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Capillary gel electrophoresis of single-stranded DNA fragments with UV detection☆

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ABSTRACT

Capillary gel electrophoresis has proven to be a powerful tool in biomedical research. We report our investigation of some of the critical parameters affecting separations of single-stranded DNA fragments as monitored by ultraviolet (UV) absorbance detection. Although not as sensitive as laser-induced fluorescence (LIF), UV absorbance detection allows one to calculate quite accurately, and inexpensively, the molarity of each separated DNA fragment and, moreover, the signal "fading" effect normally observed with LIF detection can be, in many cases, substituted for fluorescence to detect the many different single-stranded DNAs, as well as for detection of sequencing reactions.

INTRODUCTION

Capillary gel electrophoresis (CGE), a new analytical and micropreparative technique, is being rapidly developed [1–6]. Included among the advantages of CGE are ultraresolution, extremely high efficiency, rapid separation time, easy quantitation and amenability to automation. Many interesting applications in the biomedical field are just now emerging. One of the newly attractive applications of CGE is separation and identification of DNA sequencing mixtures, where the fragments have one common 5′-endpoint and differ in successive length by a single nucleotide. Because of the very small sample band volumes (a few to tens of nl) and extremely low molar concentrations of separated DNA bands, a highly sensitive detection scheme is required. Laser-induced fluorescence (LIF) detection is, thus far, one of the most sensitive methods [7–13], that is applicable to molecules having at least one fluorescent label attached. However, the exploration of alternative detection schemes in the separation and identification of DNA sequencing samples is also interesting. We herein demonstrate that CGE coupled downstream with UV absorbance detection is likewise a very sensitive, accurate, and relatively inexpensive scheme for the separation and detection of single-stranded DNAs consisting of homopolymeric deoxyadenosine, deoxythymidine and dideoxythymidine terminated sequencing reaction derived from the single-stranded becteriophage, M13.

EXPERIMENTAL

Polyacrylamide gel-filled capillaries were prepared according to the procedure described by Karger and co-workers [1,2]. Fused-silica capillary (Polymicro

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Technologies, Phoenix, AZ, USA), 150 μm I.D. × 360 μm O.D., with a detection window at 30 to 60 cm from the inlet were used in most of the experiments. A 30-kV direct current power supply (Model MJ30N400; Glassman, Whitehouse Station, NJ, USA) was used to generate the electrical field across the capillary gel column. A UV absorbance detector (Model 757; Applied Biosystems, Ramsey, NJ, USA) with a capillary holder was used as an on-column detector, generally set at 260 nm. The UV absorbance output was monitored with a dual-channel chart recorder (Model BD41; Kipp & Zonen, Netherlands) and also acquired and stored on an IBM PC/AT computer using an analog/digital interface (Model 970; Nelson Analytical, Cupertino, CA, USA).

Tris(hydroxymethyl)aminomethane (Tris), boric acid, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), ammonium persulphate (APS) and urea were purchased from Fluka (Buchs, Switzerland). N,N,N′,N′-Tetramethylenediamine (TEMED) was supplied by Merck (Darmstadt, Germany). Methacryloxypropyltrimethoxysilane was supplied by Sigma (St. Louis, MO, USA). Premixed acrylamide–N,N′-methylenebisacrylamide (29:1) was purchased from Schwarz/Mann Biotech (Cleveland, OH, USA).

Polydeoxyadenylic acids [poly-d(A)] were purchased from Boehringer Mannheim (Indianapolis, IN, USA). The sample containing the oligodeoxythymidilic acids poly-d(T) 20–160 was synthesized at Genentech (South San Francisco, CA, USA). Electrophoresis buffer solutions were filtered through a Supelco (Gland, CH, USA) nylon 66 filter unit with a pore size of 0.25 μm before use.

Single-stranded M13 mp18 (Pharmacia, Piscataway, NJ, USA) was annealed with a universal sequencing primer (−40; New England Biolabs, Beverly, MA, USA), extended with deoxynucleotides (dNTPs) using T7 polymerase (US Biochemicals Corp., Cleveland, OH, USA) or Bst polymerase (Bio-Rad, Richmond, CA, USA) and terminated with dideoxythymidine. The molar ratio of dTTP to dideoxythymidine (ddTTP) was generally 20:1. Reactions were terminated by addition of one-tenth volume of 0.5 *M* EDTA, followed by phenol–chloroform extraction, ethanol precipitation, and lyophilization. Samples were resuspended in deionized formamide, 2 m*M* EDTA, and heated to >85°C for 3–5 min immediately prior to column application.

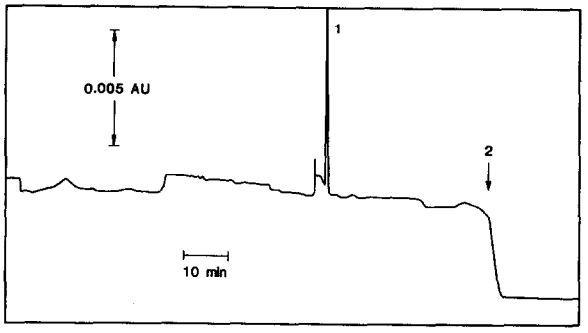

Fig. 1. A typical background UV absorbance trace recorded during pre-electrophoresis of a fresh gel-filled column: 1 = unidentified peak; 2 = sharp drop in UV absorbance. Capillary is 150 μm I.D.; 75 cm total length, 50 cm to detector. Gel composition is 4%T, 3.3%C. Electrolyte: TBE, 7 *M* urea; running voltage: 6 kV; UV absorbance at 260 nm.

RESULTS AND DISCUSSION

Pre-electrophoresis of gel-filled column

All columns were pre-run electrophoretically before any injection of DNA samples. Two phenomena were observed during pre-electrophoresis. First, at constant voltage the current steadily decreased until after a period of time (about 2 h under our experimental conditions), it reached a stable level. Secondly, the baseline UV absorbance is initially stable and then decreases sharply and finally stabilizes at a new lower level. These observations suggest that a mixture of ions from the polymerization, at least one of which is UV absorbing (acrylate?) are being eluted from the column. These small ionic substances occur along the entire length of a fresh gel-filled column. During pre-electrophoresis, they migrate toward the anode. With continuous depletion, the conductivity of the entire gel-filled column decreases resulting in the decrease of the current. When the plug of ions has finally migrated past the detector, a sharp drop in UV absorbance is observed. Fig. 1 gives a typical UV absorbance trace of a pre-run. For the gel-filled capillary with 150 μm I.D., 4%T and 3.3%C[a], 50 cm to detector at electrical field strength of 80 V/cm, the mean (n = 4)

[a] C = g N,N′-methylenebisacrylamide (Bis) /%T; T = (g acrylamide + g Bis)/100 ml solution.

migration rate was $1.0 \cdot 10^{-4}$ cm^2/V s. The mean (n = 4) background UV absorbance change at 260 nm was 4.5 mAU for the capillary with 150 μm I.D. (calibrated light path is 120 μm) or equivalent to 0.38 AU for 1 cm light path. Due to this large change in background of UV absorbance, the pre-run procedure is necessary.

Concentrating the sample during introduction in CGE

In CGE, the dimensions of the electrophoretic channel are very small, and it is difficult to introduce sample into the inlet of the capillary gel by a mechanical method. Instead, the electrokinetic injection method is usually employed; the sample ions are migrated into the column under the influence of an electrical field. A sample concentrating phenomenon occurs during electrokinetic injection when DNA fragments are prepared in electrolytes with low ionic strength. Fig. 2 demonstrates this "concentrating" effect during electrokinetic injection. A poly-d(T) sample is prepared in either deionized water or in 90 mM Tris–borate, 2.5 mM EDTA (TBE) running buffer, and then electrokinetically loaded at 15 kV for 5 s. In Table I, the UV absorbance of five consecutive peaks in the calibrated light path of the capillary is used to quantitate the extent of the concentration. It shows about 22 times more of the sample is injected when DNA is prepared in water than when it is prepared in 90 mM running buffer. This phenomenon can be explained as follows. At a constant injection voltage, the current passing the gel column is determined predominantly by the huge resistance of the gel column (*ca.* 500 MΩ in our experiments). Anions in sample solution have to migrate into the inlet of the gel column to maintain the current level. When the DNA sample is prepared in running electrolyte, there are relatively many borate anions in solution. During electrokinetical injection, the amount of DNA molecules that get into the column depends on the mole ratio and mobility ratio of DNA anions to all other anions. Since the DNA is of very low concentration compared to 90 mM borate, only a small portion of DNA can get into column during injection. When the DNA sample is prepared in pure water, the amount of other anions is much lower (depends on the desalting process used for the DNA sample). Therefore a much higher portion of DNA can get into the column. The advantage of low ionic strength load solution is that it can reduce the difference between sample volume and sample injected volume. In other words, it improves the detection since the concentration in the sample band is increased.

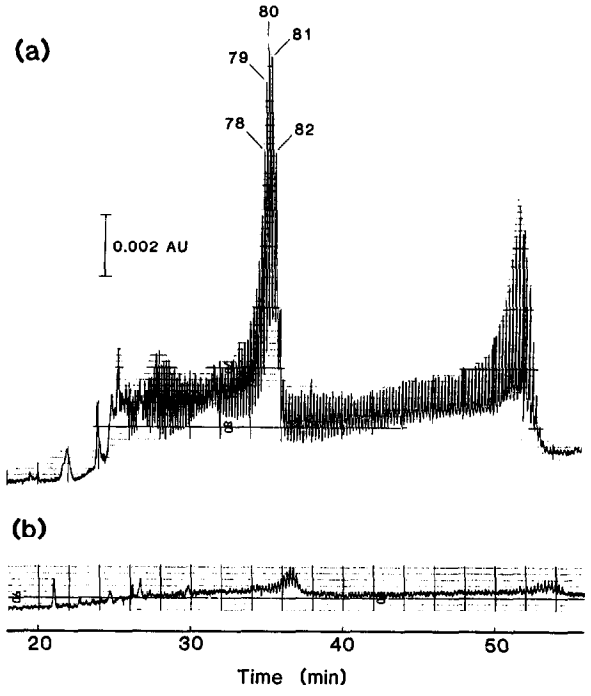

Fig. 2. Comparison of amount of sample injected between sample prepared (a) in water and (b) in running electrolyte; 200 V/cm (32 μA), Sample: synthetic poly-d(T) 20–160. Injection conditions: 15 kV, 5 s, 1 μl sample volume. Other conditions as in Fig. 1.

TABLE I

RELATIVE PEAK HEIGHT RATIOS FOR SAMPLES PREPARED IN WATER AND IN RUNNING BUFFER

Peak order	Absorbance (mAU)		Ratio
	in water	in buffer	
78	5.3	0.25	21.2
79	6.6	0.31	21.3
80	7.5	0.36	21.2
81	7.3	0.34	21.5
82	5.7	0.25	22.8
Mean			21.6

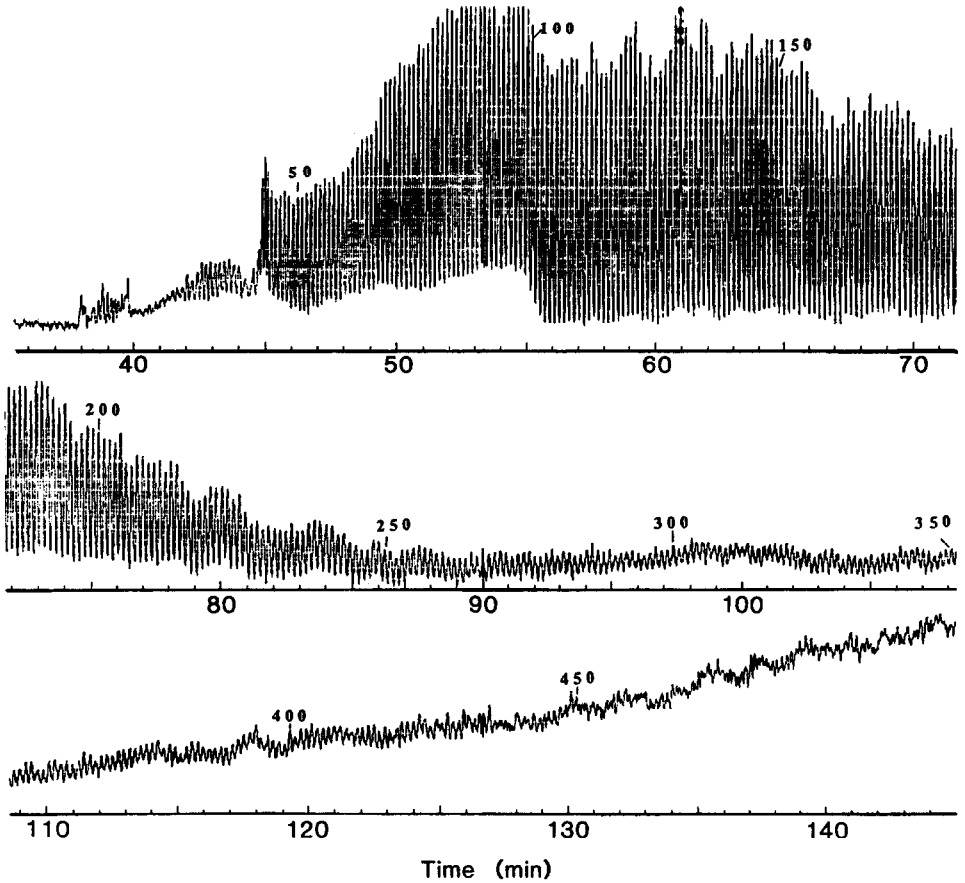

Fig. 3. Electropherogram of poly-d(A) sample. Capillary is 100 cm total length, 70 cm to detector. Gel composition is 3%T, 3.3%C; injection conditions: 6 kV, 3 s, 3 μl sample volume. Running voltage: 15 kV. Other conditions as in Fig. 1.

The migration behavior of single-stranded DNA

A difference between slab gel and CGE is that in CGE with on-column detection, all of the components injected into the column migrate the same distance before they are detected and recorded. In a slab gel autoradiography, during the same time period, the small fragments migrate further than the large ones. Thus, the pattern of migration rate of peaks in these two methods may not be same. One of the advantages of CGE with on-column detection is that it provides faster and more accurate electropherograms. Fig. 3 is a typical electropherogram of poly-d(A) sample. In order to determine the order of peaks, a slab gel purified known poly-d(A) was spiked as an internal DNA length marker. Since this marker is well defined, it is helpful in determining the numbers of bases for each peak. The migration time for the individual peaks is plotted in Fig. 4a and shows the relationship between poly-d(A) base number and migration time. It is almost a perfect linear relationship. Based on migration time, detection distance and electrical field strength, the migration rate can also be calculated. Fig. 4b shows the relationship between poly-d(A) base number and the migration rate. The curve fits well with a second-order polynomial relationship ($r = 0.998$).

A very interesting problem on the separation of single-stranded DNA is how to achieve good resolution between the nth fragment and the $(n + 1)$th fragment of a DNA polymer. Since poly-d(A) and poly-d(T) have no severe secondary structure problem, we thought it might be a useful tool for

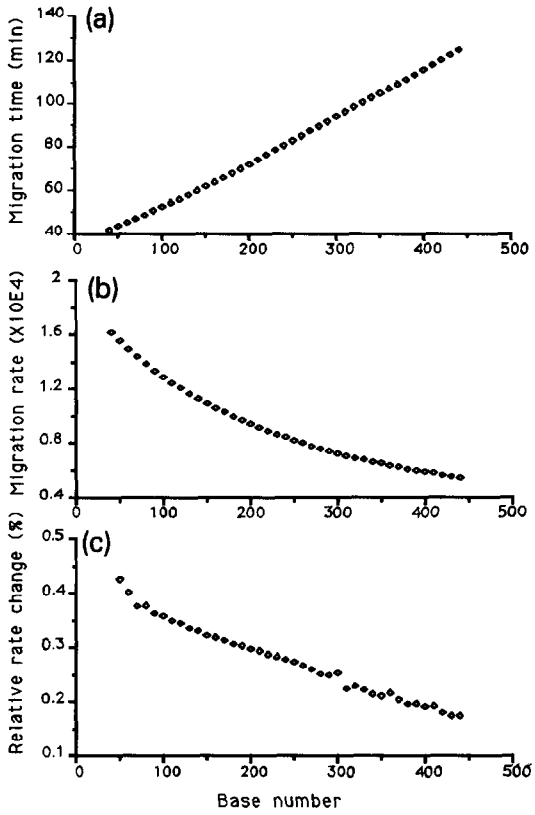

Fig. 4. (a) Plot of migration time *vs.* base number. (b) Plot of migration rate (in cm^2/V s) *vs.* base number. (c) Plot of relative migration rate change (R_c) *vs.* base number.

evaluating the capillary gel column. The most challenging problem is to separate high-molecular-weight single-stranded DNA. Fig. 4c shows the relationship between relative migration rate change and DNA base number. The relative migration rate change, R_c, is calculated using the equation:

$$R_c = \frac{\mu_{10n} - \mu_{10(n+1)}}{\frac{1}{2}(\mu_{10(n+1)} + \mu_{10n})}$$

Here, μ is migration rate of DNA fragments and n is an integer between 4 and 44. It is cleat, that with an increase in DNA size, the relative migration rate change is decreased. Since the resolution of the DNA depends upon the relative migration rate change, the resolution for large DNA molecules become increasingly difficult.

UV absorbance detection of M13 sequencing products terminating in ddT

CGE with UV detection can be used to separate DNA sequencing samples. Since the molar concentration of DNA fragments in sequencing reactions is very small, it is difficult to obtain a good signal to noise ratio for the DNA fragments. To address this problem, several measures were taken. Firstly, a more "concentrated" sequencing reaction was prepared. Secondly, a thorough desalting of the sample in 70% ethanol was performed to enhance the injection process. Thirdly, an injection using higher current for an increased period of time was attempted to ensure improved transfer of the DNA sample into the gel-filled column. Finally, a larger capillary (150 μm) was utilized to obtain longer optical path. Fig. 5 shows an electropherogram of a typical M13 T track. It is remarkable that using CGE with UV absorbance detection, peak heights do not decrease as much with increasing molecular weight as they do with LIF detection. In other words, the signal "fading" phenomenon is not significant in UV absorbance detection. In UV absorbance detection, with an increase in the length of the DNA molecule, the molar absorbance is also increased. This compensates to some degree the decrease of molar concentration with long DNA fragments. Since UV absorbance detection gives concentration information, this method can be employed to calculate the molar concentration of individual DNA fragments in each band. Table II shows the calculated DNA concentration and mass quantity of different DNA fragments in some selected bands. The data in the table give an estimation of the amount of DNA

TABLE II

CALCULATED MOLAR CONCENTRATION AND MASS QUANTITY OF THE SINGLE-FRAGMENT BANDS OF M13 T TRACK SEQUENCING PRODUCT

Base number	DNA concentration (n*M*)	Mass of DNA (fmol)
102	5.1	0.29
149	5.2	0.29
252	2.7	0.15
311	1.2	0.07
344	1.7	0.10
399	1.5	0.08

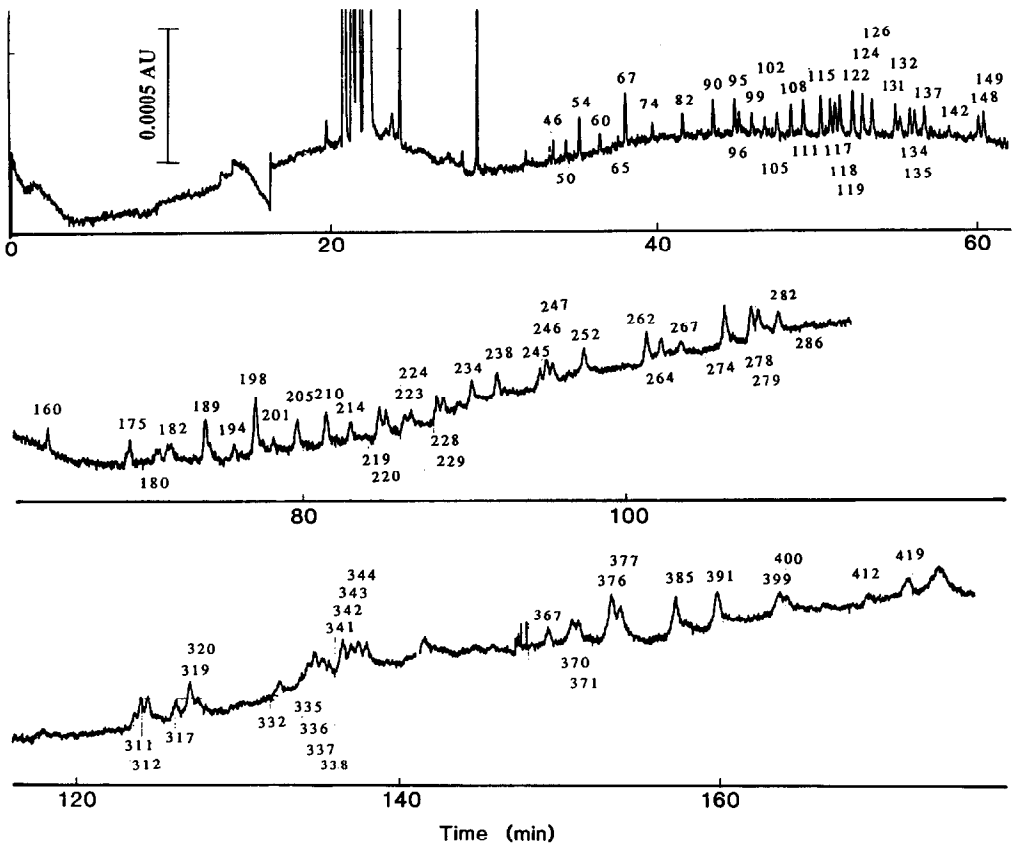

Fig. 5. Electropherogram of M13 T track sequencing product. Capillary is 85 cm total length, 50 cm to detector. Injection conditions: 12 kV, 10 s, 4 μl sample volume. Running voltage: 12 kV. Running temperature: 60°C. Other conditions as in Fig. 1.

sequencing sample injected under our experiment conditions. The mass quantity of the DNA fragments in each band ranges from 0.08 fmol of T399 mer to 0.44 fmol of T149 mer. The low detection limits obtained are quite impressive showing that, using a commercially available UV detector, it is possible to detect single-stranded DNA fragments at the sub-femtomole level.

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