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Separation of DNA sequencing fragments up to 1000 bases by using poly(ethylene oxide)-filled capillary electrophoresis

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Abstract

We have demonstrated that DNA bases up to 1000 base pairs (bp) in a sequencing ladder can be separated using poly(ethylene oxide)-filled capillary electrophoresis (resolution of raw data=0.5 at 966 bp). Separation performance of this sieving matrix has been tested under different experimental conditions. It was found that the electric field strength played a critical role in the onset of reptation and thus the separation efficiency. Optimized gel composition and concentration is required for good separation, but the total gel concentration should lie between 2.5 and 3.0%. We observed that the capillary length influences the number of theoretical plates and the maximum readable length of DNA. For sequencing up to 500 bp, relatively nonviscous solutions can be used, greatly facilitating the replacement of the sieving matrix in between runs.

Keywords: Poly(ethylene oxide) gels; Capillary columns; DNA

1. Introduction

Capillary gel electrophoresis (CGE) for the separation of DNA has become a major tool in many research areas including biochemistry, molecular biology, and clinical chemistry. Such studies include polymerase chain reaction (PCR) analysis [1–3], DNA restriction fragment analysis [4–6], DNA fingerprinting [7–9], and DNA sequencing [10–17]. CGE for DNA sequencing is a promising approach to meet the goals of the Human Genome Project because of the capability of high speed and high throughput analysis. This is the direct result of the use of small-I.D. capillaries, typically around 50–100 μm. Joule heating caused by high electric fields can be dissipated efficiently. While conventional slab gel electrophoresis (SGE) can be used only up to 50 V/cm, CGE has been successfully used at 1200 V/cm, providing 20–30-fold increase of separation speed [18].

In CGE, the choice of the polymer solution is very important because the sieving power and the migration behavior of DNA are primarily determined by the properties of gel material. Initially, gel medium that had been used in SGE was employed in CGE for DNA sequencing. Hence, the separation mechanism, buffer composition, and sequencing chemistry were all derived from the already-proven SGE system. It was reported that the analysis of DNA fragments up to 700 bases was carried out in about 230 min with conventional cross-linked polyacrylamide gel [19]. Despite its successful application to CGE, cross-linked polyacrylamide gel has become less popular due to bubble formation, gel inhomogeneity, and short lifetime of the capillary. Replaceable polymer solution (e.g. linear polyacrylamide, LPA) was later employed for DNA sequencing [17,20]. It was shown that the use of the replaceable polymer

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solutions can extend the lifetime of the column and reduce the formation of the bubbles under high electric field. Also, an essentially fresh medium can be provided for each sequencing run. In addition to LPA, cellulose derivatives were found to be good sieving matrices for DNA sequencing in a microchip format [21]. However, long read length has yet to be achieved. Quite recently, new types of matrices were introduced for DNA sequencing in CE. A material that consisted of polyethylene glycol end-capped with micelle-forming fluorocarbon tails is reported to self-assemble in water into equilibrium network structures with a well-defined mesh size [22]. A resolution limit of 450 base pairs (bp) in 75-μm capillaries at 200 V/cm was reported. Also introduced is a temperature-sensitive polymer matrix for CGE and SGE [23]. Single-base resolution of sequencing fragments up to 150 bp was obtained with this matrix.

One important consideration for DNA sequencing is to achieve long reads of the DNA bases in a reasonable amount of time. To read a long fragment from short sequences, some sequencing regions must be overlapped. Therefore, the time-consuming process to clone the short fragments derived from a large fragment need to be repeated many times. Long reads per sequencing run can significantly reduce the preparation steps, resulting in an increase of the net speed of sequencing. At present, there has only been occasional success in separating DNA up to 1000 bp in a sequencing run using linear polymer solutions in CGE [24].

In 1995, our group reported the use of a replaceable linear polymer, poly(ethylene oxide) (PEO), for double- and single-stranded DNA analysis [10,25]. It was easy to prepare homogeneous solutions to provide highly reproducible separation performance with good resolution. In fact, PEO is a material that has been extensively studied in polymer science. Its phase behavior [26], morphology [27], solution dynamics [28], micelle formation [29], and rheological phenomena [30] were well characterized. In this paper, we demonstrate the separation of single-stranded DNA up to 1000 bases using PEO gel. Optimizing conditions including PEO concentration, PEO composition, electric field strength, and capillary length are addressed.

2. Experimental

2.1. Laser-excited fluorescence detection

The experimental setup is similar to that described before [10]. Briefly, a high-voltage power supply (Trek 20/20, Medina, NY, USA) was used for electrophoresis. For safety, the electrophoresis and detection systems were enclosed in a sheet-metal box with a high-voltage interlock. A 1.5-mW He–Ne laser with 543.6 nm output (Melles Griot, Irvine, CA, USA) was used for excitation. Two RG610 filters were used to block scattered laser light. The fluorescence signal was transferred directly through a 10-kΩ resistor to an A/D interface board (Hewlett-Packard, Wilmington, DE, USA) and stored in an IBM compatible computer at 4 Hz.

2.2. Capillary and reagents

Fused-silica capillaries with 75 μm I.D. and 365 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). All chemicals for preparing buffer solutions were obtained from ICN Biochemicals (Irvine, CA, USA). Formamide for DNA denaturation was from Sigma (St. Louis, MO, USA). PEO and hydroxypropyl cellulose (HPC) were obtained from Aldrich (Milwaukee, WI, USA). Fuming hydrochloric acid was obtained from Fisher (Fairlawn, NJ, USA). PGEM/U DNA samples were prepared at the Nucleic Acid Facility (Iowa State University, Ames, IA, USA) by using cycle sequencing, terminator labeled, AmpliTaq FS polymerase, and standard ABI reagents.

2.3. Gel and buffer preparation

The buffer solution was prepared by dissolving 89 m*M* tris(hydroxymethyl)aminomethane (THAM), 89 m*M* boric acid, 2 m*M* ethylenediaminetetraacetic acid (EDTA), and 3.5 *M* urea in deionized water. The gel was prepared by gradually adding proper amounts of M_r 8 000 000 PEO and M_r 600 000 PEO in 10 ml of buffer at room temperature. The polydispersity of the polymer preparations is not known, but our previous work [25] found that polydispersed solutions are in fact advantageous. During the addi-

tion of PEO, a magnetic stirring rod was used at a high speed setting to get a homogeneous solution. After addition, the solution was typically stirred for another 12 h. Then, the resulting gel was degassed by leaving it on the bench top for 3 h.

2.4. Methods

Bare capillaries were prepared with effective lengths of 25, 50, 70, and 100 cm, and total lengths about 5 cm beyond that. The capillary was flushed with 2.5 m*M* HCl solution for 0.5 h before PEO gel was introduced. The selected PEO solution was pushed into the capillary with high pressure (400 p.s.i.; 1 p.s.i.=6894.76 Pa) for 20–40 min. A 1× TBE buffer containing 3.5 *M* urea was placed on both ends of the capillary. The pH of the buffer was 8.2 for these salt concentrations. The capillary was electrophoretically equilibrated for 10 min before sample injection. The DNA sample was denatured by heating in a denaturing solution (formamide–20 m*M* EDTA, 1:1) at 95°C for 3 min. Injection was performed at 150 V/cm for 15–20 s. After each run, the capillary was flushed with 2.5 m*M* HCl solution with high pressure for 15–30 min, and then new PEO gel was introduced again. The cycle time is roughly 2 h. For base identification, the electropherogram was compared with data provided from the Nucleic Acid Facility at Iowa State University and matched with the known sequencing of PGEM.

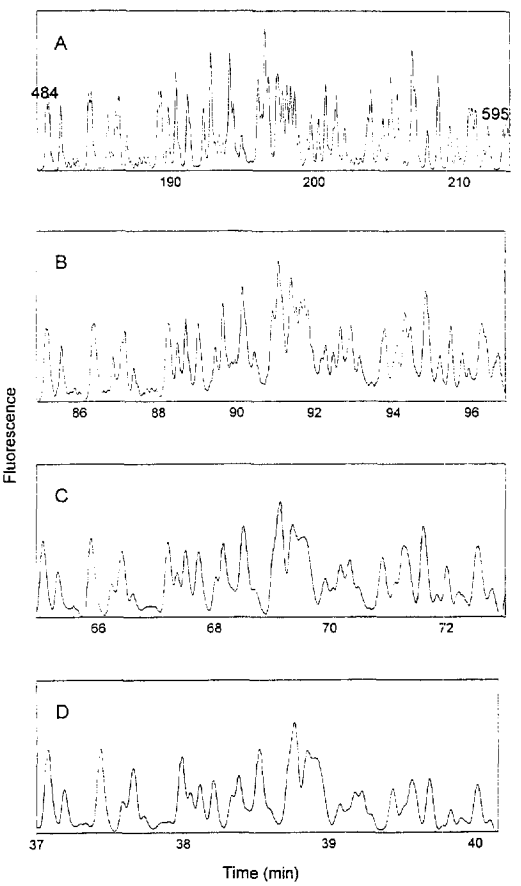

Fig. 1. Electropherograms of PGEM/U DNA sequencing sample between 484 and 595 bp (counting from the primer peak) at various electric field strengths: (A) 75 V/cm, (B) 150 V/cm, (C) 200 V/cm, and (D) 300 V/cm. Conditions: 1×TBE buffer with 3.5 *M* urea, pH 8.2; 55 cm total length; 50 cm effective length; 75-μm I.D. fused-silica capillary; excitation at 543 nm by 1.5-mW He–Ne laser. Sample injection: 20 s at running voltage.

3. Results and discussion

3.1. Effect of electric field strength

Fig. 1 shows the separation of PGEM/U DNA sequencing ladder with different electric fields. These data were obtained at 75, 150, 200, and 300 V/cm using a 50-cm capillary (effective length) in the mixture of 1.5% M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO. The electropherograms show that, at high electric field strength, rapid migration was obtained but with reduced resolution of larger DNA fragments. At low electric field strength, both the resolution and the applicable size limit (as determined from the compression band at the end of the electropherogram) were increased. These features are clearly seen in Fig. 2, where the migration times vs. DNA base number were plotted for each electric field strength. For 75 V/cm, DNA bases up to 980 could be called, while a limit of 540 bases was reached at 300 V/cm. We also investigated the separation of the same DNA sample in a mixture of 2.0% M_r 8 000 000 and 1.4% M_r 600 000 for the same set of electric field strengths. Similar trends were observed for the separation resolution and the applicable size limit, except that the upper size limit

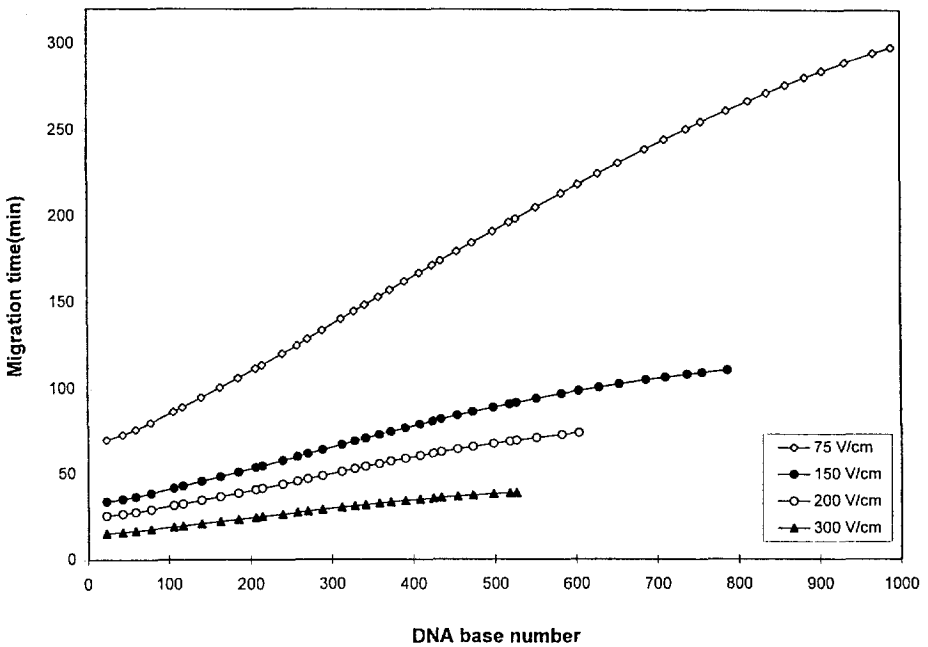

Fig. 2. Migration time vs. DNA base number for various electric field strengths: ◇, 75 V/cm; ●, 150 V/cm; ○, 200 V/cm; and ▲, 300 V/cm. Other conditions are as in Fig. 1.

was always lower for a given field strength for the higher concentration polymer solution.

Joule heating, which leads to radial temperature gradients and faster axial diffusion, can explain the loss of resolution for the smaller DNA fragments at high field strengths. Transition between the Ogston and reptation theories [31] can also be used to explain the observations for the larger DNA fragments. When the DNA size is smaller than, or comparable to, the pore diameter of the gel, the migration behavior follows Ogston theory, which is dominated by the molecular sieving mechanism. However, if the size of the DNA is larger than that of the pores, it moves like a snake between the pores (reptation) and its mobility depends on the shape of DNA. Since the shape of DNA in the gel is determined by both the field strength and the gel concentration, it is important to optimize those conditions to increase the resolution and the applicable size limit. We have investigated these factors by using a reptation plot (Fig. 3), where mobility times the base number of DNA was plotted as a function of DNA base number. The reptation plot is the most effective way to estimate the shape of DNA at various effective field strengths [32,33]. In Fig. 3, a dramatic slope change was observed at about 250 bases, indicating a transition from the Ogston regime to the reptation regime. Also, at low electric field strengths, larger DNA fragments can be resolved because the value of mobility times the base number of DNA changes its slope at a point much later than that at higher electric field strengths. With increasing field strength, stretching of the DNA fragments by a high electric field (biased reptation) resulted in poor resolution for the larger DNA fragments, as depicted by the smaller slopes in Fig. 2.

The separation performances at different field strengths are shown in Fig. 4. The selectivity is defined as the ratio of the mobility difference for two adjacent DNA fragments (1 bp difference) compared to the average mobility. The higher the selectivity, the larger the band spacing is, which enhances the ability to call the DNA bases. It was observed that up to 320 bases the selectivities were almost identical for any field strength. This means that higher fields can be employed to achieve faster sequencing

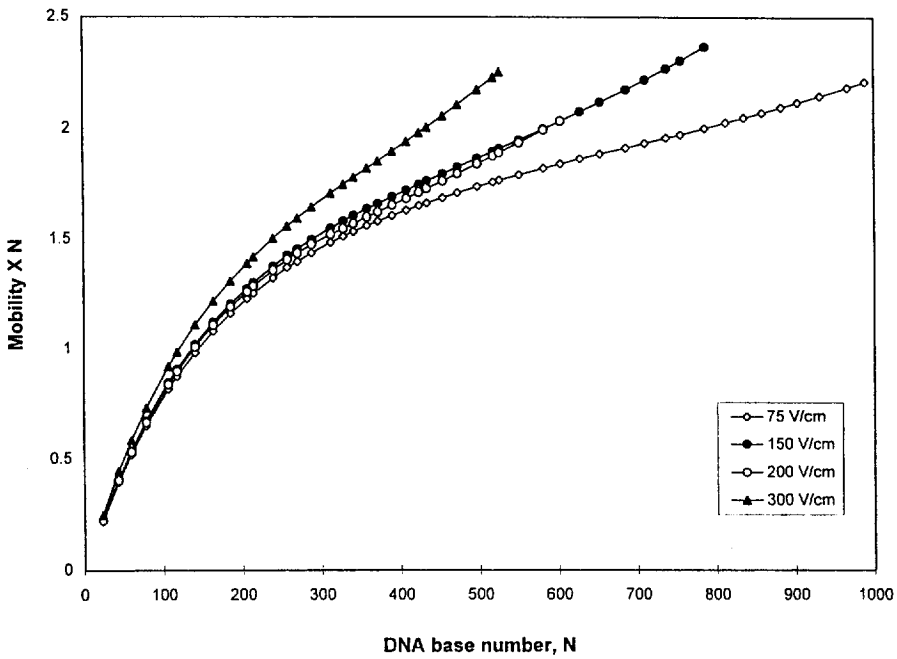

Fig. 3. Reptation plot of mobility times DNA base number vs. DNA base number. Onset of reptation occurs at the point of slope change. Each curve is identified in the inset. Other conditions are as in Fig. 1.

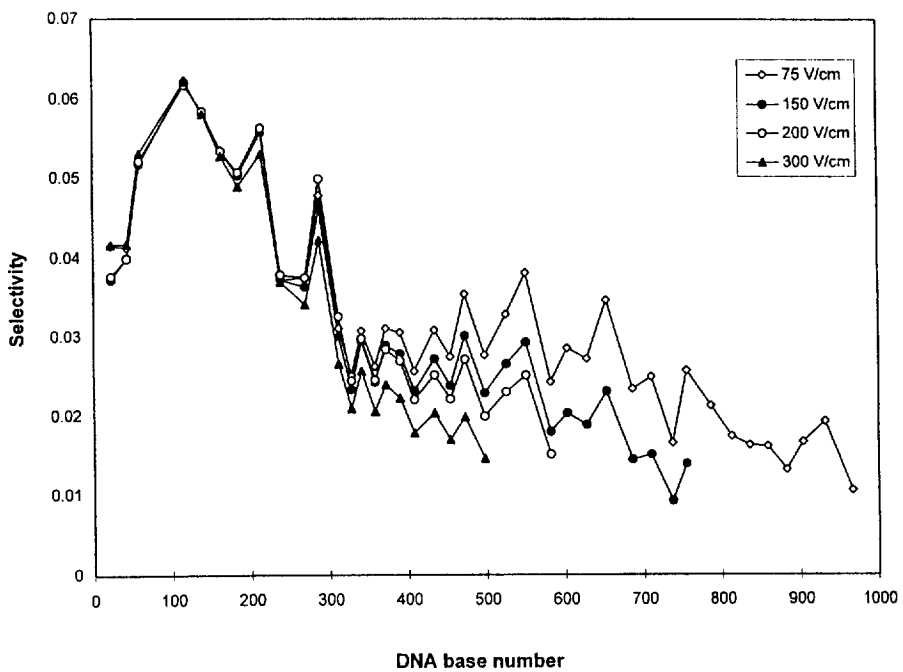

Fig. 4. Selectivity (mobility difference between two peaks divided by the average mobility) vs. DNA base number for various electric field strengths. Other conditions are as in Fig. 1.

up to that DNA size. Due to the biased reptation behavior of DNA fragments, an electric field <150 V/cm was employed in subsequent experiments.

3.2. The effect of polymer concentration on DNA separation

We investigated the performance of PEO gel with respect to PEO M_r and concentration. Also, in separate studies, a different kind of polymer was mixed together with PEO for DNA separation. Electropherograms obtained under the above conditions are shown in Fig. 5. We note that similar separation efficiencies were achieved in Fig. 5A and Fig. 5B. This means that a broad range of polymer concentrations is suitable for sequencing applications. Because of the high tolerance for the gel concentration, the use of PEO should be more advantageous over polyacrylamide in bare fused-silica capillaries. The only difference between Fig. 5A and Fig. 5B is the starting base number (lower size limit) that is resolved. This means that it is necessary to increase the concentration of the short-chain polymer in the separation matrix to provide better separation of the smaller DNA fragments. This is because short-chain polymers interact preferentially with small DNA while long-chain polymers interact preferentially with large DNA [34]. Also, more pores with smaller diameters will be formed by the short-chain polymers. Therefore, increasing the concentration of short-chain PEO is required to read the very beginning of the DNA sequencing sample. This feature becomes more important when one is dealing with unknown DNA samples. If the front part of the sequence is lost, then it requires more front- and back-end work to overlap the various regions. When a single polymer size was used for DNA separation (Fig. 5C), up to 110 bases of information at the beginning of the sequence were lost and reduced resolution was observed. This further supports the explanation described above. The short-chain polymer is needed for separating the smaller DNA fragments.

Mixed polymer systems were tested in our studies using cellulose derivatives. Improved resolution has been reported with a two-component system for separating pharmaceuticals and bioparticles [35]. However, to our knowledge, two-component systems

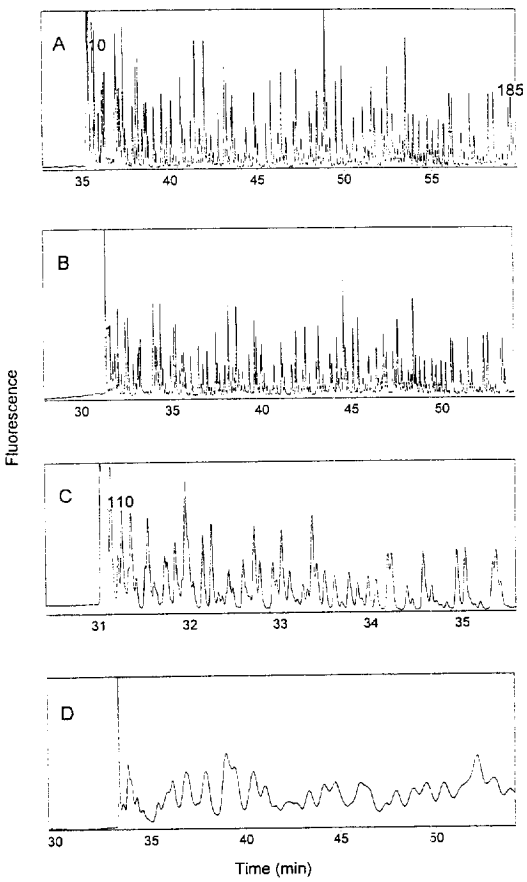

Fig. 5. Electropherograms of PGEM/U DNA sequencing sample up to 185 bp (counting from the primer peak) at various gel compositions: (A) 2.0% M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO, (B) 1.2% M_r 8 000 000 PEO and 1.8% M_r 600 000 PEO, (C) 1.5% M_r 8 000 000 PEO only, and (D) 2.0% M_r 8 000 000 PEO, 1.0% M_r 600 000 PEO, and 0.6% M_r 1 000 000 HPC. Conditions: 1×TBE buffer with 3.5 M urea; 50 cm effective length; 150 V/cm field strength.

have not been applied to CE for DNA sequencing. A 0.6% M_r 1 000 000 hydroxypropyl cellulose (HPC) was added to PEO and the resulting electropherogram is shown in Fig. 5D. The resolution was very poor, such that it was impossible to read any DNA bases. This poor resolution might come from poor miscibility of the two types of polymers even though they are both hydrophilic. It is known that cellulose derivatives are very stiff in nature and their chain length is shorter than that of PEO for a given molecular weight [34]. PEO is a very flexible chain,

and its interaction with HPC might be thermodynamically unfavorable, resulting in local aggregates of each type of polymer, which will degrade the resolution. Further studies are required to understand this mixing behavior in gel solutions, particularly with respect to polymer length, type, polydispersity, and concentration.

Fig. 6 shows the latter part of the electropherograms of the DNA sequencing ladder with three different compositions of PEO. Again, a similar separation efficiency was obtained in Fig. 6A and Fig. 6B. An interesting fact is that good resolution was also observed with a single polymer composition, while the total migration time was about two-times shorter (Fig. 6C). This polymer solution is not applicable to DNA sequencing because of its inability to separate small DNA fragments (Fig. 5C), but can be applied to other biologically important situations. If one is interested in, e.g. PCR analysis for DNA sizes between 100 and 500 but needs single base resolution, this single M_r but low viscosity PEO solution can be employed to reduce the total analysis time while retaining a high efficiency.

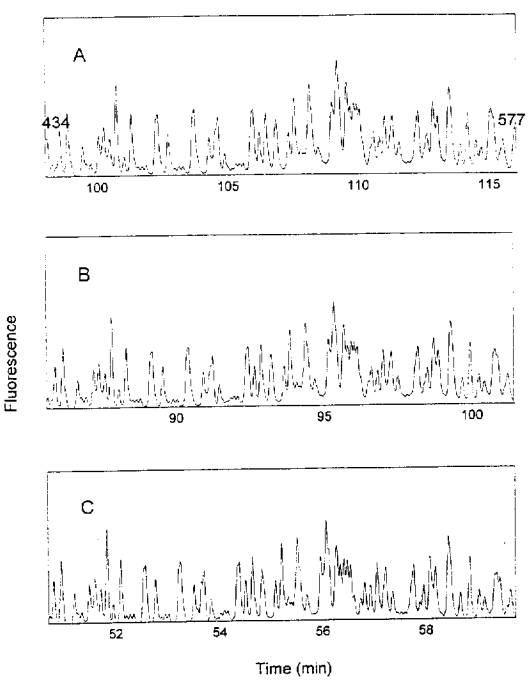

Fig. 6. Electropherograms of PGEM/U DNA sequencing sample between 434 and 577 bp at various gel compositions: (A) 2.0% M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO, (B) 1.2% M_r 8 000 000 PEO and 1.8% M_r 600 000 PEO, and (C) 1.5% M_r 8 000 000 PEO only. Other conditions are as in Fig. 5.

An even broader range of PEO concentrations was studied and the migration behavior of DNA fragments was plotted in Fig. 7. Between 0.8 and 1.5% for M_r 8 000 000 and between 1.0 and 2.2% for M_r 600 000, good separation performance was obtained. As long as the total concentration of polymers is close to 3%, good resolution was observed. The performance of 2.4% M_r 8 000 000 PEO was close to other mixtures of PEO, but the resolution of the first 10 bases was lost. Again, the implication is that it is necessary to add short-chain PEO for separating the whole range of sizes in the sequencing ladder.

For repeated use of a capillary, capillary regeneration after each electrophoresis run is a key issue in DNA sequencing applications [10]. Especially when multiple capillaries are bundled together to achieve high-throughput sequencing [6], this regeneration process is essential to avoid the high cost of replacing the bundle of capillaries after a few runs. We have found that the rate of deterioration of the capillary increased with increasing gel concentrations. It is known that for polymer solutions, aggregates can be formed at the surface of the fused-silica capillary during electrophoresis and this results in degradation of the resolution [36]. It will thus be beneficial to use lower concentrations of PEO and solutions with lower viscosity. In our experiments, we found that 1.3% M_r 8 000 000 with 1.0% M_r 600 000 showed similar separation performance compared to other gel compositions. The total concentration is much lower than 3.0% and the viscosity is lower than those of other gel compositions. This particular gel may therefore help extend the longevity of the capillary for repeated use.

3.3. The effect of capillary length

It is known that the performance of a capillary zone electrophoresis system is not dependent on the capillary length at a constant applied voltage when diffusion is the major source of band broadening [37]. For polyacrylamide gel capillary electrophoresis, it was suggested that in order to reduce the diffusion-induced band broadening, a high electric field is required for the fast separation of DNA

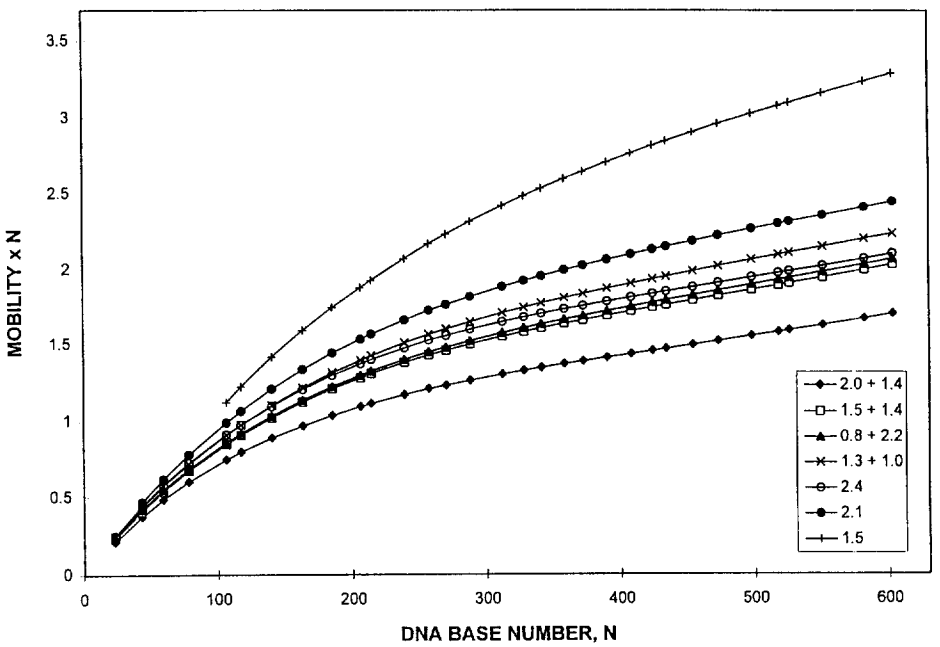

Fig. 7. Reptation plot for various gel compositions. Each composition is indicated in the inset of the figure. The two-component gel was made from M_r 8 000 000 PEO and M_r 600 000, respectively. Otherwise the gel was made from M_r 8 000 000 only. Other conditions are as in Fig. 6.

fragments up to 200 bases [19]. We tested the performance of PEO gel with respect to capillary length. The separation efficiency of electrophoresis as a function of DNA base number is plotted in Fig. 8 for each capillary (effective) length. The number of theoretical plates for each length was normalized to 1 m of the capillary. It can be clearly seen that a longer capillary showed a better efficiency for all sizes of DNA, which was not predicted by the standard theory [37]. We attribute this effect to the special pore structure and gel dynamics of PEO, which is known to be more flexible and longer than polyacrylamide for the same average molecular weight. Fig. 8 also implies that diffusion is not the limiting factor in these separations.

We note, however, that there is a trade-off between the capillary length and the maximum readable length of DNA and the resolution. According to the theory derived by Slater and Drouin [38], it is predicted that the maximum readable length of DNA (convergence limit at large bp, M^*) is proportional to the square root of the capillary length. Fig. 9 shows the measured and predicted values of M^* for each capillary length. We observed that the deviation became larger as the capillary length increased.

3.4. DNA sequencing up to 1000 bases with optimized conditions

Fig. 10 shows the separation of PGEM/U sequencing ladder up to 1000 bases in the mixture of 1.5% M_r 8 000 000 and 1.4% M_r 600 000 PEO at 75 V/cm with a 70-cm effective length capillary. When a higher field strength (150 V/cm or higher) was used, only up to 700 bp were readable, even with a longer capillary (70 cm or longer). Even though the separation in Fig. 10 takes about 7 h to complete, it can actually provide genomic DNA sequencing with at least the same speed as similar systems employing other types of gels. For instance, if one can sequence 350 bases in a run that lasts 1.5 h, it will require at least four runs to read 1000 bases. Then, the total processing time will be 7.5 h including 0.5 h for capillary regeneration and gel reloading after each run. In addition, there will need to be extra sample preparation steps to sequence shorter fragments and

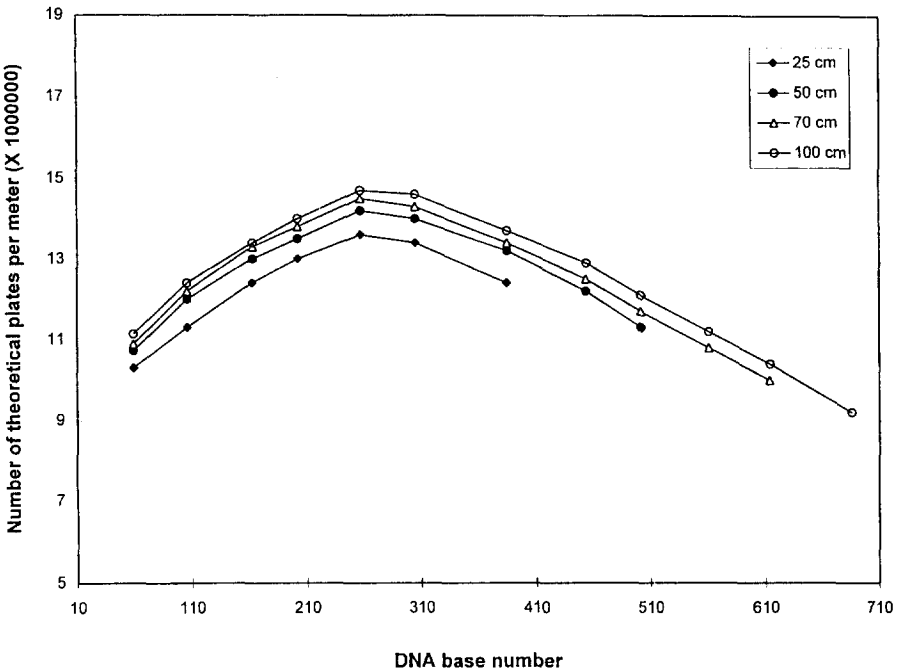

Fig. 8. The number of theoretical plates vs. DNA base number for various capillary lengths. The calculation of the number of theoretical plates is normalized to a 100-cm capillary. Each curve is identified in the inset. Conditions: 1×TBE buffer with 3.5 *M* urea; 150 V/cm field strength; gel concentrations at 1.5% M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO.

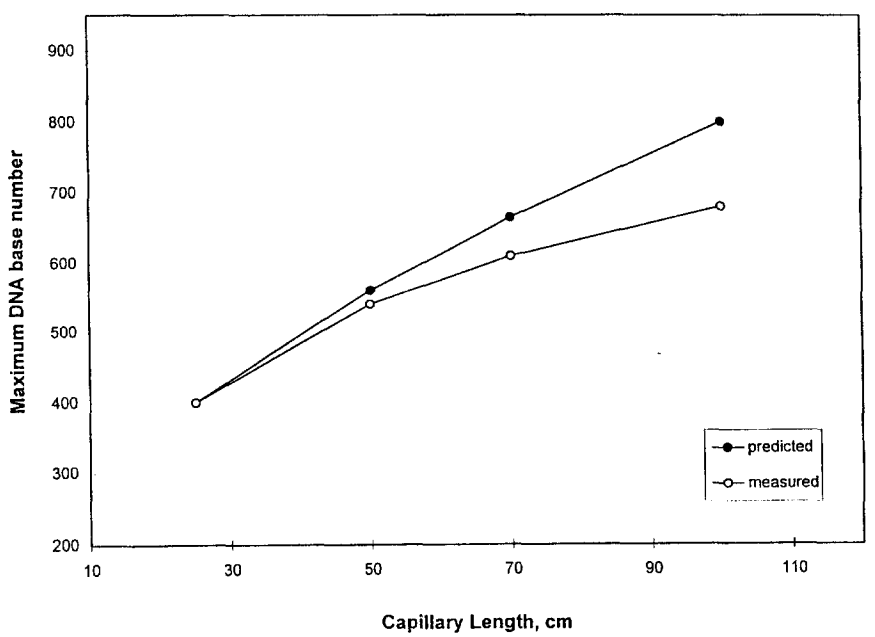

Fig. 9. Maximum readable length of DNA base (compression limit for large fragments) vs. capillary length. See the text for details.

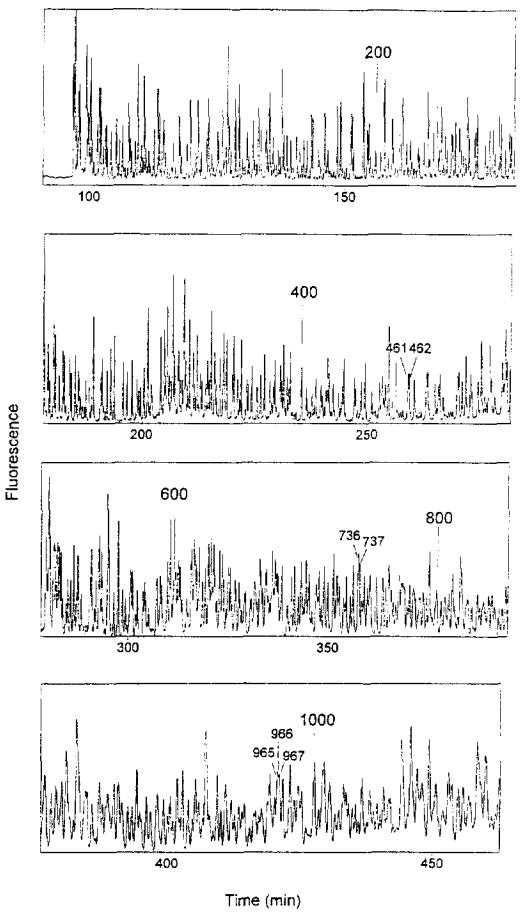

Fig. 10. Electropherograms of PGEM/U DNA sequencing sample up to 1000 bp (counting from the primer peak). Conditions: 1×TBE buffer with 3.5 *M* urea; 75 V/cm field strength; gel concentrations at 1.5% M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO; 70 cm effective length; 30 s injection at running voltage.

then extra information processing to order them. Comparing with our earlier results on 2-wavelength base calling [39], Fig. 10 shows that PEO gel can be used to call bases up to 1000 without further data smoothing or peak fitting. These results should be compared to Fig. 6 in [24], which was plotted after data smoothing and peak fitting.

High-speed, high-throughput DNA sequencing is required to meet the goals of the Human Genome Project. Here we suggest other factors that can allow further optimization of the PEO gel to satisfy that application. First, if PEO larger than M_r 8 000 000 is available, it may help to extend the size limit for resolution. Since M_r 8 000 000 is the largest one that is commercially available at present, new or elaborate synthetic approaches should be studied to extend the PEO size limit. Second, temperature programming can be employed during electrophoresis in order to reduce molecular orientation of the larger DNA and to reduce the total migration time. Also, DNA renaturation during a run could be avoided when hot injection is coupled with temperature programming. Third, since molecular orientation can be controlled by external field modulation [40–43], pulsed fields can be employed to this system to extend size limit. It was predicted that voltages larger than 5000 V and pulse times shorter than 1 ms will help reduce molecular orientation [38]. Work towards evaluating these factors is under progress in our lab.

Acknowledgments

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