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Capillary gel electrophoresis for DNA sequencing

Laser-induced fluorescence detection with the sheath flow cuvette

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ABSTRACT

Capillary polyacrylamide gel electrophoresis separation of dideoxycytidine chain-terminated DNA fragments is reported. A post-column laser-induced fluorescence detector based on the sheath flow cuvette was used to minimize background signals due to light scatter from the gel and capillary. A preliminary mass detection limit of 10^{-20} mol of fluorescein-labeled DNA fragments was obtained. The system was used to analyze an actual DNA sequencing sample. Theoretical plate counts of $2 \cdot 10^6$ were produced. Gel stability limits the performance of the current system.

INTRODUCTION

Capillary gel electrophoresis promises significant performance improvements when compared with conventional slab-gel electrophoresis. These advantages arise from the large surface-to-volume ratio of capillaries, providing rapid dissipation of Joule heat generated during electrophoresis. This efficient heat transfer allows use of high electric field strengths to generate fast, efficient separations. Early work with capillary-dimension electrophoresis media was performed by Edstrom[1,2], in the 1950s, who described the use of very fine cellulose fibers (5-μm diameter) for electrophoresis of nucleic acids from single cells at an electric-field gradient of 125 V/cm. In 1965, Matioli and Niewisch[3] studied hemoglobin from single cells on fine polyacrylamide fibers of 50-μm diameter. Grossbach[4] reported the use of gel-filled glass capillaries of 50-μm diameter in 1974. Slightly larger scale capillary polyacrylamide gel electrophoresis was reported in 1970 by Neuhoff *et al.*[5] who used 5-μl capillaries for the study of ribonucleic acid polymerase. In 1983, Hjertén[6] reported the use of a 150-μm I.D. capillary polyacrylamide electrophoresis separation of several samples

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derived from bovine serum albumin. Beginning in 1987, Karger and co-workers[7–10] have reported capillary polyacrylamide gel electrophoresis for the separation of proteins, chiral amino acids and polydeoxyadenylic acid fragments. Karger and Cohen[11,12] received two patents describing both the use of a bifunctional reagent to chemically bind polyacrylamide to the capillary surface and the use of a polymeric additive to enhance the stability of gel-filled capillaries. Swerdlow and Gesteland[13] have described the use of capilllary polyacrylamide gel electrophoresis with on-column fluorescence detection to analyze actual DNA sequencing samples.

Recently, interest in the Human Genome Project has led to the study of more rapid DNA analysis. The application of capillary polyacrylamide gel electrophoresis to DNA sequencing appears to offer three advantages compared with conventional slab-gel electrophoresis. First, and most importantly, the high electric field that can be applied to the capillary system should increase the resolution of the separation, allowing longer segments of DNA to be analyzed in a single run. Second, the capillary electrophoresis instrument offers improved potential for automation. Third, the high electric field strength utilized in the capillary system should increase the speed of separating DNA sequencing samples. However, this last advantage can only be exploited if multiple capillary gels can be simultaneously run in the same instrument.

The application of capillary polyacrylamide gel electrophoresis to DNA analysis is currently limited by two problems. First, although partially addressed by Karger and Cohen[11,12], the routine production of reliable, bubble-free gels that can be operated at high electric field strength remains problematic. Second, the nature of the DNA sequencing reactions requires a high-sensitivity detection system[14]. Recent advances in automated DNA sequencing rely on fluorescence of labeled DNA fragments that have been separated by slab-gel electrophoresis[14,15]. However, application of laser-induced fluorescence detection to capillary gel electrophoresis is not trivial. On-column fluorescence detection apparently suffers from a significant component of scattered light generated both in the gel and from the capillary walls[15].

To overcome these detector limitations, we have turned to the sheath flow cuvette, commonly employed as a detection chamber in the biomedical technique of flow cytometry[16] and proven as a high-sensitivity fluorescence detector for capillary zone electrophoresis[17–19]. In the cuvette, a sample stream is injected, under laminar flow conditions, in the center of a surrounding sheath stream, generally of the same refractive index. Our cuvette, taken from an Ortho cytofluorograph, is made of quartz, has optically flat windows and has a 250-μm square inner cross-section. Because the sample stream flows in the center of the sheath fluid in the high-optical-quality flow chamber, the contribution to the background signal due to light scatter from the windows is negligible. Extremely high-sensitivity fluorescence detection has been produced with the cuvette approaching single molecule detection[20–24].

Application of the sheath flow cuvette to capillary polyacrylamide gel electrophoresis could suffer from a subtle problem. In capillary zone electrophoresis, the electroosmotic mobility of the solvent is normally significantly greater than the electrophoretic mobility of the analytes. As a result, the analytes are swept from the capillary to the cathodic detector by the solvent flow produced by electroosmosis. In gel-filled capillaries, by comparison, electroosmosis is greatly reduced; the electrophoretic mobility of the analyte is appreciably larger than the electroosmotic mobility of the solvent. Furthermore, in the application of capillary gel electrophoresis to

DNA separations, the polarity of the power supply is reversed compared with conventional zone electrophoresis; bulk solvent flow is directed from the detector (anode) toward the injection end (cathode). This bulk flow from the detection cuvette could distort the hydrodynamic characteristics of the sheath flow cuvette[25]. It is not at all clear that the sheath flow cuvette will work well when used with capillary gel electrophoresis.

This paper demonstrates that the sheath flow cuvette functions well as a detector for capillary gel electrophoresis, independent of the relative magnitudes of electroosmosis and electrophoresis and independent of the direction of electroosmosis. Sheath flow cuvette-based fluorescence detection is employed for the analysis of an actual DNA sequencing sample.

EXPERIMENTAL

Materials

All solutions were prepared in filtered HPLC-grade water. Capillaries, 50 μm I.D. and 200 μm O.D. from Polymicro Technologies (Phoenix, AZ, U.S.A.) were used as received with no pre-treatment. Acrylamide, N,N'-methylenebisacrylamide (Bis) and N,N,N',N'-tetramethylethylenediamine (TEMED) were electrophoresis-purity reagents from Bio-Rad (Mississauga, Canada). Tris base and urea were ultrapure reagents (ICN, Montreal, Canada). Boric acid and EDTA were analytical-reagent grade (BDH, Edmonton, Canada) and ammonium persulfate was ultrapure electrophoresis grade (Boehringer Mannheim, Laval, Canada).

The DNA sequencing sample was prepared as described previously[13]. The 20-, 21-, 100- and 101-nucleotide long oligodeoxythymidylic acid marker DNAs were prepared on a Model 380A DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) using amino link 2 dye attachment reagent (Applied Biosystems) as the 5'-terminal monomer. Fluorescein isothiocyanate (400 μg) was reacted with 100 μg of the deprotected oligomer in 25 μl of 50 m*M* carbonate buffer at pH 9. The labeled oligonucleotides were purified chromatographically on a Sephadex G-25 column and by electrophoresis on a conventional 10% sequencing gel. Bands on the gel were identified by their fluorescence, eluted from the gel, concentrated by ethanol precipitation, and concentration determined in a fluorimeter.

Methods

A stock 10 × TBE (pH 8.3) solution was prepared by dissolving 108 g Tris, 55 g boric acid and 40 ml of 0.5 *M* EDTA in water to a final volume of 1 l. Capillaries (50 cm long) were filled with a degassed 6% T, 5% C[a] acrylamide/Bis, 8 *M* urea, 0.07% (w/v) ammonium persulfate, 0.07% (v/v) TEMED, solution prepared in a 1- to 10-dilution of the stock TBE buffer (1 × TBE final), and allowed to polymerize[13].

Electrophoresis was driven by a 30-kV power supply (Spellman, Plainview, NY, U.S.A.). The laser-induced fluorescence detector, based on a sheath flow cuvette, was identical to that described previously, except that the sheath fluid was 1 × TBE at a

[a] C = g N,N'-methylenebisacrylamide Bis/(g Bis + g acrylamide); T = g acrylamide + g Bis/100 ml solution.

flow-rate of 0.5 ml/h[17]. Samples were loaded on the capillary electrophoretically as described previously[13].

RESULTS AND DISCUSSION

In capillary gel electrophoresis, as applied to DNA sequencing, the electroosmotically induced bulk flow is directed from the detection cuvette to the injector. Thus it is not clear that the sheath flow cuvette will operate effectively. To test this possibility a mixture of fluorescein and fluorescein-labeled 20-, 21-, 100- and 101-nucleotide long oligodeoxythymidylic acid marker DNAs was injected onto a 50-cm long 6% T, 5% C, 8 *M* urea, polyacrylamide gel-filled capillary and electrophoresed at 200 V/cm. The instrument performed well; the electropherogram is displayed in Fig. 1. The first peak, at 35 min, is due to fluorescein, followed by the 20- and 21-mer doublet, and the 100- and 101-mer doublet. It appears that the electrophoretic velocity of the analyte is sufficiently high to inject the eluted sample quantitatively into the sheath stream, in spite of the fact that for the same sample electroosmotic mobility dominates over electrophoretic mobility in capillary zone electrophoresis (CZE; data not shown). Although these peaks are nearly baseline resolved, the plate count for the separation is rather low, about $1 \cdot 10^6$. We believe this is due to chemical inhomogeneity in the synthesized marker DNAs and not to inherent limitations of the system.

We estimated the mass detection limit for the 20-nucleotide long marker by two methods; the first based on the calculated injection volume and the known concentration of the sample, the second based on the signal observed for a standard solution of fluorescein and the area of the eluted peak. The detection limit of the sheath flow cuvette-based detector by both methods is about 10^{-20} mol of DNA injected on the capillary. This mass detection limit is about an order of magnitude poorer than that obtained for fluorescein thiocarbamyl derivatives of amino acids, but significantly better than other reported limits for fluorescent DNA sequencing[13–15]. The poor detection limit may arise from two sources. First, the fluorescence detection electronics were operated with a short time-constant, about 0.3 s, which was optimized for CZE separations. The time constant should be increased by about an order of magnitude to match the peak widths of the slower DNA analyses. Second, the low sample stream flow-rate, and its retrograde direction, results in a smaller sample-stream

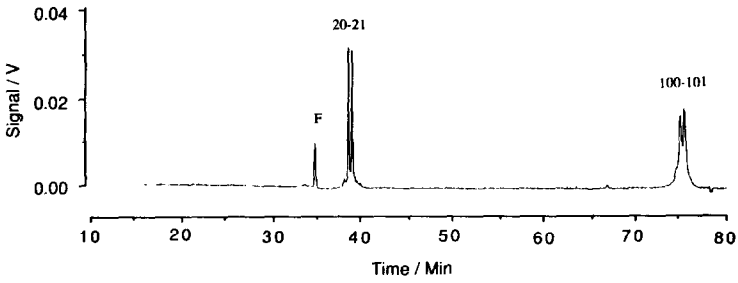

Fig. 1. Capillary gel electropherogram of synthetic fluorescently labelled oligodeoxythymidylic acid marker DNA molecules. F = Fluorescein. The other four peaks are oligodeoxythymidilic acid; numbering corresponds to the length in nucleotides.

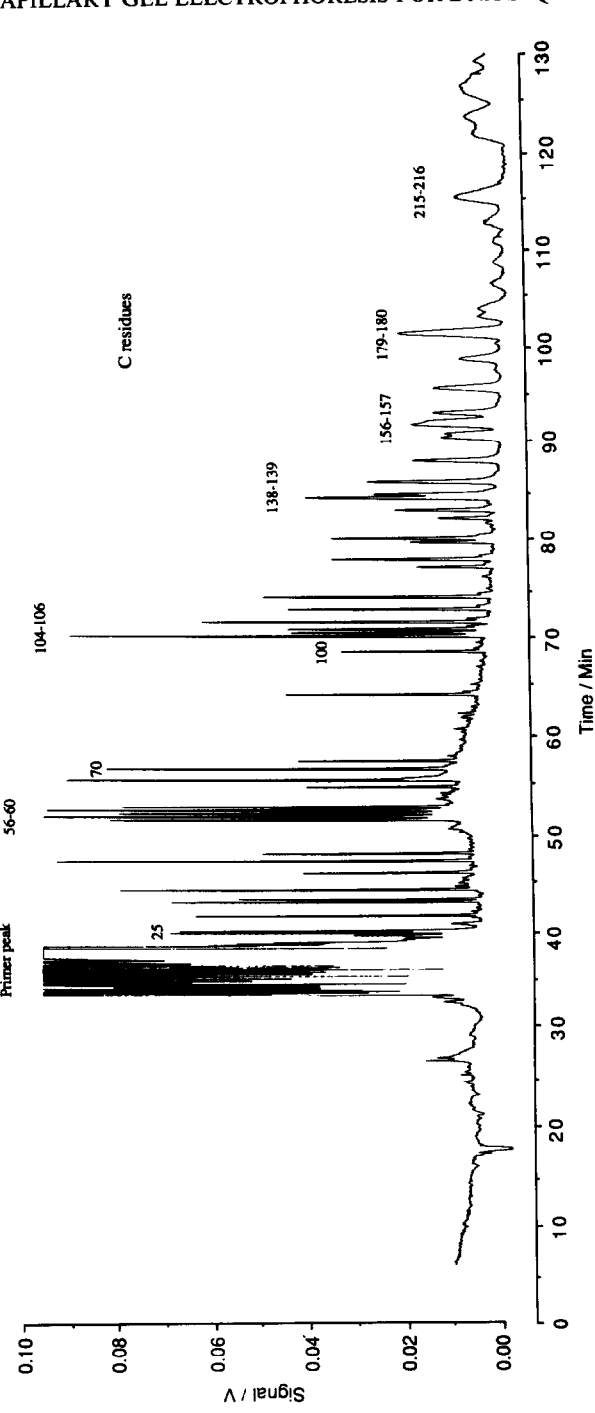

Fig. 2. Capillary gel electropherogram of a DNA sequencing reaction terminated with dideoxycytidine triphosphate. Each peak following the peak labeled 25 corresponds one-to-one to a C residue in the known DNA sequence. Peak numbering is according to total length of oligonucleotide fragments.

diameter passing through the detection region. Improved detection limits should follow by optimizing the sheath flow-rate to match the size of the image of the sample stream to the size of the pinhole in the collection optical train.

To assess the capabilities of our system for DNA sequencing, an actual DNA sequencing sample was run on a gel-filled capillary and visualized with the sheath flow cuvette-based detector (Fig. 2). A fluorescently labeled 18-nucleotide primer was chain extended, on a complementary template, in the presence of dideoxy cytidine triphosphate[13]. The resulting C-terminated oligonucleotides were electrophoresed in an identical manner to the separation of Fig. 1; the electropherogram is displayed in Fig. 2. The pattern of peaks at the beginning of the electropherogram are associated with the fluorescently labeled primer, it appears that this commercially produced primer is not pure but instead contains at least 25 minor components. The remaining peaks in Fig. 2 correspond directly to C residues in the known DNA sequence. The separation of DNA fragments is quite good in the region 25–140 bases. In particular, five consecutive cytidine residues, 56–60 nucleotides in length, are very well resolved in this analysis. The number of theoretical plates in the separation maximizes at about $2 \cdot 10^6$ in the range 25–100 bases.

Two rather significant problems appear to limit resolution of DNA oligomers in our system. First, the chemical inhomogeneity in the primer peak probably contributes significantly to broadening of the other peaks; the expected 25-nucleotide long oligonucleotide appears as a multiplet, although for larger peaks the subspecies do not resolve. Second, the separation degrades very rapidly for fragments longer than 140 bases. The origin of this degradation has been correlated with formation of one or more small bubbles at the injection end of the capillary during the separation run. This bubble formed after the start of the separation. Analytes that had passed the region where the bubble formed were not affected by its presence, whereas slower analytes suffered from reduced plate count due to eddy diffusion. Curiously, Guttman *et al.*[10] has observed poor resolution past 150 nucleotides, also on a 6% T, 5% C column. He attributes this effect to restricted migration through the gel matrix for large-molecular-weight DNA molecules. Restricted migration cannot explain the observation, however, as separations performed on 6% T, 5% C gels in both capillary and slab-gel electrophoresis, yield satisfactory resolution to at least 300 bases[13–15].

The separations reported in this paper did not use gels that had been covalently attached to the walls. Karger's results with bifunctional silane reagents offer an excellent example of the potential of capillary gel techniques. Hopefully, by increasing the stability of the gel-filled capillaries, DNA sequence analysis that exploits the full separation properties of capillary gel electrophoresis will become a reality.

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