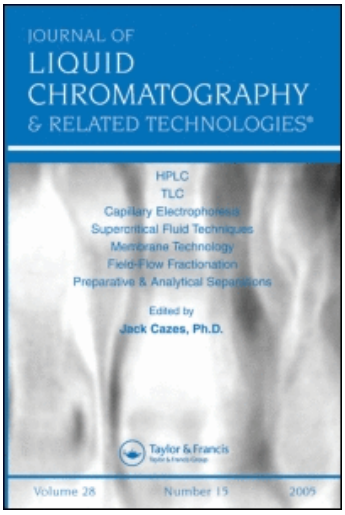

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SIZE-SELECTIVE CAPILLARY ELECTROPHORESIS (SSCE) SEPARATION OF DNA FRAGMENTS

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ABSTRACT

A capillary electrophoretic separation of DNA fragment standards in the range of 50 to 10^5 base pairs was achieved using methylcellulose as a size-selective agent. All 23 fragments in a 1 kb DNA ladder can be resolved in under 25 minutes. Capillary wall deactivation, using a siloxane-bonded polyacrylamide, greatly improved resolution and reproducibility. The concentration effect of the size-selective agent and the applied electric field strength on DNA fragment separation was determined. Instability of the wall coating limits capillary lifetimes to approximately 30 runs. Considerations in using SSCE for the analysis of DNA fragments produced by the PCR technique are discussed.

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INTRODUCTION

Slab gel electrophoresis can provide size-selective separations of DNA fragments suitable for identification, however, this technique is time consuming and labor intensive. A more rapid, automated technique is needed to meet the demands of polymerase chain reaction (PCR) technology (1) for DNA oligonucleotide synthesis and subsequent identification. One approach has been to adapt the current slab gel sieving technology for use in the more rapid technique of Capillary Electro-phoresis (CE) using in-situ cross-linking of polymer gel within the capillary (2-8). However, polymerization of stable, efficient and reproducible rigid gels within the small diameter capillaries has proven to be difficult to achieve. Inhomogeneities in the gel result in capillary failure via bubble formation when the high potential is applied. Our experience has shown that capillary gels, suitable for good resolution of DNA containing 10 to 200 base pairs (bp), totally retain large (>10^5 bp) DNA fragments. Periodic sacrifice of the first few millimeters of the gel-filled capillary is then required to remove the collapsed gel. The requirement for in-situ gel synthesis also severely restricts the choice of applicable polymers for CE.

A better approach uses free-zone CE with a buffer containing a soluble polymer, size-selective agent (9-13) for the separation of DNA fragments. The problems associated with gel inhomogeneity and large fragment retention found in gel CE are eliminated by loading a uniform solution of the size-selective buffer into the capillary prior to each electropherogram. Aqueous solubility is the only requisite that candidate polymers must meet for testing in size-selective CE.

The potential for application of SSCE to a wide range of size-selective separation problems appears great. Since the discrimination between external and in-situ synthesis of the size-selective polymer is arbitrary, we hereby propose the term Size Selective Capillary Electrophoresis (SSCE) to describe both the use of in-situ gels and soluble polymers as sizing agents in CE.

In this paper, we investigate the use of methylcellulose as a size-selective agent for the CE separation of standard DNA fragment mixtures in the size range of 50 to 10^5 base pairs. We found that high separation efficiency and reproducibility for DNA separation by SSCE requires the deactivation of capillary silanol groups. A siloxane-linked linear polyacrylamide was evaluated for this purpose. Instability of this bonded-phase in the separation buffer was studied to determine its useful life in routine use. The effects of the concentration of size-selective agent and the effect of field strength on DNA fragment resolution were studied. We hypothesize that diffusion of the DNA through transient pores in the polymer network plays a role in the separation mechanism of SSCE. The applicability of the technique to the determination of PCR-amplified DNA fragments is also considered.

EXPERIMENTAL

Materials:

Commercially-available DNA mixtures, a 1 kb DNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) and a DNA Molecular Weight Marker V (Boeringer Mannheim, Indianapolis, IN) were used to determine fragment identities by comparison to the pattern of separation achieved by slab gel electrophoresis. The

CE separation buffer was prepared to contain 100 mmol/L TRIS base, 100 mol/L boric acid, 2 mmol/L disodium EDTA at a pH of 8.0. The size-selective polymer tested was methylcellulose (Polysciences, Warrington, PA), MW = 86,000 diluted to concentrations of 0.2, 0.4 and 0.6% (w/v) with the buffer.

Apparatus and Methods:

Fused silica capillaries of two dimensions were tested. Capillaries of 100 μm i.d. and 50 cm length (length to detector = 45 cm) and 75 μm i.d. and 100 cm length (length to detector = 80 cm) were used for the bare silica and deactivated silica SSCE experiments. Siloxane-bonded polyacrylamide was used for capillary wall deactivation. The coating was prepared by polymerization of acrylamide to a silane-bonded methacrylate, following the procedure of Hjerten (14). A batch of 4 to 6 capillaries were successively pretreated by aspirating under low vacuum with 1 N NaOH for 15 minutes, deionized water for 15 minutes, methanol for 15 minutes, 0.4% methacryloxypropyltrimethoxysilane/ 0.4% glacial acetic acid in methanol for 60 minutes, methanol for 15 minutes and deionized water for 15 minutes. The polymerization of a surface layer of polyacrylamide occurred in-situ using a freshly prepared solution mixture containing 5 mL of 3.5% acrylamide, 80 mL of 10% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) and 20 mL of 10% (w/v) ammonium persulfate. The mixture was aspirated into the capillaries for 5 minutes, and left to polymerize without flow for 45 minutes. Polyacrylamide which had not reacted with the silylated surface was removed by flushing the capillaries with deionized water for 15 minutes. The capillaries were stored in deionized water when not in use.

Two commercially available capillary electrophoresis systems were employed, one with pressure electrolyte loading and the other with vacuum loading. It was necessary to use extended capillary loading times for the viscous methylcellulose buffers. Times varied from 2 minutes for the 0.2% methylcellulose buffer in the 100 μm diameter capillary to over 30 minutes for the 0.6% methylcellulose buffer in the 75 μm diameter capillary. UV/visible absorbance detection was used on-column at 260 nm.

RESULTS AND DISCUSSION

Double-stranded DNA is composed of repeating units of 4 major nucleic acids, all of which have similar size and equal charge. Thus, the size-to-charge ratio of large and small DNA fragments are similar. The resulting differences in electrophoretic mobilities are too small to permit DNA fragment separation using CE without the use of size-selective gels or soluble polymer agents. In bare silica capillaries with positive applied voltage, the anionic DNA molecules migrate against the electroosmotic flow. Addition of soluble polymer agents to the CE electrolyte can provide a size-selective separation by carrying the larger fragments along with the electroosmotic flow. Small DNA fragments can migrate with less inhibition against the flow by moving through the pores between the polymer chains. Partial separation of the 23 fragment 1 kb DNA ladder mixture can be obtained using 0.2% methylcellulose as the size-selective agent in a bare silica capillary, as shown in **Figure 1**. However, separations conducted in uncoated capillaries were not reproducible and provided only limited resolution of many fragments.

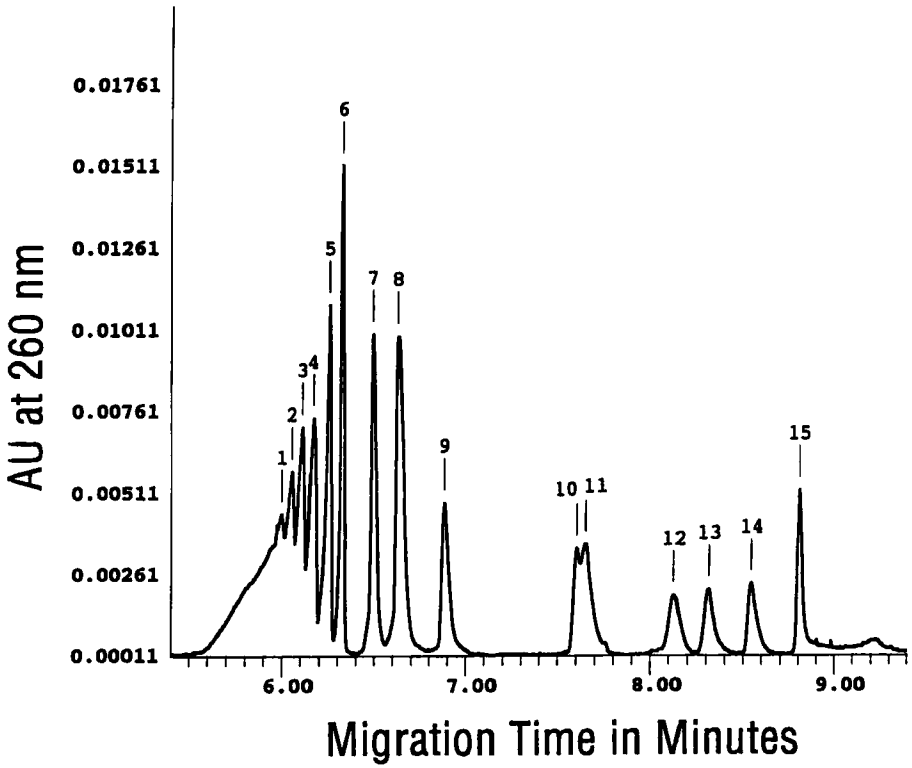

FIGURE 1:
Separation of 1 kb DNA Ladder Using 0.2% Methylcellulose Buffer in a Bare Fused Silica Capillary. Conditions: Capillary 75 μm x 50 cm (L = 45 cm); Voltage +20 kV.

Loss of resolution was attributed primarily to low efficiency caused by solute/capillary interaction, possibly through ionic adsorption of the DNA anions onto a double layer of electrolyte cations adsorbed on the silica walls. Separation irreproducibility of SSCE on bare silica persisted despite numerous attempts at conditioning the capillaries with sodium hydroxide and buffer between runs.

The efficiency of the separation was greatly improved by reducing capillary wall interaction through deactivation of the silica silanol groups. A siloxane-bonded layer of linear polyacrylamide was tested as one approach to deactivate capillaries for SSCE. Since the electrically neutral polyacrylamide coating eliminates electroosmotic flow, it is necessary to reverse polarity and elute the components solely by their electrophoretic mobilities. In this separation format, the addition of a size-selective agent results in a separation where small DNA fragments migrate most rapidly through the pores between polymer chains, while larger fragments are retarded by retention in the pores. With the coated capillary the order of migration by size is opposite to than obtained in bare silica. The separation of the 1 kb ladder performed in the polyacrylamide bonded capillary, using 0.4% methylcellulose is shown in **Figure 2**. Using this approach, the separation of individual ladder fragments is excellent, and provides results very similar to those previously reported using crosslinked gel-filled capillaries (8) and methylcellulose as the soluble polymer (9).

The influence of the concentration of methylcellulose on the migration times of the 1 kb ladder DNA fragments are shown in **Figure 3** for 0.2, 0.4 and 0.6% of the agent. The shape of these three curves deviate somewhat from the classic "S" shaped response commonly obtained in gel permeation liquid chromatography (GPLC) that show size independent regions for very small and very large molecules. In contrast, the SSCE experiment shows migration time differences for the wide range (75 to 12,216 bp) of the DNA fragment sizes in the 1 kb mixture.

The separation mechanism for DNA using soluble polymer agents in CE has yet to be determined. Cer-

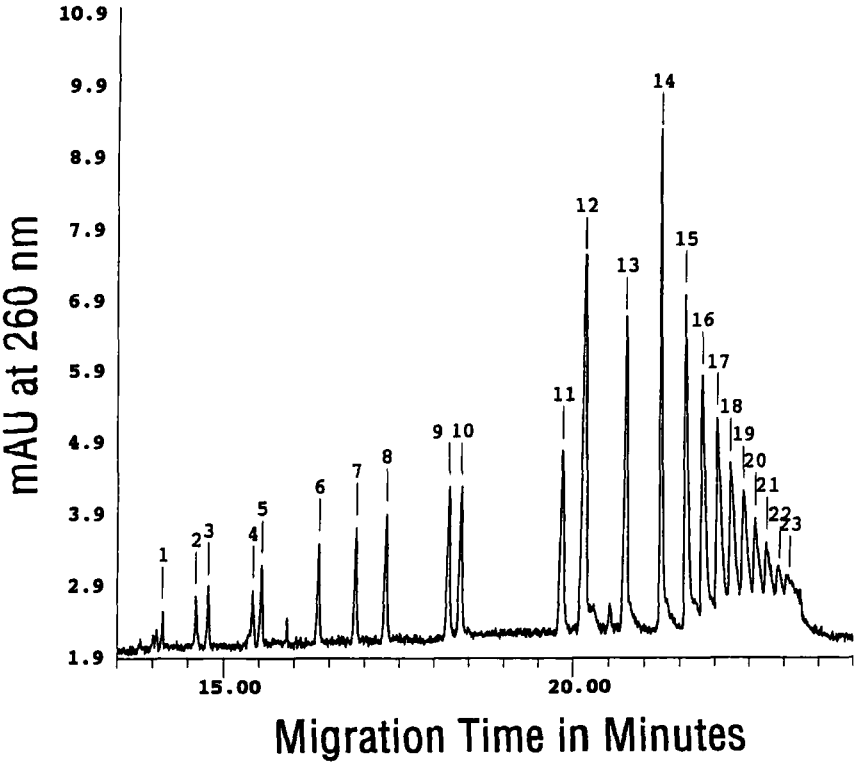

FIGURE 2:
Separation of 1 kb DNA Ladder Using 0.4% Methylcellulose Buffer in a Polyacrylamide Deactivated Capillary. Conditions: Capillary 75 μm x 100 cm (L = 80 cm); Voltage = -30 kV. Peak Identities in Number of Base Pairs: (1) 75; (2) 134; (3) 154; (4) 201; (5) 220; (6) 298; (7) 344; (8) 396; (9) 506; (10) 517; (11) 1018; (12) 1636; (13) 2036; (14) 3054; (15) 4072; (16) 5090; (17) 6108; (18) 7126; (19) 8144; (20) 9126; (21) 10180; (22) 11198; (23) 12216.

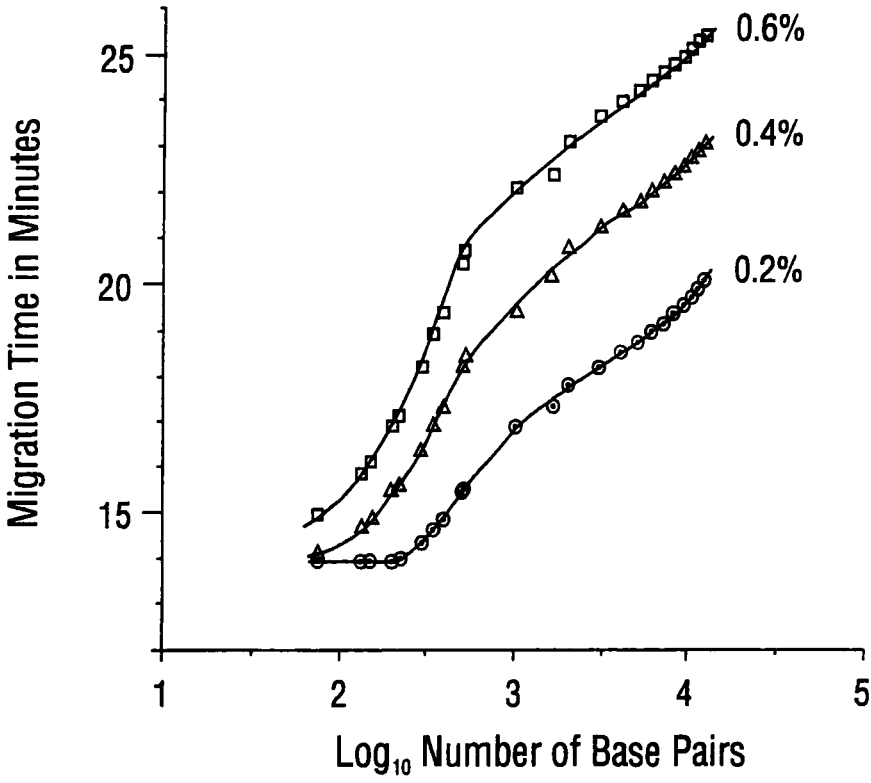

FIGURE 3:
Variation of the DNA Fragment Migration Time with the Log_{10} Number of Base Pairs of the 1 kb Ladder Using 0.2, 0.4, 0.6% Methylcellulose on a Coated Capillary. Conditions: same as Figure 2.

tainly, the formation of transient pores, similar in function to the permanent pores that are found in gels, should be a factor in the size-selective separation. However, it is difficult to justify the wide range of size selectivity found with soluble polymers using a model that considers only a single pore diameter. Multiple effective pore diameters with a broad distribution of size must be present in the polymer network.

As expected, the effective pore diameters should decrease with increasing agent concentration, providing improved separation of small fragments. The small DNA fragments in the 1 kb ladder exhibit significant improvement in resolution by increasing the methylcellulose concentration from 0.2 to 0.6% (**Figure 3**). Although the 0.6% gives the best resolution for the entire range of fragments in this mixture, the 0.6% methylcellulose buffer is too viscous to be easily loaded into the capillary with the current generation of commercial instruments, necessitating fill times on the order of 30 minutes. We found the 0.4% methylcellulose buffer to be the maximum concentration that can be used without excessive capillary fill times.

We studied the use of the 0.4% methylcellulose buffer for resolution of fragments in the range of 50 to 600 bp. This range is crucial to DNA fragment typing using the PCR amplification technique. The separation of a small fragment standard, DNA V with the 0.4% buffer is shown in **Figure 4**. Resolution is excellent for all fragments larger than 50 bp and efficiency exceeded 1 x 10^6 plates/m. We prepared a calibration plot for the 0.4% buffer using both the 1 kb ladder and DNA V fragment standard mixtures. **Figure 5** shows the plot of the migration time versus number of base pairs. Despite the small differences in migration that might be attributed to the differing base sequence of these bacterial DNA fragment mixtures, a good linear correlation (r^2 = 0.992) is found. Fragments differing by as little as 5 bp can be separated and identified in this size range with this buffer composition. The calibration plot is quite similar to the results found for the separation of a repetitive fragment PCR product mixture in the range of 50 to 350 bases on polymer gels (15).

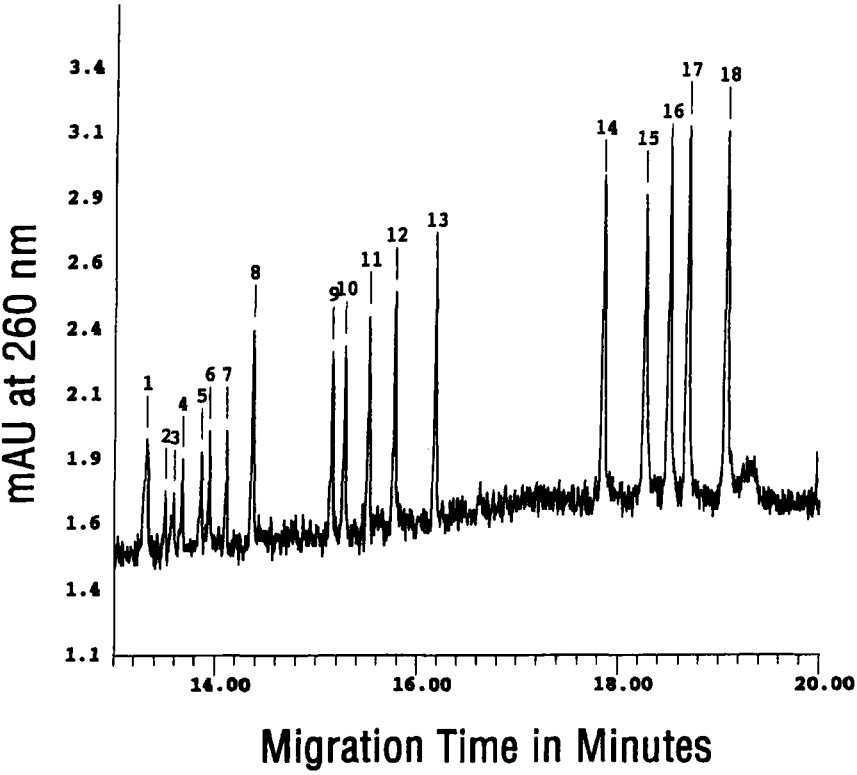

FIGURE 4:
Separation of Small Fragment DNA V Mixture Using 0.4% Methylcellulose Buffer. Conditions: same as Figure 2. Peak Identities in Number of Base Pairs: (1) 8, 11, 18, 21, (2) 51; (3) 57; (4) 64; (5) 80; (6) 89; (7) 104; (8) 123, 124; (9) 184; (10) 192; (11) 213; (12) 234; (13) 267; (14) 434; (15) 458; (16) 504; (17) 540; (18) 587.

In addition to the mechanical size-selective mechanism, other factors specific to the electrophoretic experiment must influence the separation of DNA fragments using the soluble agent. Stoichiometric association between the methylcellulose polymer and the DNA may enhance the apparent size difference of fragments.

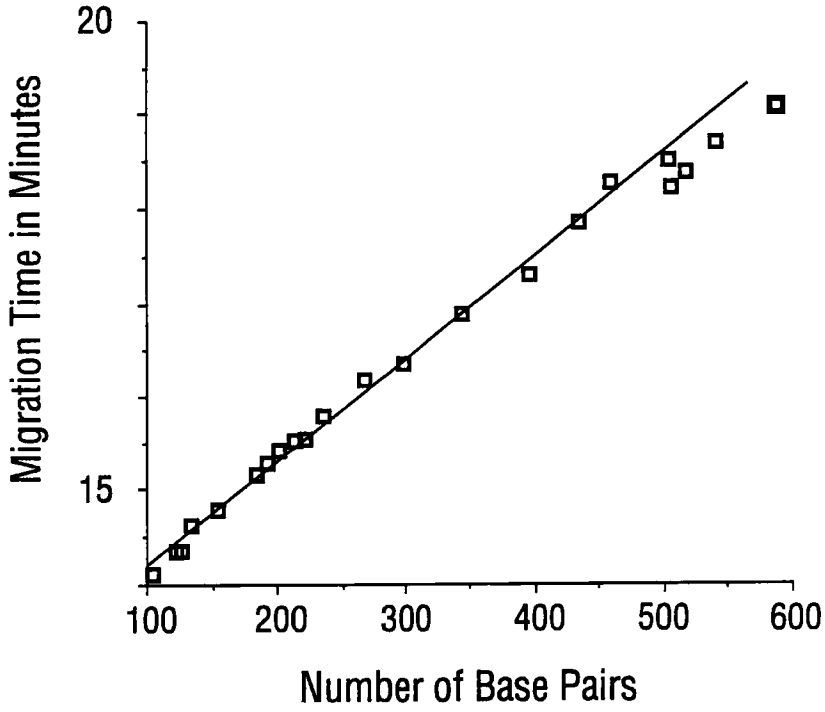

FIGURE 5:
Variation of the Migration Time with the Number of Base Pairs for Small DNA Fragment Standards. Conditions: same as Figure 2; samples same as Figures 2 and 4.

Addition of high concentrations of methylcellulose to the electrolyte may also change the activity of water molecules available for association with the hydration sheath of the DNA, modifying the effective Stokes' radius. These additional factors may help to explain the unexpected improvement in resolution of the large fragments found at the higher methylcellulose concentrations. In conventional gel sieving methods, decrease in the diameter of the pores improves resolution of small fragments at the expense of the resolution of

the large fragments, which are totally excluded. In contrast, the SSCE resolution of both small and large fragments is improved with increasing polymer concentration. Further work to elucidate the unique mechanisms of the SSCE separation is needed.

In order to further characterize the SSCE separation the influence of electric field strength on resolution was determined. For analytes migrating in the absence of confounding interactions, Giddings' equation (16) describes the resolution in free-zone CE:

$$R_s = (N^{1/2}/4)\ (\Delta t/t_{ave}) \qquad (1)$$

where N is the number of theoretical plates, Δt the time difference between two zones, and t_{ave} the average elution time of the two zones. Although this equation may not explicitly describe resolution in CE with the size-selective interaction, it does provide useful insight into the SSCE separation process. To evaluate the effect of the field strength on the resolution we chose to examine the separation of the 506 and 517 bp DNA fragments in the 1 kb ladder. The results obtained for the 506/517 pair, using the 0.2% methyl cellulose on a 100 μm diameter coated capillary, are shown in **Figure 6.** The resolution shows a broad maximum at 250 kV/cm. Since resolution should increase linearly with field strength in free-zone CE, we investigated the deviations found for the two contributing factors in equation (1), the efficiency and relative velocity difference. The dependance of $N^{1/2}$ and $\Delta t/t_{ave}$ on the field strength is shown in **Figure 7.** The efficiency of this separation increases as expected with fields up to about 350 V/cm. However, at a field of 400 V/cm, band broadening results from excessive Joule heating of the air cooled capillary. The relative velocity difference shows a maximum value at approximately 250 V/cm and

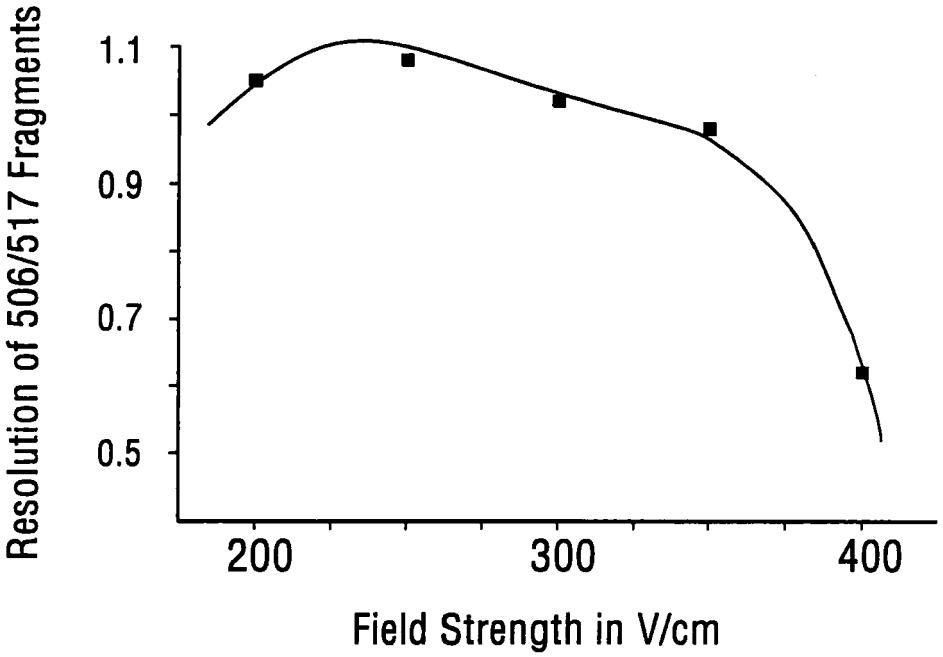

FIGURE 6:
Variation of the Resolution of the 506/517 DNA Fragments with the Applied Field for the 0.4% Methylcellulose Buffer.

then declines at higher fields. In free-zone electrophoresis, $\Delta t/t_{ave}$ is expected to be independent of electric field strength (17). However, unlike free-zone CE, which depends on electrophoretic mobility alone, the separation in the SSCE experiment may also depend on interaction with transient pores that form between polymer strands. Since the capillary length was fixed, the time scale of the separation decreases linearly with the applied field for each fragment peak. This effect is removed from the reduced velocity difference by dividing by the average migration time. However, time dependant diffusive processes that affect

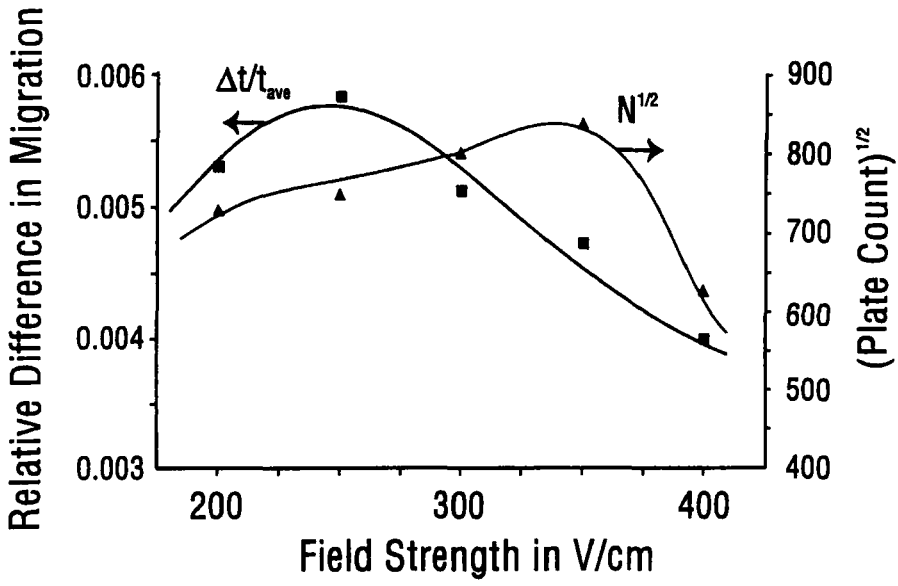

FIGURE 7:
Variation of the Factors Contributing to the Resolution of the 506/517 Fragments with the Applied Field for the 0.4% Methylcellulose Buffer.

the value of Δt will cause $\Delta t/t_{ave}$ to vary with the field strength. At high fields, the restricted time for the diffusive interaction of the fragments with the polymer pores decreases the relative velocity difference that may be achieved compared to lower fields. This provides evidence that time-dependant diffusive processes in SSCE contribute to the deviations from the expected non-interactive free-zone behavior.

The combination of the resolution maximum at about 350 V/cm and the relative velocity difference maximum at 250 V/cm contribute to the broad resolution maximum that was found in **Figure 6.** Joule heating and dif-

fusive time dependance of SSCE may contribute to the observed deviations from equation (1).

We evaluated the day-to-day and capillary-to-capillary reproducibility for migration time of fragments in the 506 to 4072 bp range using the deactivated capillaries. Overall reproducibility was better than 1.5% RSD. However, loss of the siloxane-linked polyacrylamide coating by base-catalyzed hydrolysis (18) in the pH 8.0 buffer limits the useful lifetime of the capillary to approximately 30 runs (9). As the wall coating fails, fragment migration times increase, and after approximately 50 runs, a discernible loss in efficiency is noted.

A preliminary attempt to apply the SSCE separation to the analysis of DNA fragments produced by the PCR technique was only partially successful. A peak that corresponded to a migration time expected for an easily amplified bacterial marker fragment of 500 bp was easily detected, however, no additional peaks could be detected for other amplified fragments. Although the PCR technique amplifies very small amounts of DNA, the final concentrations of test fragments may be too low to detect using SSCE with on-column UV absorbance detection. Since the PCR reaction mixture has much higher electrolyte concentrations than the SSCE buffer, the use of large injected sample volumes cannot be used to overcome this difficulty. Desalting the samples prior to SSCE, which permitted large injection volumes, shows promise as a means of solving the sensitivity problem for PCR product analysis using the current on-column absorbance detection mode.

CONCLUSIONS

The SSCE technique, using methylcellulose, works well as a separation technique for DNA fragment iden-

tification. However, several hurdles remain before routine application to PCR-produced DNA can be assured. Hydrolytically-stable coatings for deactivation of the silica capillaries are required. Improved CE instrumentation, designed to load viscous electrolytes such as the 0.6% methylcellulose, is needed. Increased detection sensitivity using longer pathlength absorbance or tagged fluorescence can provide the increased sensitivity required for PCR product determination.

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