated or eliminated by using a disposable chip or by replacing the sieving medium in between runs [14]. For microfluidic applications, reducing the cost per analysis requires a means of replacing the sieving medium and reusing the chip [16]. The separation performance of the entangled linear polymer for sieving is affected by the polymer concentration, chain length, rigidity, as well as any interaction between the polymer matrix and DNA [24-27].

The viscosity of linear polymer additives, which varies for different preparations, determines the difficulty of introducing and replacing the material in a capillary channel [28].

An alternative to linear polymers and covalent polymer gels is the use of materials with a thermally responsive viscosity, such as matrices based on co-block polymers and grafted copolymers [29, 30]. These materials are loaded into the capillary under conditions of low viscosity, but can be thermally switched to a different viscosity more suitable for DNA separations [28, 31, 32]. Most materials require slightly elevated temperatures to decrease viscosity [31]. However, some materials used to separate DNA fragments ranging from 100 to 1,000 base pairs, such as pluronics [29], polyacrylamide grafted with poly(N-isopropylacrylamide) [33], and poly-N-alkoxyalkylacrylamides [34] are less viscous below room temperature. Drawbacks to implementing these thermo-responsive materials have included limited reproducibility of the preparations or the lack of inexpensive or commercially available materials. Materials based on spontaneous self-assembly of commercially available components hold potential to reduce the effort and cost necessary to prepare a custom sieving additive for CE [35, 36]. Ideally, a self-assembled sieving matrix would be quickly mixed, rather than synthesized, using inexpensive starting materials, to form a stable matrix with reproducible separation performance.

Aqueous phospholipid preparations comprised of DMPC and DHPC are self-assembled materials with a tunable viscosity. The viscosity of the material reported in the literature varies [37-40]; however, we have previously demonstrated with a microfluidic device, that the material is a shear thinning non-Newtonian fluid [41]. The apparent viscosity of a 20% w/v preparation with [DMPC]/[DHPC] = 2.5 changes 480-fold with only a 6 °C temperature increase and 1,300-fold decrease in shear rate, from 24,000 cP to 50 cP [41]. The phospholipid preparation offers a reduction in loading viscosity and an increase in operating viscosity when compared to other linear gels used in CE. For instance, the reported viscosity of a 4.5% solution of PVP is 27 cP [15], while a 6.5% solution of PDMA is 75 cP [42], and a 2% w/v solution of hydroxypropyl cellulose (HPC) is 11,000 cP [43]. Aqueous preparations of DMPC-DHPC also have a better working viscosity profile relative to other non-Newtonian fluids, for which viscosity is dependent upon shear rate. For example, Barbier and Viovy noted that 2% w/v LPA has a viscosity of 260,000 or 27,000 cP at zero-flow or a shear rate of 1.32 s^{-1} [27].

The tunable viscosity of DMPC-DHPC preparations is due to the formation of self-assembled aggregates of varying dimensions. The long chain DMPC lipids form bilayers in which the hydrophobic alkyl chains align on the interior (see Figure 3-1). The short chain DHPC lipids stabilize the exposed hydrophobic chains at the edges [44]. Low viscosity conditions are attributed to bilayered disks, called bicelles or nanodisks [45]. At appropriate phospholipid

concentration and molar ratios of DMPC and DHPC, the nanodisks reconfigure to form elongated ribbons or wormlike micelles [38, 46, 47]. These thermally triggered changes in morphology have been suggested based on observations with various analytical techniques [47-51]. However, limitations of the methods used for characterization, such as radioactive reagents [52], alignment induced by the analytical measurement [53], or covalent labeling that holds potential to impact the self-assembled structure [49].

Figure 3-1: Phospholipid additives composing DNA sieving matrix

Figure 3-1: (A) Molecular structures of DMPC and DHPC. **(B)** Cross-sectional view of a portion of the DMPC bilayer stabilized by the short-chain phospholipid, DHPC. The phospholipids self-assemble into different geometries including discs and ribbons in response to temperature. Reproduced from [1].

Additional insight into phospholipid morphology is gained by using DNA to probe the apparent pore size, and hence fluid structure, of an unlabeled phospholipid preparation. The migration of DNA through sieving media is well developed and methods to estimate the pore size of sieving materials are well documented [54-56]. The utility of the preparation has not been determined for sieving even though the viscosity of aqueous DMPC-DHPC preparations has been harnessed for CE separations based on subtle differences in hydrodynamic size [57, 58], and to steer fluids in microfluidic channels [39, 41]. Previous evaluation of phospholipid preparations with $[DMPC]/[DHPC] = 1.5, 2.0, \text{ and } 2.5$ at concentrations ranging from 5-15% demonstrated that a preparation of $[DMPC]/[DHPC] = 2.5$ at 10% w/v was more effective than the other preparations for the separation of branched glycans [57]. Separations of discrete DNA fragments using the preparation provided insight into the material morphology. In addition, the potential of the phospholipid additives for capillary sieving of DNA fragments is determined by evaluating the migration time, reproducibility, and peak resolution. From a practical standpoint, the compatibility of the medium with fluorescence detection was also confirmed with both labeled primers and intercalating dye. The goals of this work were to determine the apparent pore size of the aqueous phospholipid preparation, delineate the suitability of the material to separate DNA fragments, and to demonstrate the compatibility with fluorescent detection of STRs utilized for human identification and clinical biomarkers of drug sensitivity.

3.1 Materials and Methods

3.1.1 Chemicals and Reagents

The 50 bp DNA ladder (part # N3236S) and fluorescent DNA ladder (part # MM-1000-FAM) were purchased from New England BioLabs (Ipswich, MA) and BioVentures (Murfreesboro, TN), respectively. Methanol (MeOH) was purchased through Calbiochem/ EMD (Gibbstown, NJ). DMPC and DHPC phospholipids were purchased through Avanti Polar Lipids (Alabaster, AL). The 3-(N-morpholino)-propanesulfonic acid (MOPS) was purchased from Alfa Aesar (Ward Hill, MA). Sodium hydroxide (NaOH) was obtained from Sigma-Aldrich (St. Louis, MO). Deionized water (diH_2O) was obtained using an ELGA PURELAB ultra water filtration system (Lowell, MA). The phospholipid additive was prepared as described previously [59] to obtain molar ratios of $[\text{DMPC}]/[\text{DHPC}] = 0.5$ and 95% w/v hydration (5% w/v phospholipid,) and $[\text{DMPC}]/[\text{DHPC}] = 2.5$ at concentrations ranging from 6-12% w/v phospholipid. An aqueous solution of 100 mM MOPS buffered to pH 7 was used to prepare the phospholipid additives and for capillary rinses and was the solution in the anodic and cathodic reservoirs during electrophoretic separations.

3.1.2 PCR Amplification

The PCR Amplification for the analyses of STRs used for human identification, the primer sequences used in the PCR for D3S1358, THO1, D21S11, D18S51, and Penta E were identical to those in the PowerPlex® 16 STR Kit manufactured by Promega® (Madison, WI). The primer sequences were obtained from the STR DNA database website (<http://www.cstl.nist.gov/strbase/PP16primers.htm>) and were synthesized by Integrated DNA Technologies (Coralville, IA). Five of the primers were 5'-labeled with FAM. The DNA template standard (K562), PCR reaction buffer (5X), and dNTP mix (each dNTP at a concentration of 10 mM, catalog # U1511) used in the amplification reaction were purchased from Promega®. PCR was performed with a thermal cycler (PTC-200, Bio-Rad, formerly MJ Research, Hercules, CA) using the following conditions: initial denaturation at 95 °C (11 min) and 96 °C (1 min), ten cycles 94 °C (30 sec), 60 °C (30 sec), and 70 °C (45 sec), 22 cycles of 90 °C (30 sec), 60 °C (30 sec), and 70 °C (45 sec), and a final incubation at 60 °C for 30 minutes. PCR products were purified with gel electrophoresis using a 15 x 10 cm, 2% OmniPur® agarose (Calbiochem/EMD Chemicals, Gibbstown, NJ) gel in 1X TBE buffer (G Biosciences, St. Louis, MO). The slab gel separation was accomplished with an applied voltage of 110 V for 3 hours. Alternate gel lanes were stained using crystal violet (Matheson Coleman and Bell, Cincinnati, OH). Purified PCR products in unstained gel lanes could then be excised and isolated using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit manufactured by GE Healthcare (Buckinghamshire, UK) prior to analysis on the Beckman/ Coulter

P/ACE MDQ Capillary Electrophoresis Instrument (Beckman Coulter, Fullerton, CA). PCR of the SNPs relevant to warfarin sensitivity was accomplished as previously described [19].

3.1.3 Capillary Electrophoresis

Separations were performed on a Beckman/Coulter P/ACE MDQ CE equipped with either a UV-visible absorbance (UV-vis) detection module operated at 254 nm or a LIF detection module equipped with an air cooled argon ion laser ($\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 520 \text{ nm}$). The fused silica capillary had an o.d. of 360 μm and an i.d. of either 25 or 50 μm (Polymicro Technologies, Phoenix, AZ). In preparation for electrophoresis, the capillary was subjected to a rinse procedure at 140 kPa (20 psi) consisting of 1N NaOH for 30 min, diH₂O for 15 min, MeOH for 15 min, and diH₂O for 15 min. Following the rinse protocol, the inner surface of the capillary was passivated in order to suppress EOF, using a previously characterized method based on phospholipids [60]. This was accomplished by coating twice at 140 kPa with a semi-permanent coating consisting of 5% phospholipid of [DMPC]/[DHPC] = 0.5 containing 1.25 mM CaCl₂ for 20 min followed by a 2 min flush with 100 mM MOPS pH 7. The capillary was then filled at an ambient temperature of 19 °C with [DMPC]/[DHPC] = 2.5 at the appropriate hydration. Once filled with the phospholipid additive, the capillary temperature was increased to the desired separation temperature. Between each separation the capillary was flushed at 140 kPa for 3 min with 5% [DMPC]/[DHPC] = 0.5,

2 min 100mM MOPS pH 7, and 3 min $[DMPC]/[DHPC] = 2.5$. Sample was injected by first introducing a pre-plug of run buffer at 6.9 kPa (1 psi) for 7 sec, then applying a reverse polarity electrokinetic injection of the DNA for a specified time, and finally providing a post-plug of run buffer introduced at 3.4 kPa (0.5 psi) for 5 sec. The purpose of these pre- and post-plugs, described previously [57], is to facilitate sample stacking and improve the theoretical plate count (N). Separations were achieved with reverse polarity. Data collection and analysis were performed using 32 Karat Software version 7.0 (Beckman Coulter), and theoretical plates were calculated using the “USP plates” criterion.

3.2 Results and Discussion

3.2.1 Estimation of Apparent Pore Size

To apply the medium to sieve DNA, the apparent pore size was characterized for different concentrations of preparations of $[DMPC]/[DHPC] = 2.5$. Separation of small DNA fragments through a gel or nanogel matrix is best accomplished with the linear migration provided by Ogston sieving. For this type of migration the velocity of the DNA fragments, which are approximated as incompressible spheres, is size-dependent and reflects the probability of unimpeded motion through the gel. When the size of the DNA-sphere approaches the apparent pore size of the sieving matrix, the DNA assumes a more linear form that is capable of traversing through the pores of the sieving matrix (i.e. reptation). For

linear gels of different viscosity, the range in size of DNA fragments that display Ogston sieving is established using a Ferguson plot [61], in which the migration of different sized DNA fragments is related to the tortuosity of the sieving medium. For a linear polymer this is accomplished by determining the migration of DNA fragments as the polymer concentration is changed. The migration velocity is plotted for different sizes of DNA yielding a linear relationship defined by intercept and slope. Larger fragments that migrate via reptation converge at an intercept and slope that differs from that obtained for migration based on Ogston sieving. The transition from Ogston to reptation sieving defines the apparent pore size of the sieving medium. This is visualized by plotting the retardation coefficient, derived from the slope of a Ferguson plot, versus the radius of gyration of the DNA.

A DNA ladder ranging from 50 to 1,350 bp was used to characterize the separation performance of the phospholipid medium and estimate the apparent pore size. The Ferguson study was performed with a preparation for which $[DMPC]/[DHPC] = 2.5$ at four different lipid concentrations described by the percentage of the total weight of phospholipid to the volume of aqueous buffer. The phospholipid concentrations: 6, 8, 10, and 12% w/v, were used to separate DNA at 25 °C. An example of a Ferguson plot obtained with phospholipid nanogel is shown in Figure 3-2. UV-vis is used to detect the DNA because utilizing covalent and intercalating labels could potentially affect the structure or properties of the DNA fragments. The radius of gyration (R_g) is calculated as

$R_g^2 = pL/3(1-p/L + p/L(\exp(-L/P)))$ [62], where L is the contour length equal to 0.34 nm per bp, and p is the persistence length estimated at 50 nm.

Electropherograms such as that shown in Figure 3-2 A are used to determine relative mobility as described previously by Holmes and Stellwagen [55]. The logarithm of each relative mobility was plotted against the concentration of the nanogel (see Figure 3-2 B). These data were fit using linear regression and the correlation coefficients ranged from 0.90 to 0.99 for all runs for hydration values ranging from 6 -12%. Figure 3-2 B summarizes the linear relationship between mobility and nanogel concentration for fragments ranging from 100 to 1,350 bp. The retardation coefficient (K_r) was derived from the slope of relative mobility versus percent phospholipid for 6-12%. The graph in Figure 3-2 C reveals that the transition from Ogston to reptation transport is between R_g ranging from 42 to 45 nm. Using the relationship $R_g = 1.4 \xi$ [63], the apparent pore size (ξ) of a 6-12% preparation of [DMPC]/[DHPC] = 2.5 is in between 30 and 32 nm.

Figure 3-2: Mobility of DNA through phospholipid matrix

Figure 3-2: (A) The mobility of 17 unlabeled DNA fragments that comprise the 50 base pair ladder ranging from 50 to 1,350 bp is determined from electropherograms obtained using an electric field strength of 100 V/cm and phospholipid additive with 6%, 8%, 10% (as shown in panel A) or 12% hydration. The size of the peak labeled with the asterisk is not known, and this peak is not used to estimate the apparent pore size. **(B)** Ferguson plot showing the relationship between mobility and phospholipid hydration. **(C)** Plot of the retardation coefficient determined from panel B against RMS gyration. The transition between Ogston sieving and reptation, visualized as the change in slope in panel C, is used to estimate the apparent pore size (30–32 nm) of the sieving material. Reproduced from [1].

Figure 3-2D: Mobility of DNA through phospholipid matrix

Figure 3-2: (D) Plot of the retardation coefficient with error bars determined from panel B against RMS gyration for the separated DNA fragments. The transition between Ogston sieving and reptation, visualized as the change in slope in panel C and D, is used to estimate the apparent pore size (30–32 nm) of the sieving material. Reproduced from [1].

3.2.2 Optimization of Separations of Double Stranded DNA

The investigation of material performance reveals that the [DMPC]/[DHPC] = 2.5 material is most useful for size based separations of dsDNA below 500 bp. The most efficient separations obtained from the Ferguson analysis (Table 3-1) were achieved with a 10% solution operated at 25 °C.

Table 3-1: Figures of Merit 350 bp dsDNA, $n \geq 5$

Phospholipid Concentration	Time (CV) minutes	Area (CV) $\times 10^3$	Plates
12%	27.9 (0.1)	0.8 (40)	260,000
10%	25.7 (0.4)	1.7 (10)	440,000
8%	23.1 (0.7)	0.5 (100)	360,000
6%	21.2 (2)	1.0 (20)	400,000

¹[DMPC]/[DHPC] = 2.5, Separations are accomplished in a 50 μm id capillary with a total length of 40.2 cm capillary and effective length of 30.2 cm, E 100 V/cm. The DNA base ladder is detected with UV-vis absorbance at 254 nm.

Table Reproduced from [1].

Separations were performed using a 50 μm i.d., 40 cm total length (L_{tot}), 30.2 cm effective length (L_{eff}) capillary. However, the material is compatible with a 25 μm i.d. capillary. Separations performed with an electric field strength (E_{app}) of 100 V/cm in 50 or 25 μm i.d. capillary produced a separation current of 6 or 1 microampere (μA), respectively. The low separation current obtained in either diameter capillary is achieved by using a zwitterionic buffer (100 mM MOPS buffered to pH 7) as the background electrolyte to support electrophoresis. The optimal injection parameters and the effects of separation distance (L_{eff}) and

applied voltage (V_{app}) were determined using a 50 bp DNA ladder, which was detected with UV-vis absorbance. The injection size is important when the extra-column band broadening associated with injection is significant relative to the band broadening produced by the separation. When the size of the injection plug is small, the theoretical plate count is high, but the reproducibility of the amount of injected analyte is worse. Based on the data in Table 3-2, a 2 kV 6 sec injection provided some compromise between high plate count and reproducibility in peak area.

Table 3-2: Effect of Injection Time 150 bp

Injection	Area (CV)	Plates (CV)
2kV 2 sec	300 (40)	900,000 (20)
2kV 4 sec	800 (30)	800,000 (20)
2kV 6 sec	1,600 (10)	460,000 (20)
2kV 8 sec	2,200 (8)	330,000 (10)
2kV 10 sec	2,800 (9)	270,000 (20)
2kV 12 sec	3,100 (9)	230,000 (20)
2kV 14 sec	3,600 (10)	210,000 (20)
2kV 16 sec	4,700 (10)	150,000 (10)

[DMPC]/[DHPC] = 2.5, 10% phospholipid. Separations ($n = 5$) are accomplished in a 25 μm id capillary with a total length of 40.2 cm capillary and effective length of 30.2 cm, with $E_{app} = 100 \text{ V/cm}$. The DNA base ladder is detected with UV-vis absorbance at 254 nm.

Table Reproduced from [1].

3.2.3 Effects of Length and Electric Field

The applied field strength (E_{app}) was varied from 50-250 V/cm for a 40 cm long capillary and a 95 cm long capillary to determine the effect of length and electric field on the separation performance of the 50 bp DNA ladder. The distance to the detection window (L_{eff}) used for UV-vis detection for the 40 and for the 95 cm capillary was 30.2 cm and 85.2 cm, respectively. The results obtained with UV-vis absorbance detection are summarized in Table 3-3 and Figure 3-3. For both separation lengths, the decrease in migration time is directly proportional to the increase in electric field. Migration time decreases 5-fold for separations performed at 50 V/cm and 250 V/cm, respectively. The theoretical plate count achieved using the 95 cm capillary is larger than that obtained with the 40 cm capillary. When the efficiency is normalized to separation length (i.e. reported as plates/m), the plate count is ~3-fold higher for the 95.2 cm capillary as compared to the 40 cm capillary. For CGE the resolution of DNA fragments is directly proportional to the effective length of the separation channel [64]. The resolution between DNA fragments differing by 50 bp is calculated using the equation $R_s = 0.589(\Delta t/w'_{1/2av})$, where Δt is the difference in migration time of the peaks and $w'_{1/2av}$ is the average peak width at half height. The data summarized in Figure 3-3 A and Table 3-3 confirm that the resolution improves by a factor of 3 when the separation length is increased by a factor of ~3 (i.e. from L_{eff} of 30.2 cm to 85.2 cm). At constant separation length of either 30.2 or 85.2 cm, the theoretical plate count and resolution of the 150 and 200 bp fragments are statistically

similar for electric field strength ranging from 50 to 250 V/cm (see Table 3-3). However, for the 350 and 400 bp fragments the plate count and resolution decrease with higher applied field strengths. For example, at an electric field strength of 250 V/cm, the resolution of the 350 and 400 bp fragments decreased to half of that obtained at 100 V/cm (See Figure 3-3 A and Table 3-3). The alignment of the 350 and 400 bp fragments in higher electric fields, distorts the radius of gyration, which is significant as these sizes of DNA approach the transition from Ogston sieving to reptation.

Table 3-3: Effects of Length and Field Strength on Resolution and Migration Time ¹									
E V/cm	² L _{effective} cm	150 base pair DNA				350 base pair DNA			
		³ R _s (CV)		Time (CV) minutes		³ R _s (CV)		Time (CV) minutes	
50	30.2	8	(10)	40.7	(1)	7.5	(10)	49.1	(1)
	85.2	24	(6)	124	(8)	25	(20)	164	(10)
100	30.2	9	(20)	22.5	(3)	9.2	(7)	28.4	(3)
	85.2	27	(40)	56.6	(2)	23	(20)	73.1	(2)
150	30.2	8	(10)	13.9	(0.7)	4.8	(5)	16.8	(0.9)
	85.2	25	(20)	37.0	(1)	19	(10)	47.1	(2)
200	30.2	7	(10)	10.6	(0.7)	4.5	(10)	12.7	(0.6)
	85.2	28	(20)	30.0	(3)	15	(20)	38.6	(3)
250	30.2	6	(4)	8.3	(0.6)	3.9	(10)	9.8	(0.6)
	85.2	21	(30)	22.7	(2)	10	(20)	28.7	(2)

¹[DMPC]/[DHPC] = 2.5, 10% phospholipid. Separations are accomplished at 25 °C in a 25 µm id capillary with a total length of 40.2 cm capillary and effective length of 30.2 cm (sample injected 2kV, 6 seconds), or a 25 µm id capillary with a total length of 95.2 cm and effective length of 85.2 cm (sample injected 6kV, 6 seconds). The DNA base ladder is detected with UV-visible absorbance at 254 nm.

²For data collected with L_{effective} = 30.2 and 85.2, n = 5 and n = 3, respectively

³Chromatographic resolution, R_s, is calculated $0.589(\Delta t/w_{1/2av})$, where Δt is the difference in migration time of the 150 and 200 or the 350 and 400 base pair fragments and $w_{1/2av}$ is the average peak width at half height.

Table Reproduced from [1].

Figure 3-3: Effect of field strength on migration time and resolution of 150 and 350 bp DNA

Figure 3-3: Effect of field strength summarized for a 25 μm i.d. capillary with a total length of 40 cm and effective length of 30.2 cm, or for a 25 μm i.d. capillary with a total length of 95 cm and effective length of 85.2 cm. Both the 150 base pair and 350 base pair fragments are assessed for (A) resolution as well as (B) migration time. Reproduced from [1].

3.2.4 Applications of Phospholipid Sieving

The high performance of the phospholipid additives and the benefit of a thermally-reversible nanogel make the material promising for routine separations of DNA fragments. Although UV-vis detection is preferable for material characterization and optimization, fluorescence detection is more practical for routine analyses over other strategies because of the low limits of detection and the ease of incorporating fluorescent tags into DNA. The most prevalent applications of CGE for DNA are in human identification. Analysis of STR markers is central to genetic analyses. For human identification, these markers are used to test paternity, kinship, as well as for forensic analyses and are detected using fluorescent labels conjugated to the primers utilized for PCR amplification. The fluorescently labeled 5'-primers target a particular locus. Following PCR amplification, the products are conveniently separated with commercial CGE systems using multi-wavelength detection. Instruments such as the ABI prism 310 genetic analyzer provide one base resolution within 30 minute separations [3] when used with commercial STR kits. These kits employ linear polymers, such as POP-4™, which is a PDMA polymer separation matrix. Although the total amount of gel required to fill a separation capillary ranges from 0.25 to 8 µL for 100 cm long columns of 25 µm or 100 µm i.d., the lower cost of the phospholipid additive over commercial sieving materials is an additional consideration. At the time this paper was submitted, the cost of the POP-4™

gel was \$70/mL [65]; while the cost of the phospholipid preparation was \$23/mL [66, 67].

To demonstrate the effectiveness of phospholipid additives for typical DNA separation based analyses, five STR markers were analyzed that contain the FAM, for which $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$, labeled primer set in the PowerPlex16™ kit manufactured by Promega®. The PCR amplified products were obtained using the DNA template standard K562. The resulting STR fragments for the five selected loci are D3S1358 (131 bp; 16 repeats), THO1 (179 bp; 9.3 repeats), D21S11 (a tri-allelic locus for K562 template with 223, 227, and 231 bp; 29, 30, and 31 repeats), D18S51 (318 and 322 bp; 15 and 16 repeats), and Penta E (379 and 424 bp; 5 and 14 repeats). The separation of the PCR products with phospholipid additive is shown in Figure 3-4. The separation is accomplished in approximately 30 minutes under non-denaturing conditions, using a 95 cm capillary and an electric field strength of 250 V/cm. The resolution of the amplified STR markers, calculated from the three peaks obtained for D21S11, is 1 for the 223 and 227, as well as, for the 227 and 231 bp fragments. These results confirm that the phospholipid additive performs as well as state-of-the-art linear gels required for STR analyses, yet are easily expelled and replaced.

Figure 3-4: FAM labeled STRs from PowerPlex® 16 kit

Figure 3-4: Electropherogram containing short tandem repeats relevant to human identification. The primers used for PCR amplification of the DNA template standard K562 are covalently modified with a fluorescent label (FAM). The PCR amplified products target 5 STR loci: D3S1358 (131 base pairs), THO1 (179 base pairs), D21S11 (223, 227, and 231 base pairs), D18S51 (318 and 322 base pairs), and Penta E (379 and 424 base pairs). The PCR products are separated using a 25 μ m i.d. capillary with an effective length of 85.2 cm, $E = 250$ V/cm, 30° C, 10% phospholipid with $[DMPC]/[DHPC] = 2.5$. Sample injection is performed at 10 kV for 3 s. Reproduced from [1].

Clinical applications of separation based CGE assays are also prevalent [2].

Fluorescently labeled primers may also be used for clinical biomarkers although intercalating dyes are effective for the detection of dsDNA [2]. Recently a multiplex PCR and microchip electrophoresis assay was described to identify

three SNPs that confer greater sensitivity to the potent anticoagulant drug warfarin [19]. Pharmacogenetic testing for warfarin sensitivity is a particularly relevant example of how rapid DNA analyses may improve patient treatment outcomes. With warfarin genotyping, patient hospitalizations over a 6 month period were reduced by almost one-third [68]. The assay utilizes four primers per SNP locus: a forward outer primer, a reverse outer primer, a forward inner primer, and a reverse inner primer. The four different primers are designed for each of the three loci to produce up to 9 different PCR products (110, 133, 154, 170, 206, 235, 286, 307, 340 bp) depending on whether each of the three loci are wild type/wild type, wild type/mutant, or mutant/mutant. The forward outer and reverse inner primers yield product for wild type CYP2C9*2, CYP2C9*3, and for mutant for VKORC1, whereas the forward inner and reverse outer primers yield product for mutant CYP2C9*2, CYP2C9*3, and for wild type VKORC1. The clinical sample is genotyped based on the pattern of amplicon sizes as determined with CGE separation.

The data shown in Figure 3-5 are a result of a PCR amplification of a clinical sample performed with the multiplexed tetra-primer assay. The primers are not fluorescently labeled. Therefore, to detect the PCR products using LIF, the intercalating dye SYBR® Green 1 is injected as a small plug into the 25 µm i.d. separation capillary and pushed past the detection window prior to the electrophoretic separation. The DNA fragments traverse the band of cationic dye and are then detected at the window. This strategy was utilized to separate and

detect the PCR amplicons using a 40 cm capillary with an effective length of 30.2 cm and electric field strengths ranging from 100 V/cm to 500 V/cm. As the electric field strength increases, the separation time decreases and, as summarized in Figure 3-5 A, the resolution also decreases. The electropherogram in Figure 3-5 B is obtained using an intermediate electric field strength of 300 V/cm. Calibration of amplicon size is not possible using multi-wavelength detection with the instrumentation as it is currently configured. Stringent size standardization utilizing standard ladders in multi-capillary instruments has been critically assessed in the literature. In a single capillary format, integrating a size standard in the sample can be used to perform size calibration in a single run rather than performing serial runs of a size ladder, followed by the sample. A standard containing the 250 and 350 bp fragments was co-injected with the sample and separated. Using these size standards to create a linear fit, the calculated size of the amplicons, in bp, was estimated to be 104 ± 3 , 135 ± 2 , 150 ± 2 , 241 ± 0.2 , 310 ± 1 , and 340 ± 1 . These results obtained with a 10 minute CGE separation are suitable to distinguish the PCR products and genotype the clinical sample, which in this case was confirmed to be wild type/wild type for all the SNP targets (CYP2C9*2, CYP2C9*3, and VKORC1) [19]. Separations accomplished at 30°C yielded similar results, as seen in Figure 3-6.

Figure 3-5 A: Relationship Among Applied Field, Separation Current and Resolution for Separations Performed at 23 ° C

Figure 3-5 B: SYBR Green 1 Labeled PCR Amplified Primers for Warfarin Genotyping

Figure 3-5: (A) Plot summarizing the effect of field strength ranging from 150 to 500 V/cm on separation current and resolution of PCR markers of warfarin sensitivity. **(B)** Electropherogram containing PCR markers of warfarin sensitivity. The double stranded DNA markers are detected using the intercalating dye SYBR Green 1. The sample is wild type/wild type for all of the SNP targets: CYP2C9*2 (154 bp from forward primer out, reverse primer in and 307 bp from forward primer out, reverse primer out), CYP2C9*3 (110 bp from forward primer out, reverse primer in and 340 bp from forward primer out, reverse primer out), and VKORC1 (133 bp from forward primer out, reverse primer in and 235 bp from forward primer out, reverse primer out). The separation is obtained using a 25 μ m i.d. capillary with an effective length of 30.2 cm, $E = 300$ V/cm, 25 ° C, 10% phospholipid with [DMPC]/[DHPC] = 2.5. Sample injection is performed at 6 kV for 6 s. Reproduced from [1].

Figure 3-6 A: Relationship Among Applied Field, Separation Current and Resolution for Separations Performed at 30 ° C

Figure 3-6 B: SYBR Green 1 Labeled PCR Amplified Primers for Warfarin Genotyping

Figure 3-6: (A) Plot summarizing the effect of field strength ranging from 150 to 500 V/cm on separation current and resolution of PCR markers of warfarin sensitivity. (B) Electropherogram containing PCR markers of warfarin sensitivity. The double stranded DNA markers are detected using the intercalating dye SYBR Green 1. The sample is wild type/wild type for all of the SNP targets: CYP2C9*2 (154 bp from forward primer out, reverse primer in and 307 bp from forward primer out, reverse primer in), CYP2C9*3 (110 bp from forward primer out, reverse primer in and 340 bp from forward primer out, reverse primer in), and VKORC1 (133 bp from forward primer out, reverse primer in and 235 bp from forward primer out, reverse primer in). The separation is obtained using a 25 μ m i.d. capillary with an effective length of 30.2 cm, $E = 300$ V/cm, 30 ° C, 10% phospholipid with [DMPC]/[DHPC] = 2.5. Sample injection is performed at 6 kV for 6 s. Reproduced from [1].

Sieving separations of STRs and SNPs with CGE provide several benefits over slab gel separations including reduced sample consumption, improved quantification, as well as, rapid and automated analyses. However, one advantage of slab gel over CGE is that a highly cross-linked stacking gel can be cast at the site of injection in addition to the separation gel. The retarded mobility in the higher cross-linked gel serves to concentrate the DNA bands at the beginning of the separation lane. Currently, such gradients cannot be achieved in CE with solution based gels because laminar flow and diffusion prohibit the formation of a discrete stacking region. With the thermally responsive phospholipid preparation, this can be achieved by injecting DNA at a higher temperature to create a more viscous medium. After the separation has been initiated, the temperature is then reduced. A fluorescently labeled DNA base ladder was electrokinetically injected (2kV 6 sec) into the phospholipid nanogel. For the 25°C isocratic separation, sample was injected at 25°C. For the 30°C isocratic separation, as well as the gradient separations, sample was injected at a temperature of 30°C. A thermal gradient from 30°C to 25°C was implemented using a 40 cm long capillary, with an effective length of 30.2 cm, and an electric field strength of 100 V/cm. Isocratic separations performed at 25°C (Figure 3-7 A) and 30°C (Figure 3-7 B), as well as the temperature gradient separations where the onset for temperature change occurs at 32 min (Figure 3-7 C) and 22 min (Figure 3-7 D), are all shown in Figure 3-7. Analyte peak areas increased with temperature. For example, at 25°C the 150 bp fragment had an area of 990, whereas at 30°C it was 12,000. The thermal gradient separations, shown in

Figure 3-7 C, yielded a peak area of 15,000 for the 150 bp fragment and a theoretical plate count of 590,000. The chromatographic resolution (R_s) between the 150 and 200 bp fragments obtained with a thermal gradient with a change in temperature onset at 22 min was 20. This resolution performance is similar to that obtained when using the 30°C isocratic separation ($R_s = 19$) and is better

Figure 3-7: Isocratic and Temperature Gradient Separations

Figure 3-7: Electropherogram of a fluorescently conjugated base ladder comprised of 23 discrete DNA fragments ranging from 50 to 1,000 base pairs is performed using an (A) isocratic separation at 25 ° C and (B) isocratic separation at 30 ° C. Then, a gradient separation from 30 to 25 ° C for which sample is injected at 30 ° C and the separation is initiated. For gradient separations a temperature change of 1 ° C / min is initiated at (C) 22 min and at (D) 32 min. Reproduced from [1].

than that obtained for a 25°C isocratic separation ($R_s = 15$) or with the gradient with a change in temperature onset at 32 min ($R_s = 17$). These results demonstrate that the use of thermal gradients with phospholipid pseudo gels provides a new advance to sieving based separations with CGE.

3.3 Conclusions and Future Directions

The phospholipid nanogel provides efficient separations of DNA performing with single bp resolution within a 30 minute separation. The switchable viscosity of the material simplifies introduction into the channel and provides a means to create a gel gradient within the capillary by changing the temperature. The separations demonstrate the dependence of migration time and resolution as well as the effect of capillary length and applied voltage. These findings are particularly relevant to microfluidic separations of DNA as thermally responsive nanogels are easier to replace and extend the useful lifetime of the chip. The low cost and simpler preparation of the material ultimately increases the accessibility of sieving-based assays.

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Chapter 4

Reversible Phospholipid Nanogels for Deoxyribonucleic acid Size Determinations up to 1500 Base Pairs and Integrated Sample Stacking

Reproduced from [1]:

Durney, B.C., B.A. Bachert, H.S. Sloane, S. Lukomski, J.P. Landers, and L.A. Holland, *Reversible Phospholipid Nanogels for DNA Fragment Size Determinations up to 1,500 Base Pairs and Integrated Sample Stacking*. accepted Analytica Chimica Acta, 2014.

4.0 Introduction

Estimating the size of DNA is critical in genetic analysis associated with human identification [2], species identification [3, 4], detecting personal biomarkers [5], analyzing food [6], and categorizing pathogenicity [7]. Moreover, following sample preparation, accurate determination of the size of DNA fragments is a mandatory analytical step in sophisticated high-throughput sequencing techniques. It is also a critical endpoint for methods based on PCR amplification, where the length of the product is used to determine the presence of a specific target sequence in the DNA template. Confirmation of size is achieved by comparing the fragment migration time during electrophoretic sieving using various sieving agents. Slab gel electrophoresis, the workhorse of modern analytical science, is a commonly used tool for DNA sizing due to the simplicity and low cost of the method. Sizing is achieved by comparing the migration time of the targeted DNA with a DNA ladder that contains a mixture of fragments of known size. The DNA ladder standard is run in a separate lane. A serious disadvantage of slab gels is the poor separation efficiency and the reduced throughput even when completed in parallel lanes in the gel [8, 9]. Although more expensive than slab gel methods, CGE provides a size discrimination that is improved substantially, and higher sample throughput. Sequencing of the human genome provides direct proof of this [10].

CGE separations of DNA fragments are accomplished using polymer solutions that provide a size-based separation of DNA fragments by creating networks or matrices that form dynamic pores for sieving [11-13], or separation through DNA entanglement [14]. DNA fragments ranging from 50 to 1,500 bases are often present in an electropherogram, but the resolution obtained for the larger DNA fragments is poor and accurate sizing is limited to DNA fragments that are shorter than 500 bases in length. Ultra-high resolution separations were achieved with 0.75% HEC used to size a 256 bp DNA fragment [15]. Although larger fragments could not be sized, 1,078 bp and 1,353 bp DNA were baseline resolved [15]. A commercial system utilized HEC to separate 50 bp to 10 kbp DNA and to accurately size 275 to 815 bp DNA [16]. In another case, a matrix of 2% LPA was used to sequence a DNA fragment 1,300 bases long [17]. Several polymer properties dictate the utility of the material and the useful size range of the separation [18]. Increasing the upper fragment size limit of the separation can be accomplished by increasing the length of the polymer and maintaining the concentration of the gel at or above the entanglement threshold [11]. This increase in concentration is accompanied by an increase in viscosity [12], which provides challenges for the introduction to or replacement of, the gel in the separation capillary.

Temporary gels, which rupture and reform during the separation, are an exciting alternative to permanent gels because the upper size limit increases when the rate of gel rupture is optimized [19]. Wormlike micelles form large self-assembled structures that dynamically rupture and reform. Aqueous solutions of the long-chain phospholipid DMPC and the short-chain phospholipid DHPC are known to form wormlike micelles [20, 21]. A dynamic sieving matrix of 10% phospholipid with $[DMPC]/[DHPC] = 2.5$ was optimized for single-base pair resolution that was adequate for detection of STRs in selected loci utilized for human identification using a 100 cm long capillary within a 30 minute run time [22]. This is comparable to separations on commercial CE instruments for forensic STR analyses [23]. What makes this particularly attractive, is that the phospholipid nanogel is substantially more cost-effective at roughly one-fourth the cost of commercial gels utilized for human identification [24-26]. A significant advantage of the dynamic sieving matrix is the thermally-responsive viscosity. The sieving matrix has low viscosity below the gel-phase transition temperature of the phospholipids and becomes gel-like at higher temperatures. As a result, the sieving agent is easily introduced into or expelled from the narrow bore separation capillary at a temperature of 19 °C. Prior to the separation, the matrix is transformed into a viscous gel in-capillary at a separation temperature of 30 °C.

While a phospholipid additive has been previously described for the separation of DNA fragments below 500 bp [22], we show that the DNA sizing range of the separation can be extended by reducing the concentration of wormlike micelles in solution. The size, shape, and stability of the phospholipid structures depend upon the phospholipid concentration, the ratio of DMPC and DHPC, and the temperature. The effect of these parameters on the size limit of DNA separations was evaluated, and the linear range for size separation was extended to 1,500 bp by tuning the concentration of the phospholipid nanogel and the ratio of [DMPC]/[DHPC]. This preparation was used to compare the size of the DNA fragments to internal standards. The effectiveness of the sieving matrix was demonstrated with the analysis of PCR-amplified DNA sequences specific to select pathogens. The difference between the true size of a DNA fragment and the size calculated from internal standards is less than 2.3% for fragment sizes between 200 to 500 bp. For fragments that range from 600 to 1,500 bp this difference between the true size and measured size is less than or equal to 4%. Different properties of the high and low concentration phospholipids were exploited by integrating both dynamic sieving matrices into a single capillary to create a discontinuous gel in-capillary used to concentrate DNA samples through gel stacking.

4.1 Materials and Methods

4.1.1 Chemicals and Reagents

The fluorescently labeled DNA ladder (catalog # MM-1000-FAM, 50 to 1,000 bp) was purchased from BioVentures (Murfreesboro, TN). The fluorescent intercalating dye SYBR® Green 1 nucleic acid gel stain (catalog # S-7567), 1 Kb Plus (catalog # 10787-018, 100 to 12,000 bp), and 100 bp (catalog # 15628-019, 100 to 2,072 bp) DNA ladders were purchased from Life Technologies (Grand Island, NY). The MeOH was purchased through Calbiochem/EMD (Gibbstown, NJ). The DMPC and DHPC phospholipids were purchased through Avanti Polar Lipids (Alabaster, AL), and the MOPS was purchased from Alfa Aesar (Ward Hill, MA). NaOH was obtained from Sigma-Aldrich (St. Louis, MO). All dH_2O used was obtained from an ELGA PURELAB ultra water filtration system (Lowell, MA).

4.1.2 Preparation of phospholipid mixture

The phospholipid preparations were made as described previously [27] to obtain molar ratios of $[\text{DMPC}]/[\text{DHPC}] = 0.5$ and $[\text{DMPC}]/[\text{DHPC}] = 2.5$. The phospholipids were stored at $-20\text{ }^{\circ}\text{C}$ and thawed before being weighed out. Once they were weighed, an aqueous buffer of 100 mM MOPS (pH 7.0) was added to obtain the appropriate concentration of phospholipid per volume of aqueous diluent (% w/v). The solution was then mixed using a vortex mixer for several minutes to dissolve the phospholipids. The phospholipid medium was

then exposed to three rapid freeze thaw cycles using liquid nitrogen followed by centrifugation (10,000 rpm at 4 °C) for 10 minutes. The matrix was then portioned into working volume aliquots of 200 µL [DMPC]/[DHPC] = 0.5 (5% w/v) and 100 µL [DMPC]/[DHPC] = 2.5 (10% w/v). The [DMPC]/[DHPC] = 2.5 lipids were then diluted with the same 100 mM MOPS (pH 7.0) buffer to obtain the 7.5, 5.0, and 2.5% phospholipid used for electrophoresis. No freeze thaws were done after the dilution from the 10% phospholipid. The additional phospholipid matrices with [DMPC]/[DHPC] = 3.0, 4.0, and 5.0 used in the study were prepared the same manner. The MOPS buffer was also used for capillary rinses between runs and served as the solution in the anodic and cathodic reservoirs during electrophoresis separations.

4.1.3 PCR Amplification

Primers were based on *sc/2* genes with the following gene bank accession numbers and genome locations; M3: AE014074, location 791329-793050; M12: CP000259, location 862738-863880; M28: CP000056, location 781169-782437. Forward primers specific for each *sc/2* allele, are as follows: M3- Sc/2.3_CLflank_2F, 5'-AGGCATACAAGATCATGTCCTTGA-3'; M12- Sc/2.12_CLfl_1F, 5'-AAGAGTGGGATATCTTTAGGC-3'; M28-Sc/2.28_CLfl_1F, 5'-CTACAGGAACGAGAACAAGC-3'. A single reverse primer Sc/2.3_CLflank_2R, 5'-TTTGGTGTATGTGCTGCGGT-3', targeting a conserved 3' region of *sc/2* gene was paired with each forward primer. Genomic DNA was

purified and isolated as described previously [28]. PCR reactions were carried out using a C1000 Touch Thermal Cycler (BioRad, Hercules, CA) and 1.5 units of *Taq* DNA polymerase (Qiagen, Germantown, MD) diluted to a final volume of 50 μ L per reaction and DNA template in the nanogram range. The manufacturer's PCR buffer was supplemented with 0.2 μ M concentration of each primer and 0.2 mM dNTPs (Bioline, Taunton, MA). Amplification conditions consisted of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 51 °C, and 1 min 45 s at 72 °C, followed by a final extension step at 72 °C for 7 min. The amplicons were analyzed by agarose gel electrophoresis and the true size of each *sc*/2-PCR product was determined by Sanger DNA sequencing (Eurofins Genomics, Huntsville, AL). The DNA sequences were assembled and analyzed with the Lasergene 11 Genomics Suite (DNASTAR, Inc., Madison, WI) software [29]. Isolation of genomic DNA and purification from fungal cultures, PCR primers (Eurofins Genomics, Huntsville, AL), amplification conditions, and verification of conserved amplicon sizes for *acI*-based detection of *Aspergillus* species were previously established [30].

4.1.4 Gel extraction of DNA ladder standards

Fragments from the two unlabeled DNA ladders were separated using a VWR horizontal gel box Midi Plus 15x10cm connected to a Hoefer hsi (San Francisco, CA) power supply using a 1% agarose (CalbioChem/EMD Chemicals) in 1X TBE buffer (G Biosciences, St. Louis, MO) gel. Slab gel electrophoresis was accomplished using an applied voltage of 110 V for 3 hours, and alternate gel lanes were stained using ethidium bromide (EtBr) (part # 15585011, Life Technologies) for UV visualization. Extraction and purification of DNA bands in unstained lanes was done using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit manufactured by GE Healthcare (Buckinghamshire, UK) prior to analysis on the P/ACE MDQ CE instrument (Beckman Coulter, Fullerton, CA). DNA that was isolated and recovered from the agarose gel was then utilized as an internal standard to size PCR amplicons. The 600, 1,000, 1,500, and 1,650 bp fragments were used as internal standards to size *S. pyogenes* and *B. anthracis* amplicons, whereas the 150, 450, and 500 bp were used to size *Aspergillus* spp. amplicons.

The size of the PCR amplicons was calculated using internal standards purified from the DNA ladders listed in the materials section. Sizing of fragments was accomplished using two DNA standards that bracketed the size of the PCR amplicon. All DNA fragments were co-injected and a linear fit was used to relate the migration time to the known size in bp of the DNA standards. Using this fit,

the size of each PCR amplicon was calculated based on the migration time. Resolution between fragments was calculated using the expression $R_s = 0.589 \cdot (\Delta t / w_{1/2av})$, where Δt is the difference in migration time between the two peaks of interest, and $w_{1/2ave}$ is the average width at half height for the same two peaks. Resolution in base pairs was calculated by dividing the difference in the number of base pairs for the two fragments (Δbp) by the calculated resolution. Therefore, base pair resolution can be expressed as $R_{s_{bp}} = (\Delta bp) / (0.589 \cdot (\Delta t / w_{1/2av}))$. Data collection and analysis were performed using 32 Karat Software version 7.0 (Beckman Coulter), and peak width at 50% height as well as theoretical plate count were calculated using the “USP” criterion.

4.1.5 Pre – treating the separation capillary

Separations were performed using fused silica capillary with an o.d. of 360 μm and an i.d. of 25 μm (Polymicro Technologies, Phoenix, AZ). The separation capillary had an L_{tot} of 40 cm, and an L_{eff} of 30.2 cm. New capillary was conditioned with a 4-step rinse procedure performed at 140 kPa with (1) 1N NaOH for 30 min, (2) diH₂O for 15 min, (3) MeOH for 15 min, and (4) diH₂O for 15 min. The conditioned capillary was then passivated with a semi-permanent phospholipid coating to suppress EOF as previously described [31]. This was accomplished using a 2-step rinse at 140 kPa with (1) 5% phospholipid of [DMPC]/[DHPC] = 0.5 containing 1.25 mM Ca²⁺ for 20 min and (2) a 2 min flush with 100 mM MOPS buffered to pH 7. This passivation rinse is repeated once (i.e., 4-steps). The capillary was then

filled with phospholipid nanogel at an ambient temperature of 19 °C. Once filled with the phospholipid, the capillary temperature was increased to the desired separation temperature. A reverse polarity voltage of 20 kV was applied for 5 min prior to the first run of the day.

4.1.6 Capillary Electrophoresis sample introduction and separation

Sample was injected by first introducing a pre-plug of run buffer at 6.9 kPa for 7 sec, then applying a reverse polarity electrokinetic injection of DNA for the specified time, and finally by inserting a post-plug of run buffer at 3.4 kPa for 5 sec. The purpose of these pre- and post-plugs was to facilitate stacking and improve the theoretical plate count [32]. Separations were performed at 4 kV under reverse polarity conditions. Between each separation the capillary was flushed at 140 kPa for 3 min with 5% [DMPC]/[DHPC] = 0.5, 2 min MOPS (pH 7), and 3 min with the phospholipid preparation used to separate DNA.

4.1.7 Fluorescence detection and analysis of DNA

The Beckman Coulter P/ACE MDQ was equipped with an LIF detection module and an air cooled argon ion laser (λ_{ex} =488 nm and λ_{em} =520 nm). The optimization of concentration and composition of phospholipid nanogel was accomplished using a fluorescein end-labeled DNA ladder. The intercalating dye SYBR® Green 1 was utilized for separations of PCR amplicons. SYBR dye was

diluted to a concentration of 20 μM SYBR in phospholipid preparations of $[\text{DMPC}]/[\text{DHPC}] = 2.5$ at a concentration equal to that used for the separation. The concentration of the plug was calculated based on a literature estimate of 20 mM stock concentration from the manufacturer [33]. Unless otherwise stated, a 7 second 34.5 kPa plug of phospholipid modified with dye was pushed toward the detection window in the separation capillary prior to sample injection. Under an applied field, the positively-charged dye and negatively-charged DNA migrated toward each other and the two bound effectively upon contact as the K_d is 3.1 nM [34]. Any variation in the ratio of dye and DNA in a separation will change the migration time of the DNA. Reproducible run times are achieved by utilizing a dye concentration compatible with the detector output, in this case an output of approximately 1-5 RFU was achievable with a plug of 20 μM SYBR Green 1. As summarized in Table 4-1, the reproducibility in migration time is less than or equal to 5% for separations with a 7 second dye plug. The use of internal size standards in each separation further mitigates any bias in migration time due to variation in the ratio of dye to DNA.

Table 4-1 Effect of SYBR Green 1 Concentration on Migration Time of 650 bp DNA

Concentration	Injection size	Migration Time in minutes	(CV)
2 μM	5 psi 7 sec	27.9	(4)
20 μM	5 psi 7 sec	27.8	(2)
100 μM	5 psi 7 sec	26.1	(5)

[DMPC]/[DHPC] = 2.5, 2.5% phospholipid. Separations ($n = 5$) are accomplished in a 25 μm id capillary with a total length of 40 cm capillary and effective length of 30.2 cm, with $E_{\text{applied}} = 100 \text{ V/cm}$. The DNA base ladder (1 Kb + DNA ladder, Life Technologies) is detected with LIF at 520 nm using SYBR green 1 nucleic acid stain at different concentrations positioned before the detection window.

Table Reproduced from [1].

4.2 Results and Discussion

4.2.1 Effects of gel concentration on the separation

The total lipid concentration (% w/v) determined the number of 'ribbons' or 'disks' in solution and the degree of long-range interaction, including the extent of lipid entanglement of the nanogel. Using a fluorescently-labeled DNA ladder, the separations were examined in the 500 to 1,000 bp range to quantify the effect of phospholipid concentration on separation performance. Our previous literature reported the effect of concentrations of phospholipid ranging from 5-15% for separations based on subtle differences in hydrodynamic volume [27] and from 6-12% for separations based on sieving [22]. For both studies [22, 27] a 10% phospholipid concentration generated the most effective separation efficiency and peak resolution. In the current study, concentrations of 2.5, 5.0, 7.5, and 10% were used to evaluate the effect of phospholipid concentration on the useful upper size limit for DNA separations. Preparations below 2.5% phospholipid did not generate reproducible DNA separations, presumably because wormlike micelles are not stable under these conditions, and consequently, these were not assessed. The peak resolution summarized in Table 4-2 was measured for a set of DNA fragments at 450 and 475 bp as well as a set at 900 and 950 bp. There was no significant improvement in resolution of DNA fragments with a length of 450 and 475 bp for separations accomplished at 23 °C. When the separation was performed at the optimum temperature for each lipid preparation, which was 25 °C for 5% nanogel, and 30 °C for both 7.5% and 10% nanogel, the resolution

of DNA fragments smaller than 500 bp was similar when comparing a 2.5% to a 10% phospholipid nanogel (see Table 4-3). The resolution between the DNA fragments with a length of 900 and 950 bp improved substantially (2.3-fold) when comparing a nanogel of 2.5% phospholipid to one of 10% phospholipid. When the separation was performed at the optimum temperature for each lipid preparation (i.e., 25 °C for 5% nanogel, and 30 °C for both 7.5% and 10% nanogel), the resolution between fragments of 900 and 950 bp improved 1.8-fold (see Table 4-3). These results indicated that the lipid preparations should be maintained at a concentration of 2.5% lipid.

Table 4-2: Effect of Concentration on Resolution ¹

Phospholipid (%)	Resolution 450/475 bp DNA		Resolution 900/950 bp DNA	
	Resolution (CV) ²	in bp ³	Resolution (CV) ²	in bp ³
2.5	5.0 (6)	5	6.4 (7)	8
5.0	4.6 (10)	5	4.3 (10)	12
7.5	4.5 (9)	6	3.4 (10)	15
10.0	4.4 (10)	6	2.8 (9)	18

¹ Separations ($n = 5$) of DNA base ladder MM-1000-FAM are achieved at 23 °C with [DMPC]/[DHPC] = 2.5 in a 40 cm long, 25 μ m id capillary. The effective length is 30.2 cm and $E_{app} = 100$ V/cm.

² Resolution is calculated as $0.589(\Delta t/w_{1/2av})$, where Δt is the difference in peak migration times and $w_{1/2av}$ is the average peak width at half height

³ Resolution in base pairs is calculated as the difference in base pairs/resolution.

Table Reproduced from [1].

Table 4-3: Effect of Phospholipid Concentration on Resolution¹

Hydration (%)	Temp (°C)	Resolution 450/475 base pair DNA ²		Resolution 900/950 base pair DNA ³	
		Resolution (CV) ²	in bp ³	Resolution (CV) ²	in bp ³
2.5	23	5.0 (6)	5	6.4 (7)	8
5.0	25	5.1 (10)	5	4.5 (10)	11
7.5	30	5.4 (7)	5	4.2 (20)	12
10.0	30	6.2 (7)	4	3.6 (20)	14

¹Separations ($n = 5$) are achieved at the specified temperature with [DMPC]/[DHPC] = 2.5 in a 40 cm long, 25 μ m id capillary. The effective length is 30.2 cm and $E_{app} = 100$ V/cm.

²Resolution is calculated as $0.589(\Delta t/w_{1/2av})$, where Δt is the difference in peak migration times and $w_{1/2av}$ is the average peak width at half height

³Resolution in base pairs is calculated as the difference in base pairs/resolution.

Table Reproduced from [1].

4.2.2 The effect of temperature and composition, factors that impact nanogel morphology, on separation

The ratio of long-to-short-chain lipid determines the shape of the self-assembled aggregate formed in solution. When the relative amount of long chain lipid increases, the aggregates are transformed from a bilayer disk conformation with a small diameter, to disks with larger diameters and 'ribbons' [20, 21]. The temperature also impacts the transformation from nanodisks to nanoribbons and a substantial change in apparent viscosity is observed over a temperature range specific to the preparation [35-37]. The morphologies adopted by the matrix were dependent on the ratio of long-to-short chain lipid as well as the temperature of the preparation. At a total lipid concentration of 2.5%,

Table 4-4: Effect of [DMPC]/[DHPC] Ratio on Resolution at 23 °C ¹

	Resolution 450/475 bp DNA		Resolution 900/950 bp DNA	
	[DMPC] [DHPC]	Resolution (CV) in bp	Resolution (CV) in bp	
	2.5	5.0 (6) 5	6.4 (7) 8	
	3.0	4.4 (10) 6	5.2 (10) 10	
	4.0	4.8 (10) 5	5.5 (10) 9	
	5.0	1.1 (20) 23	1.2 (20) 42	

¹ Separation conditions, calculation of resolution, and calculation of resolution in bp are identical to that reported in Table 3-2.

Table Reproduced from [1].

Table 4-5: Effect of [DMPC]/[DHPC] on Resolution ¹

	Temp (°C)	Resolution 450/475 base pair DNA ²		Resolution 900/950 base pair DNA ³	
		[DMPC] [DHPC]	Resolution (CV) ² in bp ³	Resolution (CV) ² in bp ³	
	23	2.5	5.0 (6) 5	6.4 (7) 8	
	21	3.0	5.1 (8) 5	5.3 (10) 9	
	21	4.0	5.5 (10) 5	5.5 (10) 9	
	21	5.0	1.4 (20) 18	2.6 (20) 36	

¹ Separations ($n = 5$) are achieved at the specified temperature with [DMPC]/[DHPC] = 2.5 in a 40 cm long, 25 μ m id capillary. The effective length is 30.2 cm and $E_{app} = 100$ V/cm.

² Resolution is calculated as $0.589(\Delta t/w_{1/2av})$, where Δt is the difference in peak migration times and $w_{1/2av}$ is the average peak width at half height

³ Resolution in base pairs is calculated as the difference in base pairs/resolution.

Table Reproduced from [1].

preparations were examined with a ratio of long-to-short chain lipid of 2.5, 3.0, and 4.0. The results, summarized in Table 4-4, revealed that the lipid composition had no significant effect on the resolution of DNA fragments under these conditions. When the ratio of long-to-short chain lipid was 5.0, the separation performance decreased. The resolution improved marginally for nanogels with different lipid composition when the optimum performance was observed at a temperature different from 23 °C (see Table 4-5).

Although, the performance of the separation matrix was tolerant of changes in lipid composition, the preparation made with a 2.5 ratio of long-to-short chain lipid was used for analytical separations and the effect of temperature on separation performance was tested. The results for a 2.5% w/v preparation composed of [DMPC]/[DHPC] equal to 2.5 are summarized in Table 4-6. The best resolution for both the 450 and 475 bp DNA and the 900 and 950 bp DNA was obtained at 23 °C. At this temperature 450 and 475 bp DNA fragments were separated with 5 bp resolution, and 900 and 950 bp DNA fragments were separated with 8 bp resolution. For both size domains the resolution obtained using a 2.5% nanogel is 1% when normalized by dividing the resolution (in bp) by the size of the separated DNA fragments. This is a substantial increase over the 10% resolution obtained with a commercial system that utilizes a hydroxypropylmethylcellulose (HPMC) matrix for DNA shorter than 1,000 bp [16]. This improved sizing obtained with the phospholipid nanogel is an extraordinary

advantage for DNA analyses with sieving that require accurate size determinations.

Table 4-6: Effect of Temperature on Resolution¹

Temperature (°C)	Resolution 450/475 base pair DNA ²		Resolution 900/950 base pair DNA ³	
	Resolution (CV) ²	in bp ³	Resolution (CV) ²	in bp ³
19	1.2 (20)	22	1.8 (20)	27
20	3.2 (10)	8	5.3 (10)	9
21	4.5 (9)	6	5.7 (10)	9
22	4.5 (8)	6	5.8 (10)	9
23	5.0 (6)	5	6.4 (7)	8
24	2.7 (10)	9	4.2 (20)	12
25	2.5 (10)	10	3.3 (20)	15

¹Separations ($n = 5$) are achieved with 2.5% [DMPC]/[DHPC] = 2.5 in a 40 cm long, 25 μm id capillary. The effective length is 30.2 cm and $E_{\text{app}} = 100 \text{ V/cm}$.

²Resolution is calculated as $0.589(\Delta t/w_{1/2\text{av}})$, where Δt is the difference in peak migration times and $w_{1/2\text{av}}$ is the average peak width at half height

³Resolution in base pairs is calculated as the difference in base pairs/resolution.

Table Reproduced from [1].

4.2.3 Expanded size range on sieving using wormlike nanogel

These optimization studies confirmed that decreasing the concentration of the lipid preparation separated a wider range of DNA fragments than previously reported. This is demonstrated in Figure 4-1, which depicts the separation of the

1kilobase DNA ladder. The separation performance obtained with the DNA size ladder improves by successively decreasing the viscosity of the nanogel used for sieving DNA. Figure 4-1 trace A is of 10% lipid with [DMPC]/[DHPC] equal to 2.5 at 30 °C, which is the higher viscosity formulation and provides accurate sizing for DNA fragments smaller than 500 bp in length. When the temperature of the 10% nanogel matrix is decreased the viscosity is decreased as well. The trace in Figure 4-1 B obtained using a 10% nanogel matrix at a separation temperature of 23°C demonstrates some improvement in the resolution of larger fragments ranging from 1,000 to 12,000 bp in length. The trace in Figure 4-1 C is obtained using a 2.5% nanogel matrix at a separation temperature of 30°C. This material has a lower viscosity than the 10% nanogel matrix and relative to the 10% nanogel the resolution is improved for the fragments ranging from 1,000 to 12,000 base pairs. Figure 4-1 trace D is of the optimized preparation of 2.5% lipid with [DMPC]/[DHPC] equal to 2.5 at 23 °C. This nanogel has the lowest viscosity and generates the best resolution for DNA fragments larger than 500 bp.

Figure 4-1: Sieving performance for DNA fragments larger than 500 bp

Figure 4-1: Electropherograms of a 1 kilobase DNA ladder are detected using SYBR Green 1 fluorescent dye using a phospholipid nanogel with [DMPC]/[DHPC] = 2.5: **(A)** at a concentration of 10% and temperature of 30 ° C, **(B)** at a concentration of 10% and temperature of 23 ° C, **(C)** at a concentration of 2.5% and temperature of 30 ° C, and **(D)** at a concentration of 2.5% and temperature of 23 ° C. The DNA fragments in the ladder (Life Technologies, #10787-018) range from 100-12,000 bp and the size in bp specified by the manufacturer is indicated above each corresponding peak. Reproduced from [1].

For DNA fragments shorter than 500 bp, the performance of the optimized preparation was similar. The resolution of 450 and 475 bp fragments decreased from 6.2, using the 10% phospholipid preparation with [DMPC]/[DHPC] = 2.5 at 30 °C (see Table 4-3), to 5.0, using the optimized preparation of 2.5% phospholipid with [DMPC]/[DHPC] = 2.5 at 23 °C. This slight decrease in resolution performance to 78% of the previously reported value of 6.2, did not limit the application of the material to analyze and accurately calculate the size of these PCR amplicons.

4.2.4 Application to assess length polymorphism of PCR amplicons derived from pathogenic bacteria

PCR amplicons were identified using bioinformatics to predict unique DNA biomarkers of different strains of *Streptococcus pyogenes* ranging from 634 to 1,332 bp in length. Over 700 million infections annually are attributed to this pathogenic bacterium associated with common throat and skin diseases with more than 0.5 million deaths resulting from invasive and autoimmune complications [38]. Strains are typically characterized epidemiologically by serological typing of the M protein or, more recently, by sequencing of the *emm* gene [39-41]. However, epidemiological surveillance during outbreaks of infection requires additional tools to distinguish between strains of the same M-type. The *scf2* gene, encoding a common *S. pyogenes* surface protein, exhibits significant length polymorphism between strains of the same M-type, due to the presence of repetitive Gly-X-Y motifs in the collagenous domain [29, 42]. Strain

typing by length variation in collagen-like genes has been successfully demonstrated for the *bcl* genes of *Bacillus anthracis* [43] and is a viable strategy for typing *S. pyogenes*. The *sc*/2 alleles from strains of M-types 3, 12, and 28 were selected here as epidemiological markers since these are among the most prevalent M-types causing infection in the United States and globally [44, 45]. Importantly, M3-type strains are associated with invasive diseases like necrotizing fasciitis and streptococcal toxic shock syndrome [44, 46-48].

Primers were designed to generate PCR amplicons of *sc*/2 alleles associated with each M-type. The 2.5% phospholipid preparation was utilized to evaluate the applicability of the method for the analysis of PCR amplicons. Three amplicons were separated, as shown in Figure 4-2 A, and sized with internal standards of 600 and 1,000 bp. The 634 bp and 715 bp amplicons, which were sequenced in the genome of three different M12-type strains, and the 842 bp amplicon, which was sequenced in the M28-type strain, were detected and sized using the phospholipid nanogel. The remaining amplicons (1,071, 1,152, and 1,332 bp) sequenced in the M3-type strains were detected and sized (see Figure 4-2 B) using internal standards of 1,000 and 1,500 bp. The extended sizing range of the phospholipid nanogel enabled detection of larger *sc*/2-derived amplicons to generate unique DNA markers that distinguish strains of *S. pyogenes*. To further demonstrate the effectiveness of the phospholipid nanogel for size determination, a previously reported amplicon of *bcl* gene that is 1,446

bp in length [43] was analyzed using internal standards of 1,000 and 1,650 bp as shown in Figure 4-2 C.

Figure 4-2: Separation of PCR amplicons obtained with the phospholipid nanogel optimized for DNA up to 1500 bp in length

Figure 4-2: Electropherograms detected using SYBR Green 1 fluorescent dye and a phospholipid nanogel with [DMPC]/[DHPC] = 2.5 at a concentration of 2.5% and temperature of 23 ° C. The true sizes are specified above each peak while the measured sizes are denoted in the brackets. The peaks for each marker are labeled to indicate: **(A)** the three *sc/2* strains designated 6166, 6140, 6180 **(B)** the three *sc/2* strains designated 9716, 10118, 9842, and **(C)** *bcl/C*. The separation is accomplished using a capillary that is 40 cm long, 25 μ m id, with an effective length of 30.2 cm and E_{app} = 100 V/cm. Reproduced from [1].

4.2.5 Characterization of the difference between true and measured size (bias)

To demonstrate that the phospholipid preparation optimized for the separation of large DNA fragments remained effective for the analysis of DNA fragments below 500 bp in size, unique biomarkers of several *Aspergillus* species were analyzed (see Figure 4-3). This fungus leads to increased mortality among immunocompromised patients suffering from invasive aspergillosis [49-52] and is also associated with crop contamination [53]. Detection of *Aspergillus* through unique DNA sequences is significant because PCR methods are faster and more conclusive than the traditional means of identifying *Aspergillus* species based on morphology observed in microbiology laboratory cell cultures. Unique sequences in *Aspergillus* collagen-like genes (*acI*) found in five pathogenic species were targeted by PCR resulting in amplicons of distinct lengths: *A. nidulans* 227 bp; *A. terreus* 262 bp; *A. niger* 297 bp; *A. flavus* 402 bp; and *A. fumigatus* 338 and 489 bp. Using internal standards of 150 and 450 bp to calculate the size, these six biomarkers were resolved in a capillary with 30.2 cm effective length and a separation additive containing 2.5% phospholipid preparation of [DMPC]/[DHPC] equal to 2.5 at 23 °C. This separation additive optimized for larger DNA fragments estimated the size with a bias ranging from 0-6 bp (see Table 4-7), which represented a size accuracy within 2.5% of the true fragment size. Previously, these biomarkers were separated using a 40 cm long capillary and a 10% phospholipid preparation of [DMPC]/[DHPC] equal to 2.5 at 30 °C [30]. In that study, the amplicon size was estimated using three size standards of 150,

250, and 350 bp with a bias ranging from 1-7 bp, which represented a size accuracy within 2% of the true fragment size (see Table 4-7).

Table 4-7: Performance Comparison of 10% vs 2.5% for *Aspergillus* biomarkers

Species	True Size in bp ¹	2.5%		10%	
		Measured Size in bp ²	(CV)	Measured Size in bp ³	(CV)
<i>Aspergillus nidulans</i>	227	227	(0.3)	226	(0.4)
<i>Aspergillus terreus</i>	262	256	(0.4)	259	(0.8)
<i>Aspergillus niger</i>	297	301	(0.3)	296	(0.3)
<i>Aspergillus fumigatus</i>	338	333	(0.5)	336	(0.6)
<i>Aspergillus flavus</i>	402	402	(0.1)	396	(0.8)
<i>Aspergillus fumigatus</i>	489	495	(0.4)	482	(1)

¹ True size established as described in [30]. *A. fumigatus* derived amplicons of gene *acIF1-5'* and *-3'* were sequenced and represent true sizes of 338, 489 bp. Amplicons derived from *A. terreus* (gene *acIT2-5'*), *A. nidulans* (gene *acINi-3'*), and *A. niger* (gene *acINi-5'*) were based on bioinformatics and were also partially sequenced with forward, but not with reverse, primers that were used to generate these amplicons. The amplicon from *A. flavus* (gene *acIFL-3'*) was sequenced in full and the size of this amplicon is 402 bp.

² Separations conditions are identical to those reported in Table 3-2. Size standards of 150 and 450 bp are used for linear fit.

³ Separations ($n = 5$) reported from reference [30] are achieved with 10% [DMPC]/[DHPC] = 2.5 in a 40 cm long, 25 μ m id capillary operated at 30 °C. The effective length is 40.2 cm and $E_{app} = 100$ V/cm. Size standards of 150, 250, and 350 are used.

Table Reproduced from [1].

Figure 4-3: Separation of *Aspergillus* species markers

Figure 4-3 : Separation of **(A)** 5 amplicons unique to different species of *Aspergillus* as well electropherograms that contain internal standards of 150, 450, and 500 base pairs as well as a single amplicon for **(B)** *A. nidulans* (227 bp); **(C)** *A. terreus* (262 bp); **(D)** *A. niger* (297 bp); **(E)** *A. fumigatus* (338 and 489 bp); **(F)** *A. flavus* (403 bp). Reproduced from [1].

The analysis of DNA biomarkers for strains of *S. pyogenes* revealed that the phospholipid nanogel generated a relative bias in size accuracy less than 1% (2 to 4 bp bias) of the total fragment size for 634, 715 and 842 bp, and increased to 2%, 3%, and 4% (19 to 48 bp bias) of the total fragment size for 1,071, 1,152, and 1,332 bp, respectively (Table 4-8). The analysis of the 1,446 bp PCR amplicon for the *bcl C* gene of *Bacillus anthracis* shown in Figure 4-2 C had an estimated size of $1,449 \pm 8$ bp ($n = 5$), generating a size bias of less than 1%.

Table 4-8: Performance of 2.5% Phospholipid for *Streptococcus* biomarkers

Strain , type	True Size in bp ¹	Measured Size in bp ²	(CV)	Bias in bp ³
6166 , M12	634	630	(0.2)	4
6144 , M12	715	717	(0.4)	2
6180 , M28	842	844	(0.5)	2
9716 , M3	1071	1090	(0.4)	19
10118 , M3	1152	1185	(0.3)	33
9842 , M3	1332	1380	(0.2)	48

¹ True size established using DNA sequencing as reported in reference [29].

² Separations conditions are identical to those reported in Table 3-2. For strains 6166, 6144, and 6180 the size is determined using 600 and 1,000 bp standards. For strains 9716, 10118, and 9842, the size is determined using 1,000 and 1,500 bp standards

³ Bias is calculated as the difference between the true fragment size and the measured size.

Table Reproduced from [1].

Each single Gly-X-Y repeating unit in *scf2* or *bcl* is encoded by 9 bp. Thus, the phospholipid nanogel will separate two distinct *scf2* or *bcl* alleles differing only by one Gly-X-Y repeat at the ~500-850-bp range and by 1-5 Gly-X-Y repeats at the ~1,000-1,400-bp range. The phospholipid nanogel performance is similar to the values reported for a commercial system that utilizes hydroxyethyl cellulose, which generated a size bias ranging from 0-7% for 119 to 1,270 bp DNA fragments [16]. Clearly, the material can be used for DNA fragments approaching 1,500 bp. This is a valuable assay to be used alone or in combination with other epidemiological markers to trace the emergence of new strains with a higher discriminatory power. Accurate size evaluation for any extended repeating sequence will also be an important tool assisting in long-range genomic assemblies produced from short (50 to 150 bp) DNA sequences obtained using next generation sequencers [54].

4.2.6 Advanced material properties enable in – capillary sample stacking

Continuous nanogels were used to generate the data in Figures 4-1 and 4-2 as well as Tables 4-1 through 4-8; however, the separations in Figure 4-1 confirmed that DNA had a faster mobility in the 2.5% phospholipid preparation than in the 10% preparation, which also has a substantially higher apparent viscosity. The different viscosity of the 10% vs the 2.5% phospholipid nanogels was integrated

to create a discontinuous nanogel to stack DNA within a small plug of the highly viscous 10% phospholipid nanogel and then separate the DNA in the low viscosity 2.5% nanogel that yielded higher separation performance. These two nanogel preparations were introduced serially into the separation capillary. DNA was stacked using a 10 kV, 30 second injection in the discontinuous nanogel as shown in Figure 4-4 A. At a temperature of 19 °C, the capillary was first filled with the 2.5% nanogel for 3 minutes at 140 kPa (20 psi). This was followed by a small plug of 10% nanogel introduced for 10 seconds at 69 kPa (10 psi), and finally a pre-plug of aqueous buffer was introduced for 15 seconds at 34 kPa (5 psi). The temperature was raised to 30 °C to increase the viscosity, which caused the 10% phospholipid to form a highly viscous nanogel. The DNA was electrokinetically-injected at 30 °C and stacked. The temperature was then dropped to 23 °C and the separation proceeded. The enhancement in peak area achieved with stacking is apparent by comparing the electropherograms shown in Figure 4-4 A with 3-4 B and 3-4 C. The DNA in the electropherogram in Figure 4-4 B, obtained with a 10 kV, 2 second injection in a discontinuous nanogel, was difficult to detect. The peaks were broad when the large 10 kV, 30 second injection was performed in the absence of the stacking gel as shown in Figure 4-4 C. The plate count of the 600 bp peak in Figure 4-4 C (40,000 theoretical plates) was 12-fold lower than that obtained with stacking in Figure 4-4 A (500,000 theoretical plates). Integrating both materials offered a means to harness advantages of each medium for unique processes of injection and separation of large DNA fragments.

Figure 4-4:

A) Stack and Separate

B) Inject (10kV 2 sec), Separate

C) No Stacking Gel

Figure 4-4: Depicts the process of **(A)** discontinuous phospholipid nanogels in which the DNA is stacked in the 10% phospholipid and separated in the 2.5% phospholipid, **(B)** discontinuous phospholipid nanogels in which the 10 kV 2 sec injection is too short to stack DNA, and **(C)** a continuous nanogel using a 10 kV 30 sec injection without a stacking plug. The separation is accomplished using a capillary that is 40 cm long, 25 μm id, with an effective length of 30.2 cm and $E_{\text{app}} = 100 \text{ V/cm}$. Reproduced from [1].

4.3 Conclusions

A practical alternative to slab gels was developed to separate and size DNA fragments as large as 1,500 bp, with a size accuracy ranging from 1-4%. The material was compatible with the higher concentration medium reported previously [22] and was suitable for thermally controlled gel-stacking and separations. By decreasing the concentration of the phospholipid preparation from 10% to 2.5%, the net cost of the dynamic sieving gel was reduced. The ease with which the dynamic sieving nanogel was introduced into capillary channels was ideal for microscale separations because the low flow resistance required only nominal pressures for loading. This holds potential to benefit microfluidic separations as well because when the separation device can be used repeatedly, the lifetime of the device is increased, which in turn decreases the overall cost of the analytical measurement.

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Publications

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Presentations

B.C. Durney*, L.A. Holland, "Tunable DNA Sieving With Thermally Responsive Nanogels", PITTCON® 2014, Chicago, IL March 4, 2014.

B.C. Durney*, L.A. Holland, "Self-Assembled Nanogels for Capillary Electrophoresis Separations of DNA", PITTCON® 2013, Philadelphia, PA, March 21, 2013.

B.C. Durney*, A. Kadiyala, M. Srungarapu, J. Dawson and L.A. Holland, "Multi-layer Microfluidic device for Human Identification Using Capillary Gel Electrophoresis and a Photonic Crystal for Detection" 2012 AIChE Annual Meeting, Pittsburgh, PA, October 31, 2012.

B.C. Durney*, L.A. Holland, "Capillary Electrophoresis Separations of DNA Using Phospholipid Additives", Research Horizons Day, Morgantown, WV, April 5, 2012.

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Reversible Phospholipid Nanogels: An Alternative Matrix for Tunable High Resolution DNA Sieving with Enhanced Separation Efficiency and Accurate Extended Range Sizing by Capillary Gel Electrophoresis

By: Brandon Charles Durney

**Dissertation submitted to The Eberly College of Arts and Sciences
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for the degree of**

Doctor of Philosophy in Chemistry

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