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Separation of DNA restriction fragments by high performance capillary electrophoresis with low and zero crosslinked polyacrylamide using continuous and pulsed electric fields

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ABSTRACT

This paper presents results on the separation of DNA restriction fragments by high performance capillary electrophoresis (HPCE). Capillaries containing polyacrylamide with low amounts of crosslinking agent (*i.e.* 0.5% C) were first studied. The greater molecular accessibility offered with columns of low crosslinking, relative to higher crosslinked gels (*e.g.* 5%C), permitted high efficiency separations of double stranded DNA fragments up to 12 000 base pairs in length. Capillaries containing no crosslinking agent, *i.e.* linear polyacrylamide, were then examined. Ferguson plots (*i.e.* log mobility *vs.* %T) were used to assess the size selectivity of linear polyacrylamide capillaries. In another study, it was determined that the relative migration of DNA species was a strong function of applied electric field and molecular size. Lower fields yielded better resolution than higher fields for DNA molecules larger than about 1000 base pairs, albeit at the expense of longer separation time. Based on these results, we have examined pulsed field HPCE and have demonstrated the use of this approach to enhance separation.

INTRODUCTION

The separation of DNA species by high performance capillary electrophoresis (HPCE) is an area of growing activity. This interest follows from the use of polyacrylamide or agarose slab gel electrophoresis as a standard method for this field[1]. Slab gel electrophoresis, however, is slow, difficult to quantitate, and not easily amenable to automation. HPCE, an instrumental approach to electrophoresis, is able to overcome many of these problems[2–5].

When separating oligonucleotides or DNA restriction fragments, a sieving medium is most often employed since it is known that mobility of these species is nearly independent of molecular size in free solution[6]. While open-tube HPCE has been examined[7,8], high-resolution separation of nucleic acids by this approach is limited. Accordingly, we have examined polyacrylamide gel-filled capillaries for the separation

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of oligonucleotides[9,10]. These capillaries have been shown to yield ultra-high efficiency, in excess of $30 \cdot 10^6$ theoretical plates/m (ref. 10). Capillaries with relatively large amounts of crosslinking agent (*e.g.* 5% C) yield stable columns with good size selectivity for single stranded oligonucleotides and DNA sequencing reaction products. (See ref. 11 for definition of %T and %C.)

As a continuation of this work, we describe the application of low or zero crosslinked polyacrylamide filled capillaries for the separation of DNA restriction fragments up to 12 000 base pairs. The analysis of such species is important, for example, in restriction fragment mapping[12], in analysis of restriction fragment length polymorphisms (RFLPs) for diagnostic[13], clinical[14] and forensic[15] assays, in polymerase chain reaction (PCR) product applications[16] and in genomic mapping[17].

Linear polyacrylamide has been employed for the separation of proteins in the slab gel format[18–21]; however, the minimum monomer concentration required to achieve sufficient anticonvective character for slab operation was approximately 10 %T[21]. The anticonvective properties of the narrow capillaries in HPCE[22] permit use of a much broader range of linear polyacrylamide compositions. While dissolution of hydrophilic polymers has been used in HPCE to improve DNA fragment separations[23,24], polymerization within the capillary will be shown to offer advantages in terms of size selectivity. Finally, we will demonstrate the influence of the applied electric field on the mobility of DNA fragments and the use of pulsed field[25–27] HPCE to enhance separation.

EXPERIMENTAL

Apparatus

The basic HPCE instrumentation has been previously described in detail[10]. A 60-kV direct-current power supply (Model PS/MK 60; Glassman, Whitehouse Station, NJ, U.S.A.) was used to generate a potential drop across the capillary for continuous field electrophoresis. For pulsed field experiments a 20-kV operational amplifier (Model PO434; Trek, Medina, NY, U.S.A.) controlled with a function generator (Model 185; Wavetek, San Diego, CA, U.S.A.) was employed. A UV–VIS spectrophotometer (Model 100; Spectra-Physics, San Jose, CA, U.S.A.) was used at 260 nm to detect the DNA fragments. Column cooling was achieved by air convection using a laboratory fan. Each end of the capillary was placed in buffer reservoirs (3 ml) with platinum wire electrodes. In some cases, placing polyacrylamide in the reservoirs was found to be beneficial in extending column lifetime. Electrophoresis was performed in fused-silica tubing (Polymicro Technologies, Phoenix, AZ, U.S.A.) of 75 μm I.D. and 375 μm O.D., which had a 2–4-mm portion of the polyimide coating removed for spectroscopic detection. The electropherograms were acquired and stored on an IBM PC/XT (Boca Raton, FL, U.S.A.) via an analog-to-digital converter interface (Model 762SB; PE/Nelson, Cupertino, CA, U.S.A.).

Procedures

Capillary preparation followed procedures previously described[9,10]. Methacryloxypropyltrimethoxy silane (Petrarch Systems, Bristol, PA, U.S.A.) was first covalently bound to the fused-silica capillary walls. Polymerization of polyacrylamide was next accomplished by filling the capillary with degassed, low viscosity acrylamide solution.

Polymerization was initiated using ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED). Columns containing linear polyacrylamide concentrations between 3 and 14% T were prepared for study. Polyacrylamide gels crosslinked with N,N′-methylenebisacrylamide (bis) were used at compositions 3% T, 5% C and 3% T, 0.5% C. Acrylamide solutions were prepared in a buffer of 100 m*M* Tris base, 100 m*M* boric acid, 2 m*M* EDTA, pH 8.3. For single-stranded oligonucleotide separations the buffer also contained 7 *M* urea. Samples were injected electrophoretically with specific injection conditions provided in the figure captions.

Chemicals

Samples of ϕX174 DNA digested with restriction enzyme *Hae* III were obtained from Pharmacia (Piscataway, NJ, U.S.A.) and New England Biolabs. (Beverly, MA, U.S.A.), and were used at a sample concentration of 500 μg/ml in 10 m*M* Tris–HCl (pH 8.0) and 0.1 m*M* EDTA. Vectors M13mp18 and dephosphorylated pBR322, both digested with *Eco*R I, were obtained from New England Biolabs. These samples were used at a concentration of 100 μg/ml in 10 m*M* Tris–HCl (pH 7.5) and 1 m*M* EDTA. Polydeoxyadenylic acids, $p(dA)_{20}$ and $p(dA)_{40-60}$, purchased from Pharmacia, were dissolved in water and used at 1 μg/ml and 30 μg/ml, respectively. A 1000-base pair ladder (up to 12000 base pairs) was supplied by Bethesda Research Labs. (Gaithersburg, MD, U.S.A.). This sample was used at a concentration of 920 μg/ml in 10 m*M* Tris–HCl (pH 7.6), 50 m*M* NaCl and 1 m*M* EDTA. Ultra-pure Tris base, urea and EDTA were obtained from Schwartz/Mann Biotech (Cleveland, OH, U.S.A.). Acrylamide, bis, TEMED and APS were purchased from Bio-Rad (Richmond, CA, U.S.A.). All buffer and acrylamide solutions were filtered through a 0.2-μm pore size filter (Schleicher and Schuell, Keene, NH, U.S.A.).

Each fragment of the Hae III digest of ϕX174 was isolated from 1.8% SeaKem GTG agarose (FMC, Rockland, ME, U.S.A.) slab gels by standard methods[1] and were spiked into the mixture of HPCE peak identification. Similar isolation of the 1000-base pair ladder fragments was performed using 0.45% and 0.8% agarose slab gels. PCR was conducted using a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.). Twenty five cycles were run using the buffers, primers, λ DNA template, Taq polymerase, and protocol as supplied by Perkin-Elmer Cetus[28].

RESULTS AND DISCUSSION

3% T, 0.5% C polyacrylamide capillary columns

Previous work from this laboratory has concentrated on the use of crosslinked polyacrylamide gels for the HPCE separation of oligonucleotides[9,10]. Gels of composition 3% T, 5% C were demonstrated to be particularly well suited to the high resolution separation of single stranded oligonucleotides. However, the pores were generally too small for restriction fragments to migrate efficiently. Specifically, introduction of fragments larger than about 700 base pairs resulted in distorted peak shapes and poor resolution.

The separation of DNA fragments would appear to require a more open pore structure of the gel. An increase in the pore size can be achieved by reducing the amount of crosslinking agent for a given percentage T[29]. We have found that a ten-fold reduction in the percentage of bis crosslinker (*i.e.* from 5 to 0.5% C) resulted

in good resolution of DNA restriction fragments, as illustrated in Fig. 1. This electropherogram shows the separation of the *Hae* III restriction digest of ϕX174 DNA and *Eco*R I digests of both pBR322 DNA and M13mp18 DNA. The ϕX174 digest consisted of eleven fragments ranging from 72 to 1353 base pairs, and the pBR322 and M13mp18 digests consisted of single fragments of 4363 and 7250 base pairs, respectively. All thirteen fragments are well-resolved in less than 18 min. Peak identification was made by spiking each peak with slab gel-isolated fragments and, as expected, size-dependent migration was achieved. The separation power of this polyacrylamide capillary is evidenced by the resolution of species 5 and 6, which differ in length by only 10 base pairs (*i.e.* 271 and 281 base pairs). Efficiency is further noted

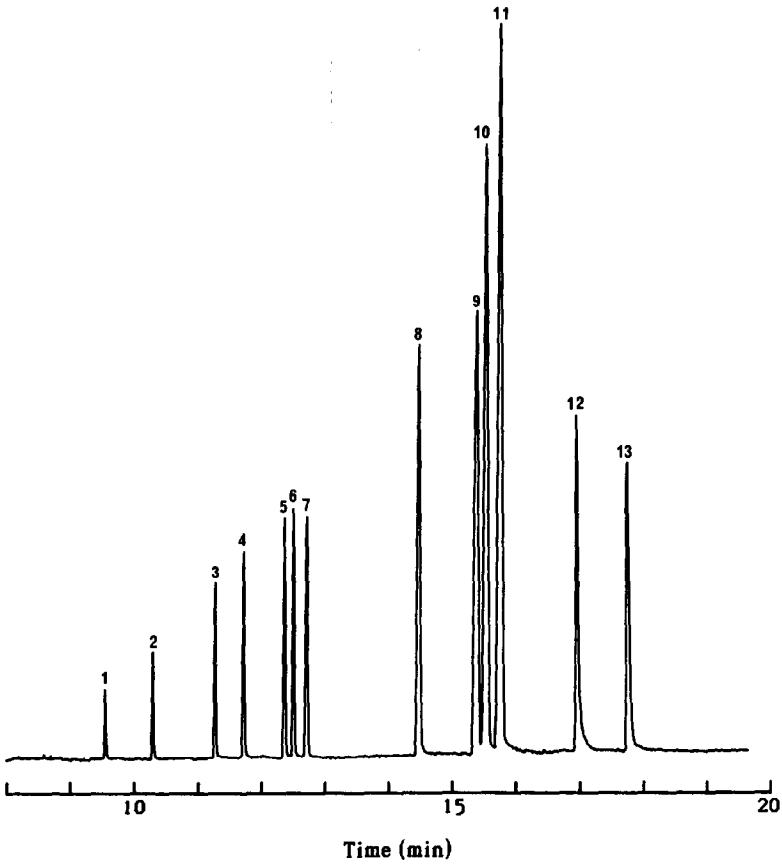

Fig. 1. Separation of *Hae* III digest of ϕX174 DNA, *Eco*R I digest of pBR322 and *Eco*R I digest of M13mp18 on a 3% T, 0.5% C polyacrylamide capillary column. The 11 fragments of the ϕX174 digest have been identified as the first 11 peaks in the electropherogram, followed by pBR322 and M13mp18. Identification was made by spiking each peak with slab gel isolated species. Peaks: 1 = 72; 2 = 118; 3 = 194; 4 = 234; 5 = 271; 6 = 281; 7 = 310; 8 = 603; 9 = 872; 10 = 1078; 11 = 1353; 12 = 4363 and 13 = 7253 base pairs. Conditions: effective length, *l*, was 30 cm, total length, *L*, was 40 cm, applied field, *E*, was 250 V/cm, current generated, *i*, was 12.5 μA, and sample was injected electrophoretically at 10 kV for 0.5 s. Buffer: 100 m*M* Tris–borate (pH 8.3), 2 m*M* EDTA.

by the large number of theoretical plates per meter: $5 \cdot 10^6$ for the first peak (72 base pairs) and $1 \cdot 10^6$ for the last peak (7250 base pairs). The theoretical plates were calculated using the appropriate expressions which take into account peak asymmetry[30].

We next examined the performance of a 3% T, 0.5% C capillary for the separation of DNA restriction fragments up 12 000 base pairs. The electropherogram shown in Fig. 2 demonstrates the rapid separation of a 1000-base pair DNA ladder up

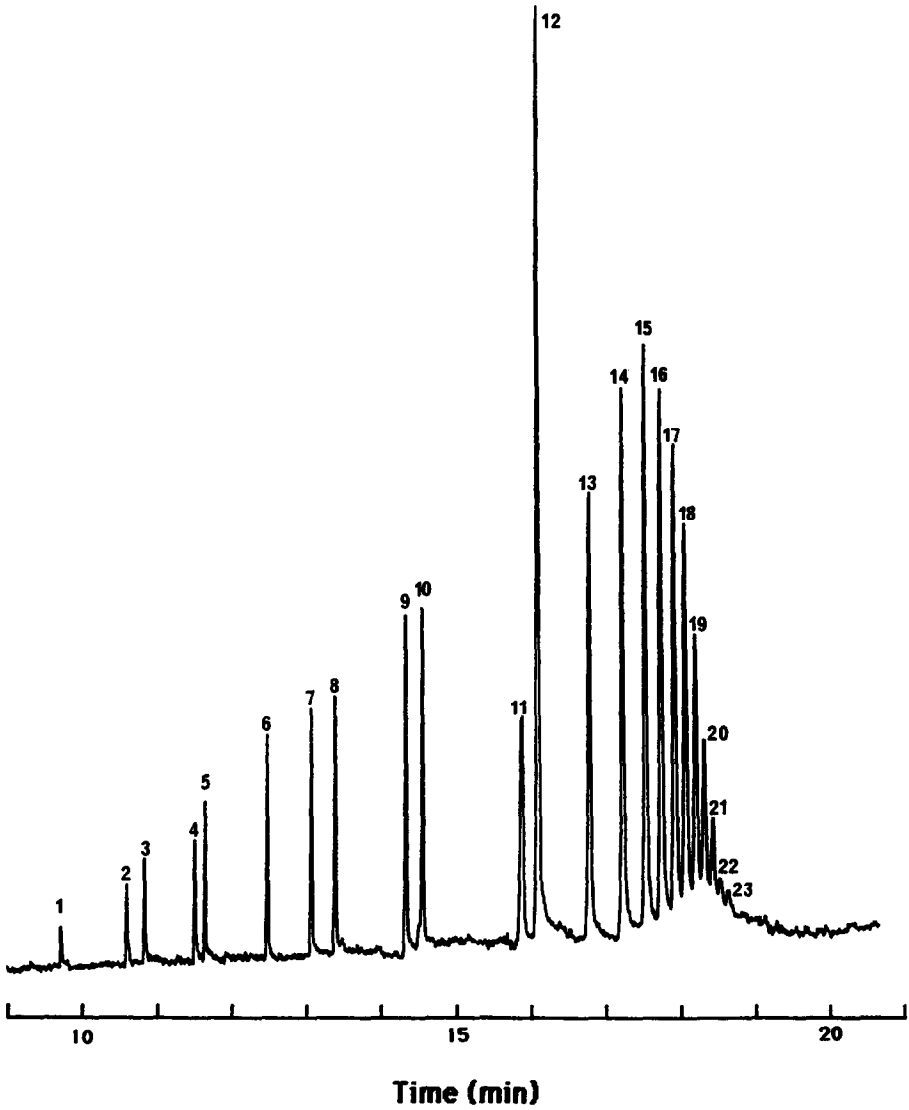

Fig. 2. Separation of 1000-base pair DNA ladder on a 3% T, 0.5% C polyacrylamide capillary. Fragment lengths range from 75 to 12 216 base pairs. All peaks were identified by spiking with slab gel isolated species. Peak legend: 1 = 75; 2 = 142; 3 = 154; 4 = 200; 5 = 220; 6 = 298; 7 = 344; 8 = 394; 9 = 506; 10 = 516; 11 = 1018; 12 = 1635; 13 = 2036; 14 = 3054; 15 = 4072; 16 = 5090; 17 = 6108; 18 = 7126; 19 = 8144; 20 = 9162; 21 = 10 180; 22 = 11 198; 23 = 12 216 base pairs. All conditions as in Fig. 1.

to 12 000 base pairs (*i.e., ca.* 1000, *ca.* 2000, *ca.* 3000, ... *ca.* 12 000 base pairs). This complex mixture consisted of 11 species in addition to the 12 species from the ladder. The additional fragments, ranging from 75 to 1636 base pairs arise from enzymatic digestion of the cloning vector which was used in the preparation of the sample. A total of 23 fragments ranging from 75 to 12 216 base pairs were thus expected and were observed. Peak identification was again made by spiking all the peaks with slab gel isolated fragments. Notably, the electropherogram is similar to that observed from slab gel electrophoresis, except fragments 9 and 10, which differ by only 10 base pairs, are usually not separated on the slab[31].

Linear polyacrylamide capillary columns

With the success of low crosslinked polyacrylamide capillaries, we next turned to linear polyacrylamide (*i.e.* no crosslinking) in the composition range of 3% T to

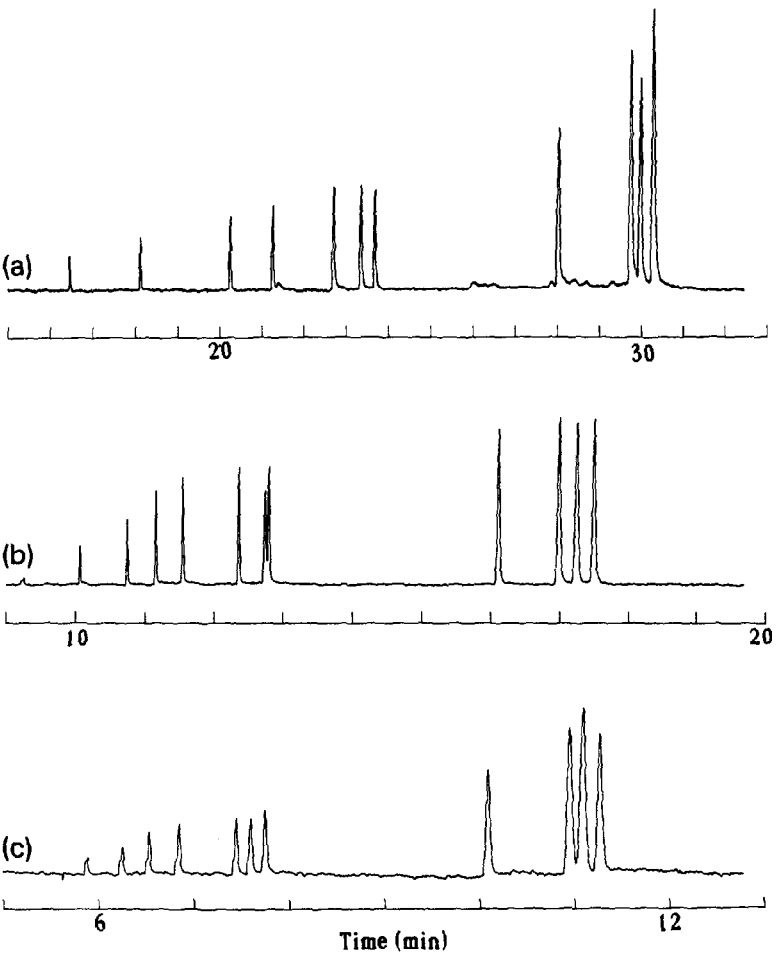

Fig. 3. Separation of *Hae* III restriction fragment digest of ϕX174 DNA on linear polyacrylamide columns of composition: (a) 6, (b) 9 and (c) 12% T. Effective lengths of these capillaries were 35 cm, 15 cm and 10 cm, respectively. E = 300 V/cm, injection at 150 V/cm for 3 s. Currents generated were (a) 10.5 μA, (b) 9.1 μA, and, (c) 7.8 μA. All other conditions as in Fig. 1.

14% T. The viscous character of such compositions range from nearly liquid-like at 3% T to that of a gelatinous material at 14% T. The separation performance of several different linear polyacrylamide compositions (*i.e.* 6, 9 and 12% T) is shown in Fig. 3, using the ϕX174 *Hae* III digest as a test mixture. These electropherograms indicate that size selectivity is a function of polymer concentration. For a given electric field and base pair size range, shorter column lengths can be utilized with increasing acrylamide concentration due to the greater sieving capability of the higher percentage T. On the other hand, as described previously for crosslinked capillaries gels[10], the reduced sieving power at lower polyacrylamide concentrations can be compensated by increasing the effective length of the capillary. This approach can result in a broader molecular size range, encompassing larger kilo base pair fragment sizes.

The currents generated at 300 V/cm in the 6, 9 and 12% T capillaries were, respectively, 10.5 μA, 9.1 μA and 7.8 μA. These low currents are a direct consequence of the high viscosity of the polyacrylamide media. The power generated in a typical capillary (total length 40–80 cm) at 300 V/cm is well below 0.5 W/m, and significant Joule heating of the column is not expected[32]. As seen in Fig. 3, high fields and short effective length columns can be employed for rapid analysis.

The mechanism of sieving in linear polyacrylamide has been suggested to be similar to that occurring in crosslinked polyacrylamide gels[18,19,33]. This model views the molecules migrating through "dynamic pores" which are formed by the fluctuating polymer chain network. As shown both here and by others[19,21], the extent of sieving is greater with increased polymer content. Since viscosity is proportional to polymer content, a system which polymerizes inside the column is advantageous relative to that obtained by simple dissolution of a polymer in a bufffer. The viscosities of the medium can be much greater with *in situ* polymerization. Further, the difficulties of handling viscous solutions are minimized.

The linear polyacrylamide capillaries described here were stable for at least several weeks without degradation of performance. In addition, the 9% T linear polyacrylamide capillaries exhibited an absolute day-to-day reproducibility in migration time (for the 1358-base pair species from the ϕX174 *Hae* III mixture) of 1.7% ($n = 10$) and a capillary-to-capillary reproducibility of 2.3% ($n = 21$). The day-to-day variation in the relative migration time for the 1358-base pair using the 72-base pair species as an internal standard was 0.9% ($n = 10$).

We have found the higher %T linear polyacrylamide capillaries also useful for single stranded oligonucleotide separations, as illustrated in Fig. 4 with a 9% T column. The sample in this figure consisted of a polydeoxyadenylic acid 20-mer [$p(dA)_{20}$] spiked into a mixture of polydeoxyadenylic acids ranging from 40 to 60 bases [$p(dA)_{40-60}$], each differing in length by a single residue. The high resolving power of this capillary is evidenced not only by the separation of each oligonucleotide, but also by the appearance of shoulders on the major peaks which have been identified as the dephosphorylated form of each oligonucleotide[34]. While comparable resolution in shorter time has been previously achieved for these species on 3% T, 5% C gel columns[10], the linear polyacrylamide is useful for resolving a broader range of fragment sizes.

Ferguson plot

The size selectivity of linear polyacrylamide capillaries can be characterized by

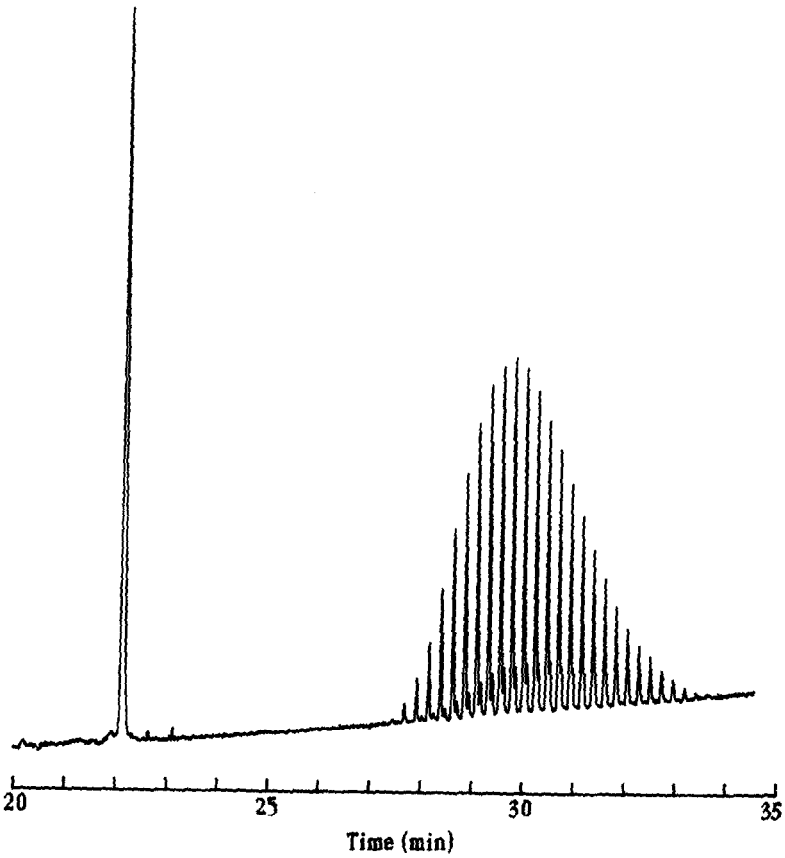

Fig. 4. Single base resolution of deoxyoligonucleotides on 9% T linear polyacrylamide. Sample mixture: $p(dA)_{20}$ and $p(dA)_{40-60}$. Conditions: $E = 308$ V/cm, $i = 8.8$ μA, $l = 45$ cm, $L = 60$ cm, injection at 10 kV for 2 s. Buffer contained 7 *M* urea. All other conditions as in Fig. 1.

means of a Ferguson plot[35,36]. This plot of log mobility *versus* %T at constant %C is a linear function as follows:

$$\log \mu = \log \mu_0 - k_R (\%T) \tag{1}$$

where μ is the measured mobility ($cm^2/V\,s$), μ_0 is the mobility in free solution, k_R is the retardation coefficient, and %T is the acrylamide concentration. Fig. 5 shows the Ferguson plots for ϕX174 *Hae* III fragments from 273 to 1358 base pairs on capillaries containing between 6 and 14% T. The slope and intercept for each fragment was calculated by least squares analysis. As expected, the intercept (*i.e.* 0% T) of each line is essentially identical since, as noted earlier, the mobility of DNA molecules becomes increasingly independent of molecular size in free solution[6]. It is interesting to note that the measured free solution mobility of all fragments in the same buffer was $3.56 \cdot 10^{-4}\,cm^2/V \cdot s$ ($\log \mu = -3.45$). This value is roughly 35% higher than the intercept of the Ferguson plot. It appears that the Ferguson plot may exhibit upward curvature at

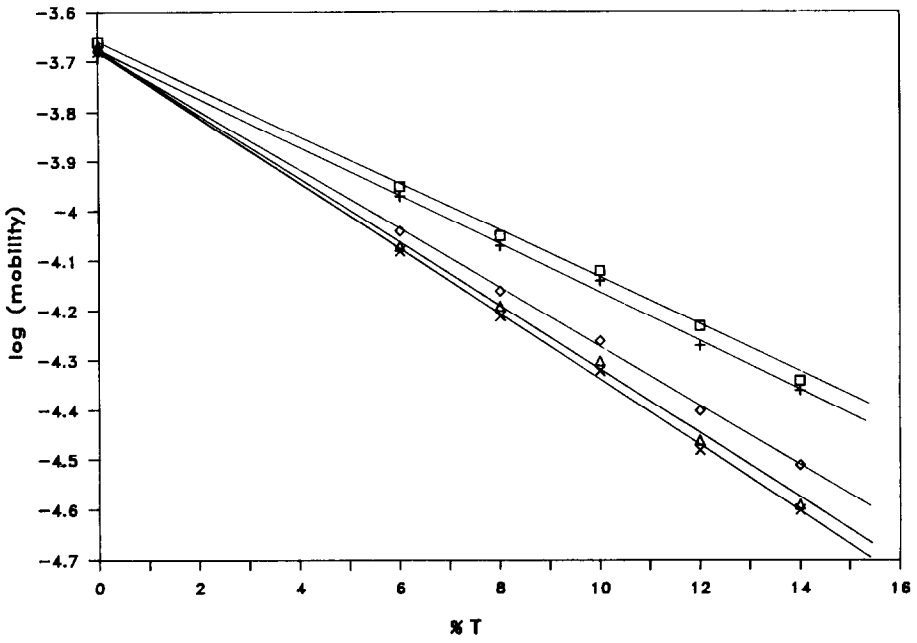

Fig. 5. Ferguson plots for linear polyacrylamide. The lines represent the log mobility of various ϕX174 *Hae* III fragments as a function of monomer composition. □ = 273; + = 310; ◇ = 603; △ = 872; and × = 1358 base pairs. Conditions: E = 300 V/cm, l = 15 cm, injection at 5 kV for 3 s. All other conditions as in Fig. 1.

low %T. Such non-linearity has already been described on slab[21]. The values of the retardation coefficient, k_R, range from $-4.6 \cdot 10^{-2}$ for the 273-base pair fragment to $-6.6 \cdot 10^{-2}$ for the 1358-base pair fragment. The steeper slope for the largest fragment is expected in the sieving mechanism. Caution must be exercised in the quantitative application of the Ferguson plot to the determination of fragment size dimensions and matrix structure, since, as shown below, the mobility of DNA species can be field dependent.

Polymerase chain reaction product analysis

One potential use for linear polyacrylamide HPCE is the fast analysis of PCR products[16]. The PCR method is capable of rapidly increasing the concentration of the target DNA 10^5 times or more. Since this method is susceptible to errors, such as amplification of spurious segments of DNA[16], a method of analysis and possible purification is necessary. Currently, the predominant method is slab gel electrophoresis; however, capillary electrophoresis can be advantageously exploited as well.

Following standard protocols[28], we used PCR to amplify a 500-base region of λ DNA from an initial template concentration of 0.001 ng/μl. The electropherogram in Fig. 6 was obtained by direct injection of the PCR reaction mixture without further sample treatment. The separation reveals several peaks, with a major band appearing at 19 min. This band migrates at the expected velocity of a 500-base pair fragment, as shown in the inset of Fig. 6. Here, a calibration curve of migration time *versus* fragment size is presented, using the previously identified ϕX174 *Hae* III fragments as

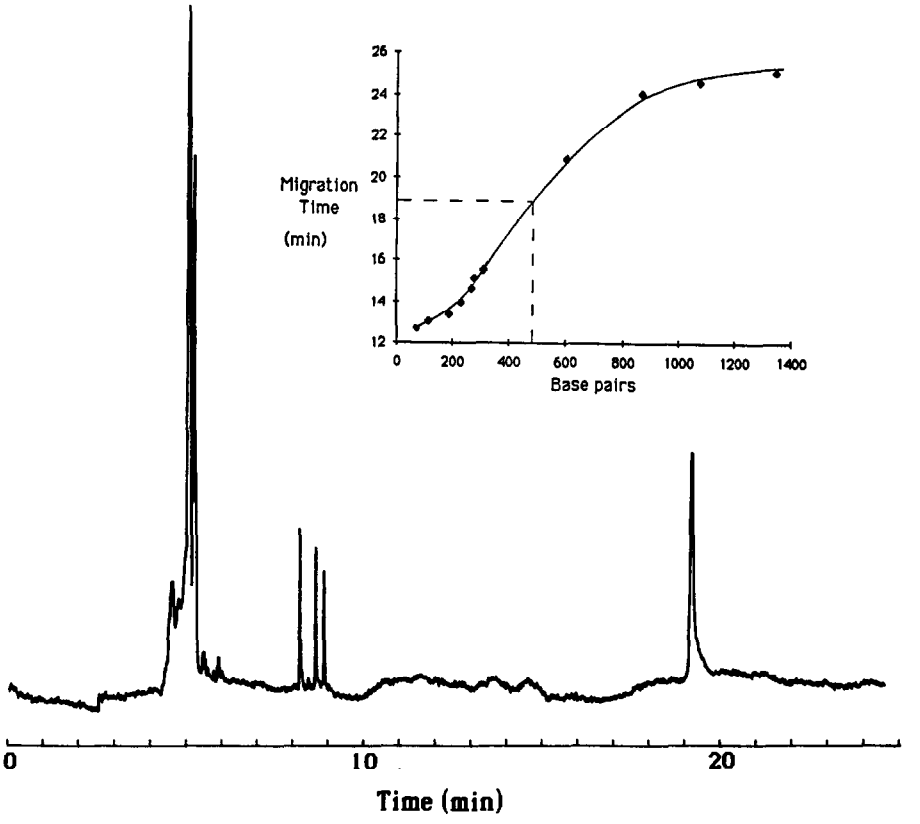

Fig. 6. Polymerase chain reaction (PCR) product analysis. Injection of PCR amplified 500-base pair fragment directly from reaction mixture onto a 9% T linear polyacrylamide column. Conditions: E = 300 V/cm, i = 9.0 μA, l = 15 cm, L = 40 cm, and injection at 300 V/cm for 10 s. All other conditions as in Fig. 1. Inset: size calibration curve for identification of PCR product. The fragments of the ϕX174 *Hae* III digest were electrophoresed as above, and migration time plotted as a function of fragment length. The dashed line indicates size of PCR product.

size standards. As noted by the dashed line, the migration time of the peak at 19 min compares well to a 500-base pair fragment. It is interesting to note that the shape of the calibration curve is similar to that found for DNA fragments in slab gels[37].

The fastest migrating species in Fig. 6, eluting at 5 min, are believed to be unincorporated nucleotides. The other peaks at 8–9 min are thought to be primers and possibly dimers of the primers which are known to form[28]. Absolute identification requires further study. Nonetheless, the results in Fig. 6 demonstrate the potential of polyacrylamide HPCE for PCR product assignment and purity analysis. It is apparent that direct injection of PCR products is possible. Sample desalting to increase electrophoretic injection and preconcentration by isotachophoresis[38,39] for analysis of sample concentrations more dilute than in this example can be used. Moreover, micropreparative collection of purified material can be achieved, if desired[10].

Effect of applied electric field on mobility

The magnitude of the applied electric field is known to influence migration of DNA species in slab gels, particularly species larger than 15 000–20 000 base pairs[26].

While the causes of this phenomenon are not completely understood at present, it is known that DNA molecules can align parallel to the applied field, depending on the size of the fragment and the magnitude of the field[40]. Migration of aligned species is thought to lead to a snake-like motion, called reptation[41], which often results in movement in the gel matrix of fragments much larger than expected from the sieving mechanism of Ogston[42]. Reptation of aligned species also leads to size-independent mobilities.

In this work, we examined the influence of the applied field on the mobility of the DNA fragments shown in Figs. 1 and 2. The plot in Fig. 7 shows the electrophoretic velocity as a function of field for the 75- and 11 000-base pair fragments from the 1000-base pair ladder, on a 3% T, 0.5% C capillary column. The small fragment shows

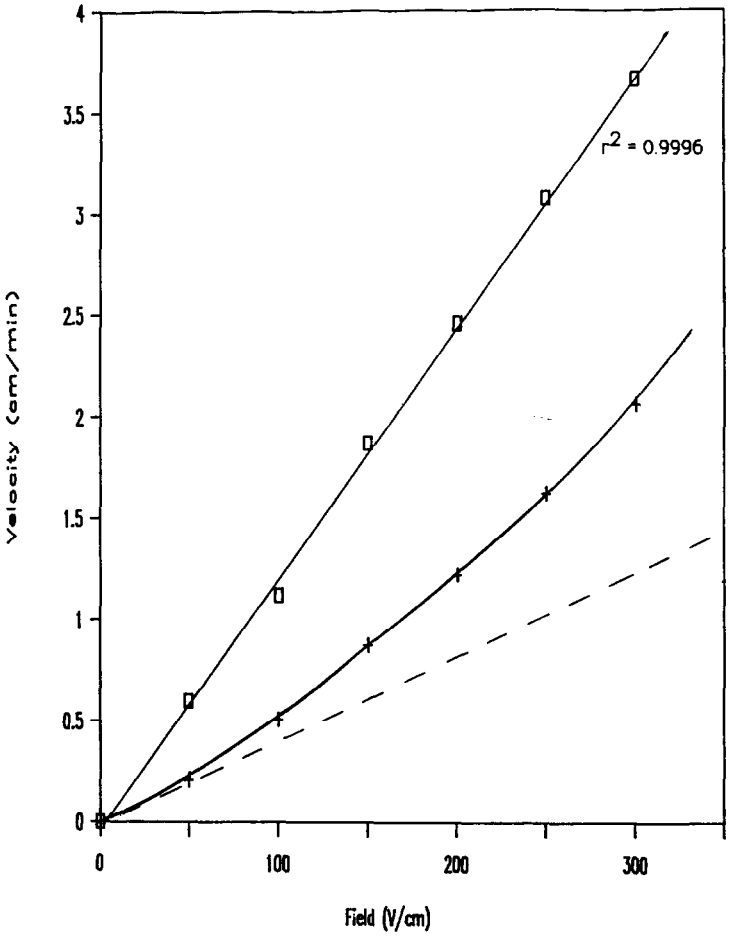

Fig. 7. Effect of applied electric field on linear velocity of various DNA fragments. □ = 75-base pair fragment; + = 11 198-base pair fragment. Solid line denotes experimental curve, dashed line denotes expected curve. Conditions: 3% T, 0.5% C polyacrylamide, l = 30 cm, L = 40 cm, injection at 10 kV for 0.5 s. Power generation at 300 V/cm was 0.37 W/m. All other conditions as in Fig. 1.

the expected linear trend with field[2]; however, a non-linear behavior is observed for the large fragment, with higher than expected velocity at elevated fields. This latter behavior is consistent with known field effects on mobility of DNA molecules[26]. Joule heating within the capillary can be eliminated as the cause of this behavior since only the largest fragment exhibits anomalous behavior and the power generated at the highest field is less than 0.4 W/m[32]. The upward curvature in velocity exhibited by the larger DNA molecules can eventually result in faster migration of the larger species than smaller ones[26]. Such anomalous migration would be most prominent at both high electric fields and high %T.

Field effects were also found on a 12% T linear polyacrylamide capillary for the largest fragments of the *Hae* III digest of ϕX174 . Fig. 8 shows the separation of this mixture at 250 V/cm (Fig. 8a) and 125 V/cm (Fig. 8b). The smaller fragments are observed to have lower resolution at 125 V/cm, probably as a consequence of

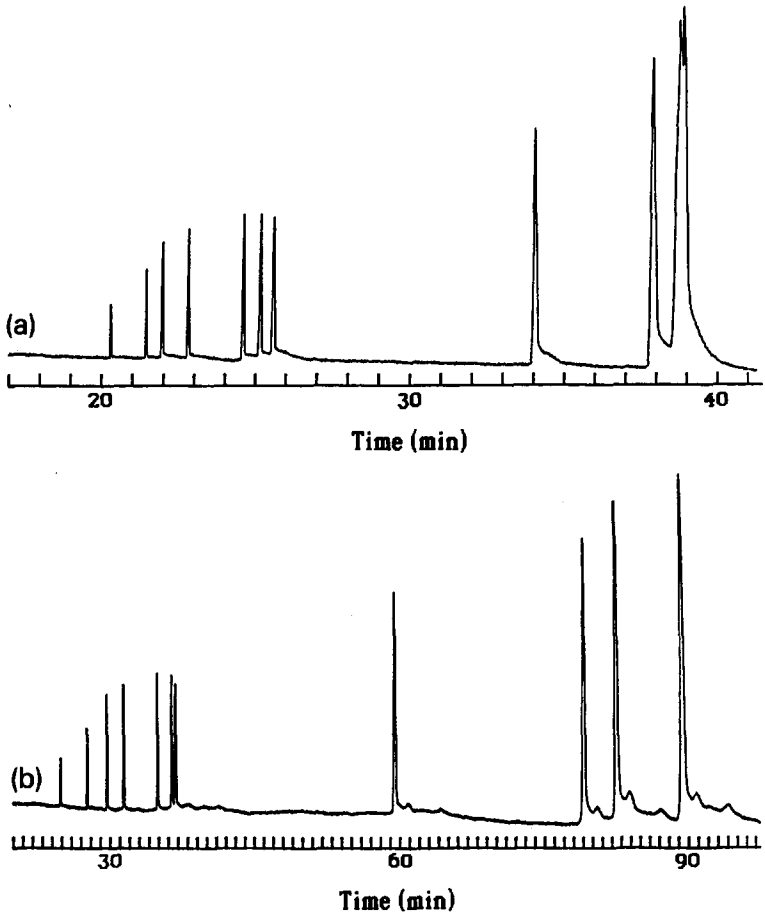

Fig. 8. Effect of applied electric field on separation of *Hae* III digest of ϕX174 DNA. (a) 250 V/cm. (b) 125 V/cm. Note loss of resolution at high field for the largest fragments. Conditions: 12% T linear polyacrylamide, l = 20 cm, L = 40 cm, injection at 10 kV for 1 s. Current: (a) 5.4 μA, (b) 2.9 μA. Power generated: (a) 0.14 W/m, (b) 0.04 W/m. All other conditions as in Fig. 1.

diffusional band broadening; however, a dramatic increase in resolution is seen for the largest fragments of roughly 1000 base pairs. It is believed that these species are less aligned at the lower applied field and as the molecules adopt a more random coil structure, size discrimination is increased.

Another manifestation of the influence of the applied field on resolution is shown in Fig. 9 for the 4363 and 7253-base pair species (see Fig. 1) on a 6% T, 0% C capillary. Calculation of resolution (R_s) for the two fragments was based on the standard equation, $R_s = 2[(t_2 - t_1)/(w_1 + w_2)]$, where t is the migration time at the peak height maximum and w is the baseline peak width in time units. The subscripts 1 and 2 denote the two species in question. As can be seen, there is a substantial improvement in resolution at lower fields, albeit at the expense of analysis time. Again, the low currents in the polyacrylamide capillary columns used in these studies result in minimal power generation and thus minimal Joule heating.

The field effects on mobility observed in Figs. 7–9 are occurring at much smaller fragment sizes than generally found in slab gel operation. A significant difference between the capillary and slab procedures, however, is the use of an applied field that is at least an order of magnitude higher in the capillary format. Since alignment is field dependent, it is not surprising that these effects are observed for molecules only a few thousand base pairs in length. Thus, manipulation of the applied field and proper selection of polyacrylamide composition are important parameters in the optimization of DNA separations.

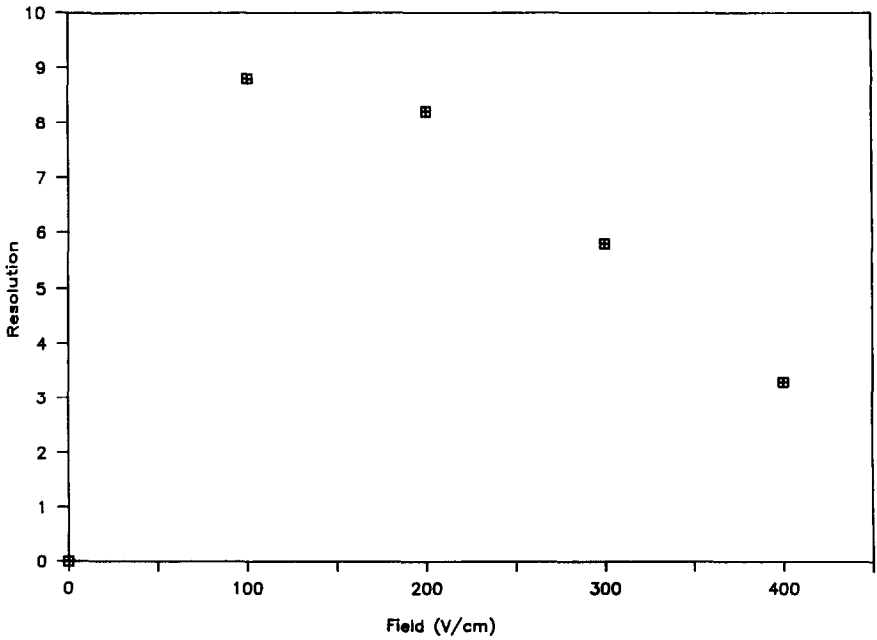

Fig. 9. Effect of applied electric field on resolution. Resolution calculated for DNA fragments 4363 and 7253 base pairs in length on a 6% T linear polyacrylamide capillary. Conditions: l = 15 cm, L = 28 cm, injection at 7.4 kV for 0.5 s. Current at 400 V/cm was 13 μA. All other conditions as in Fig. 1.

Pulsed field HPCE

One approach to manage the loss of size dependent mobility, manifested in Figs. 7–9, is electric field programming. This discontinuous electric field method, originally described for sample collection[10] and for enhancing sensitivity of radioactive detection[43], can be used to optimize separation of appropriate mixtures. For example, for the separation of Fig. 8, one could employ a high field initially to separate the small species with high efficiency, followed by a step or continuous gradient decrease in applied field to separate the larger species. While high resolution of both small and large species can be obtained using this approach, this benefit is offset by longer analysis time.

An alternative and more general method for optimization of DNA separations is the use of pulsed field electrophoresis[25–27]. This technique is used to confer size-dependent mobility by periodically changing the direction and magnitude of the applied field, essentially relaxing molecular elongation. Both unidirectional[44] and field-inversion methods[27] are compatible with the capillary format. In this work we have examined the use of unidirectional pulsing to increase separation of the 4363- and 7253-base pair fragments of Fig. 1. With the apparatus described in the experimental section, a waveform consisting of a symmetric square wave between 0 and 300 V/cm was applied across a 6% T linear polyacrylamide capillary. Waveform frequency was varied incrementally between 0.1 and 1000 Hz.

Fig. 10 shows a plot of peak separation of the two species as a function of frequency. Here, a maximum was observed at 100 Hz; an optimum in frequency specific to the molecular size of the analyte is well expected[44]. Interestingly, frequencies of 0.1 and 1 Hz resulted in the same separation as continuous field operation. The data points in Fig. 10 were independent of the sequence of measurements, *i.e.* no hysteresis in the curve was observed when pulsing frequencies were chosen in random order.

A 20% increase in peak separation was observed in pulsed-field (100-Hz) operation relative to continuous field operation. It is anticipated that greater

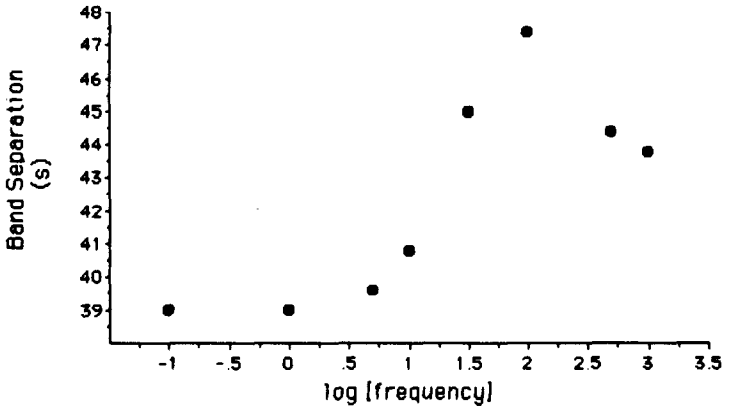

Fig. 10. Pulsed field HPCE of 4363- and 7253-base pair fragments. Plot of peak separation as a function of frequency of unidirectional pulse waveform. Maximum represents optimum frequency for separation of these species. Conditions: symmetric square wave of amplitude 0 to 300 V/cm, 6% T linear polyacrylamide, l = 30 cm, L = 50 cm. All other conditions as in Fig. 1.

variations will result when larger DNA species are studied[26]. Further, as in slab gel operation, separation of larger species may only be achievable by employing pulsed fields. In addition to frequency, a number of other parameters can be manipulated to optimize separations, including field amplitude, type of waveform (*e.g.* field inversion or combination waveforms) and separation medium. These parameters are currently under investigation.

CONCLUSION

We have demonstrated the resolving power of polyacrylamide capillary columns with little or no crosslinking agent. The columns were stable and could be used repeatedly for long periods of time. The manipulation of monomer concentration permitted optimization of separation with respect to resolution and speed. Analysis of PCR products and restriction fragment mapping are applications well suited to these capillaries. Finally, pulsed field HPCE was shown to be feasible, and it is expected that this method will prove useful for the separation of DNA fragments.

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