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DNA sequencing with hydrophilic and hydrophobic polymers at elevated column temperatures

Read length in DNA sequencing by capillary electrophoresis at elevated temperatures is shown to be greatly affected by the extent of hydrophobicity of the polymer separation matrix. At column temperatures of up to 80°C, hydrophilic linear polyacrylamide (LPA) provides superior read length and separation speed compared to poly(*N*,*N*-dimethylacrylamide) (PDMA) and a 70:30 copolymer of *N*,*N*-dimethylacrylamide and *N*,*N*-diethylacrylamide (PDEA30). DNA-polymer and polymer intramolecular interactions are presumed to be a major cause of band broadening and the subsequent loss of separation efficiency with the more hydrophobic polymers at higher column temperatures. With LPA, these interactions were reduced, and a read length of 1000 bases at an optimum temperature of 70°–75°C was achieved in less than 59 min. By comparison, PDMA produced a read length of roughly 800 bases at 50°C, which was close to the read length attained in LPA at the same temperature; however, the migration time was approximately 20% longer, mainly because of the higher polymer concentration required. At 60°C, the maximum read length was 850 bases for PDMA, while at higher temperatures, read lengths for this polymer were substantially lower. With the copolymer DEA30, read length was 650 bases at the optimum temperature of 50°C. Molecular masses of these polymers were determined by tandem gel permeation chromatography-multiangle laser light scattering method (GPC-MALLS). The results indicate that for long read, rapid DNA sequencing and analysis, hydrophilic polymers such as LPA provide the best overall performance.

Keywords: Capillary electrophoresis / DNA sequencing / Elevated column temperature / Hydrophobicity / Polymer solutions / Separation matrices EL 4896

1 Introduction

A working draft of the Human Genome [1, 2] was recently completed by means of commercially available, automated capillary electrophoresis sequencing instrumentation. These instruments use separation matrixes based on linear polyacrylamide (LPA) and poly(*N*,*N*-dimethyl acrylamide) (PDMA). Polymers, such as hydroxyethylcellulose (HEC) [3], poly(ethylene oxide) (PEO) [4, 5], poly(vinylpyrrolidone) (PVP) [6], and others [7], have also been used in DNA sequencing experiments but with less success. Most of these polymers, in contrast to LPA, are somewhat hydrophobic in nature.

In previous work, the DNA separation properties of several polymer matrixes with different levels of hydrophobicity were tested at 44°C [8]. It was found that the performance of polymer matrixes for DNA sequencing decreases with an increase in the hydrophobicity of the matrix. In addition, in the case of LPA, it has been shown that elevated column temperature is beneficial for separation of ssDNA sequencing fragments [9, 10]. Additional thermal energy is assumed to mitigate the stretching and biased orientation of DNA molecules caused by the electric field, thus increasing the separation selectivity for longer fragments and, consequently, the read length, as well as reducing band compressions. However, for the best performance with respect to read length, it is important to find an optimum sequencing temperature for each polymer matrix. In this paper, we explore temperature effects on the DNA separation ability of several different polymers.

The separation properties of LPA and its use for long read length DNA sequencing by capillary electrophoresis has been systematically studied in previous work [9–16]. It was found that in a highly optimized system, with a matrix containing 2% w/w LPA of molecular mass above 10 MDa and 0.5% w/w of the same polymer with the molecular mass 270 kDa, at column temperature 70°C and electric field 125 V/cm, up to 1300 bases can be sequenced in 2 h with an accuracy of 98.5% [16]. With that same accuracy, a read length of 1000 bases is routinely obtained in less than 1 h at 60°C and 200 V/cm, using a similar separa-

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Abbreviations: **DEA**, *N*,*N*-diethylacrylamide; **DMA**, *N*,*N*-dimethylacrylamide; **GPC**, gel permeation chromatography; **LPA**, linear polyacrylamide; **MALLS**, multiangle laser light scattering; **PDEA30**, 70:30 copolymer of DMA and DEA; **PDMA**, poly(*N*,*N*-dimethylacrylamide)

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tion matrix containing 0.5% w/w 50 kDa LPA instead of 270 kDa [15]. PDMA was introduced as a matrix for DNA sequencing because of its ability to absorb to glass surfaces and hence "self-coat" the capillary surface as a result of its hydrophobicity, and its relatively low viscosity (75 cP), which allowed facile polymer matrix replacement [17]. However, in order to maintain viscosity of the 6.5% w/w PDMA solution at this low level, the molecular mass of the polymer was kept below 220 kDa, and the resultant read length was limited to 600 bases in 2 h. For longer reads, higher molecular mass polymers are needed [10], and in the case of PDMA, lead to attainable read lengths of more than 800 bases in 96 min [18].

For this study, we prepared LPA and PDMA with weight-average molecular masses in the range of 3.9–5.6 MDa. We also synthesized a random copolymer of *N*,*N*-dimethylacrylamide (DMA) and *N*,*N*-diethyacrylamide (DEA) 70:30 (PDEA30) that is more hydrophobic in chemical nature than PDMA. With these three polymers, we were able to distinguish and characterize the effects of polymer hydrophobicity on DNA separation by capillary electrophoresis at elevated column temperatures.

2 Materials and methods

2.1 Instrumentation

The design of a single-capillary instrument with detection based on laser-induced fluorescence (LIF) was similar to an instrument we have previously described [19]. Fluorescence emission was collected with a microscope objective (Model 13600; Oriel, Stamford, CT, USA), and the spectra of the labeled sequencing fragments were acquired in 16 channels in the range from 500 to 660 nm by a CCD camera (Model NTE/CCD-1340/400-EMB, Roper Scientific, Trenton, NJ, USA). The laser was 5 mW multilane argon ion (Ion Laser Technologies, Salt Lake City, UT, USA). Details on other components are the same as previously [14, 19]. The CE columns were fused-silica capillaries of 75 μm ID, 365 μm OD (Polymicro Technologies, Phoenix, AZ, USA), covalently coated with polyvinyl alcohol (PVA) [20]. The effective capillary length (distance from injection point to the detector window) was 30 cm, with a total length of 45 cm. The sample was injected for 10 s at constant current of 0.7 μA, and electrophoresis was performed at 200 V/cm.

2.2 Chemicals

Acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate and urea were purchased from ICN Biomedicals (Costa Mesa, CA, USA). Acrylamide for solution polymerization was purchased from Amresco (Solon, OH, USA). DMA and DEA of high purity (>99.5%) were obtained from Monomer-Polymer and Dajac Labs (Feasterville, PA, USA), and V-50 (2,2'-azobis (2-amidinopropane) dihydrochloride) was purchased from Wako Chemical USA (Richmond, CA, USA). Tris, TAPS, and EDTA were from Sigma (St. Louis, MO, USA). All reagents were either electrophoresis or analytical grade, and no further purification was performed. Span 80 emulsifier and petroleum special with a boiling range from 180 to 220°C were purchased from Fluka Chemicals (Milwaukee, WI, USA). Water was deionized with a Milli-Q purification system to 18.2-MΩ grade (Millipore, Worcester, MA, USA).

2.3 Polymer synthesis and characterization

One batch of LPA (LPA1) with weight-average (M_w) molecular mass 5.6 MDa was prepared in powder form using inverse emulsion polymerization, as described previously [13]. After polymerization, the LPA powder was washed with acetone and vacuum dried. A second batch of LPA (M_w 4.6 MDa, LPA2), as well as PDMA (M_w 3.9 MDa) and a PDEA30 (M_w 3.6 MDa), were prepared by solution polymerization in water [8], and these polymers were dialyzed against water for removal of low-molecular-mass components and unreacted monomers following by lyophilization. Molar mass distributions of all polymers used in this study were characterized by tandem gel permeation chromatography (GPC) – multiangle laser light scattering (MALLS), according to the methods described in the previous paper [8].

2.4 Preparation of the separation polymer solutions

For this work, polymer solutions containing 2.5% w/w LPA1 or LPA2 or 4% w/w PDMA or DEA30 were utilized to separate the DNA sequencing reaction products. To prepare an LPA1, LPA2, PDMA, or DEA30 solution (20 g), appropriate amounts of dry polymer, 8.4 g urea (7 M), 10 × buffer concentrate (500 mM Tris: 500 mM TAPS: 20 mM EDTA) and water were added in a glass jar and slowly stirred with a magnetic bar. The solutions were usually homogenized after two days and ready for use. Fresh polymer solution was replaced in the capillary before each run using a gas-tight syringe, and the voltage was applied for 5 min before injection. LPA1 powder and other dried polymers are stable and have almost unlimited shelf life. The working polymer solutions could be stored in the refrigerator at 4°C for up to 3 months. DNA sequencing reactions were performed using standard cycle sequencing chemistry with AmpliTaq-FS and BigDye (-21) M13

universal primers (Applied Biosystems, Foster City, CA, USA) on an M13mp18 single-stranded template (New England Biolabs, Beverly, MA, USA). The temperature cycling protocol for this sequencing chemistry was made on a PTC200 thermocycler (MJ Research, Watertown, MA, USA), consisting of 15 cycles of 10 s at 95°C, 5 s at 50°C and 1 min at 70°C, followed by 15 cycles of 10 s at 95°C and 1 min of 70°C. After completion of a reaction, the samples were heated for 5 min at 100°C in order to inactivate the enzymes prior to the clean-up procedure.

2.5 Purification of the reaction products

Sequencing reactions were purified using a method described previously [12] with some modifications. Template DNA (M13mp18) was removed using spin columns with a polyethersulfone ultrafiltration membrane, molecular weight cutoff of 300 000 (MWCO 300K, Pall Filtron, Northborough, MA, USA), which was first pretreated with a 0.005% w/w solution of LPD with a molecular mass of 700–1000 kDa. The filtrate was dried under vacuum and dissolved in 50 μL of deionized water. The reconstituted template-free sequencing samples were then desalted using prewashed Centri-Sep 96 (gel filtration) plates (Princeton Separations, Adelphia, NJ, USA). The desalting procedure was performed twice per sample, after which the sample volume was adjusted to 55 μL. A 5 μL aliquot of the purified sample was diluted with 20 μL of deionized water prior to injection. The specific injection conditions are described in the figure captions. The purified sequencing samples were stored at –20°C in deionized water.

2.6 Base-calling software

An expert system developed in this laboratory was used for base-calling [15]. Data processing was begun by determining the primer dye spectra from the relatively intense peaks in the data, performing color separation by a least-squares fit to these spectra and applying a median filter to remove high frequency noise. The electropherogram was divided into sections, each containing approximately 20 bases, and the fifth percentile value among the amplitudes of all data points in each section was computed. This calculation established the background at the center of each section, and elsewhere it was derived by linear interpolation. After background subtraction, a search was conducted for long runs of peaks with similar height, with the run having highest median height being selected as the starting point for sequence determination. Peak width, separation, and dye mobility shifts were determined at this point. A set of empirical rules was then employed to find peak boundaries and estimate the number of bases in each peak. Sequencing data obtained from the base-caller were processed for presentation using Origin 6.0 software (Microcal, Northampton, MA, USA).

3 Results and discussion

In a previous paper [8], the effect of polymer hydrophobicity on DNA sequencing read length at fixed column temperature was explored. In this work, we continued the comparative study of DNA separation properties of polymers with increasing hydrophobicity at various temperatures. The theory of the dynamic polymer networks and DNA separation in the entangled solutions of linear polymers will be reviewed first, followed by the discussion of experimental data.

3.1 Theoretical background

Molecules of water-soluble linear polymers are hydrated and form an entangled dynamic network in an aqueous solution [16, 21–29] at concentrations above entanglement threshold (c^*) [30]. According to a physical model of the noncross-linked polymer networks [23], the network fluctuates, and its dynamic pores (*i.e.*, voids in the entangled polymer, or "blobs") dissociate and reform at a rate determined by the reptation time of the polymer. For the same polymer, the average mesh size depends on the concentration of the solution and on the polymer nature through the coil density. Polymers with significant hydrophobicity, due to their relatively high coil density in aqueous solution [8], tend to have intramolecular hydrophobic interactions prevailing over interaction with neighboring polymers. As a result, even at ambient temperature, networks of such polymers have fewer intermolecular entanglements per chain than a network of more hydrophilic polymers at the same concentration (for chains of similar degrees of polymerization). At elevated temperatures, intramolecular hydrophobic interactions become stronger and coil density increases, further disrupting intermolecular entanglements. If the polymers are sufficiently hydrophobic, they may exhibit a volume phase transition at a particular temperature, which can be exploited to reversibly control polymer solution viscosity [31].

The electrophoretic migration behavior of single-stranded DNA molecules through a polymer network depends on the size of the DNA molecule. Single-stranded DNA molecules less than 100 bases in length [29] generally migrate in the Ogston mode [32]. Longer DNA molecules, which in the native form are larger than an average blob size, are partially stretched under the influence of the electric field

and migrate through the network in the biased reptation mode [24, 27]. In this mode, the mobility of the DNA molecules is inversely proportional to the DNA size. With further molecular size increase, stretching and orientation also increase up to the point at which the DNA molecules became fully stretched and migrate together, with no size separation. This point, called the onset of biased reptation, can be shifted towards longer DNA molecules by lowering the polymer concentration or the electric field [10].

Single-stranded DNA molecules have hydrophobic properties that increase with DNA size [33], such that the effects of hydrophobic DNA-polymer interactions may effectively be stronger for long DNA fragments. It was shown elsewhere that reptating DNA molecules may drag polymer fibers under the influence of the electric field, so the possibility of such dragging increases with increase in the column temperature [34]. In addition, a hydrophobic fluorescent dye label on the DNA molecule can also contribute to these interactions [8]. Hydrophobic DNA-polymer interactions, being non-size-specific, can lead to band broadening, lower resolution and subsequent shortening of the resultant read length. It is important to note that in DNA sequencing by CE, extremely high efficiencies of millions of theoretical plates are achieved, and at this high level, even subtle hydrophobic interactions can cause a substantial decrease in efficiency and in the resolution. These interactions, similar to polymer-polymer interactions, are expected to be stronger at elevated column temperatures, given that they are also related to the hydrophobic effect. At the same time, high temperatures, up to at least 75°C, have been shown to help to improve DNA separation selectivity with LPA [9, 10, 15, 16], because the onset of biased reptation increase with temperature increase and, in addition, high temperature helps to resolve compressions by destabilizing DNA secondary structure. Based on these theoretical considerations and on previous experimental works, we decided to investigate the physical and DNA separation properties of polymers with increasing hydrophobicity at various temperatures.

3.2 Physical properties of the polymers

Several physical parameters of the polymers were determined using GPC-MALLS, as shown in Table 1. We calculated the weight-average radii of gyration for PDMA and the PDEA30 copolymer, normalized to the real molecular mass of LPA2, using the physical scaling law [8]

$$R_g = kM_w^{0,6} \quad (1)$$

Table 1. Physical characteristics of hydrophilic and hydrophobic polymers[a)]

Polymer	M_w (MDa)	R_g (nm)	Calculated R_g for M_w = 4.6 MDa (nm)	Calculated R_g for a polymer of 50 000 monomers[e)]
LPA1[b)]	5.6	209	177[d)]	130
LPA2[c)]	4.6	157	157	130
PDMA[c)]	3.9	122	134	141
PDEA30[c)]	3.6	114	132	137

a) Data obtained from GPC-MALLS measurements
b) Prepared by inverse emulsion polymerization
c) Prepared by solution polymerization
d) Value calculated for the actual molecular mass
e) Data excerpted from [8]

where k is a constant specific to the polymer-solvent system, R_g is the weight-average radius of gyration and M_w is the weight-average molecular mass of the polymer. This constant was estimated for each polymer using the experimental values of M_w and R_g shown in Table 1. The experimentally determined R_g for LPA1 was unexpectedly larger than the value of this parameter which is predicted from Eq. (1) using the constant k of LPA2. Given that the M_w of LPA1 is at the upper limit, in our experience, of LPA chains that can be properly fractionated by GPC, it is likely that this value is somewhat inaccurate (perhaps, by 10%). The limitations of tandem GPC-MALLS for polymers in this size range will be discussed in an upcoming paper [35]. For the other three polymers, experimentally determined R_g values decreased with increase of hydrophobicity. With normalization by the polymer molecular mass, R_g values of PDMA and the copolymer, however, were almost identical. This data is in agreement with the model outlined above, and, as a result, more hydrophobic polymer coils should occupy smaller volume in the aqueous solution than the less hydrophobic ones.

Since LPA has the smallest monomer mass of the other polymers in Table 1, LPA with the same chain length should also have the smallest molecular mass. In previous work [8], R_g was estimated using the scaling law (Eq. 1) for ideal polymers of 50 000 monomer units long. It was found that when of equal degree of polymerization, both PDMA and PDEA30 copolymer molecules would occupy a slightly larger volume in solution than a hydrophilic LPA molecule. However, when the substantially higher monomer molar mass of PDMA and more particularly of the PDEA30 copolymer was accounted for, molecules of these polymers were shown to adopt much denser coils in solution than LPA, as expected [8].

The polydispersities of all polymers used in this study were similar; however, the molecular mass distribution of inverse emulsion-polymerized LPA1 was substantially narrower than that of all solution synthesized polymers (data not shown). This is likely due to the fact that in the inverse emulsion polymerization process, the highly exothermal reaction occurs within very small aqueous droplets dispersed in a continuous oil phase, and intensive stirring of the emulsion provides for efficient heat dissipation in the reaction volume, thus making the reaction conditions identical in all parts of the emulsion [13]. As a result, preparation of acrylamide polymers by inverse emulsion polymerization is a quite reproducible process. In addition, the resultant polymer is obtained in a powder form that is easy to store and handle. In the case of solution polymerization, heat dissipation is much less effective due to slow stirring and the high viscosity of the reaction mass. Consequently, a temperature gradient from the center of the reactor toward the walls may be formed, leading to uneven reaction conditions in different regions of the solution.

3.3 Concentrations of the polymer solutions

In our previous work, we found that the optimum concentration of LPA for long read DNA sequencing was 2.5% w/w [15, 16], comprising 2% w/w LPA with molecular mass 10–17 MDa and 0.5% w/w short-chain LPA (50–270 kDa). In the current work, for comparison purposes, we used single-mass 2.5% w/w LPA1 and LPA2 solutions because other polymers were also single-mass. It was expected that hydrophobic polymers, due to their smaller actual R_g and higher density of polymer coil, should form less entangled and less stable networks at the concentration that was optimum for LPA. Using an approach similar to that employed for LPA concentration optimization, we optimized the concentration of PDMA and PDEA30 at column temperature 50°C for maximum read length. In this initial study, sequencing of the ssDNA samples was performed at several concentrations ranging from 3% to 6% w/w, and the longest read length for both polymers was achieved at 4% w/w concentration (data not shown). At optimum and sub-optimum concentrations, PDMA generated longer read lengths than PDEA30. At higher concentrations, both polymers produced almost identical read lengths, thus indicating that DNA separation was limited by the blob size of the polymer network (causing full orientation of migrating ssDNA molecules at relatively low base numbers) rather than by the level of the polymer hydrophobicity. The blob size is the average coil volume adapted by a polymer chain subunit [36]. Based on this study, we used both PDMA and PDEA30 at 4% w/w concentration in our experiments.

3.4 Separation resolution and column temperature

One of the most important parameters affecting read length in DNA sequencing by CE is electrophoretic resolution [16]. This factor is a function of separation selectivity and efficiency and can be expressed as [36]:

$$R_s = \left(\frac{\mu_1 - \mu_2}{\mu_1 + \mu_2}\right)\frac{\sqrt{N}}{2} \quad (2)$$

where μ_1 and μ_2 are mobilities of two adjacent peaks (2 migrating slower), and N is number of theoretical plates. In the case of LPA, the selectivity of separation, especially for longer fragments, increases with increasing temperature, while efficiency becomes lower [15, 16]. Due to the nonlinear relationship between selectivity and efficiency, relatively small increases of selectivity may compensate for higher decreases in efficiency [38].

With each polymer solution, DNA sequencing was performed over a wide range of temperatures from 30 to 85°C. Resolution plots for LPA1, PDMA and DEA30 are shown in Fig. 1A–C. For clarity of the presentation, these data for LPA2, which were similar to that of LPA1 though just somewhat reduced in resolution and read length, are not shown in the Figs. 1 and 2, but are summarized in Table 2. At 40°C, the maximum resolution for the PDEA30 copolymer solution occurs at around 250 bases (Fig. 1A).

Table 2. Migration time for the ssDNA fragments and read length in solutions of hydrophilic and hydrophobic polymers at optimum temperatures[a)]

Polymer matrix[b)]	Optimum temperature (°C)	Migration time for 600 bases (min)	Maximum read length (bases)	Migration time for the longest DNA fragment (min)
LPA1, 2.5% w/w	70	38.8	1055	58.1
LPA2, 2.5% w/w	75	42.8	972	63.8
PDMA, 4% w/w	60	50.9	857	67.5
PDEA30, 4% w/w	50	49.6	652	52.4

a) For experimental conditions, see text.
b) See Table 1 for polymer characteristics.

In the region of small base numbers, T-terminated DNA generated broader peaks with higher mobility shifts than other terminations in the same base region, as previously noted [8]. T-terminated DNA fragments were labeled with dROX as acceptor dye, which was the most hydrophobic dye in the dye set, and its interaction with the polymer may have a greater effect on the mobility shifts of these short fragments than other, less hydrophobic dyes. Thus, despite the higher resolution attained, read length at this

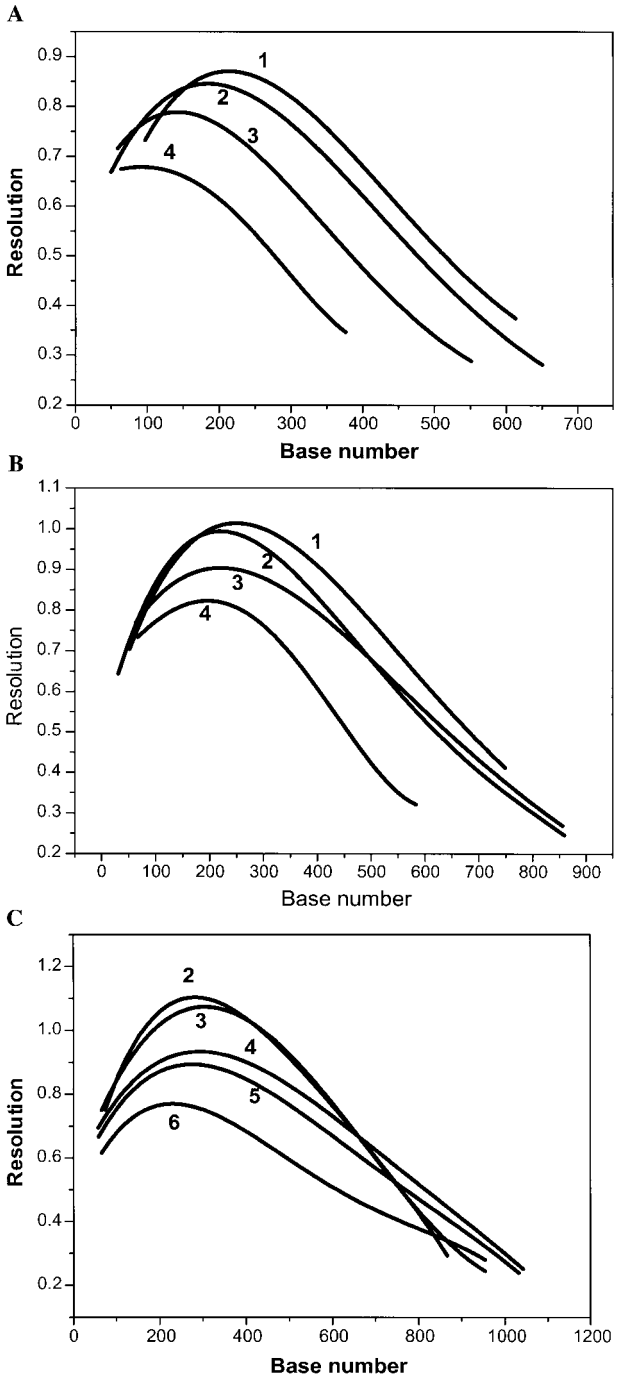

Figure 1. Resolution of DNA sequencing fragments in (A) 4% w/w PDEA30, (B) 4% w/w PDMA, and (C) 2.5% w/w LPA1 at temperatures (1) 40°C, (2) 50°C, (3) 60°C, (4) 70°C, (5) 75°C, and (6) 80°C. For experimental conditions, see text.

temperature was lower than at 50°C. With each column temperature, resolution was observed to be lower for all fragments. In addition, the resolution maximum shifted to lower base numbers as the column temperature rose, probably due to the fact that hydrophobic DNA-polymer interactions became stronger, and the network entanglements became weaker. As a result, the polymer fibers were more easily dragged by shorter DNA molecules. Subsequently, the resolution rapidly dropped to a level below which base-calling was not possible, thus resulting in shorter read length. Changes in resolution were in large part determined by the dramatic drop in efficiency with the temperature increase.

Compared to the copolymer PDEA30, at any given temperature, resolution of DNA sequencing fragments was higher for 4% PDMA solution (Fig. 1B). A shift in the position of maximum resolution to lower base numbers with the temperature increase was also observed but was less significant. These differences in the polymers' intrinsic DNA separation properties could be attributed to the fact that the PDMA network was more strongly entangled than PDEA30, and hydrophobic DNA interactions with the polymer fibers were weaker than that of the copolymer. However, similar to the more hydrophobic copolymer, selectivity in the base-called region did not improve with column temperature increase.

With LPA1, the maximum resolution was obtained for fragments of 200–300 bases at temperatures up to 85°C (Fig. 1C). While this maximum value decreased with the temperature increase, the resolution of fragments longer than 600 bases was substantially higher at temperatures 60–70°C than at lower temperatures. With further temperature increase, resolution became lower; however, in the late part of the electropherogram, the slope of the resolution plot was shallower at the higher temperatures. This improvement in resolution was mainly due to the increase in the selectivity in this region (data not shown). To illustrate the higher resolving power of hydrophilic polymers at high column temperatures, we plotted the efficiency for all three polymers at their optimum temperatures (Fig. 2). From this figure, it can be clearly seen that only LPA matrices can best utilize the advantages of high temperature for DNA separation. Other advantages of moderately hydrophobic polymers, such as PDMA and DMA/DEA copolymers, including their self-coating ability and their ability to provide a temperature-controlled "viscosity switch", respectively, must be weighed against this intrinsic physical limitation.

3.5 Temperature effects on read length and separation speed

The read lengths achieved with LPA1, LPA2, PDMA, and PDEA30 solutions at temperatures from 30 to 85°C are summarized in Fig. 3. Interestingly, for each polymer, we find that there is an optimum temperature range in which

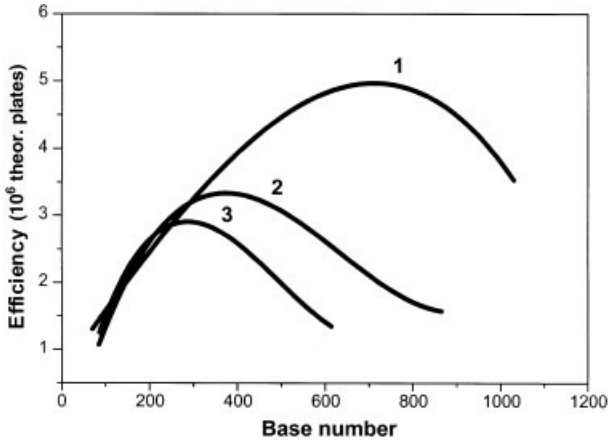

Figure 2. Separation efficiency for DNA sequencing fragments in (1) 2.5% w/w LPA1 at 70°C, (2) 4% w/w PDMA at 60°C, and (3) 4% w/w PDEA30 at 50°C. For experimental conditions, see text.

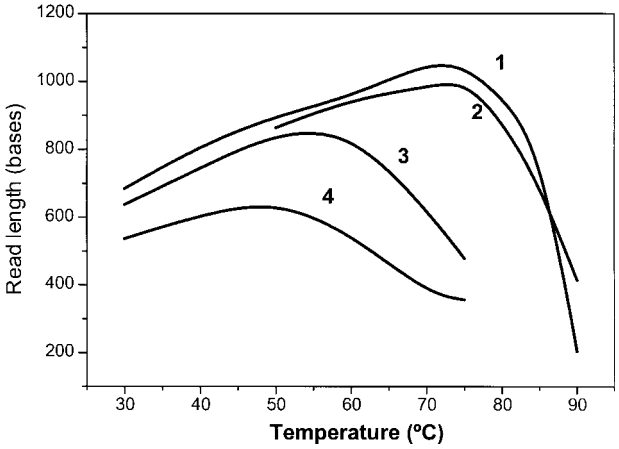

Figure 3. Effect of column temperature from 30°C to 90°C on resultant read length in DNA sequencing with (1) 2.5% w/w LPA1, (2) 2.5% w/w LPA2, (3) 4% w/w PDMA, and (4) 4% w/w PDEA30 polymer solutions. For experimental conditions, see text.

a maximum read length is achieved. For both LPAs, this temperature was between 70 and 75°C; for PDMA the maximum read length was between 50 and 60°C, and the most hydrophobic polymer, PDEA30, had the lowest optimum temperature, in the range of 40 and 50°C. The latter polymer also had the lowest read length at any given temperature, while PDMA produced intermediate read length. The longest read length was obtained with LPA, the most hydrophilic polymer, and was independent of the polymerization method (see also Table 2). While both LPAs and PDMA gave a substantial increase in read length when the column temperature was raised from 30°C to the corresponding optimum temperatures, PDEA30 showed only a small improvement in read length in this temperature range. However, all polymers exhibited a sharp decrease in read length at column temperatures higher than the optimum.

Separation speeds of DNA sequencing fragments at temperatures optimum for each polymer are compared in Table 2, where migration times for the ssDNA fragment 600 bases long and for the maximum base-called fragment are shown. Both LPA1 and LPA2 provided the longest read length and the fastest separation speed. LPA1 was synthesized by inverse emulsion polymerisation, a process which generates a polymer with a much narrower molecular mass distribution than a monomer polymerized by free radical solution polymerisation, such as LPA2. Higher molecular mass and lower polydispersity index (PDI) values for LPA1 means that there is a greater degree of larger polymer molecules in the solution when compared to LPA2. With a larger fraction of longer polymer chains, LPA1 generates a more robust entangled network in solution than LPA2, while at the same time having a larger blob size (greater chain length between entanglements, or a more "open" network). Therefore, larger DNA fragments can be separated more rapidly and with high resolution on LPA1 relative to LPA2, see Table 2.

PDMA separated ssDNA about 20% slower than LPA1, and its maximum read length was also 19% shorter. With PDEA30, separation speed at its optimum temperature was almost identical to that of the PDMA solution for the first 600 bases, but the maximum read length was much shorter, comprising only 62% of LPA1. With all matrices, migration times decreased with a temperature increase from 30° to 70°C due to a decrease in solution viscosity, as well as a decrease in the strength of network entanglement. Only LPAs were tested at temperatures higher than 70°C, and it is interesting to note that with further temperature increase, separation of ssDNA became slower, and at 90°C, only 400 bases could be sequenced in 40 minutes (data not shown).

As presented elsewhere (Kotler *et al.*, submitted), partial urea decomposition at temperatures above 75°C had deleterious effects on the separation of DNA sequencing fragments and led to substantial decrease in the read length and separation speed at these temperatures. We conducted our experiments at a constant electric field and observed a current drop during the run at temperatures above 80°C. This can be attributed to accumulation of microbubbles in the polymer solution due to the formation of gaseous products in the decomposition of urea.

With both LPAs and PDMA, column temperature had a strong effect on decrease of the migration time between 30 to 60°C; with PDEA30, the biggest drop in separation

time was between 60 and 70°C. This is a clear effect of the polymer-polymer intramolecular hydrophobic interactions that likely caused disentanglement of the network and formation of aggregated polymer domains in the aqueous solutions of hydrophobic polymers at high temperatures.

4 Concluding remarks

The results of this work indicate that polymer hydrophilicity is an important parameter in achieving long sequencing reads at elevated column temperatures. As shown earlier [9, 10, 15, 16], separation at elevated column temperature is beneficial for longer reads because DNA molecules are more thermally energetic at high temperature, and DNA onset of biased reptation is shifted toward higher base numbers. In addition, in solutions of the hydrophilic polymers, the efficiency of separation decreased slower with column temperature increase because DNA-polymer hydrophobic interactions were reduced, relative to the other polymers. Finally, the optimum concentration of the hydrophilic LPA was lower than that for the polymers with greater hydrophobicity, thus resulting in faster separation run times. While separation properties of LPA1 and LPA2 were similar, LPA1 prepared by inverse emulsion polymerization had more uniform molecular mass distribution and provided somewhat better results on this basis. With this method, reproducibility of the polymer synthesis and separation matrix preparation were also higher [13].

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