ELSEVIER

Journal of Chromatography B, 669 (1995) 113–123

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Poly(ethyleneoxide) for high-resolution and high-speed separation of DNA by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) with polyacrylamide gels has already been demonstrated to allow single-base resolution of single-stranded DNA. However, linear polyacrylamide is not an ideal matrix because of a high viscosity and difficulties in preparing the polymer with well defined pore sizes. Alternatively, poly(ethyleneoxide) (PEO) with a large range of molecular masses from 300 000 to 8 000 000 is available commercially. In addition, it is easy to prepare homogeneous solutions to provide highly reproducible separation performance with sufficient resolution. Single-base resolution of double-stranded DNA between 123 and 124 base pairs can be achieved by the use of homogeneous matrices prepared from PEO (2.5% M_r 8 000 000), and even better resolution is achieved by using mixed polymer matrices. With further work, it should be possible to change the fractions and the total amounts of polymers to achieve even higher resolution for different samples with different size ranges of fragments. Another advantage of mixed polymer matrices is that relatively high resolution can be obtained while maintaining a relatively low viscosity compared to linear polyacrylamide with identical contents of formamide and urea, which makes it easier to fill these matrices into small capillaries.

1. Introduction

The need to increase speed and accuracy for the determination of DNA sequences is driven by the Human Genome Project (HGP) [1–3]. With current techniques, it is not possible to sequence the entire genome without substantial time and cost. So far, the most promising method based on known technology that can substantially increase the speed of DNA sequencing is the use of capillary gel electrophoresis (CGE) with multiplexed operation. With low current during runs and efficient heat dissipation, high electric fields (up to 500 V/cm) can be applied to DNA separations in CGE. Since the first reports on the separation of DNA mixtures, CGE [4] progressed to the point where it has been applied successfully in DNA sequencing [5–7] and the separation of restriction fragments [8,9] and PCR products [10]. Traditionally, crosslinked polymers such as polyacrylamide were used as gel matrices in CGE because of their known utility in slab gels for the separation of proteins and DNA. The separation performance is controlled by the pore structure of the gel matrices, which is based on the amount and characteristics of the monomer and crosslinking agents. However, due to the instability over time, irreproducibility in the polymerization processes, and the fragile nature of the medium, crosslinked gels are not suitable for large-scale DNA sequencing, especially in multiplexed operation.

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0378-4347/95/$09.50
SSDI 0378-4347(95)00044-5

Alternatively, low- to moderate-viscosity entangled polymers have been used to overcome the above problems [11–21]. Unlike crosslinked gels, they are replaceable and more stable for use at higher temperatures and electric field strengths. Linear polyacrylamide (0% C) has been used for the size separation of DNA or proteins by sieving [5,11–13]. In addition, methyl cellulose [14,15], hydroxyalkyl cellulose [16–18], polyhydroxy- and polyethyleneglycol methacrylate [19], and polyvinylalcohol [20,21] also have been employed for DNA separations. Entangled polymers are also useful for the separation of biological samples in capillary zone electrophoresis, since they can coat onto the capillary wall to suppress electroosmotic flow and minimize the interaction between the capillary wall and solutes [15].

One approach to further increase the speed and throughput for DNA sequencing with CE is to find better matrices. To meet these goals, new matrices must provide the following advantages: high separation efficiency, high speed, low background signal in absorbance and fluorescence, ease in preparation, good reproducibility and high tolerance when run in high electric fields and at high temperatures. In this article, we discuss the use of a novel entangled polymer, poly(ethyleneoxide), PEO, for the separation of DNA fragments. Factors such as molecular mass (M_r), concentration of polymers and electric field strength are evaluated with reference to the separation performance.

Separation of the nested set of fragments from the Sanger reaction for DNA sequencing is particularly challenging since a large range of sizes are involved. It is difficult to maintain single-base resolution out to high base numbers. One possibility is to use a gradient mode of separation. In capillary zone electrophoresis (CZE), pH [22–24], temperature [25], voltage [26], and flow [27] gradients have been used to enhance the resolution of separation. The above gradient approaches are not applicable here because separation of macromolecules such as proteins and DNA is via a sieving mechanism [28,29]. Several different gradient methods have been developed to enhance the resolution in a slab gel system. Denaturing gradient gel electrophoresis (DGGE) employs a gradient of denaturing solvent in a polyacrylamide gel and has been demonstrated for the separation of two DNA fragments different by a single base pair [30,31]. Temperature-gradient gel electrophoresis (TGGE) has also been used to enhance the separation performance by taking advantage of the abnormal temperature dependence of mobilities of some DNA fragments [32–34]. Fawcett et al. [35] and Wheeler and Chrambach [36] have developed transverse agarose pore gradient gel electrophoresis to separate DNA fragments with a large range of sizes. However, these schemes are difficult to implement in CE. In the studies described herein, mixed polymer matrices prepared from poly(ethyleneoxide) with different molecular masses were used to enhance the separation of DNA fragments over a large range of sizes. In these mixtures, a large range of matrix pore sizes exist over the entire column to facilitate sieving of DNA fragments of varying sizes.

2. Experimental

2.1. Absorption detection

A commercial instrument (Model 3850 ISCO; Lincoln, NE, USA) was used for all absorption studies. The detection wavelength was set at 260 nm. A 50 μm I.D., 360 μm O.D. DB-1 coated GC capillary (J&W Scientific, Folsom, CA, USA) with 0.2 μm coating thickness was used without any further modification. The capillary length was 50 cm total and 32 cm effective. Electromigration injection was performed throughout for 2 s at the running voltage (-10 to -30 kV).

2.2. Laser-excited fluorescence (LIF) detection

The experimental setup is similar to that described in Ref. [37]. Briefly, a high-voltage power supply (Glassman High Voltage, Whitehorse Station, NJ, USA) was used to drive the electrophoresis. The entire electrophoresis

and detection system were enclosed in a sheet-metal box with a HV interlock. An argon-ion laser with 488 nm output from Uniphase (San Jose, CA, USA) and 1-mW He–Ne laser with 543.6 nm output from Melles Griot (Irvine, CA, USA) were used for excitation. For the study of DNA-TOTO (with argon-ion laser), one 535-nm interference filter was used to block scattered light and to allow the emitted light to reach the phototube (PMT). For DNA-EthB (for both types of lasers) and DNA-TOTO (with He–Ne laser), one RG 610 cutoff filter and one 630-nm interference filter were used. The fluorescence signal was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 4 Hz (Justice Innovation, Palo Alto, CA, USA; Model DT 2802) and stored in a computer (IBM, Boca Raton, FL, USA; Model PC/AT 286).

2.3. Capillary and reagents

Capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm I.D. and 365 μm O.D. were used for fluorescence studies after they were coated with γ-methacryloxypropyltrimethoxysilane and polyacrylamide by Hjerten's method [38]. All chemicals for preparing buffer solutions and for coating capillaries were purchased from ICN Biochemicals (Irvine, CA, USA), except that acrylamide and formamide were from Sigma Chemical (St. Louis, MO, USA) and poly(ethyleneoxide) was obtained from Aldrich Chemical (Milwaukee, WI, USA). EthB (ethidium bromide) was purchased from Sigma. TOTO [1,1-(4,4,7,7-tetramethyl-4,-7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide] was obtained from Molecular Probes (Eugene, OR, USA). The concentrations of dyes in the running buffer were 1 μg/ml. ϕX 174 DNA-Hae III restriction fragment digest was purchased from United States Biochemical (Cleveland, OH, USA). pBR 322 DNA-Hae III, pBR 328 DNA-Bgl I + pBR 328 DNA-Hinf I restriction fragment digests were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA).

2.4. Methods

The buffer solution (TBE) contained equimolar amounts of tris(hydroxymethyl)aminomethane (THAM) and boric acid, with ethylenediaminetetraacetic (EDTA) as a chelating reagent for divalent cations. The pH of the 1× TBE buffer was 8.2 without any further adjustment. The matrix was prepared with PEO and TBE buffer solution to the desired concentration. Briefly, PEO was gradually added into the TBE buffer solution in a beaker sitting in a water bath at 85 to 90°C. During the addition of PEO, a magnetic stirring rod was used at high speed to produce a well homogenized solution. After addition was complete, the solution was stirred for at least an additional 15 min.

Initially, the capillary was pressure flushed with water, methanol, and water for at least 2 cycles. Then, the capillary was filled with very low-viscosity polymer solution (e.g. 0.5% PEO) and run at −10 kV for 10 min. Finally, the capillary was refilled with the separation matrix and equilibrated at the running voltage for 15 min before any sample injection. It is quite easy to use air pressure to fill the capillary with the polymer solution and the total operation time is no more than 5 min. From our experience, the capillary can be used for over 2 weeks and more than 50 runs without any degradation.

The injected concentration of DNA for the mixed polymer separations was 0.83 μg/ml, and injection was performed at −6 kV for 3 s. Between each run, the used polymer matrix was flushed out from the capillary, then the capillary was filled with new polymer matrix. Before the injection of the analytes, the capillary was equilibrated at −10 kV for 10 min.

3. Results and discussion

3.1. Separation performance

To obtain the best separation efficiency for large molecules in CGE, it is important to have matrices with the right mesh size to obtain a suitable sieving effect for the solutes. Also,

suppression of the interaction between the capillary wall and solutes is required to improve separation efficiency. Increasing the capillary lifetime is another consideration. In this study, we found that it is very easy to have a high-quality capillary, if it is pretreated with low-viscosity polymer solution. The reason is that it is very difficult to remove bubbles from high-viscosity matrices once they are trapped at the capillary wall. Pretreatment of capillaries in this way can minimize the entrapment of bubbles. To eliminate electroosmotic flow while minimizing the adsorption of DNA fragments onto the hydrophobic coating, we found that DB-1 columns, which have a very thick coating, provide an optimal environment for DNA separations.

To form a sieving medium, the concentration of polymers has to be higher than a certain value called the overlap threshold. Polymer chains then interact with one another to form an entangled solution. The average mesh size (ξ) of the pores formed can be expressed as [39,40]

$$\xi(\Phi) \approx A\Phi^{-0.75} \quad (1)$$

where Φ is the polymer volume fraction and A is a proportionality constant. Since Φ equals $S^{-0.8}$, where S is the size of polymer chain, Eq. (1) can be rewritten as

$$\xi(\Phi) \approx A\, S^{0.6} \quad (2)$$

Hence, in order to create a small mesh, one wants to use a polymer with short chains and vice versa. Further, in order to be able to fill the capillary easily with the matrix while keeping the optimal mesh size for good separation, it is desirable to have gel matrices with as low a viscosity as possible.

The separation of DNA fragments in matrices prepared from PEO with M_r from 300 000 to 5 000 000 at different concentrations are shown in Fig. 1. For a comparable separation performance, concentrations of the polymers with small chains in solution (low M_r) always need to be higher than those with long chains. Increasing the polymer concentration (fixed M_r) generally leads to higher resolution, the cost being an increase in analysis time and increased difficulty in handling the matrix (higher viscosity). It should be noted that in Fig. 1 there are inversions in the elution order as a function of molecular mass. Since molar absorptivity is proportional to the number of base pairs, one can assign the fragment peaks based on peak area. The fragments at 271/281/310 and at 872 show anomalous migration [41].

It is worth mentioning that the resolution between the 271 and 281 pair of fragments with PEO matrices is much better than with cellulose-type gel matrices. Also, separation performed in PEO matrices provides highly reproducible results for at least 10 runs without replacement. The reproducibility among different capillaries and different batches of polymers is also excellent. Overall, PEO matrices provide several advantages, including easy preparation, better reproducibility, and longer lifetime as compared with cellulose-type matrices. Compared to home-made linear polyacrylamide, PEO is expected to be more stable since no further polymerization of these commercial preparations is expected. There are linear polyacrylamide preparations available commercially. However, they are not yet available with a wide range of M_r, which turns out to be important for DNA separations, *vide infra*.

3.2. Resolution

In this report, the resolution (R) is calculated as

$$R = \frac{(2 \ln 2)^{1/2} \Delta t_R}{HW_1 + HW_2} \quad (3)$$

where Δt_R is the difference in migration time between the two adjacent peaks, and HW is the full width at half maximum. Fig. 2 shows the change in resolution *per base pair* for consecutive pairs of fragments as a function of the polymer matrices, as calculated from Fig. 1. Matrices prepared from PEO with M_r 2 000 000 at 3% provide the best resolution for DNA fragments. However, this solution is too viscous to be easily forced into 50-μm capillaries by pressure. Overall, the best performance should be obtained from PEO with M_r 5 000 000 at a concentration

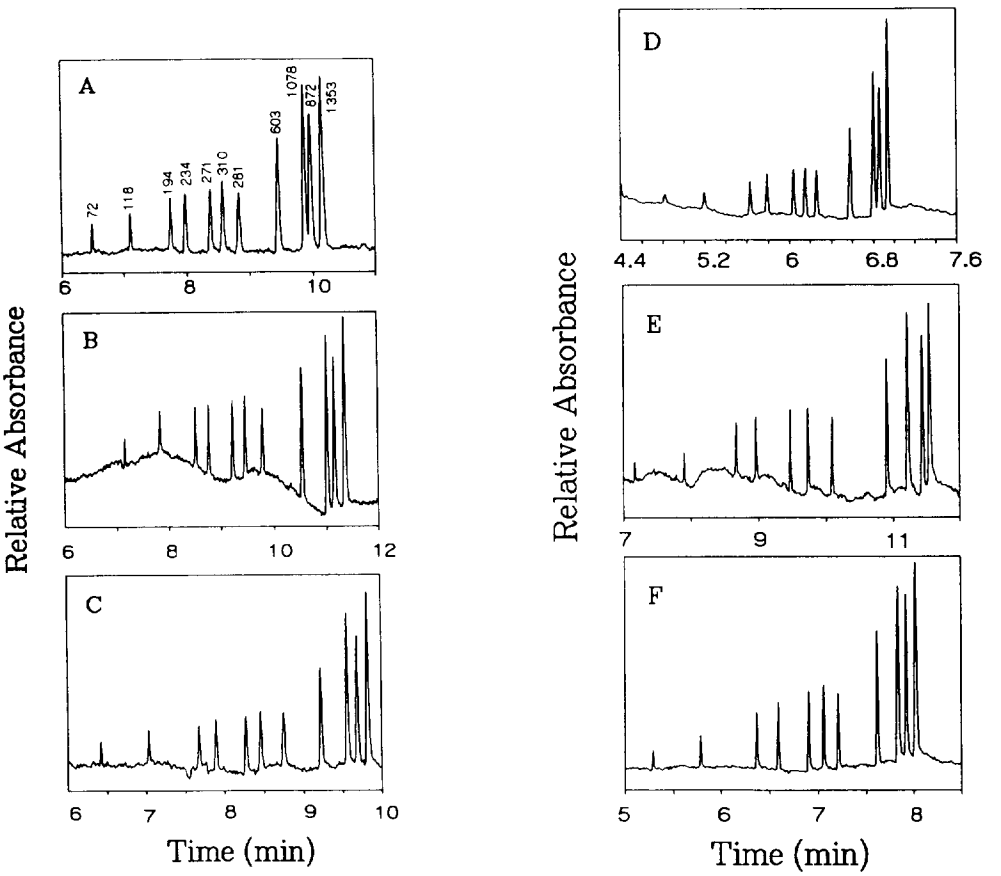

Fig. 1. Separation of 250 μg/ml ϕX 174 DNA-Hae III digest in various PEO matrices; (A) 3% M_r 300 000, (B) 3.3% M_r 300 000, (C) 2.75% M_r 1 000 000, (D) 2% M_r 2 000 000, (E) 3% M_r 2 000 000, and (F) 2% M_r 5 000 000. Conditions: 1 × TBE buffer, pH = 8.2, 50 cm total length, 32 cm effective length, 50 μm I.D. DB-1 coated capillary; absorption wavelength: 260 nm; applied potential: −26 kV.

somewhat higher than 2%. Fig. 2 shows that the maximum resolution per base pair is in the range 250 to 350 base pairs. The resolution degrades as DNA fragments become larger than 600 base pairs.

Electric field strength is also an important factor affecting separation performance in CE since the shape of DNA and the degree of disruption of the network of polymers may change. Heat generated also varies as the electric field strength changes. In terms of speed, we would like to run CE at as high an electric field strength as possible to shorten the separation time. Fig. 3 shows the effects of electric field strength on resolution. The plot is derived from electropherograms similar to Fig. 1. In the low to middle size range, the order in resolving power is $520 > 420 \approx 320 \approx 220 > 600$ V/cm. The resolution increases with electric field strength because longitudinal diffusion is minimized, except the resolution degrades substantially at 600 V/cm. For the large fragments, clearly the resolution decreases as the electric field strength increases. Possible reasons include local heating and large changes both in the shape of DNA and the mesh size of the polymer matrix at extremely high electric field strengths. The change of DNA from random coil to rod-like shape at high electric

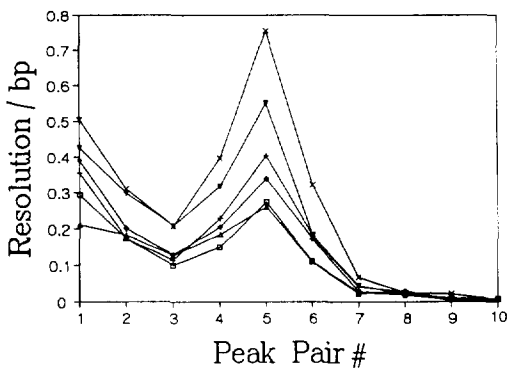

Fig. 2. Single-base resolution of consecutive DNA fragment peaks in different polymer matrices. Data are taken from Fig. 1. Symbols: □, 3% M_r 300 000; +, 3.3% M_r 300 000; ◇, 2.75% M_r 1 000 000; △, 2% M_r 2 000 000; x, 3% M_r 2 000 000; and ▽, 2% M_r 5 000 000. Ordinate represents the order of consecutive peak pairs.

field strength [42,43] and a large change in the orientation of the flexible network in low-viscosity gel matrices [44] have been reported before.

3.3. Effects of intercalating dyes

One of the advantages of using LIF to monitor DNA separations is that very small amounts of DNA can be detected. In order to maintain reasonable separation efficiency, it is critical to choose a suitable dye with the right ratio of DNA to minimize any changes in the mobilities of DNA. Fig. 4A shows the separation of the 11 DNA fragments of ϕX 174/HAE III intercalated with a bisintercalator, TOTO, for at least 20 min before the separation. Fig. 4B and C shows the separation of these DNA fragments intercalated with EthB (a monointercalator) and TOTO respectively that are incorporated into the running buffer. Fig. 4 shows that PEO matrices are compatible with intercalating dyes for the separation and detection of DNA fragments, but that EthB is to be preferred to obtain the sharpest peaks and to reduce anomalous migration behavior. The separation performance and sensitivity decreased on successive runs whenever the dyes were incorporated into the running

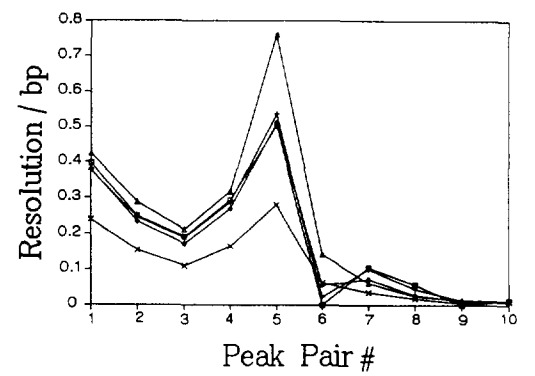

Fig. 3. The effect of electric field strength on the single-base resolution of consecutive DNA fragment peaks in PEO (2%, M_r 5 000 000) matrix. □, 220 V/cm; +, 320 V/cm; ◇, 420 V/cm; △, 520 V/cm; x, 600 V/cm. Other conditions are as in Fig. 1.

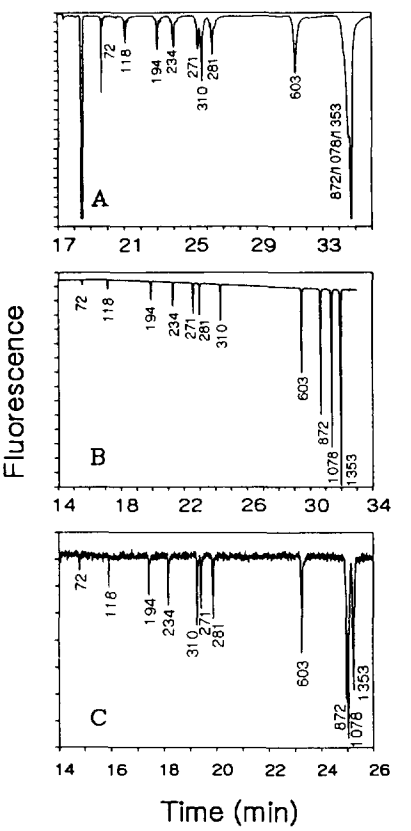

Fig. 4. Separation of 0.9 μg/ml of ϕX 174 DNA-Hae digest intercalated with (A) 9.5 μg/ml of TOTO before the separation, (B) 1 μg/ml of EthB in the running buffer, and (C) 1 μg/ml of TOTO in the running buffer. Capillary total length: 60 cm; effective length: 52 cm; applied potential: −12 kV.

buffer. Therefore, it is important that new polymer solutions be refilled into the capillaries after each run. The degradation was much more serious if TOTO was in the running buffer. A possible reason for this phenomenon is that there exist strong interactions between the dye and the polymer matrix or between the dye and the capillary wall.

3.4. Separations in mixed polymer matrices

Fig. 5A–F shows the separations of DNA fragments in polymer matrices prepared from single-M_r materials. Peaks assignments were based on relative intensities. In order to have a reasonable separation, matrices prepared from polymers with low molecular mass always require a higher concentration of polymers. Fig. 5A shows that it is impossible to separate DNA fragments less than 400 base pairs in matrices prepared from polymers with M_r 300 000. Similar results were obtained even when a high concentration, up to 15%, was used (results not shown). This is because the pore size of the matrices is too small to have a sieving effect even for those small DNA fragments. The larger fragments can presumably associate with the small polymer chains to change their effective mobilities [45]. Fig. 5 shows that the smaller fragments can be well separated in matrices prepared from individual polymers with M_r of 600 000 to 2 000 000. For DNA fragments from 80 to 400 base pairs, better resolution is achieved in matrices prepared from polymers with higher M_r. An important feature in these electropherograms is the separation of fragment 9 from fragment 10. Similar to Fig. 4B, EthB tends to minimize anomalous migration behavior, except for the highest M_r matrices. For fragments larger than 400 base pairs, the performance of all matrices degrades significantly.

In the mixed polymer matrices, a polymer network with random pore sizes is formed. Fig. 6 shows the results of the separation of the same DNA fragments in mixed polymer matrices. It is obvious that the separation performance is different from that made from single-M_r polymers. This is because the mixed matrices simultaneously provide optimum pore sizes for a large range of DNA fragments. From comparison of the results of DNA mobilities from Fig. 5 and 6, the average pore size of the mixed polymer matrices is between that made from 2.5% (M_r 8 000 000) and 3.5% (M_r 2 000 000) PEO. While the resolution for the larger fragments is worse in the matrix made from 3.5% PEO (M_r 2 000 000) compared to mixed polymer matrices, the resolution of the small fragments is better. On the other hand, the matrix prepared from 2.5% PEO (M_r 8 000 000) provides slightly higher resolution for DNA fragments from 80 to 400 base pairs compared to the mixed polymer matrices, but the resolution for the small fragments is worse. For DNA fragments larger than 400 base pairs, relatively higher resolution can be obtained in mixed polymer matrices. Although one cannot directly correlate the utility for separating double-stranded DNA fragments (this study) with that for separating single-stranded Sanger reaction products (DNA sequencing), it is clear that higher resolution for the larger fragments is always desirable.

A comprehensive comparison among the various PEO matrices is given in Figs. 7 and 8. Fig. 7A shows that for individual polymers, the low-M_r material provides a more efficient separation for the smaller DNA fragments, and vice versa. This is why mixed polymer solutions are beneficial to the separation of a broad range of DNA fragments, as depicted in Fig. 7B. Further optimization of the ratio between the amounts of low-M_r and high-M_r materials in the mixed solutions should lead to even better separation performance. Overall, it is worth noting that the mixed polymer matrix made from equal amounts of polymers (0.6%) with different molecular masses provided comparable resolution, while retaining a much lower viscosity, compared to all other matrices used in this study. Still higher resolution was obtained when mixed matrices were prepared in the same way from higher concentrations of polymers (0.7%). However, the higher viscosity may limit its utility in capillary array electrophoresis since the matrix inside the capillaries must be readily replaceable.

Fig. 9 shows the result of the separation of

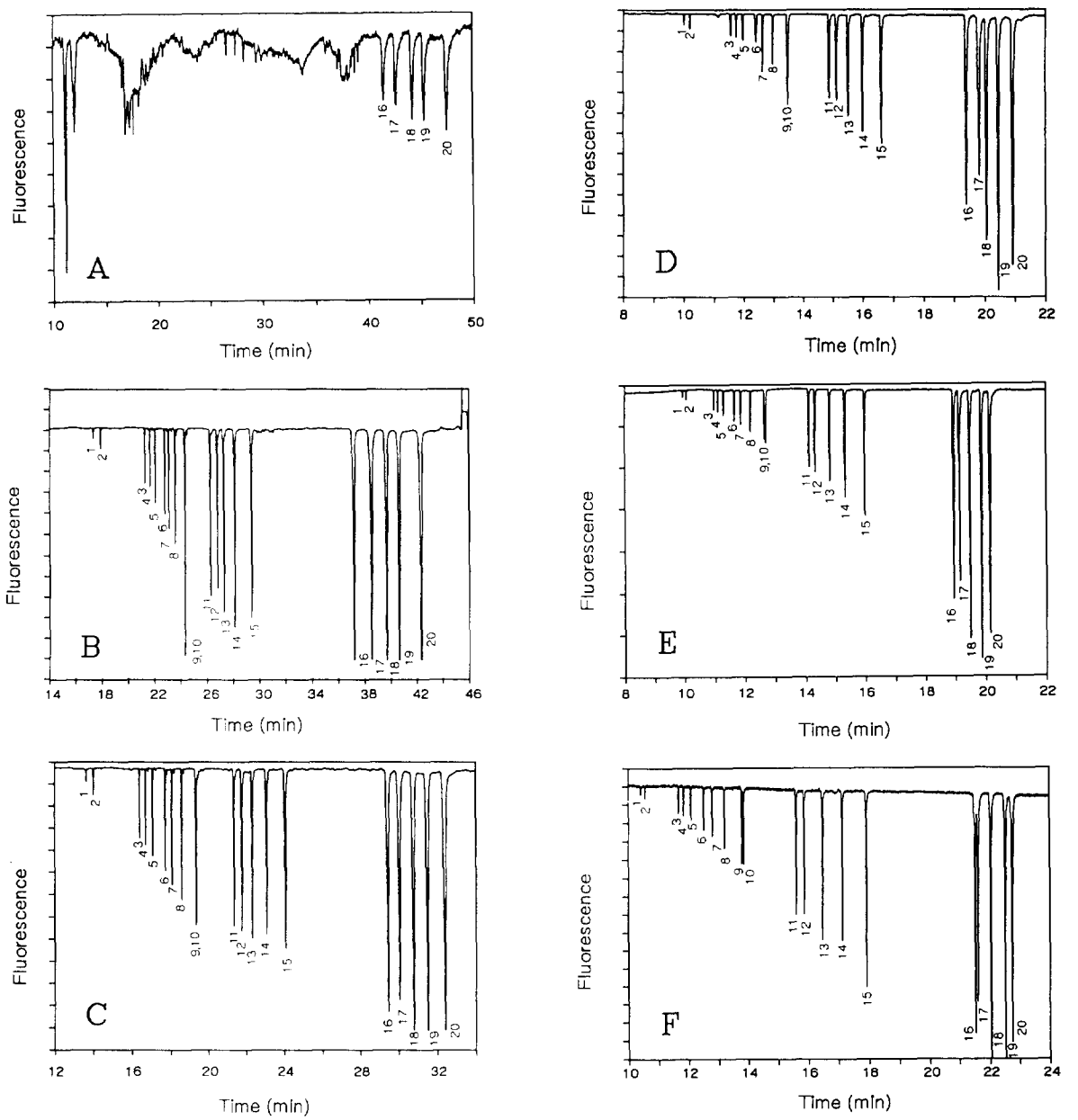

Fig. 5. Electrophoretic separation of EthB-stained pBR 322 DNA-Hae III digest: matrices were (A) 9% M_r 300 000, (B) 6% M_r 600 000, (C) 3.5% M_r 2 000 000, (D) 2.5% M_r 5 000 000, (E) 2% M_r 8 000 000 and (F) 2.5% M_r 8 000 000. Peak assignments: 1 = 18 bp, 2 = 28 bp, 3 = 51 bp, 4 = 57 bp, 5 = 64 bp, 6 = 80 bp, 7 = 89 bp, 8 = 104 bp, 9 = 123 bp, 10 = 124 bp, 11 = 184 bp, 12 = 192 bp, 13 = 213 bp, 14 = 234 bp, 15 = 267 bp, 16 = 434 bp, 17 = 458 bp, 18 = 504 bp, 19 = 540 bp and 20 = 587 bp. Buffer: 1 × TBE, pH = 8.2; capillary: 75 μm I.D., total length 50 cm, effective length 42 cm; applied potential: −10 kV.

DNA fragments in a mixture of molecular mass markers V and VI, which contain fragment sizes from base pairs 8 to 2176. All of the fragments can be separated in this matrix in less than 30 min. Fig. 9 highlights the excellent separation performance among the fragments with 434, 453

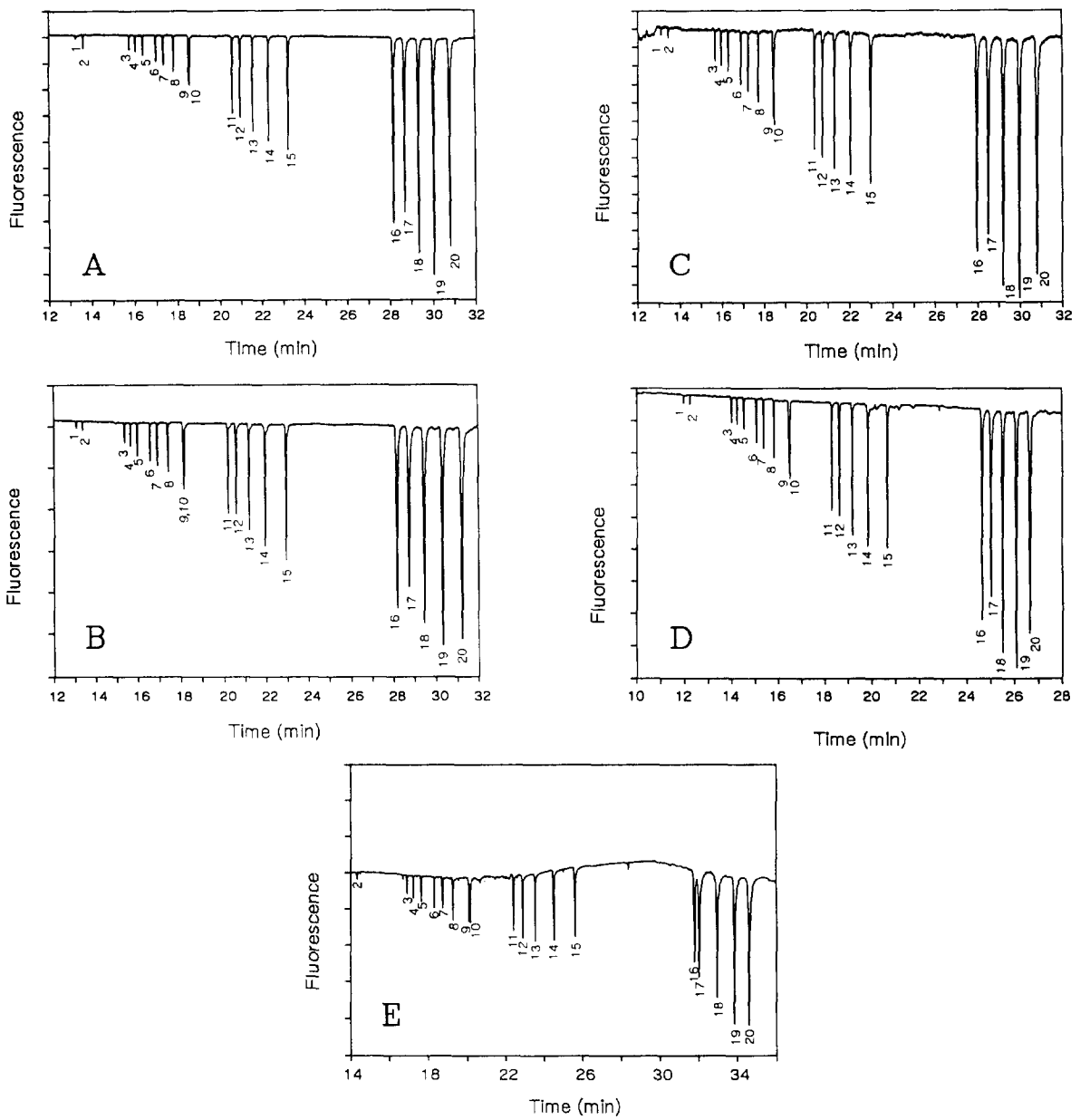

Fig. 6. Electrophoretic separation of EthB-stained pBR 322 DNA-Hae III digest: mixed PEO matrices were (A) 1.5% M_r 300 000, 1.8% M_r 2 000 000, 0.7% M_r 5 000 000 and 0.7% M_r 8 000 000; (B) 3.0% M_r 1 000 000 and 1.3% M_r 8 000 000; (C) 1.5% M_r 600 000, 1.0% M_r 1 000 000 and 1.5% M_r 5 000 000; (D) 0.6% each M_r 300 000, 600 000, 1000 000, 2 000 000, 5 000 000 and 8 000 000; and (E) 0.7% each M_r 300 000, 600 000, 1000 000, 2 000 000, 5 000 000 and 8 000 000. Other conditions and peak assignments are as in Fig. 5.

and 458 base pairs. The results also show that this matrix can be used to separate certain normal DNA samples from mutated samples, since fragments with 234 base pairs from two different samples (peaks 14 and 23) are well separated. It should be noted that DNA frag-

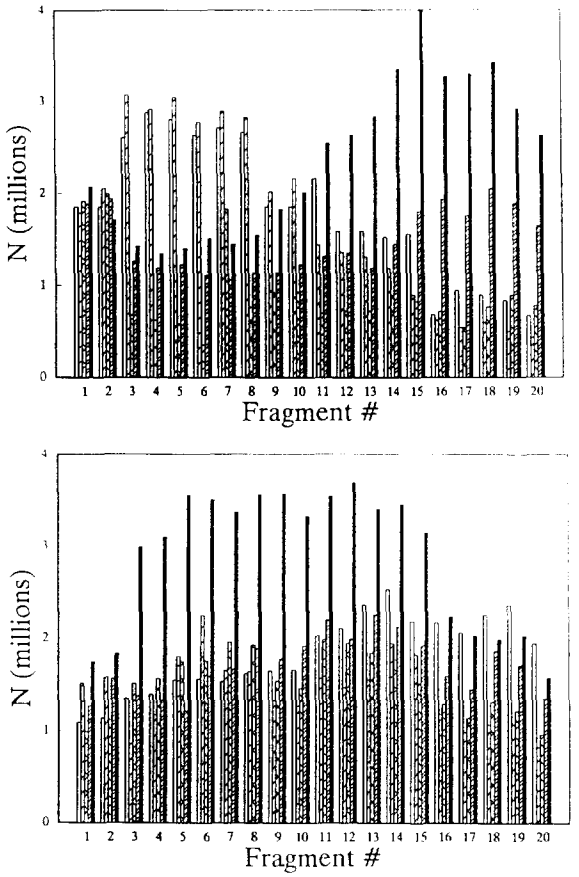

Fig. 7. Comparison of the theoretical plates (N) from the results of Fig. 5 and 6. (A) Open bar: Fig. 5B, light-shaded bar: Fig. 5C, cross-shaded bar: Fig 5D, heavy-shaded bar: Fig. 5E, and solid bar: Fig. 5F. (B) Open bar: Fig. 6A, light-shaded bar: Fig. 6B, cross-shaded bar: Fig. 6C, heavy-shaded bar: Fig. 6D, and solid bar: Fig. 6E.

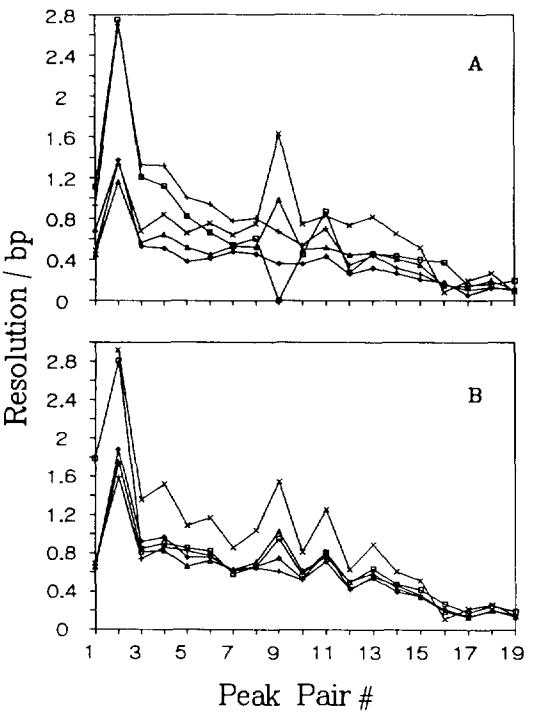

Fig. 8. Comparison of the calculated single-base resolution from the results of Fig. 5 and 6. (A) □: Fig. 5B, +: Fig. 5C, ◇: Fig. 5D, △: Fig. 5E, x: Fig. 5F; (B) □: Fig. 6A, +: Fig. 6B, ◇: Fig. 6C, △: Fig. 6D, x: Fig. 6E.

ments from standard Sanger reactions are denatured single-stranded and covalently tagged rather than double-stranded and intercalated with a fluorophore. One expects those separations to be even more efficient because there will not be a distribution of conformations or fluorophore numbers per DNA fragment. Successful application of these mixed polymer matrices in actual DNA sequencing will be published elsewhere. Independent of DNA sequencing applications, the highly reproducible polymer matrices developed here for high-resolution separation of restriction fragment digests or PCR

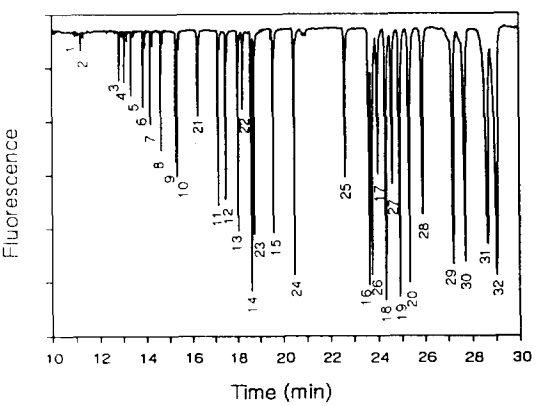

Fig. 9. Electrophoretic separation of the mixture of pBR 322 DNA-Hae III, pBR 328 DNA-Bgl I, and pBR 328 DNA-Hinf I digests. Conditions and sieving medium are identical to that in Fig. 6D. Peak assignments: 1 to 20 are as in Fig. 5, 21 = 154 bp, 22 = 220 bp, 23 = 234 bp (from pBR 328), 24 = 298 bp, 25 = 394 bp, 26 = 453 bp, 27 = 517 bp, 28 = 653 bp, 29 = 1033 bp, 30 = 1230 bp, 31 = 1766 bp, 32 = 2176 bp.

products should be of value for DNA typing [46].

Acknowledgments

The Ames Laboratory is operated for the US Department of Energy by Iowa State University under Contract No. W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Health and Environmental Research.

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