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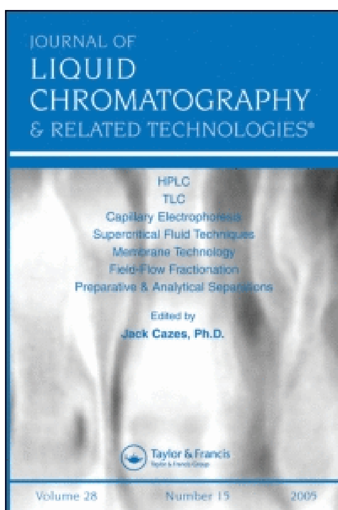

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Separation of DNA Restriction Fragments using Capillary Electrophoresis

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SEPARATION OF DNA RESTRICTION FRAGMENTS USING CAPILLARY ELECTROPHORESIS

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ABSTRACT

Gel-filled and "non-gel" capillary electrophoresis (CE) have been applied to the separation of various DNA restriction fragments. 30% HydroLink gel, polymerized inside a 75 μm i.d. fused-silica capillary, was used in the gel-filled CE. Primary results show that the HL capillary gel was simple to cast, and its stability was reasonably good under our running conditions. In the non-gel CE experiment, a buffer containing the sieving additive hydroxypropylmethyl cellulose was used to affect the size-dependent separation. The use of GC capillaries eliminates the inconvenience of separately coating the capillary walls for efficient non-gel separation. Finally, we demonstrate that it is feasible to detect native DNA fragments using indirect fluorometry in non-gel capillary electrophoresis.

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INTRODUCTION

Slab gel electrophoresis is by far the most widely used separation technique in biological research. It is used routinely in applications where size fractionation is required, such as DNA restriction mapping, mobility shift assays, and DNA sequencing (1). The capability of performing multiple-channel analysis is the major advantage of using slab gel electrophoresis. However, the disadvantages of the conventional gel system include time-consuming gel preparation, long running times, a lack of real-time detection capability, and the requirement of a large sample size.

In the past few years, capillary electrophoresis (CE) has been shown to be a fast, powerful, and efficient separation technique for a variety of compounds (2). These characteristics are the direct results of the use of high separation voltage and rapid dissipation of heat in a narrow capillary, typically 50-150 μm i.d. In capillary zone electrophoresis (CZE), a capillary is filled only with a buffer solution for the separation of analytes. Compounds with different size-to-charge ratios can usually be separated by CZE. It has been applied to the separation of a variety of biological compounds such as proteins (3-5), peptides (6-8), amino acids (9-11), and drug metabolites (12-14). However, using CZE to fractionate DNA fragments is not quite successful since each fragment has a similar size-to-charge ratio (15).

Kasper et al. (16) demonstrated that partial separation of the Hae III fragments of ϕX174 DNA can be achieved by using a cetyltrimethylammonium bromide (CTAB) micellar CE system. Another approach to separate DNA fragments involved the introduction of a sieving medium in the CE system. By using a gel-filled capillary with low and zero cross-linked polyacrylamide, Heiger et al. (17) demonstrated good separation of DNA fragments up to about 10,000 base pairs. Recently, CE approaches using a viscous buffer containing cellulose additives have been reported for the size-dependent separation of DNA fragments in coated (18-20) and uncoated capillaries (21). Since chemical gels were not used, one can refer to this type of separation as non-gel separation.

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In this paper, we will discuss results on DNA separation using gel-filled and non-gel CE. The feasibility of applying a new type of polymer matrix, the HydroLink gel, in capillary electrophoresis of DNA fragments is examined. In addition, we also investigate a non-gel CE separation strategy using a commercial GC microbore column as a coated capillary. Finally, the application of indirect fluorometry for the detection of native DNA restriction fragments in non-gel CE will be demonstrated.

EXPERIMENTAL

Materials

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DNA restriction fragments, Hind III/lambda DNA and Hae III/ϕX174, were obtained from International Biotechnologies (New Haven, CT). Hae III digests of pBR322 DNA and Bgl I/Hinf I digests of pBR328 DNA were obtained from Boehringer Mannheim (Indianapolis, IN). Electrophoresis-grade reagents, ammonium persulfate, N,N,N',N',-tetramethylethylenediamine (TEMED), and acrylamide were obtained from Bio-Rad (Richmond, CA). DS DNA HydroLink gel (HL) was supplied by AT Biochem (Malvern, PA). Hydroxypropylmethylcellulose (4000 cp and 15000 cp at 25°C for a 2% solution) were obtained either from Sigma Chemical (St. Louis, MO) or Serva Biochemicals (Paramus, NY). All other chemicals were reagent grade and supplied by Sigma. Fused-silica capillary was obtained from Polymicro Technologies (Phoenix, AZ). GC capillaries coated with a non-polar stationary phase DB-1 (50 μm i.d.) and BP-1 (100 μm i.d.) were supplied by J & W Scientific (Folsom, CA) and Scientific Glass Engineering (Austin, TX), respectively.

Instrumentation

A commercial (Isco, Lincoln, NE, Model 3850) and a home-built capillary electrophoresis system were used. UV detection at 260 nm was used for the commercial instrument while laser-induced

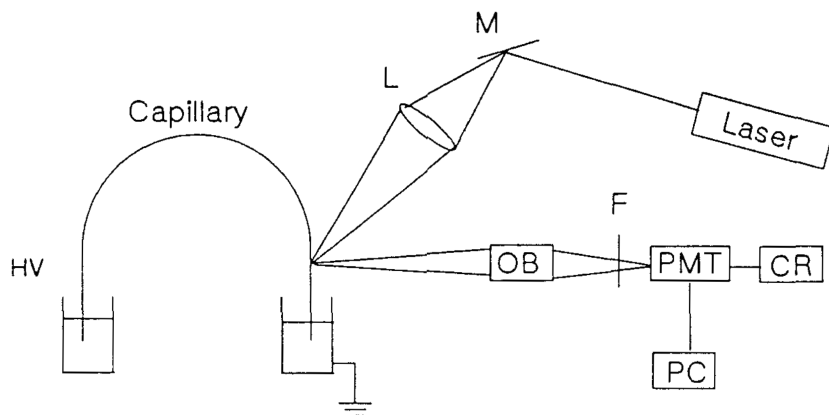

FIGURE 1. Schematic diagram of CE with laser-based detection. HV = high voltage supply, M = mirror, L = lens, OB = objective, F = filter, PMT = photomultiplier tube, CR = chart recorder, PC = computer.

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fluorescence detection was used for the home-built system. A sketch of the home-built CE system is shown in Figure 1. The ends of both the separation capillary and the platinum electrodes were immersed in buffer vials to complete the electrical circuit. The constant potential required for separation was generated by a negative polarity high-voltage power supply (Glasman High Voltage, Inc., Whitehouse Station, NJ).

For direct fluorescence detection of the ethidium bromide stained DNA, an argon ion laser operated at 488 nm (Cyonics, San Jose, CA; model 2213) was used for excitation. For indirect fluorescence detection using the background fluorophore salicylate, excitation was performed with a stabilized helium-cadmium laser operated at 325 nm (Liconix, Santa Clara, CA, Model 4240). Laser stabilization was achieved with a laser stabilizer (Cambridge Research, Cambridge, MA, Model LS100). The laser beam was focused onto the capillary with a quartz focusing lens (1 cm focal length). A 10x microscope objective was placed about 0.5 cm away from the capillary to

collect the emission. The collected light passed through a red or blue glass filter and was then detected by a silicon photodiode (Hamamatsu, Middlesex, NJ; Model HC220-01). The voltage signal from the photodiode was monitored by a strip-chart recorder (Measurement Technology, Denver, CO) or a PC-compatible computer equipped with the ChromPerfect Direct data acquisition interface (Justice Innovations, Palo Alto, CA).

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HydroLink Gel-Filled Capillary

A 75 μm i.d. fused-silica capillary was washed for 5 minutes sequentially with 1 N NaOH and water. Before gel casting, a window for on-column detection was created on the capillary by burning off a small portion of the polyimide coating. The window was then carefully washed with methanol. A 30% HL gel solution was prepared by diluting the stock HL solution with 2 mM sodium borate buffer (pH 9.1). Next, 5 μl TEMED and 150 μl freshly prepared 10% ammonium persulfate were added to the diluted HL solution. After mixing, the resulting solution was carefully loaded into the capillary with a syringe to avoid the introduction of air bubble. Polymerization of the gel inside the capillary was completed in less than 1 hour at room temperature. Before sample injection, the gel-filled capillary was prerun for 1 hour in 1 mM borate buffer containing 0.4 μg/ml ethidium bromide (EBr). EBr was not added to the buffer for absorption detection.

Non-Gel Capillary

A stock solution of approximately 1% hydroxypropylmethyl cellulose (HPMC) was prepared by dissolving HPMC in water at room temperature or as follows. 2 g of HPMC was added to 100 ml hot water (about 90 °C) and this mixture was then agitated until the cellulose particles were wet and evenly dispersed. Next, 100 ml cold water was added and the resulting mixture was continuously agitated for at least half an hour. The electrophoresis buffer was prepared by di-

luting the stock cellulose solution with the desired buffer. The separation is performed in a GC capillary having an i.d. of 50 μm (DB-1) or 100 μm (BP-1). A detection window was formed by removing a small portion of the GC capillary coating with hot concentrated sulfuric acid. This was followed by a methanol wash. To fill the capillary with the running buffer, a syringe was used. The capillary was flushed with the running buffer after each run. For both gel-filled and non-gel capillary separation, sample loadings were performed by electromigration.

RESULTS AND DISCUSSION

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HydroLink Gel-Filled Capillary Electrophoresis

Since polyacrylamide gel has already provided an excellent sieving medium for the separation of DNA fragments in slab gel electrophoresis (1), its application in capillary gel electrophoresis is straightforward. Indeed, gel-filled capillaries are commercially available. However, those capillaries are designed mainly for the purification of small DNA fragments (e.g., synthetic oligonucleotides) due to the small pore size of the gel matrix. Cohen et al. have reported using a 75 μm polyacrylamide gel-filled capillary for the separation of proteins, peptides, and nucleic acids (22-24). To enhance the stability of the gel-filled capillary, their gel casting procedure involves the covalent binding of polyacrylamide gel to the capillary wall via a bifunctional agent, (methylacryloxypropyl)-trimethoxysilane. However, routine production of reliable capillary gels was difficult (25). Nevertheless, Kasper et al. (16) used a 100 μm i.d. polyacrylamide gel-filled capillary (3% T, 5% C) to separate Hae III/ϕX174 DNA with modest success.

In this study we used a new type of gel matrix, the HydroLink (HL) gel, instead of using polyacrylamide gel as the sieving medium. HL gel was well characterized (26) and its application in slab gel DNA electrophoresis has been studied in detail (27,28). Compared to agarose and polyacrylamide gels, HL gel offers higher mechanical

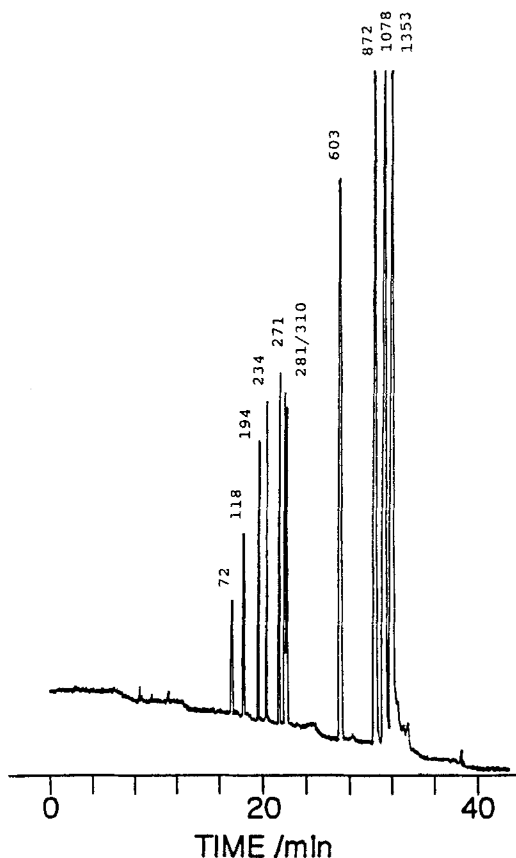

FIGURE 2. Gel-filled CE separation of Hae III/ϕX174 DNA fragments using 30% HydroLink gel. Column: 75 µm i.d. x 50 cm total length, 30-cm effective length. Buffer: 1 mM borate (pH 9.1). Sample concentration: 0.13 µg/µl. Injection: 5 s at -3 KV. Running voltage: -6 KV. Absorption detection at 260 nm. Peaks are labeled as base pairs.

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strength, ten times the sample loading capacity, and higher band resolution.

Figure 2 shows the separation of Hae III/ϕX174 DNA fragments using a 75 µm i.d., 30% HL gel-filled capillary with UV detection. It is obvious that the separation performance of this gel capil-

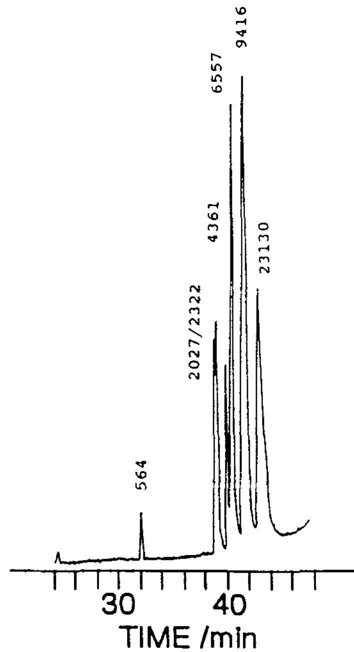

FIGURE 3. Gel-filled separation of Hind III/lambda DNA fragments using 30% HydroLink gel. Column: 75 μm i.d. x 50 cm total length, 30-cm effective length. Buffer: 1 mM borate (pH 9.1). Sample concentration: 0.18 μg/μl. Injection: 3 s at -3 KV. Running voltage: -6 KV. Absorption detection at 260 nm. Peaks are labeled as base pairs.

lary is very good. All of the 11 Hae III-digested fragments, ranging in size from 72 to 1353 bp, were well separated in about 35 minutes. Figure 3 shows the HL capillary gel separation of the Hind III-digested fragments of lambda DNA. Again, we achieved reasonably good separation of the fragments, ranging from 564 to 23130 bp, in about 45 minutes.

In slab gel electrophoresis, for high sensitivity and simplicity, DNA fragments are mostly detected by fluorescence staining. This is usually done by complexing DNA fragments with the fluorescent dye, ethidium bromide (EBr), and monitoring the red fluorescence of the

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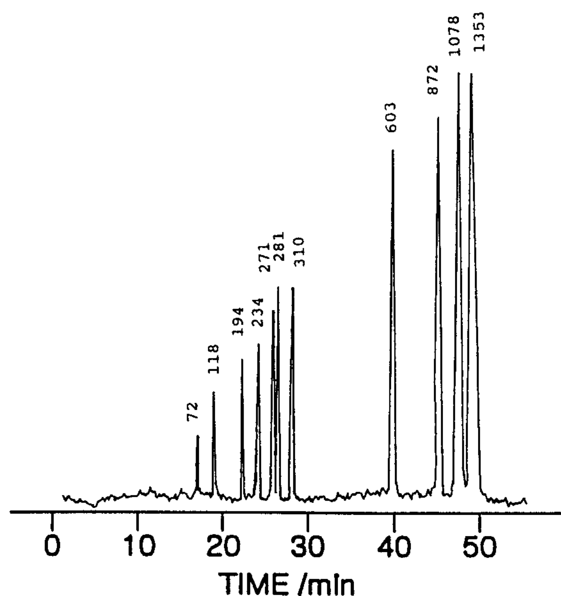

FIGURE 4. Gel-filled CE separation of Hae III/μX174 DNA fragments using 30% HydroLink gel with fluorescence detection. Column: 75 μm i.d. x 40 cm total length, 25 cm effective length. Buffer: 1 mM borate (pH 9.1) containing 0.4 μg/ml ethidium bromide. Sample concentration: 0.13 μg/μl. Injection: 1 s at -1 KV. Running voltage: -2 KV. Peaks are labeled as base pairs.

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DNA-dye complex. Figure 4 shows the electropherogram for the HL gel-filled capillary separation of Hae III-digested DNA fragments with fluorescence detection. To eliminate co-polymerization with the gel matrix, EBr was not added during the gel casting step. Instead, it was introduced into the capillary gel by electromigration when the running buffer containing the dye was used. At pH 9.1 the capillary wall is negatively-charged due to the ionization of the silanol groups on the wall surface. In an open capillary, the positively-charged ethidium ion sticks to the wall by electrostatic attraction. This results in a high fluorescence background that makes fluorescence detection of DNA difficult. In addition, any interaction between DNA

and the ethidium-coated capillary can degrade separation efficiency. Such problems did not occur in these studies. This may be due to the fact that the HL gel, like the polyacrylamide gel, can effectively shield the capillary wall to prevent its interaction with the dye.

Reproducibility in gel preparation and long-term stability are common problems in polyacrylamide gel-filled capillary separation. Our studies show that the success rate for casting a 75 μm i.d., 30% HL capillary gel is around 60%. For each good capillary, more than 15 sample injections can be performed with no significant loss in separation performance. Of course, further studies are needed to determine the factors that affect the casting reproducibility, stability, and separation performance of the HydroLink capillary gel. We also tried casting HL gel in a 50 μm i.d. capillary, but the reproducibility of gel casting and the gel stability was poor.

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Non-Gel Capillary Electrophoresis

Zhu et. al. first demonstrated that electrophoresis buffers containing cellulose additives (i.e., non-gel) such as methyl- and hydroxypropylmethyl-cellulose can provide a molecular-sieving capability for the CE separation of nucleic acids (18). Once dissolved, cellulose molecules form a linear polymer network that contributes to the viscous property of the solution. The separation mechanism for the cellulose buffers is not fully understood; however, the polymer network is believed to form dynamic pores, similar to the stationary pores found in agarose and polyacrylamide gel, which provides molecular sieving for size-dependent separation (29).

Non-gel DNA separations have been performed in linear polyacrylamide in coated (18-20) and uncoated (21) capillaries. The coated capillaries provide higher separation efficiencies. This coating serves two purposes. First, it prevents DNA from interacting with the negatively-charged capillary wall because such interaction will degrade the separation efficiency (19). Second, the coating eliminates electroosmotic flow to allow molecules to be separated mainly based on their electrophoretic mobilities. We have tried coating the walls of

different i.d. capillaries with linear polyacrylamide (30); but our experience is that under our working conditions, the run-to-run and column-to-column reproducibility of these coated capillaries was poor. In addition, the coating procedure can be difficult with a narrow capillary (e.g., 50 μm).

Most stationary phases of gas chromatography (GC) columns are composed of polysiloxane-based polymers with different functional groups. For example, both the DB-1 and BP-1 GC capillaries are coated with polymethylsiloxane polymer. These types of polysiloxane coatings have been used in microcolumn liquid chromatography to cover the negatively-charged fused-silica wall (31). The coating procedures for most commercially available GC columns are fully automated and well controlled. Therefore, variations in coating thickness, degree of polymerization, and column-to-column performance should be minimum. GC capillaries with different i.d. can be easily obtained commercially. These GC capillaries can be useful for most CE applications that require a coated capillary.

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Figure 5 shows an electropherogram using non-gel CE separation for Hae III/pBR 322 DNA. 50 μm i.d., DB-1 GC capillary was used. These DNA markers consist of 22 fragments ranging from 8 to 587 bp. Under the indicated conditions, all 21 fragments (except the 8 bp) were well separated in less than 25 minutes. The 8 bp fragment may well be separated but was not detected in this run. The response in Figure 5 is consistent with the additive contribution of each bp to absorption. The high separation efficiency of this system is highlighted by the resolution of two fragments differing by only 1 bp (i.e., the 123 and 124 bp). The average number of theoretical plates per meter for this capillary was calculated to be over one million. A high separation power is also demonstrated in Figure 6. Here, the complete separation of the 12 Bgl I/Hinf I-digested fragments of pBR328 DNA was achieved in less than 30 minutes using the same conditions.

Figure 7 shows the non-gel CE separation of the high-range DNA markers, the Hind III digest of lambda DNA. Six of the 7 fragments were separated in less than 17 minutes. The 4361 and the

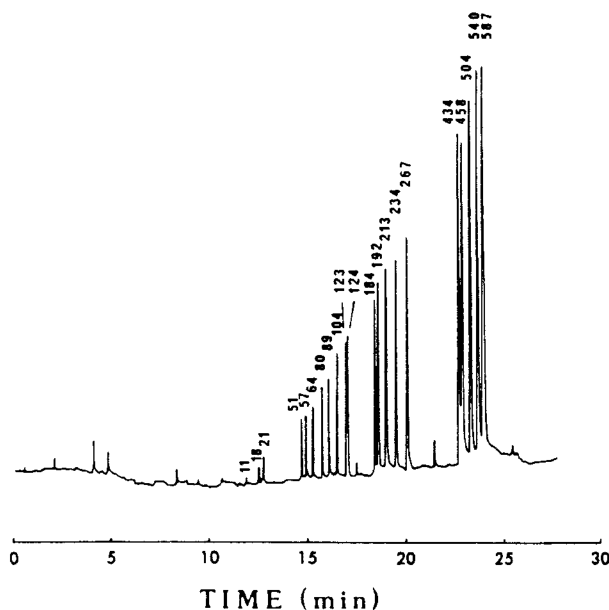

FIGURE 5. Non-gel CE separation of Hae III/pBR322 DNA fragments with fluorescence detection. Column: DB-1 GC capillary, 50 μm i.d. x 60 cm total length, 50 cm effective length. Buffer: 1 mM borate (pH 8.5) containing 0.5 μg/ml ethidium bromide and 0.5% HPMC (4000 cP). Sample concentration: 50 μg/ml. Injection: 1 s at -10KV. Running voltage: -15KV. Peaks are labeled as base pairs.

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23130 bp fragments possess the 12-base cohesive ends of the lambda DNA so that they will hybridize during storage. This is why the signal of the 4361 bp fragment peak was weak. The 2027 and 2322 bp fragments were not separated under the indicated conditions. Figure 8 shows the separation of another set of high-range markers, the EcoR I digest of lambda DNA. This set of markers consists of six fragments ranging in size from 3530 to 21226 bp. The 5643 and 5804 bp fragments were not separated at the indicated condition. In fact, it is known that resolution of fragments larger than 1000 bp using non-gel separation was relatively poor (19,20).

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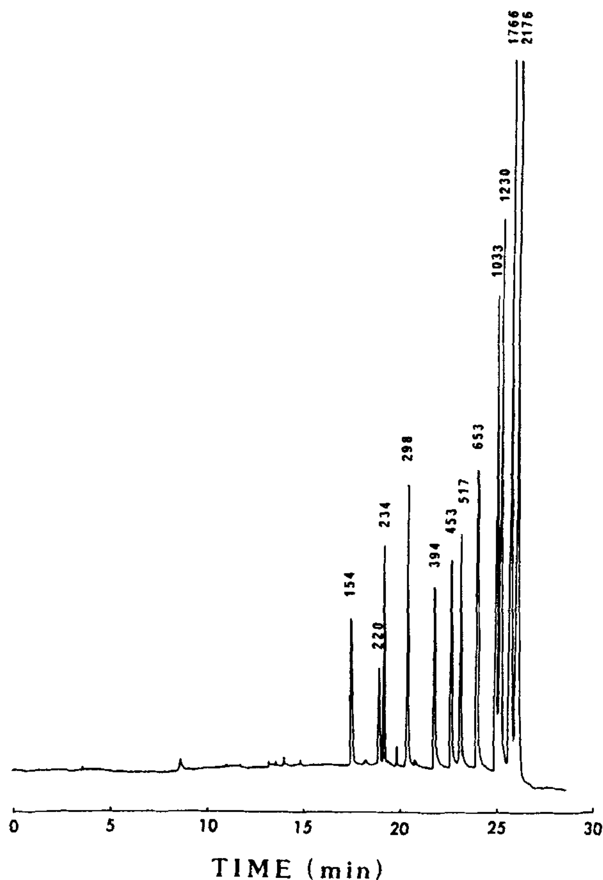

FIGURE 6. Non-gel CE separation of Bgl I/pBR328 + Hinf I/pBR328 DNA fragments with fluorescence detection. Column: DB-1 GC capillary, 50 μm i.d. x 60 cm total length, 50 cm effective length. Buffer: 1 mM borate (pH 8.5) containing 0.5 μg/ml ethidium bromide and 0.5% HPMC (4000 cP). Sample concentration: 50 μg/ml. Injection: 1 s at -10KV. Running voltage: -15KV. Peaks are labeled as base pairs.

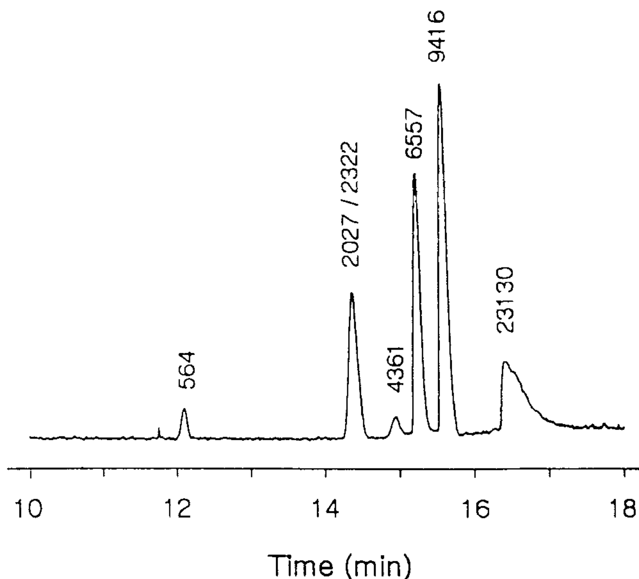

FIGURE 7. Non-gel CE separation of Hind III/lambda DNA fragments with fluorescence detection. Column: BP-1 GC capillary, 100 μm i.d. x 31 cm total length, 25 cm effective length. Buffer: 2 mM sodium phosphate (pH 7.6) containing 0.47% HPMC (15000 cP) and 0.5 μg/ml ethidium bromide. Sample concentration: 90 μg/ml prepared in the running buffer. Injection: 10 sec at -0.5 KV. Running voltage: -5 KV. Peaks are labeled as base pairs.

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The long-term stability of the GC columns is not ideal. At a buffer of pH 8.5, the DB-1 and BP-1 capillaries last for about 20 injections before degradation in performance is evident. This instability is due to the base-catalyzed hydrolysis of the siloxane bond at the silica surface (32). It will be very useful if a stable coated capillary designed for CE applications can be made commercially available in the future.

Since agarose gel has proven to be a good sieving medium for the separation of a wide range of DNA fragments (1), we also investigated the use of agarose gel-filled capillaries for DNA separation. Agarose gel cannot be cast inside bare fused-silica capillaries, since

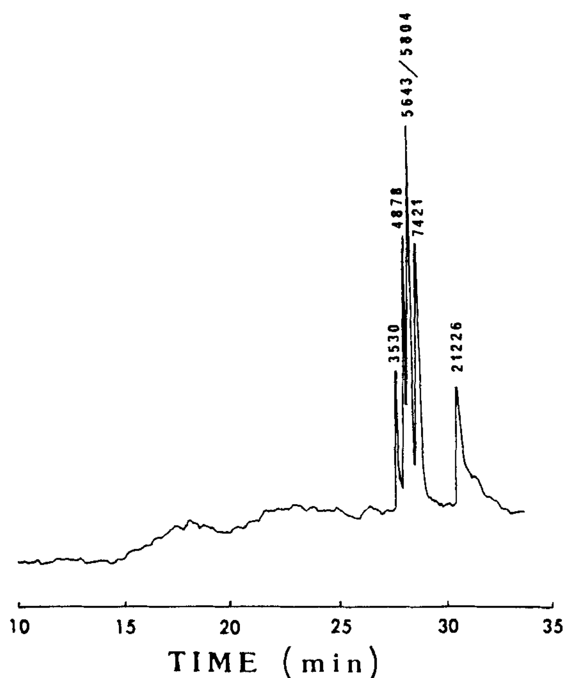

FIGURE 8. Non-gel CE separation of EcoR I/lambda DNA fragments with fluorescence detection. Column: DB-1 GC capillary, 50 μm i.d. x 60 cm total length, 50 cm effective length. Buffer: 1 mM borate (pH 8.5) containing 0.5 μg/ml ethidium bromide and 0.5% HPMC (4000 cP). Sample concentration: 50 μg/ml. Injection: 1 s at -10KV. Running voltage: -15KV. Peaks are labeled as base pairs.

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it will be washed out by electroosmotic flow. Casting of agarose gel in 50, 150, and 250 μm i.d. GC capillaries was attempted. However, most of the time DNA separation was only successful with the 250 μm capillary. The poor and irreproducible separation obtained with the other two sizes of capillaries was probably due to the formation of inhomogeneous gel matrix inside a narrow capillary.

Indirect Fluorescence Detection

As we have shown above, staining DNA fragments with ethidium bromide enables the use of laser-induced fluorescence detection,

which is one of the most sensitive detection schemes for CE today. However, this staining procedure is not effective for certain nucleic acids such as small oligonucleotides and single-stranded DNA. In addition, the ethidium ion changes the conformation and charge distribution of DNA molecules, resulting in a change in mobilities (33). These changes may cause complications in performing size determination in mapping. The presence of ethidium ion in the running buffer also can degrade detectability because of a larger background fluorescence signal.

An attractive alternative to fluorescence staining of DNA fragments is the use of indirect fluorometry. This technique has proved to be universal, efficient, sensitive, and non-destructive (34). The application of indirect fluorometry in CZE has been reviewed recently (35). Briefly, a non-interacting, fluorescing ion is added to the running buffer to create a constant fluorescence background. When a charged analyte is present, it displaces the fluorescing ion of same charge due to local charge neutrality, resulting in a decreased background signal even though the analyte does not absorb or fluoresce. This indirect method has been applied in CZE for the detection of metal ions (36,37), sugars (38), amino acids (39), peptides (40), and nucleotides (41).

The concentration detection limit (C_{lim}) of indirect detection is described by the equation (42), $C_{lim} = C_m/TR \times DR$. C_m is the concentration of background fluorophore. TR is the transfer ratio which is defined as the number of fluorophore molecules displaced per analyte molecule. DR is the dynamic reserve which is defined as the ratio between the background fluorescence signal to its noise level. To obtain the best detection limit, one can use very low ionic strength fluorophore buffer. However, separation of biomolecules with low ionic strength buffers can be difficult. As a result, the requirements for obtaining optimum detection and optimum separation oppose each other. Thus, one has to compromise both the detection and separation to make indirect detection practical.

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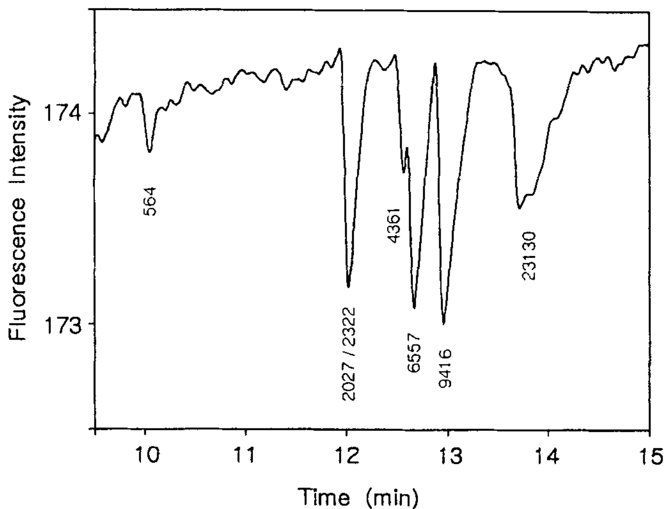

FIGURE 9. Electropherogram for the CE separation of Hind III/lambda DNA fragments with indirect fluorescence detection. Column: BP-1 GC capillary, 100 μm i.d. x 25 cm total length, 20 cm effective length. Buffer: 1 mM sodium phosphate (pH 7.6), 1 mM sodium salicylate, and 0.47% HPMC (15000 cP). Sample concentration: 0.26 μg/μl. Injection: 1 min at -0.5 KV. Running voltage: -4 KV. Peaks are labeled as base pairs.

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A typical electropherogram for the non-gel CE separation of Hind III/lambda DNA fragments with indirect fluorescence detection is shown in Figure 9. The running buffer contained 1 mM sodium phosphate (pH 7.6), 1 mM sodium salicylate, and 0.47% HPMC. Under this running condition, most of the Hind III digested fragments were separated. The observed indirect DNA peaks were confirmed by direct fluorescence detection with ethidium bromide (Figure 10) and by the indirect detection of the same sample in agarose slab gel electrophoresis (Figure 11). The concentration of DNA used in this study was comparable to that used for absorption detection. Better results can be anticipated once the conditions for separation and detection are further optimized.

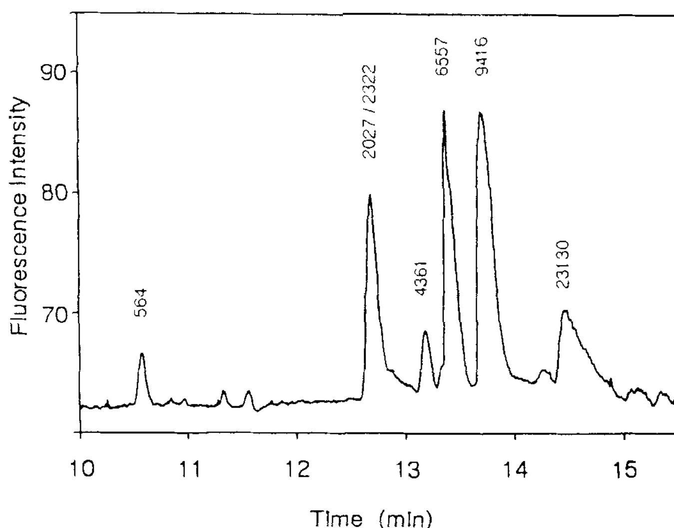

FIGURE 10. Electropherogram for the CE separation of Hind III/lambda DNA fragments with direct fluorescence detection. Column: BP-1 GC capillary, 100 μm i.d. x 25 cm total length, 20 cm effective length. Buffer: 1 mM sodium phosphate (pH 7.6), 1 mM sodium salicylate, 0.47% HPMC (15000 cP), and 0.1 μg/ml ethidium bromide. Sample concentration: 0.26 μg/μl. Injection: 30 s at -0.5 KV. Running voltage: -4 KV. Peaks are labeled as base pairs.

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CONCLUSIONS

Gel-filled and non-gel capillary electrophoresis proved to be promising methods for the separation of DNA fragments. The advantages of these methods include fast analysis time, high separation efficiency, small sample size, real-time detection, easy quantitation, and suitability for automated operation. Although CE lacks the suitability for multiple samples application as in the slab gel system, the throughput of CE can be improved by running parallel capillaries simultaneously with an array detection system (43). Compared to polyacrylamide capillary gel, the HydroLink capillary gel is simpler to prepare. Because of its high loading capacity, the HL capillary gel

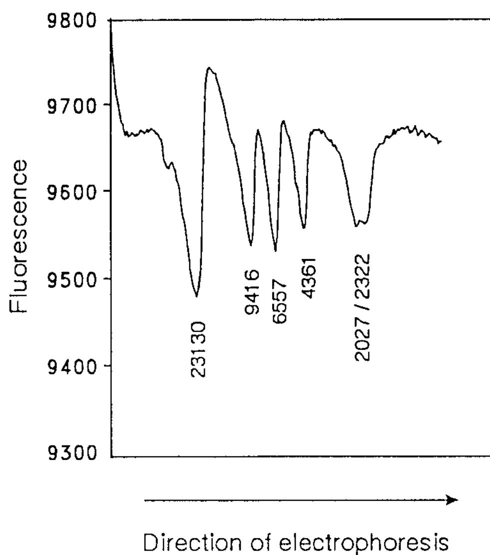

FIGURE 11. Slab gel separation of Hind III/lambda DNA fragments with indirect fluorescence detection. Buffer: 7.5 mM Tris base, 0.5 mM Tris phosphate, 0.4 mM fluorescein, 0.05 mM EDTA at pH 8.2. Gel: Bio-Rad 0.25% ultrapure grade agarose.

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is valuable in semi-preparative CE applications. To characterize the HL gel-filled capillary further, additional studies are needed to determine the factors that affect the gel preparation reproducibility, separation efficiency, and stability.

Non-gel capillary electrophoresis performed inside a GC capillary is a simple and efficient method for the separation of DNA fragments. The separation matrix can be easily replaced by flushing the capillary with the sieving buffer. The GC capillary eliminates the inconvenient steps for reproducibly coating the capillary wall. The run-to-run and column-to-column reproducibility of GC capillaries used in this study was reasonably good. The major limitation of the GC columns is a short life-span caused by the base-catalyzed hydrolysis of the siloxane bond on the capillary surface.

Our results show that detection of native DNA fragments by indirect fluorometry is feasible in non-gel capillary electrophoresis. The method is non-destructive as staining of the DNA is not required for detection. Since indirect fluorometry monitors the background fluorescence signal for detection rather than the analyte itself, dyes with different physical and chemical properties can be selected for optimum separation and detection.

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