Reversible Phospholipid Nanogels for Deoxyribonucleic Acid Fragment Size Determinations up to 1,500 Base Pairs and Integrated Sample Stacking

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Abstract

Phospholipid additives are a cost-effective medium to separate deoxyribonucleic acid (DNA) fragments and possess a thermally-responsive viscosity. This provides a mechanism to easily create and replace a highly viscous nanogel in a narrow bore capillary with only a 10 °C change in temperature. Preparations composed of dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) self-assemble, forming structures such as nanodisks and wormlike micelles. Factors that influence the morphology of a particular DMPC-DHPC preparation include the concentration of lipid in solution, the temperature, and the ratio of DMPC and DHPC. It has previously been established that an aqueous solution containing 10% phospholipid with a ratio of [DMPC]/[DHPC] = 2.5 separates DNA fragments

with nearly single base resolution for DNA fragments up to 500 base pairs in length, but beyond this size the resolution decreases dramatically. A new 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 increase in the upper size limit is accomplished using commercially available phospholipids at an even lower material cost than is achieved with the 10% preparation. The 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.

1. Introduction

Estimating the size of DNA is critical in genetic analysis associated with human identification,[1] species identification,[2, 3] detecting personal biomarkers,[4] analyzing food,[5] and categorizing pathogenicity.[6] 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.[7, 8] Although more expensive than slab gel methods, capillary gel electrophoresis provides a size discrimination that is improved substantially, and higher sample throughput. Sequencing of the human genome provides direct proof of this.[9]

Capillary gel electrophoresis 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,[10-12] or separation through DNA entanglement.[13] DNA fragments ranging from 50-1,500 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% hydroxyethyl cellulose used to size a 256 base pair (bp) DNA fragment.[14] Although larger fragments could not be sized, 1,078 bp and 1,353 bp DNA were baseline resolved.[14] A commercial system utilized hydroxyethyl cellulose to separate 50 bp to 10 kbp DNA and to accurately size 275 to 815 bp DNA.[15] In another case, a matrix of 2% linear polyacrylamide was used to sequence a DNA fragment 1,300 bases long.[16] Several polymer properties dictate the utility of the material and the useful size range of the separation.[17] 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.[10] This increase in concentration is accompanied by an increase in viscosity,[11] 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.[18] Wormlike micelles form large self-assembled structures that dynamically rupture and reform. Aqueous solutions of the long-chain phospholipid dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and the short-chain phospholipid 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) are known to form wormlike micelles.[19, 20] 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 short tandem repeats (STR) in selected loci utilized for human identification using a 100 cm long capillary within a 30 minute run time.[21] This is comparable to separations on commercial capillary electrophoresis instruments for forensic STR analyses.[22] 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.[23-25] 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,[21] 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 200-500 bp. For fragments that range from 600-1,500 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.

2. Materials and Methods

2.1 Chemicals and Reagents. The fluorescently labeled DNA ladder (catalog # MM-1000-FAM, 50-1,000 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, 100-12,000 bp), and 100 bp (catalog # 15628-019, 100-2,072 bp) DNA ladders were purchased from Life Technologies (Grand Island, NY). The methanol was purchased through Calbiochem/EMD (Gibbstown, NJ). The DMPC and DHPC phospholipids were purchased through Avanti Polar Lipids (Alabaster, AL), and the 3-(*N*-morpholino)-propanesulfonic acid (MOPS) was purchased from Alfa Aesar (Ward Hill, MA). Sodium hydroxide was obtained from Sigma-Aldrich (St. Louis, MO). All deionized water used was obtained from an ELGA PURELAB ultra water filtration system (Lowell, MA).

2.2 Preparation of Phospholipid Mixture. The phospholipid preparations were made as described previously[26] to obtain molar ratios of [DMPC]/[DHPC] = 0.5 and [DMPC]/[DHPC] = 2.5. The phospholipids were stored at -20 °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 μ L [DMPC]/[DHPC] = 0.5 (5% w/v) and 100 μ L [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.

2.3 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.[27] PCR reactions were carried out with 1.5 units of *Taq* DNA polymerase (Qiagen, Germantown, MD) in a final volume of 50 μ L per reaction and DNA template in the nanogram range using a C1000 Touch Thermal Cycler (BioRad, Hercules, CA). The manufacturer's PCR buffer was supplemented with 0.2 μ M concentration of each primer and 0.2 mM deoxynucleotide triphosphates (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 *scf2*-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, as previously described.[28] 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.[29]

2.4 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 (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 Capillary electrophoresis 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.

2.5 Pre-treating the Separation Capillary. Separations were performed using fused silica capillary with an outer diameter of 360 µm and an inner diameter of 25 µm (Polymicro

Technologies, Phoenix, AZ). The separation capillary had a total length of 40 cm, and an effective length 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) deionized water for 15 min, (3) methanol for 15 min, (4) deionized water for 15 min. The conditioned capillary was then passivated with a semi-permanent phospholipid coating to suppress electroosmotic flow as previously described.[30] This was accomplished using a 2-step rinse at 140 kPa with (1) 5% phospholipid of [DMPC]/[DHPC] = 0.5 containing 1.25 mM Ca^{2+} 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.

2.6 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.[31] 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.

2.7 Fluorescence Detection and Analysis of DNA. The Beckman Coulter P/ACE MDQ was equipped with a laser induced fluorescence 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 μ M SYBR in phospholipid preparations of [DMPC]/[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.[32] 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.[33] 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 relative fluorescent units was achievable with a plug of 20 μ M SYBR Green 1. As summarized in Table S-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.

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 $w_{1/2av}$ 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.

3. Results and discussion

3.1 Effect 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 500-1,000 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 difference in hydrodynamic volume[26] and from 6-12% for separations based on sieving.[21] For both studies[21, 26] 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 1 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 S-2 in the supporting information). 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 S-2 in the supporting information). These results indicated that the lipid preparations should be maintained at a concentration of 2.5% lipid.

3.2 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'. [19, 20] 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. [34-36] 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%, 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 2, 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 S-3 in the supporting information). 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 S-4 in the supporting information. 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 matrix for DNA shorter than 1,000 bp.[15] This improved sizing obtained with the phospholipid nanogel is an extraordinary advantage for DNA analyses with sieving that require accurate size determinations.

3.3 Expanded Size Range of Sieving Using a 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 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 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 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 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 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. 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 S-2), 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.

3.4 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.[37] Strains are typically characterized epidemiologically by serological typing of the M protein or, more recently, by sequencing of the *emm* gene.[38-40] 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.[28, 41] Strain typing by length variation in collagen-like genes has been successfully demonstrated for the *bcl* genes of *Bacillus anthracis*[42] and is a viable strategy for typing *S. pyogenes*. The *scf2* 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.[43, 44] Importantly, M3-type strains are associated with invasive diseases like necrotizing fasciitis and streptococcal toxic shock syndrome.[43, 45-47]

Primers were designed to generate PCR amplicons of *scf2* 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 2a, 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 Fig. 2b) using internal standards of 1,000 and 1,500 bp. The extended sizing range of the phospholipid nanogel enabled detection of larger *scf2*-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[42] was analyzed using internal standards of 1,000 and 1,650 bp as shown in Figure 2c.

3.5 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 S-1 in the supporting information). This fungus leads to increased mortality among immunocompromised patients suffering from invasive aspergillosis[48-51] and is also associated with crop contamination.[52] 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 3), which represented a size accuracy within 2.5% of the true fragment size. Previously, these biomarkers were separated using a 40 cm effective length and a 10% phospholipid preparation of [DMPC]/[DHPC] equal to 2.5 at 30 °C.[29] 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.

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% (2-4 bp bias) of the total fragment size for 634, 715 and 842 bp, and increased to 2%, 3%, and 4% (19-48 bp bias) of the total fragment size for 1,071, 1,152, and 1,332 bp, respectively (Table 4). The analysis of the 1,446 bp PCR amplicon for the *bcl* C gene of *Bacillus anthracis* shown in Figure 2c had an estimated size of $1,449 \pm 8$ bp ($n = 5$), generating a size bias of less than 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 1270 bp DNA fragments.[15] 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 (50-150 bp) DNA sequences obtained using next generation sequencers.[53]

3.6 Advanced Material Properties Enable In-capillary Sample Stacking. Continuous nanogels were used to generate the data in Figures 1 and 2 as well as Tables 1-4; however, the separations in Figure 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 3A. 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 3A with 3B and 3C. The DNA in the electropherogram in Figure 3B, 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 3C. The plate count of the 600 bp peak in Figure 3C (40,000 theoretical plates) was 12-fold lower than that obtained with stacking in Figure 3A (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.

4. 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[21] 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.

5. Supplementary data

Supplementary data is available that includes summaries of the migration time reproducibility as a function of intercalating dye concentration (Table S-1). Also summarized are the effects of phospholipid concentration (Table S-2) and [DMPC]/[DHPC] (Table S-3) on resolution at optimum temperatures, as this performance improves slightly for some nanogels at a temperature other than 23 °C. Table S-4 summarizes the effect of temperature (19 °C - 25 °C) on resolution for a sieving matrix of 2.5% with [DMPC]/[DHPC] = 2.5. Figure S-1 contains electrophoretic separations of PCR amplicons associated with *Aspergillus* species.

6. Acknowledgements

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7. References

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Table 1. Effect of Concentration on Resolution¹

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

¹Separations ($n = 5$) 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.

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 2. Effect of [DMPC]/[DHPC] Ratio on Resolution at 23 °C¹

	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	

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

Table 3. Performance of 2.5% Phospholipid for *Aspergillus* biomarkers

Species	True Size in bp ¹	Measured Size in bp ²	(CV)	Bias in bp ³
<i>A. nidulans</i>	227	227	(0.3)	0
<i>A. terreus</i>	262	256	(0.4)	6
<i>A. niger</i>	297	301	(0.3)	4
<i>A. fumigatus</i>	338	333	(0.5)	5
<i>A. flavus</i>	402	402	(0.1)	0
<i>A. fumigatus</i>	489	495	(0.4)	6

¹True size established as described in [26]. *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.

²Separation conditions are identical to those reported in Table 1. Size standards of 150 and 450 bp are used.

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

Table 4. 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

¹True size established using DNA sequencing as reported in reference [27].

²Separations conditions are identical to those reported in Table 1. 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

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

Figure Captions.

Figure 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.

Figure 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) *bc/ C*. The separation is accomplished using a capillary that is 40 cm long, 25 µm id, with an effective length of 30.2 cm and $E_{app} = 100$ V/cm.

Figure 3. 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 $E_{app} = 100$ V/cm.

Figure 1. Separations obtained at different phospholipid concentration and run temperature.

Figure 2. Separation of PCR amplicon obtained with the phospholipid nanogel optimized for DNA up to 1,500 bp in length.

Figure 3. DNA stacking with phospholipid additives

A) Stack and Separate

B) Inject (10kV 2 sec), Separate

C) No Stacking Gel

Graphical Abstract