Journal of Chromatography A, 652 (1993) 67–73
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 254

Optimization of capillary electrophoretic separation of DNA fragments based on polymer filled capillaries

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ABSTRACT

The separation of ΦX174RF DNA-*Hae* III fragments is studied in a 0.5% methycellulose polymer solution as a function of temperature and applied voltage. The migration times decreased with both increasing temperature and increasing field strength, as expected. However, the relative migration rates of the fragments (selectivity) did not change with temperature but are affected by the applied field. A clear transition from the Ogston mechanism to the reptation mechanism is observed. The use of 0.5% methyl cellulose and 202 m*M* borate buffer at pH 8.2 allows the separation of the 271/281 base pair fragments, even without the addition in intercalating agents. At 700 V/cm and 20°C, complete separation of all fragments is achieved in less than 4 min with an average plate number of $2.5 \cdot 10^6$/m.

INTRODUCTION

The identification and codification of the human genome is a continuing worldwide scientific goal. A committee for The Human Genome Initiative has proposed a 15-year time table for the completion of the human genome project [1]. To ultimately achieve this goal, critical reevaluation of current separation techniques must be done, to substantially increase the speed, reliability, and sensitivity in gene mapping and in DNA sequencing applications. For example, to identify the $3 \cdot 10^9$ base pairs (bp) of the human genome would require sequencing individual bases, even at a rate of 23 000 bp/h, for the next 15 years. However, completion of this task will aid us in better understanding and possibly treating the more than 4000 genetic disorders that afflict humankind [2]. Although this daunting task appears impossible, great strides have been made recently.

Slab gel separations of deoxyribonucleic acid (DNA) has been the standard technology for many years, but tends to be very time consuming [3,4]. More recently, the union of DNA separations and high-performance capillary electrophoresis (HPCE) have been achieved for separations of DNA fragments under *ca.* 12 kilobase pairs (kb) [5]. The principal advantages of HPCE in DNA separation have been increased efficiencies, shortened separation times, and reduced amounts of sample needed. Separation of DNA in HPCE has been ongoing for several years. In many cases, adaptation of existing slab-gel methodology was applied to the relatively new technique of HPCE. Examples include filling capillaries with a proven separation medium such as agarose [6,7] or cross-linked polyacrylamide [5,8,9]. A leading advantage of HPCE is the decrease in Joule heating of the separation medium, which allows for increased voltages and consequently decreasing separation times. This combination also offers improved results over slab gels such as separation efficiency.

Problems exist in filling the capillary with the traditional separation media. When an immobilized separation medium such as agarose or

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0021-9673/93/$06.00

crosslinked polyacrylamide is introduced into the capillary, care must be exercised to maintain the integrity of the gel. Formation of polyacrylamide inside a capillary is very difficult and careful polymerization must be achieved. Otherwise voids in the gel will occur and the capillary will be useless. Instead of using an immobilized matrix in capillaries, polymer networks can be used. These include liquified agarose [6,7,10], non-cross-linked (linear) polyacrylamide [5,11–13], and viscous polymers [14–18]. These polymers can emulate the sieving effect of the fixed matrix and similar separations can be achieved. The ability to replenish the medium with fresh polymer or to vary the concentration of the polymer is a definite advantage over immobilized gels.

We will describe here optimization of electrophoretic separations in methyl cellulose solutions as an alternative to immobilized gels. With this simple polymer matrix in solution, less current is drawn and higher velocities can be achieved along with the capability of achieving extremely high resolution in a relatively short column. This results in drastically reduced separation times. Viscous polymers such as hydroxypropylmethylcellulose [14,17,18], hydroxyethylcellulose [16], polyethylene glycol [14] and methylcellulose [15] have been applied to DNA separations before. In this work, the influence of temperature and field strength on the separation of DNA fragments from 72 to 1353 bp derived from ΦX174RF DNA-*Hae* III based on methylcellulose solutions is examined. Comparisons with the behavior in linear polyacrylamide solutions [12,13] will be made.

EXPERIMENTAL

Materials

ΦX174RF DNA-*Hae* III digest from United States Biochemical (Cleveland, OH, USA) was diluted to 250 ng/μl. The TBE buffer consisted of tris[hydroxymethyl]-aminomethane (THAM), boric acid and ethylenediaminetetraacetic acid (EDTA), all from Sigma (St. Louis, MO, USA). The polymer was methyl cellulose 4000 (Sigma) which has a viscosity rating of 4000 cP for a 2% solution at 25°C.

Apparatus

DB-1 coated GC capillaries, 50 μm I.D. × 360 μm O.D. (J & W Scientific, Folsom, CA, USA) with 0.2 μm coating thickness was used without any further modification. The capillary length was 43 cm total, 35 cm effective length. A SpectraPHORESIS 1000 system (Spectra-Physics, Fremont, CA, USA) was used for all separations in the constant voltage mode. Temperature control to ±0.1°C was achieved by the peltier cooling system of the SpectraPHORESIS-1000. Electrokinetic injection was used throughout at 1 s at 200 V/cm. Detection of nucleic acids were monitored at 260 nm. Because of the small width of the peaks, the time constant was set to zero on the CE instrument to prevent any biasing of peak shapes.

Methods

The buffer solution contained 100 m*M* each of THAM and boric acid, both being the free base and acid respectively, with EDTA added as a chelating agent for divalent cations that could activate DNAases. The pH of the solution at the 202 mM TBE was 8.2 and was not further adjusted. A 19.90-ml volume of buffer was mixed with 0.5% (w/w) of methyl cellulose (0.10 g) and sonicated for 15 min. When the methyl cellulose appeared thoroughly wetted the solution was stirred until it appeared homogeneous. To further clarify the solution the stirring was continued on ice for at least 15 min. This caused the solution to appear transparent, leaving only small methyl cellulose needles in solution. Filtration was performed on the cooled solution through a 0.8 μm cellulose acetate filter (Alltech, Deerfield, IL, USA) and the solution was stored at 4°C until used.

Once the capillary was pressure filled it was allowed to equilibrate at running voltages for 5 min. The polymer did not degrade significantly over 30 to 40 runs and could be easily refilled once separation times started to change significantly. Column lifetimes were in excess of 100 injections.

RESULTS AND DISCUSSION

In Figs. 1–3, we show the separation of DNA fragments at various temperatures and at various

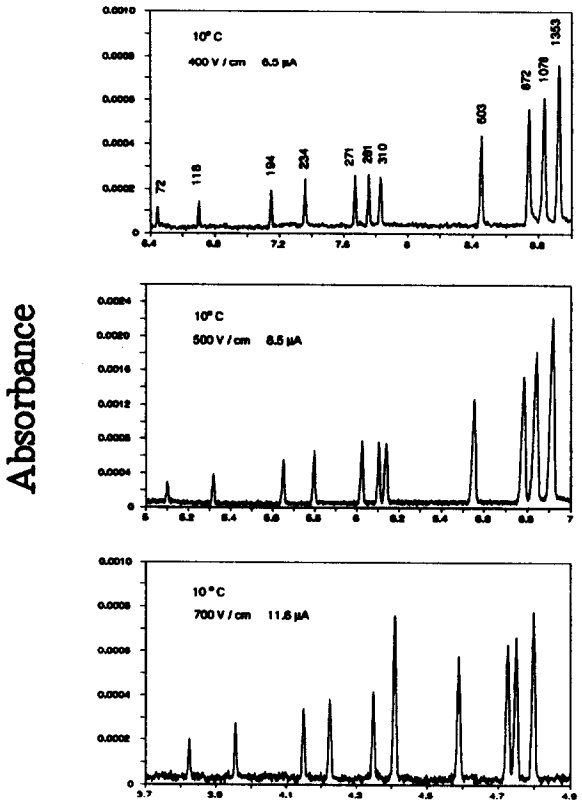

Fig. 1. Separation of ΦX174RF DNA-*Hae* III fragments at various field strengths. Conditions: 202 m*M* TBE buffer, 0.5% methyl cellulose 4000, 45 cm (35 cm to detector) × 50 μm I.D. DB-1 coated capillary, all electropherograms are at 10°C.

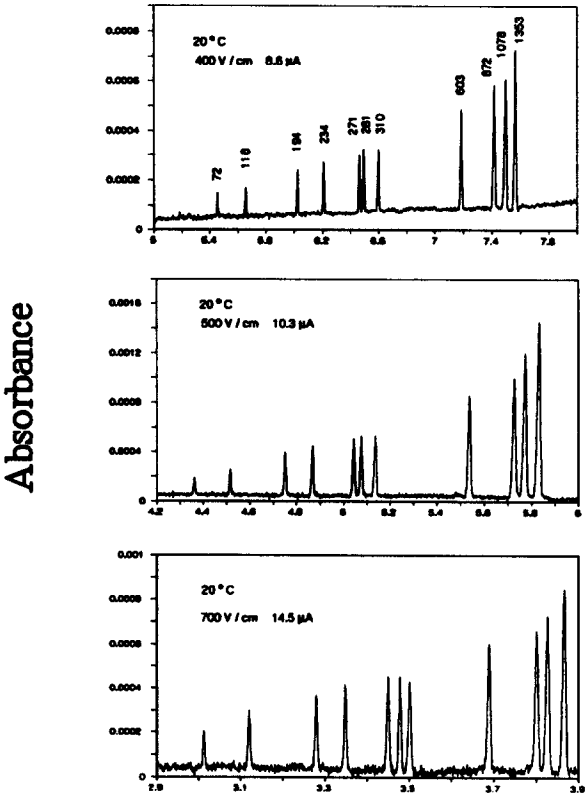

Fig. 2. Separation of ΦX174RF DNA-*Hae* III fragments at various field strengths. Conditions as in Fig. 1 except all electropherograms are at 20°C.

electric field strengths. It is clear that there are differences in migration times, efficiencies, and resolution between pairs of fragments. Several important observations can be made.

First, the migration times decrease with increasing temperature in each case. This is consistent with published results in linear polyacrylamide gel systems [12], where the viscosity of the gel–buffer system decreases with temperature. In that study, the migration times decreased roughly by a factor of 2 over a 20°C temperature range. Here, we observe a factor of 1.3 decrease over the same temperature range. In effect, the activation energy for viscous flow is smaller in methylcellulose by a factor of 0.65 [12]. Fig. 4 shows the temperature dependence of mobilities of the DNA fragments at fixed field strengths. For comparisons, the mobilities are normalized to that of the smallest (72 bp) fragment. Overall, the relative mobilities show little temperature dependence, indicating that there is negligible change in the effective pore size of the polymer matrix [19] and that the DNA conformation stayed constant over this temperature range.

Second, the separation efficiencies generally decrease with increasing fragment size, increasing temperature and increasing electric field strength, as shown in Table I. The peak widths should have negligible contributions from the injection process, since both the injection times and the injection voltages are lower. At higher temperatures and at higher applied fields, the

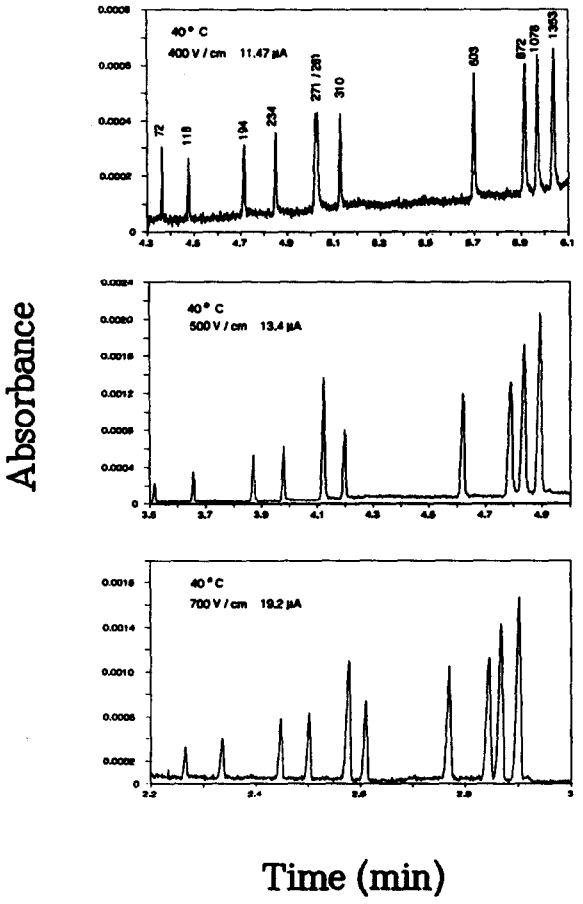

Fig. 3. Separation of ΦX174RF DNA-*Hae* III fragments at various field strengths. Conditions as in Fig. 1 except all electropherograms are at 40°C.

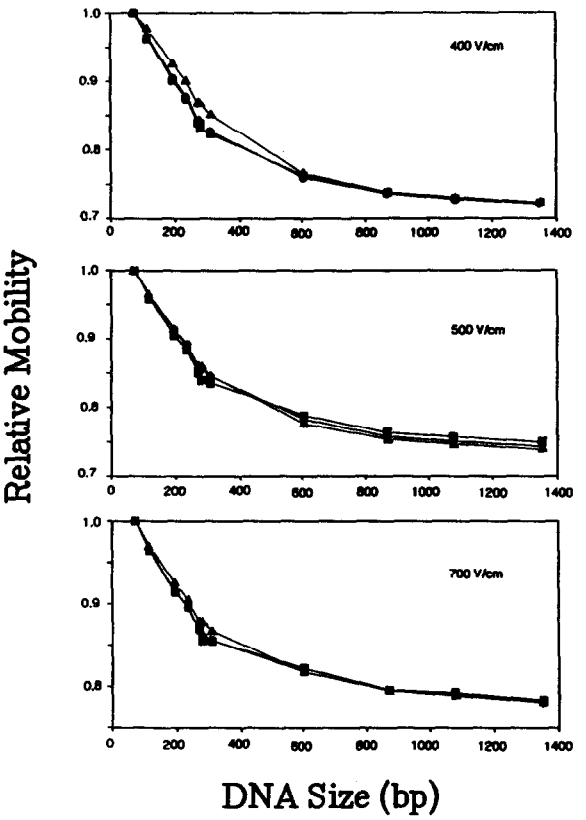

Fig. 4. Normalized mobilities *versus* DNA size for (a) 400 V/cm, (b) 500 V/cm, (c) 700 V/cm at various temperatures. ■ = 10°C; ● = 20°C; ▲ = 40°C.

half-width of the bands actually decreased for a given DNA fragment, but the shorter migration times in each case led to lower numbers of theoretical plates. The concept of theoretical plates strictly should be modified since the migration times past the detector are not constant. A closer examination reveals that the physical lengths of the zones on column are insensitive to the temperature or the electric field strength. This means that one can in general shorten the running time by increasing the temperature or the field strength without introducing band broadening. Diffusional broadening of the DNA fragment bands should be minor based on the short residence times and the large molecular masses (sizes) involved. We can conclude that the primary contributor to the observed peak widths is a mismatch in the mobilities of the fragments compared to that of the buffer ions [20], leading to skewed, triangular peaks. This is consistent with the fact that the widths of the peaks for the larger fragments, even after correction for migration times, are larger than those for the smaller fragments. In these studies, we cannot decrease the injected amounts further because of the lack of detector sensitivity. Also, the buffer ionic strength cannot be increased if joule heating is to be avoided at these high field strengths.

Third, the migration times decrease as the applied field strength is increased. This goes beyond the normal linear dependence of migration velocity on field strength, as is depicted in Fig. 5. There, the mobilities of each series of fragments had been normalized to that of the 72

TABLE I

NUMBER OF THEORETICAL PLATES (MILLIONS) FOR THE SEPARATION OF DNA FRAGMENTS UNDER VARIOUS CONDITIONS

Size (bp)	Theoretical plates ($\times 10^6$)								
	10°C			20°C			40°C		
	400 V/cm	500 V/cm	700 V/cm	400 V/cm	500 V/cm	700 V/cm	400 V/cm	500 V/cm	700 V/cm
72	2.9	1.3	1.8	3.0	1.3	1.2	5.1	1.1	1.0
118	2.6	2.3	1.5	2.0	1.2	1.4	2.1	1.1	1.0
194	2.2	1.8	1.3	1.9	1.1	1.4	1.7	1.2	0.9
234	2.3	1.9	1.5	2.3	1.1	1.4	1.8	1.2	1.3
271	2.0	1.8	1.4	2.1	1.0	1.1	1.5	1.2	0.8
281	2.2	1.7	1.2	2.7	1.1	1.2	2.3	1.2	0.8
310	1.3	1.2	1.2	1.9	0.9	1.0	1.9	1.2	1.0
603	1.6	1.4	1.4	1.8	0.7	1.0	1.9	1.0	1.0
872	1.3	1.1	1.1	1.6	0.5	1.0	1.7	0.8	0.9
1078	1.2	1.0	1.2	1.3	0.6	0.9	1.8	0.8	1.1
1353	1.2	1.0	1.3	1.3	0.5	0.9	1.8	0.8	1.1

bp fragment. The smaller fragments (<250 bp) show little dependence on field strength, a result that is similar to typical small-molecule separations in CE. The larger fragments clearly show a higher relative mobility with increased field strength. The explanation of this effect could be attributed to the mechanism which drives this separation. The Ogston or sieving mechanism which describes analyte through a porous medium states that the analyte can be viewed as a hard sphere and is immutable [21,22]. Since the porous medium is made up of a range of pore sizes, the smaller the analyte sphere the more pores become available for the analyte to travel through, hence the "sieving" effect. This explains the behavior of the smaller fragments. Obviously, DNA is not a hard sphere but more closely represented by a long chain. This is more pronounced for the larger (>250 bp) DNA fragments. When this happens the Ogston mechanism fails and another theory must be applied. The new mechanism will more closely resemble reptation that describes the DNA as snaking through a porous medium [22]. This explains the plateau-like behavior for the larger fragments in Figs. 4 and 5. When higher voltages are applied the DNA is certainly more stretched out parallel to the field that is applied. The plateaus therefore shift to larger values as the electric force per unit length of the molecule increases [22].

Fourth, we are able to resolve the 271/281 pair of fragments under certain experimental conditions (see Figs. 1–3), *i.e.* certain combinations of temperature and field strength. Such a separation is almost never achieved for a cellulose [14,17] or agarose [6,7] medium except if an intercalator such as ethidium bromide is used. This is in contrast to a linear polyacrylamide medium, where the native 271/281 fragments are well separated [5,11–13]. A possible explanation is that polyacrylamide is formed with a wide distribution in pore sizes. This allows the small difference between the two fragments to affect separation. Cellulose solutions are generally of one viscosity rating and are not diverse enough to optimize separation in the 280 bp range simultaneously with the larger and the smaller fragments. Fortunately, 280 bp lies just about at the transition point between the sieving and the reptation mechanisms (Figs. 1–3) for this polymer type, size and concentration. Since the applied field only affects the larger fragments, the migration of the 281 and larger fragments can be "tuned" to optimize their resolution from

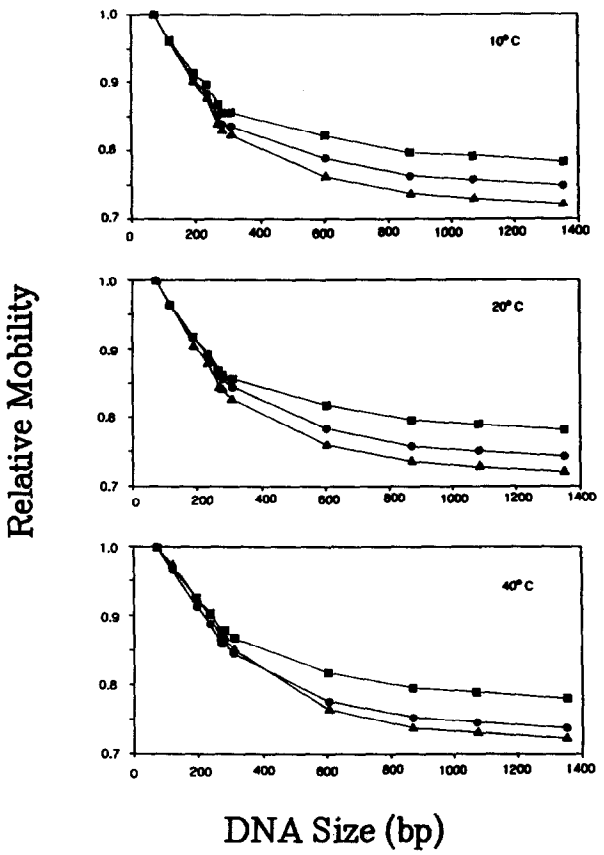

Fig. 5. Normalized mobilities *versus* DNA size for 10, 20 and 40°C at various field strengths. ▲ = 400 V/cm; ● = 500 V/cm; ■ = 700 V/cm.

the 271 and smaller fragments. This then constitutes a systematic approach for enhancing the resolution of a narrow range of DNA fragments in polymer solutions.

Fifth, there are some differences in resolution between our results and those reported in the literature. High electric field strengths have been found to degrade the resolution between the larger DNA fragments in polyacrylamide gels [5,13]. Such is not the case in Figs. 1–3, even though some of the runs are at very high fields (700 V/cm). The degradation of resolution of the larger fragments at high fields is also not always observed in polyacrylamide gels (Fig. 3 *vs.* Fig. 8 in ref. 5). One can thus rule out field-induced DNA alignment as the cause, since alignment should be matrix independent. These results may indicate some inherent differences between cellulose *versus* polyacrylamide matrixes, such as the latter being less rugged at high fields.

Sixth, because of the very high fields used, the complete separation of all ΦX174RF DNA-*Hae* III fragments was accomplished in record time. The best result along these lines is for separation at 700 V/cm and 20°C, requiring less than 4 min. The number of theoretical plates averaged better than $1 \cdot 10^6$ (*ca.* $2.5 \cdot 10^6$ plates per meter). A direct comparison is given in Table II. Even higher plate numbers have been obtained for DNA sequencing runs [23,24], but those involve run times of up to an hour.

TABLE II

SEPARATION OF ΦX174RF DNA-*Hae* III FRAGMENTS UNDER DIFFERENT CONDITIONS

MC = Methylcellulose; PA = polyacrylamide; HPMC = hydroxypropylmethylcellulose; EB = ethidium bromide; ND = not determined.

Ref.	Medium	Conditions	Total time (min)	Average plate number ($\times 10^6$)
This work	0.5% MC	20°C, 700 V/cm	3.8	1.1
13	linear PA	20°C, 400–100 V/cm	9.3	0.2
5	linear PA	20°C, 300 V/cm	12	ND
12	linear PA	40°C, 400 V/cm	14	ND
11	linear PA	25°C, 250 V/cm	16	1.2
5	crosslinked PA	20°C, 250 V/cm	16	2
13	linear PA	20°C, 200 V/cm	27	0.2
17	0.5% HPMC, EB	25°C, 175 V/cm	32	ND
14	0.5% HPMC, EB	25°C, 175 V/cm	33	1

In summary, we have demonstrated effective control of the separation of DNA fragments in a 0.5% methylcellulose polymer solution. We expect this system to be a reasonable alternative to the use of linear polyacrylamide, particularly if one can further optimize the performance towards the higher mass range by suitable combinations of polymers of different specific viscosities and molecular mass [19].

ACKNOWLEDGEMENT

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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