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Improvements in DNA sequencing by capillary electrophoresis at elevated temperature using poly(ethylene oxide) as a sieving matrix

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Abstract

DNA sequencing in poly(ethylene oxide) (PEO) matrix by capillary electrophoresis was demonstrated at high temperature. The optimal separation temperature is around 40°C. The effects of polymer concentration and types of buffers on the separation performance were investigated. A new buffer system consisting of Tris–Taps–His–EDTA works well with PEO. High-speed separation and good resolution can be fulfilled by using a single-MW PEO polymer. It offers similar separation performance as before for the small DNA fragments, but better performance for large DNA fragments. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: DNA sequencing; Poly(ethylene oxide)

1. Introduction

Capillary electrophoresis (CE) is an attractive separation technique for DNA sequencing owing to the advantages of high speed, high resolution, and low sample consumption over other techniques [1–10]. In particular, multiplexed array CE dramatically improves the throughput of DNA sequencing [6]. DNA sequencing generally demands single-base resolution of around 0.5 in order to allow accurate base calling. Polymers suitable for DNA sequencing have been reviewed recently [11,12]. In 1995, our group first successfully demonstrated the use of poly(ethylene oxide) (PEO) as a matrix for DNA sequencing by CE [6,7]. DNA bases up to 1000 bp in a sequencing ladder were separated within 7 h at room temperature using a PEO-filled capillary [8]. The separation speed is comparable to that of the slab gel, and is too slow to meet the demands of high-speed and high-throughput DNA sequencing. Faster separations to 1000 bases have been demonstrated using linear polyacrylamide (LPA) solutions [13,14], but the high viscosity puts serious constraints on reliable and rugged operation. For example, commercial array CE instruments have not duplicated this level of performance. Further optimization of the PEO gel is therefore desirable. The goal of the present work is to comprehensively study all the parameters that influence the separation of DNA fragments in PEO. The effects of separation temperature, electric field strength, buffer composition, capillary length and polymer molecular weight and concentration on the performance of DNA sequenc-

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ing are evaluated. The basic strategy for optimizing the separation conditions, to speed up the separation but maintain long reads, is discussed.

2. Theory

The migration behavior of DNA in a polymer matrix has been studied extensively and theoretical models for describing the migration of DNA have been proposed [15–22]. The Ogston sieving model [15] assumes that the polymer network is like a molecular sieve. The expression for the mobility of DNA molecules is [15],

$$\mu = \mu_0 \exp(-K_1 c\,(r + R_g)^2) \tag{1}$$

where μ_0 is the mobility of DNA in pure solvent, K_1 is a constant, c is the polymer concentration and r and R_g are the thickness of the polymer strands and the radius of gyration of DNA molecules, respectively.

However, when the radius of gyration of DNA molecule is larger than the pore radius of the polymer matrix, reptation [16] and biased reptation models (BRM) [17–22] are used to explain the separation mechanism. In reptation, the DNA molecules are confined to ''tubes'', which are formed by the polymer solution. The expression for the mobility of DNA becomes [16]

$$\mu = \mu_0 A/N \tag{2}$$

where A is a proportionality constant which depends on the relative length of DNA in a pore and N is the length of the fragment in nucleotide units.

However, reptation is based on a weak applied electric field. Large DNA molecules will be deformed in a relatively high electric field E. In BRM, the mobility of DNA molecules is [18]

$$\mu \approx K\left(\frac{1}{N} + aE^2\right) \tag{3}$$

where K is the total charge on the DNA molecule and is a function of the mesh size of the polymer network as well as its charge, and a is the persistence length of DNA molecules.

BRM predicts that above a certain size limit (N^*), DNA molecules begin to be oriented and the mobilities of different size fragments will tend to become the same and cannot be distinguished from each other under constant electric field [18–22]. The limit of sequencing (N^*) is related to the scaled electric field (ϵ) as follows,

$$N^* \sim \epsilon^{-1} \tag{4}$$

where $\epsilon = e_1 E\xi/2k_B T$, e_1 is the net electric charge per base, ξ is the average pore radius of the matrix and k_B is the Boltzmann's constant.

We can rewrite Eq. (4) as

$$N^* \sim T/(E\xi) \tag{4a}$$

The limit of sequencing (N^*) depends on separation temperature, electric field and the average pore radius of the matrix. Thus, we can control experimental conditions to get longer sequence reads.

The Ogston and BRM models were originally used for describing the migration behavior of DNA molecules in a physical gel. In the case of polymer solutions, the polymers are only entangled and not linked together, they may disentangle as addressed by Viovy and Duke [17, 19, 20]. Although the Ogston regime and BRM regime are both observed in polymer-filled capillary electrophoresis, these models are not always valid. Duke and Viovy [17] modified the BRM model and introduced the concept of constraint release (CR) in entangled polymer solutions. CR model predicts that the limit of separation in a temporary gel is given as [17, 20]

$$N^* \sim (\xi/b)^{-4}(E/E_0)^{-2} \tag{5}$$

where b is the Kuhn length of the DNA molecule and $E_0 = k_B T/e_0 b$.

PEO composition here refers to the PEO concentration and its molecular weight (M_w). The polymer concentration is related to the effective pore radius of the sieving solution as [23],

$$\xi = 1.43 R_g (c/c^*)^{-3/4} \tag{6}$$

where c and c^* are the mass concentration and the entanglement threshold concentration of the polymer and R_g is the radius of gyration of the polymer. c^* can be expressed as

$$c^* = 0.6[\eta]^{-1} = K_2 M_w^{-r} \tag{7}$$

where $[\eta]$ is the intrinsic viscosity of matrix, and K_2 and r $(0 < r < 1)$ are constants.

For PEO with molecular weight in the range $10^4 \sim 10^7$, the intrinsic viscosity can be described by an empirical equation [24],

$$[\eta] = 1.25 \times 10^{-4} M_w^{0.78} \quad (8)$$

The radius of gyration of PEO is expressed by [25]

$$R_g = 0.0215 M_w^{0.583} \text{ (nm)} \quad (9)$$

Combining Eqs. (6), (7), (8) and (9), for $c > c^*$,

$$\xi \approx K_3 c^{-3/4} \quad (10)$$

where K_3 is a constant. That is, the pore radius of the polymer is determined only by the polymer concentration.

According to the CR model, the polymer chain length that provides the optimal rate of constrain release is related to the electric field as [17],

$$R_g/b \sim (E/E_0)^{-2/5} \quad (11)$$

Combining Eqs. (5), (9) and (11),

$$N^* \sim b^{-1} c^3 M_w^{2.92} \quad (12)$$

The limit of separation is thus determined by both the molecular weight and concentration of the polymer. We can extend the read length by increasing the concentration or by using higher molecular weight polymer. In the case of PEO, two factors (c and M_w) have a similar influence on N^* (exponent terms are 3 and 2.9 for c and M_w, respectively). However, the increase in PEO concentration causes other problems. First, the higher the concentration the more viscous the polymer solution (see Eq. (8)), creating difficulties in handling. What is more, the thicker gel slows down the separation because the concentration determines the separation velocity (v) via the following scaling law [21],

$$v \sim E c^{-x} \quad (13)$$

where x ($0 < x < 1$) is constant.

The theoretical considerations above provide a basic strategy to optimize DNA sequencing in an entangled polymer matrix.

3. Materials and methods

3.1. Instrumentation

The CE instrument with laser-induced fluorescence (LIF) detection, built in house, has been described in previous work [8]. Briefly, a 5-mW He–Ne laser with 543.5 nm output (Melles Griot, Irvine, CA, USA) was used for excitation. A RG610 cutoff filter was used to block stray light. The signal from the photomultiplier tube (PMT) was transferred directly through a 10-kΩ resistor to a 24-bit A/D converter (Lawson Labs, Kalispell, MT, USA) and stored in a 486/33 computer at 4 Hz.

Fused-silica capillaries with 75 μm I.D. and 367 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). The separation capillary was enclosed in a 0.5 cm I.D. copper heating jacket. The jacket is connected to a water bath circulator (Fisher Scientific). A model HH23 thermometer was directly connected to the outside of the copper tube, and the separation temperature can be directly read by the thermometer with a precision of 0.1°C.

3.2. Chemicals and materials

PEO was obtained from Aldrich (Milwaukee, WI, USA), Tris(hydroxymethyl) aminomethane (Tris), 3-[[tris(hydroxymethyl)-methyl]propanesulfonic acid (Taps), histidine, EDTA, foramide and PVP with M_w 360 000 were from Sigma (St. Louis, MO, USA). Urea was purchased from ICN Biochemicals (Irvine, CA, USA). PGEM/U DNA samples were prepared at the Nucleic Acid Facility (Iowa State University, Ames, IA, USA) by using cycle sequencing, dye-terminator, AmpliTaq FS polymerase and standard ABI (Foster City, CA, USA) reagents.

3.2.1. Buffer and matrix preparation

Several different buffer solutions were prepared for testing the effect of buffer types on the separation. 1×TBE was prepared from 89 m*M* Tris, 89 m*M* boric acid and 2 m*M* EDTA, Tris-Taps-EDTA buffer (TTE) from 89 m*M* Tris, 89 m*M* Taps and 2 m*M* EDTA-Na, Tris-His-EDTA buffer (THE) from 89 m*M* Tris, 89 m*M* histidine and 2 m*M* EDTA-Na, and Tris-Taps-His-EDTA buffer (TTHE) from 89

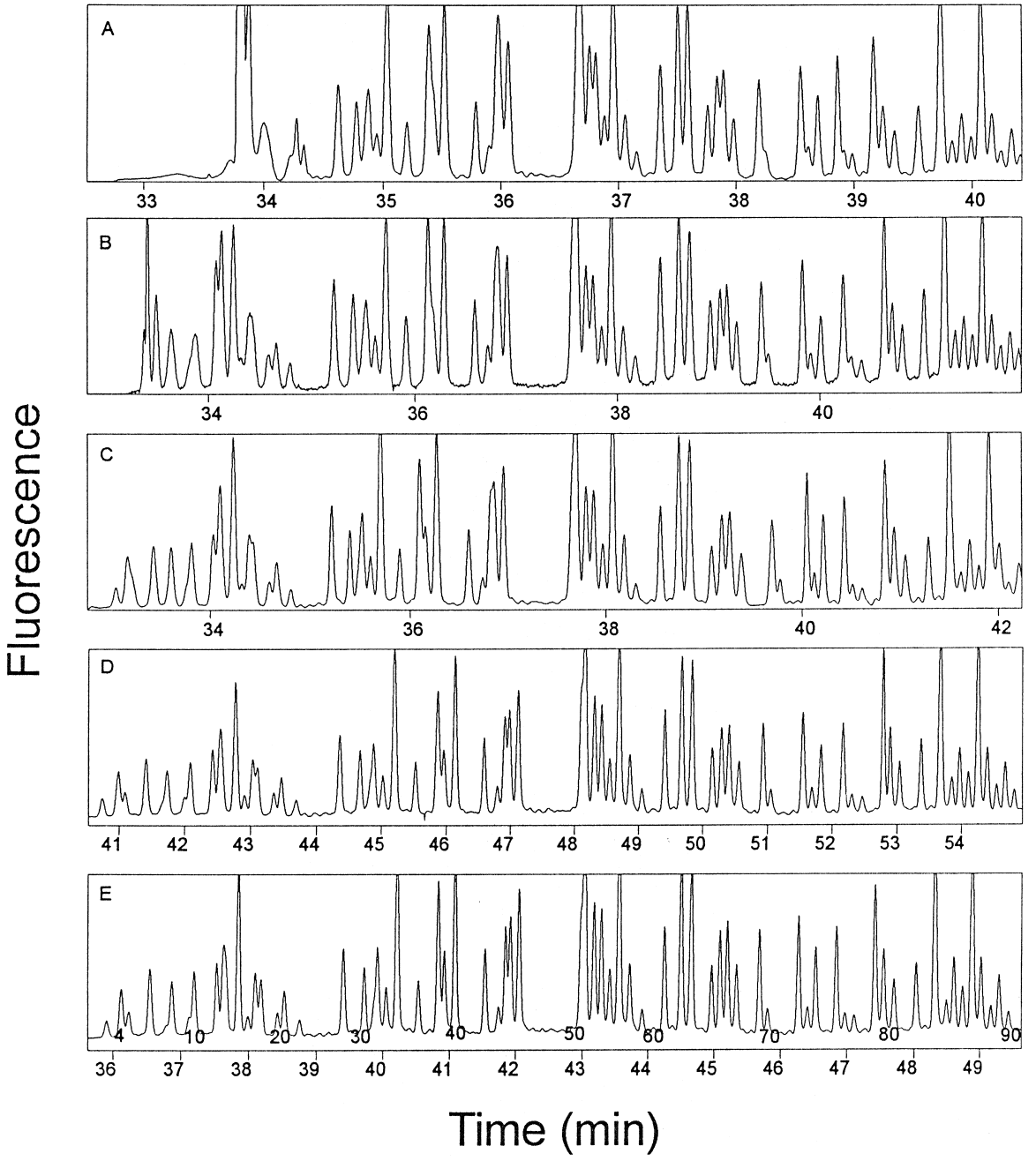

Fig. 1. Comparison of the early parts of the electropherograms of PGEM/U DNA sequencing sample at various gel compositions. (A) 1.8% M_w 8 000 000 PEO; (B) 2.0% 8 000 000 PEO; (C) 2.2% 8 000 000 PEO; (D) 2.5% 8 000 000 PEO; (E) 1.4% 600 000 and 1.5% 8 000 000 PEO. Conditions: 1×TTHE buffer with 7 *M* urea, 65 cm effective length, 150 V/cm field strength, 40°C.

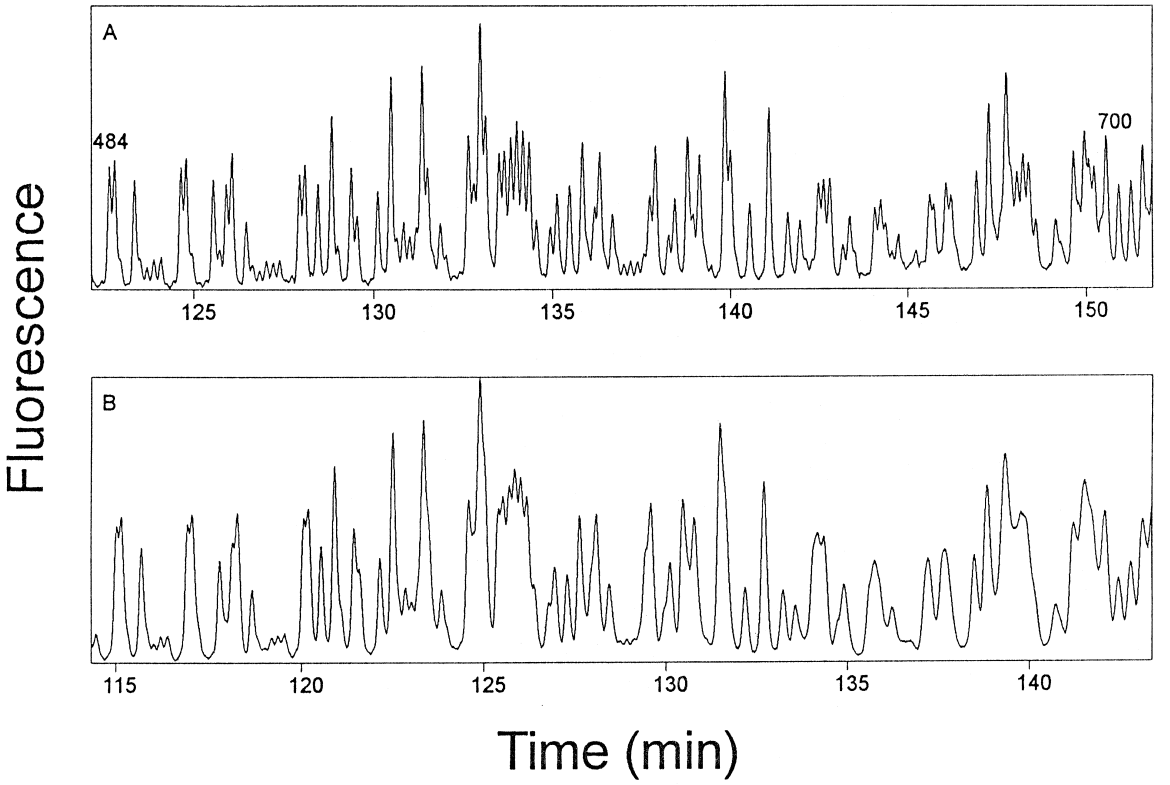

Fig. 2. Comparison of the later parts of the electropherograms of PGEM/U DNA sequencing ladder from 485 to 700 bp (counting from the primer peak). (A) 2.5% M_w 8 000 000 PEO; (B) 1.4% 600 000 and 1.5% 8 000 000 PEO. Other conditions are as in Fig. 1.

m*M* Tris, 50 m*M* Taps, 39 m*M* histidine and 2 m*M* EDTA. All buffers contain 7 *M* urea for DNA denaturation.

PEO matrix was prepared by adding proper amounts of M_w 8 000 000 or 600 000 PEO powder in 10 ml of buffer at room temperature. A magnetic stirring bar was used at a high speed setting for the first several hours. After 12 h, the stirring speed was decreased and the solution was stirred for another 12 h to obtain a homogeneous solution.

3.3. Operation conditions

New bare capillaries were flushed with deionized water for 10 min and then with 2% PVP solution for 5 min before PEO was introduced. The PEO solution was pushed into the capillary by a 100 μl syringe for 5 min. The running buffer did not contain urea. The DNA sample was denatured by heating in formamide at 95°C for 3 min, and then putting the vial into ice water. Electrokinetic injection was performed at the running voltage for 10–30 s. After each run, the capillary was flushed with water and then 2% PVP solution for 10 min before new PEO solution was pushed through again.

4. Results and discussion

4.1. Effect of PEO matrix composition

We choose PEO with M_w 8 000 000 as the basic component of the matrix because it is the largest molecular weight of commercial PEO products at present. In our previous work [8], the total PEO concentration (high plus low molecular weight polymers) is around 3% in 1×TBE buffer for acceptable matrix viscosity and sufficient resolution for the larger DNA fragments. The total concentration should be decreased to maintain a similar viscosity

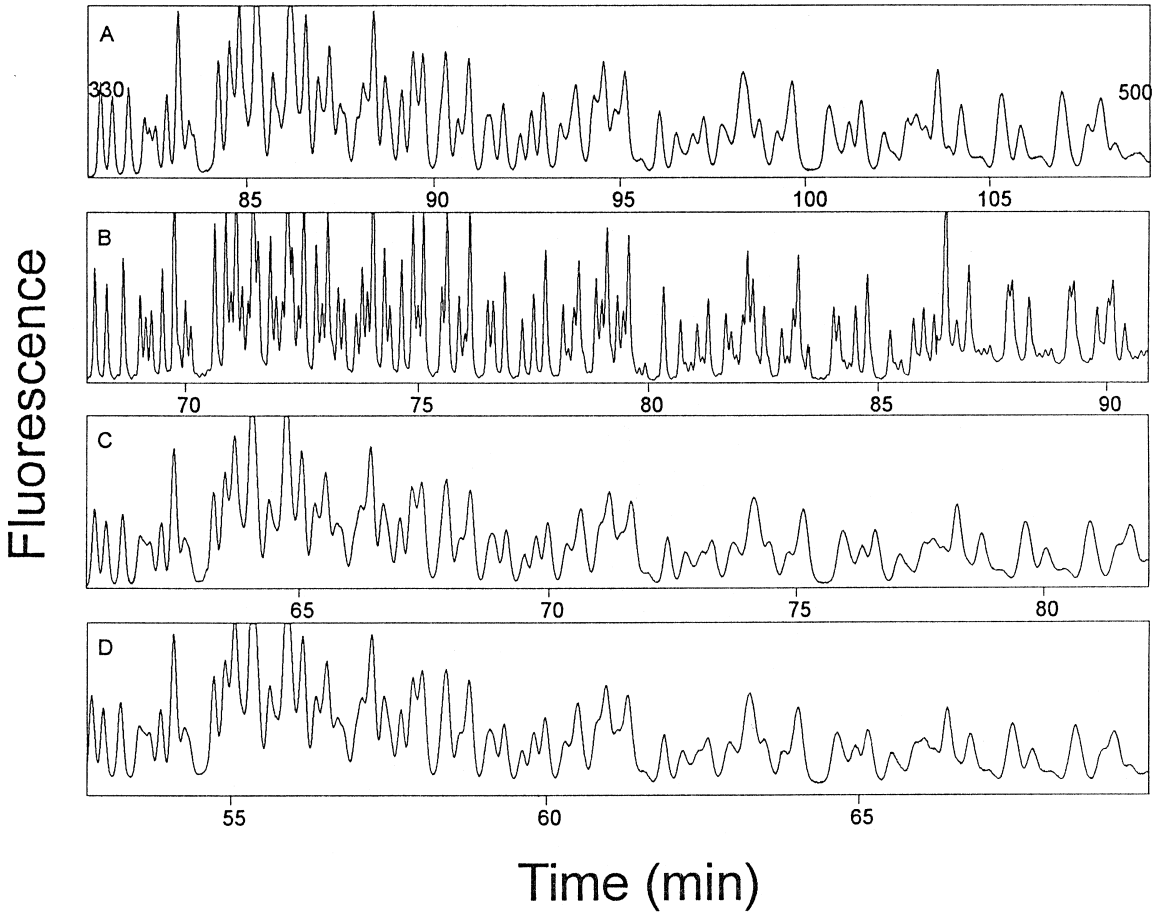

Fig. 3. Electropherograms of PGEM/U DNA sequencing sample between 330 and 485 bp at various separation temperatures. (A) 30°C; (B) 40°C; (C) 50°C; (D) 60°C. Matrix: 2.2% PEO. Other conditions are as in Fig. 1.

when a single molecular weight of polymer is used. We performed separations with different concentrations of 1.8, 2.0, 2.2, and 2.5% (w/v). In order to compare with previous work, a mixed matrix with 1.4% M_w 600 000 and 1.5% M_w 8 000 000 PEO is also used. Fig. 1 shows the early part of electropherograms from the primer peak to 80 bp after the primer peak obtained under the above conditions. We note that the resolution of fragments smaller than 50 bp was lost when the PEO concentration was less than 2.5%. 2.5% M_w 8 000 000 PEO and the 2.9% mixed PEO matrix (1.4% 600 000 and 1.5% 8 000 000 PEO) show similar resolution. The total concentration thus plays a role in keeping the resolution of smaller DNA fragments, which is different from the previous findings [8]. There, it was found that the addition of short-chain polymers is needed to resolve the early peaks [8]. A reasonable explanation for our results is that the short-chain polymers were actually formed during the preparation of the matrix. We observed that the PEO solution becomes thinner after extensive stirring. In addition, other studies in polymer physics indicate that PEO molecules degrade under high-speed stirring [26]. PEO with M_w larger than 1 000 000 will degrade to a relatively stable M_w~600 000 [27]. Thus, the solution of M_w 8 000 000 PEO alone can provide similar low M_w components after stirring.

A lower total concentration was used here than in previous work (less than 2.5% for 8 000 000 PEO).

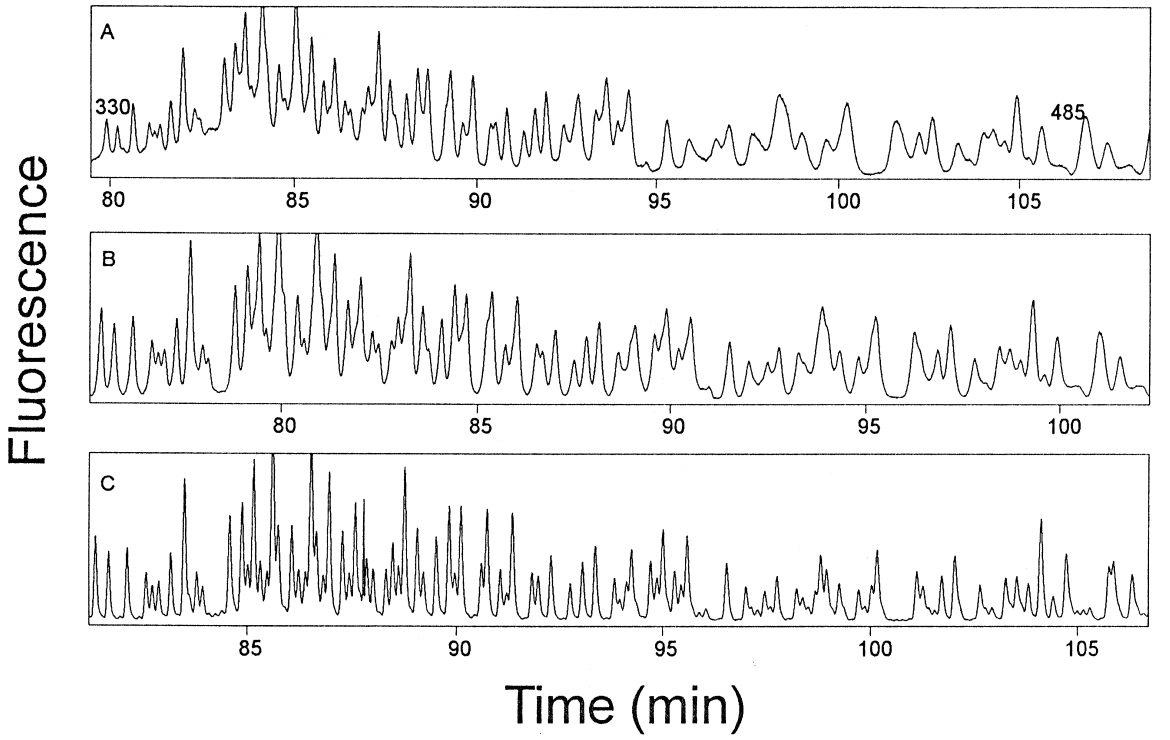

Fig. 4. Comparison of electropherograms of PGEM/U DNA sequencing sample between 330 and 485 bp at various buffer compositions. (A) 1×TTE; (B) 1×TBE; (C) 1×THE. Other conditions are as in Fig. 1.

According to theoretical predictions, the higher the concentration, the smaller the radius of gyration of the polymer. PEO is thus advantageous over other polymers for DNA sequencing because it is relatively convenient to prepare a homogeneous and resilient gel using a single polymer compared with the use of two different molecular weight polymers. Wu et al. [28] have pointed out that resilient gels produced less dispersion in the DNA bands and thus higher efficiency.

Fig. 2 shows the latter part of the electropherograms of the DNA sequencing ladder from 484 to 700 bp with 2.5% PEO and with the mixed matrix. The difference in separation for the large fragments is very striking. The matrix made of 2.5% M_w 8 000 000 PEO provides better resolution and longer read than the mixed matrix. Eq. (12) can be used to explain the results. It is apparent that the molecular weight ratio of the two polymer solutions is larger than their concentration ratio. When we tried to further increase the PEO concentration, we did not observe improved resolution or longer read. The effect of dispersion on the separation may become important due to the very slow separation speed at high concentration. Thus, for a polymer with a given molecular weight, when its concentration reaches a certain value, the limit of separation can no longer be changed by increasing only the concentration. The molecular weight will play a dominant role to extend its utility to longer bases. The single 2.5% M_w 8 000 000 PEO seems to be a compromise that improves the read length but keeps a reasonable separation speed.

4.2. Effect of separation temperature

Separation temperature is an important parameter in optimizing the resolution and speed. High temperature operation brings several advantages for separation. Firstly, the onset of biased reptation will be extended to longer base pairs at high temperature due to an increase in the thermal energy of DNA

molecules. Kelepanik et al. [29] observed extended read lengths for DNA fragments from 742 bp to 883 bp when the separation temperature changed from 40 to 60°C in replaceable LPA matrix. Secondly, high temperature operation improves the separation precision by releasing the secondary structure of DNA [30,31]. We observed improved resolution for DNA sequencing at elevated temperature in PEO in the absence of denaturant (urea) [32]. Other researchers also demonstrated similar advantages in other matrices, such as LPA [2,13,14,16,33], PDMA [3], and poly(*N*-acryloylaminopropanol) [4]. Thirdly, the separation time is shortened with the decrease of viscosity at higher temperature. However, high temperature also gives rise to two problems. It causes a change in the pore structure of the matrix [14] and the EOF becomes larger at higher temperature [3]. In order to eliminate EOF at high temperature, a 2% PVP solution is used as a dynamic coating on the capillary between runs [10]. By using this coating procedure, we performed separation at several temperatures. When the operation temperature is above or below 40°C, the resolution for DNA fragments above 330 bp is lost, as shown in Fig. 3. The broader peaks at low separation temperature may be attributed to slow separation speed and renaturing of the sample. The structure of the PEO matrix likely degraded at high temperature, causing poor separation performance above 40°C. Furthermore, at very high temperatures, the dynamic coating on the capillary wall becomes ineffective, which also leads to poor separation.

4.3. Effect of buffer composition

There is little published work on studying the effect of buffer composition on separation in DNA sequencing by CE. Following the protocols of traditional slab gel, 1×TBE (Tris-Boric acid-EDTA, pH 8.2, ionic strength 200 m*M*) buffer is typically used in CE. Karger's group [14] finds that boric acid in the separation buffer causes irregular peaks in the

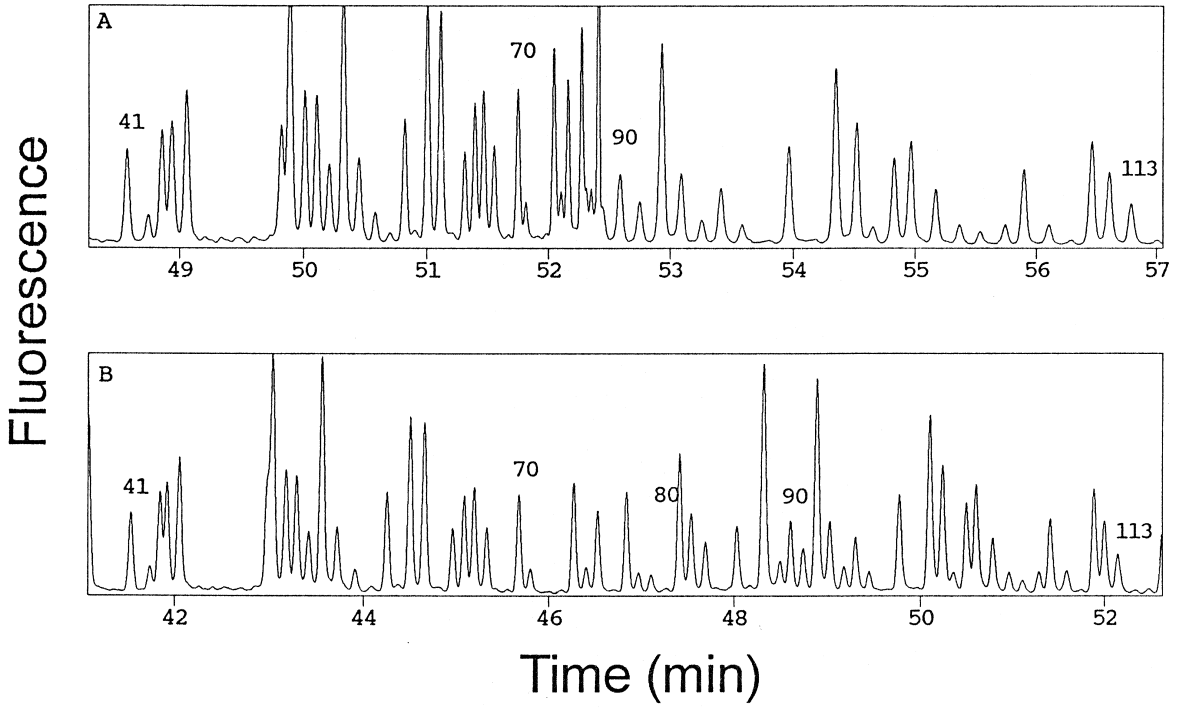

Fig. 5. Comparison of electropherograms of PGEM/U DNA sequencing sample between 41 and 113 bp in (A) THE and (B) TTHE buffers. Conditions: 1.4% 600 000 and 1.5% 8 000 000 PEO matrix, effective length 63 cm, field strength 150 V/cm, 40°C.

beginning of the electropherograms in LPA matrix. They explained that residual glycerol in the samples could complex with boric acid. Thus, Tris (50 m*M*)/Taps (50 m*M*)/EDTA (2 m*M*) (TTE) buffer was used in place of TBE buffer, which minimized the number of distorted peaks. However, when TTE buffer was used in the PEO matrix, a poor separation performance was observed, as shown in Fig. 4A. This is attributed to Joule heating due to the high conductivity of TTE buffer. In the case of 1×TBE buffer, we find the resolution for GC rich regime decreases and peaks begin to become broad after 330 bp, as shown in Fig. 4B. Thus, we decided to change the buffer composition but keep the same ionic strength as 1×TBE. 7 *M* urea was used in place of 3.5 *M* urea to enhance denaturing. Histidine is chosen to replace boric acid since the organic histidine structure unit could help to denature the sample. Fig. 4C is the electropherogram obtained in THE buffer under otherwise the same conditions. The peaks are very sharp and a better performance was observed. However, we find the peaks around 70 bp to 80 bp co-elute as a very large peak sometimes (see Fig. 5A compared with Fig. 5B) in THE buffer. We tried to decrease the histidine concentration to reduce Joule heating. When the histidine concentration was decreased from 89 m*M* to 39 m*M*, the co-eluted peaks never came out. Probably, histidine at high concentration (89 m*M*) prefers to interact with DNA fragments around this DNA region. In order to keep the same ionic strength and pH values as 1×TBE, Taps (pK_a~8.2) is added into THE

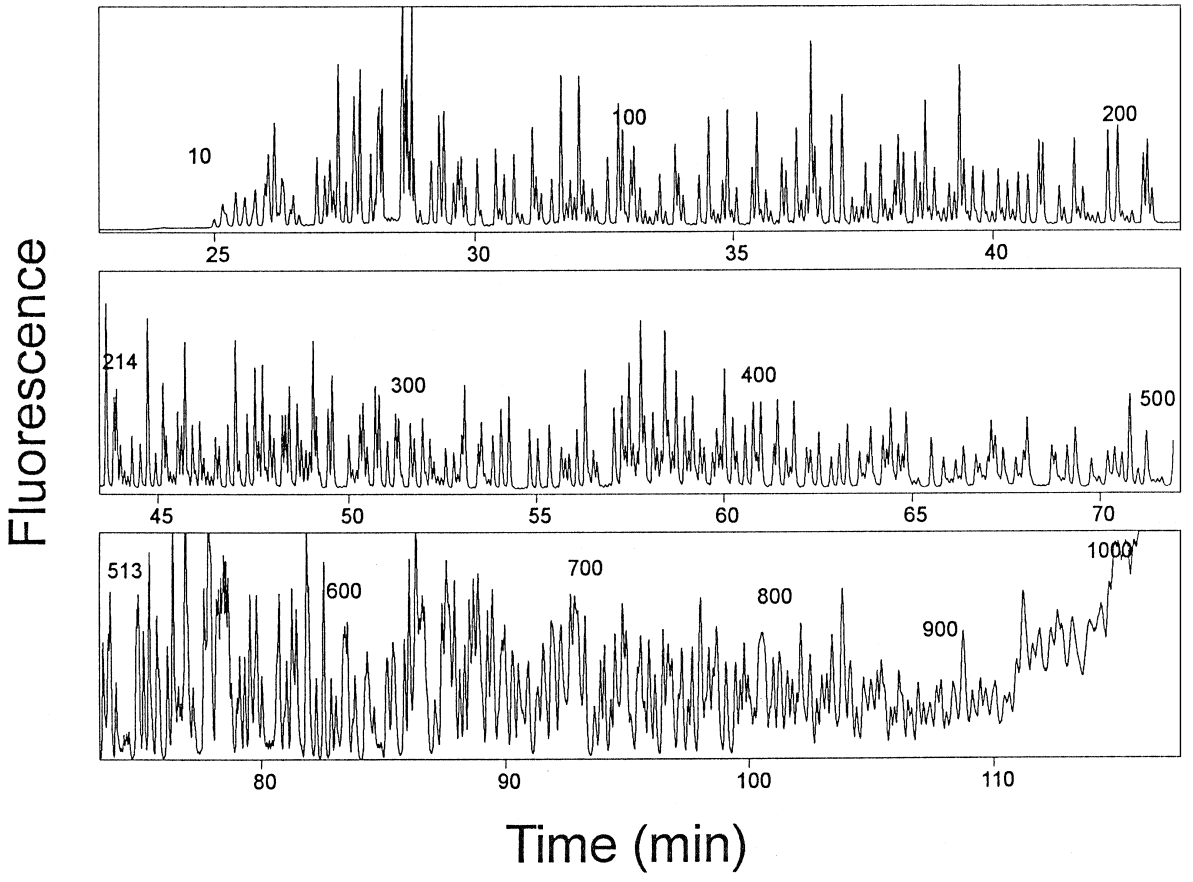

Fig. 6. Electropherogram of PGEM/U DNA sequencing sample at optimized conditions. Conditions: 2.5% M_w 8 000 000 PEO, 1×TTHE buffer, effective length 40 cm, field strength 160 V/cm, 40°C.

buffer at 50 m*M* concentration. The final pH value of TTHE is around 8.2. Fig. 5B shows the electropherogram obtained in 1×TTHE buffer under similar conditions, which represents the best performance found.

4.4. Improved performance under optimized conditions

Another two parameters, electric field strength and effective capillary length, also need to be considered. According to our previous work [8], lower electric field strength does allow longer reads since large DNA fragments are easily stretched by high field strength. But, the increase of thermal energy of DNA molecules at high temperature can offset the effect of field strength. Thus, high electric field strength can be employed at a high temperature. A long capillary contributes to long read but causes long separation time. There is a trade-off between the capillary length and the readable length depending on the purpose of separation. Fig. 6 shows the separation of PGEM/U sequencing sample in 2.5% M_w 8 000 000 PEO at 160 V/cm with 40 cm effective length at 40°C. The separation time was dramatically shortened compared to that at room temperature while the resolution and readable bases were preserved (Fig. 10 in Ref. [8]).

In summary, significant improvements in DNA sequencing in PEO matrix at elevated temperature have been obtained. Long read lengths in a short time are the keys toward high throughput and rugged sequencing technology. This paper only describes the inherent separation performance in PEO matrix. Further improvements in the maximum possible number of bases read can be expected by the development of better base-calling algorithm. Work towards this aspect is underway in our laboratory.

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