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Use of the fluorescent intercalating dyes POPO-3, YOYO-3 and YOYO-1 for ultrasensitive detection of double-stranded DNA separated by capillary electrophoresis with hydroxypropylmethyl cellulose and non-cross-linked polyacrylamide

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Abstract

Double-stranded DNA is separated by means of capillary electrophoresis with either hydroxypropylmethyl cellulose or non-cross-linked polyacrylamide as the separation medium. A high-sensitivity laser-induced fluorescence detector is used to monitor the elution of DNA that has been stained with the intercalating dyes POPO-3, YOYO-3 or YOYO-1. Detection limits are on the order of 1 zmol of dye. The dye is incorporated at a rate of 1 dye molecule for every 10 base pairs in the double-stranded DNA; as a result, detection limits are a few yoctomoles of fluorescently labeled DNA.

1. Introduction

The separation of double-stranded DNA is important for analysis of restriction endonuclease digests of genomic DNA and polymerase chain reaction products. Traditionally, agarose gels are used in a slab format [1,2]. However, the large slab gels tend to be unwieldy and difficult to automate.

Over the past few years, capillary electrophoresis (CE) has been used for the analysis of double-stranded DNA [3–17]. The capillary format offers simpler automation than slab gel systems. Furthermore, the high surface-to-volume ratio of the capillary results in efficient heat transfer, allowing use of higher electric fields and decreasing the analysis time. While early work reported successful application of cross-linked polyacrylamide [3] and melted agarose [4,5,17], attention has focused more recently on non-cross-linked polymers for the separation [6–9]. These entangled polymers appear to provide higher reproducibility than cross-linked gels. Low-concentration polymers have sufficiently low viscosity and may be pumped from the capillary after each separation.

There have been two classes of non-cross-linked polymers used for separation of nucleic

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

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acids. The earliest work used non-cross-linked polyacrylamide (0%C polyacrylamide)[a]* for the separation of proteins and nucleic acids; more recently several groups have reported the use of 0%C polyacrylamide for separation of both double- and single-stranded DNA. Also, various cellulose derivatives have been used as a sieving medium in electrophoretic separation of double-stranded DNA. These derivatives are of low viscosity and may easily be pumped into and out of a capillary.

Most CE analysis of double-stranded DNA has relied on ultraviolet absorbance for detection. Because of the large molar absorptivity of large DNA fragments, UV absorbance has produced detection limits of *ca.* 10^{-18} mol of injected DNA.

However, fluorescence detection is required for study of minute amounts of analyte. Native fluorescence of DNA at basic pH generates a useful fluorescence signal upon excitation in the UV portion of the spectrum [18]; unfortunately, excitation with UV lasers tends to be somewhat cumbersome. When samples are prepared by the polymerase chain reaction, fluorescent primers can be used to generate the sample, which automatically incorporates a fluorescent label onto the double-stranded DNA. Addition of a fluorescent label to restriction fragment digests is more problematic.

Rather than using a covalently attached dye, the DNA fragments may be labeled with a physically adsorbed fluorescent dye. Non-intercalating dyes such as Hoechst 33258 and 33342 can be added to the running buffer to achieve fluorescence emission. However, the sensitivity attained is roughly equivalent to UV detection [3]. Other dyes insert themselves between adjacent base pairs (bp) of double-stranded DNA. These dyes have planar aromatic or hetero-aromatic rings and are known as intercalating dyes. Some intercalating dyes do not fluoresce significantly unless they are inside a DNA strand, resulting in low background measurements [19]. One of the first intercalating dyes, ethidium homodimer (EthD), shows better sensitivity than non-intercalating dyes and produces picogram fluorescence detection of DNA [1].

A new line of intercalating dyes has been developed recently by Glazer [19] (see also ref. 20). These dyes are dimers of intercalating dyes that have been modified to produce high affinity for DNA. The benzothiazolium-4-quinolinium dimer (TOTO-1) and the benzoxazolium-4-quinolinium dimer (YOYO-1) are examples of these type of dyes. They have much higher fluorescent enhancement (fluorescence signal for bound dye *vs.* free dye) than EthD: EthD has enhancement of 40, TOTO-1 has enhancement of 1100 and YOYO-1 has enhancement of 3200 [19]. Due to their structural differences, absorption occurs at different wavelengths allowing excitation by different laser lines. The dyes have been used to identify DNA that has been separated by agarose slab gel electrophoresis. There have been a few preliminary reports on the use of these dyes in capillary gel electrophoresis [3]. Schwartz *et al.* [3] used ethidium bromide to improve resolution of restriction digest fragments; UV absorbance was used for detection. Guttman and Cooke [21] reported the use of ethidium bromide as a complexing ligand to improve electrophoretic resolution; UV absorbance was used in this report. Demana *et al.* [22] reported use of ethidium bromide with helium–cadmium laser excited fluorescence and velocity-modulated CE. More recently, Clark and Sepaniak [23] reported fluorescence detection for ethidium bromide and POPO-3 (dimeric benzoxazolium-4-pyridinium dye) labeled DNA. Detection limits (3σ) were 8.9 fg ($9 \cdot 10^{-20}$ mol) of a 310-bp fragment; assuming one dye molecule per four base pairs, the detection limit (3σ) for the dye molecule is $3 \cdot 10^{-19}$ mol intercalated into DNA. Goodwin *et al.* [24] have reported size determination of individual double stranded DNA molecules that had been labeled with intercalating dyes. In these experiments, a large number of intercalated dye molecules were used to label the DNA fragments; when a highly dilute solution was analyzed in a modified flow cytometry instrument, the amplitude of the fluorescent burst was used to identify the size of the DNA fragment.

In this study we show separation of DNA fragments intercalated with POPO-3, YOYO-1 and YOYO-3 (a benzoxazolium-4-quinolinium dimer with three carbon atoms bridging the

aromatic rings of the unsymmetrical cyanines) using either hydroxypropylmethyl cellulose (HPMC) or non-cross-linked polyacrylamide as sieving media in CE. High-sensitivity laser-induced fluorescence is used for detection of these fluorescently labeled analyte.

2. Experimental

2.1. Capillary gel electrophoresis and micellar electrokinetic capillary chromatography

A high-voltage power supply (CZE1000R, Spellman, Plainview, NY, USA) provides the negative high voltage for sieving CE and positive high voltage for micellar electrokinetic capillary chromatography (MECC). The power supply is controlled through a Macintosh IISI computer via a NB-MIO-16X-18 input/output board (National Instruments, Austin, TX, USA).

One end of the capillary is immersed into the running buffer or sample along with a Pt wire electrode that supplies high voltage. This end of the capillary is enclosed inside a Plexiglas box provided with a safety interlock to avoid risk of electric shocks. The other end of the capillary (from which the polyimide coating has been removed by a gentle flame for MECC) is inside the sheath flow cuvette that serves as detector.

2.2. Detector

The detector used is similar to others reported from this laboratory [25]. Briefly, a sheath flow cuvette (200 × 200 μm inner bore) is used as post-column detector. A beam from one of the following lasers, 1.8 mW at 543 nm green He–Ne laser (PMS Electro-Optics, Boulder, CO, USA), 6.0 mW at 612 nm orange He–Ne laser (PMS), or 40 mW at 488 nm argon-ion laser (Innova 90-4, Coherent, Palo Alto, CA, USA) is focused by a 6.3 × (N.A. 0.20) microscope objective (Melles Griot, Nepean, Canada) into the sheath flow cuvette. A 1.0 O.D. neutral density filter is placed between the argon-ion laser and the focusing objective to reduce the laser power to 4 mW.

The laser beam is focused about 20 μm from the tip of the capillary. The fluorescence is collected at 90° from the direction of excitation by a 60 × (N.A. 0.70) microscope objective (Model 60X-LWD, Universe Kogaku, Japan). In order to eliminate scattered light from the capillary and stray light, we used an adjustable slit which resembles the shape of the fluorescing region. Fluorescence is then directed to a 590DRLP@45° XF40 dichroic mirror (Omega Optical, Brattleboro, VT, USA). Fluorescence below 590 nm is reflected at 45° through a 580DF40 bandpass filter for 543 nm excitation or a 535DF45 bandpass filter for 488 nm excitation (Omega Optical) and is detected with an R1477 photomultiplier tube (PMT) (Hamamatsu, Middlesex, NJ, USA); this detector is referred as reflected channel. Light above 590 nm is transmitted toward a second R1477 PMT having in front a 640DF20 bandpass filter (Omega Optical) for 612 nm excitation; this is referred as transmitted channel.

PMT currents are converted to appropriate signals for data acquisition using a 1 MΩ resistor and a 10 Hz bandwidth low-pass electronic filter. Data are collected at 10 Hz in the Macintosh IISI via the input/output board.

2.3. Capillary preparation

For use with HPMC, a stock 1 × TBE buffer was prepared from 0.54 g Tris, 0.275 g boric acid and 0.100 mmol disodium EDTA, diluted to 50 ml with deionized water. Then, 10 and 32 μm I.D. capillaries (Polymicro, Phoenix, AZ, USA) were silanized by filling them with α-methacrylopropyltrimethoxysilane (Sigma, St. Louis, MO, USA) and allowed to stand for 1 h. The capillaries were filled with 2%T, 0%C acrylamide (Bio-Rad, CA, USA) solution containing N,N,N′N′-tetramethylethylenediamine (TEMED, Bio-Rad) and ammonium persulfate (Boehringer Mannheim, Montreal, Canada). This step coats the inner walls of the capillary with acrylamide, which reduces electroosmosis. After 20 min, the acrylamide solution was flushed out and 0.4% HPMC in 1 × TBE, prepared from a 4000 cP, 2% HPMC stock solution (Sigma), was introduced.

For use with non-cross-linked polyacrylamide, capillaries were silanized as described above. Stock solutions of 40%T acrylamide were prepared monthly. Non-cross-linked acrylamide was prepared daily in 5-ml aliquots by dilution of stock solutions in 1 × TBE. To initiate polymerization, 2 μl of TEMED and 20 μl of ammonium persulfate were added to the degassed aliquot. The unpolymerized solution is mixed and quickly injected into the capillary. Polymerization was performed at room temperature in the capillary.

A modified syringe is used to introduce the unpolymerized acrylamide solution to the capillary. A glass capillary connector (Polymicro) is epoxied to the end of a disposable 1-ml syringe. The capillary is press-fit into the capillary connector, and solution is pumped into the capillary by finger pressure on the syringe.

2.4. Sample preparation

Unless otherwise indicated, stock solutions of POPO-3, YOYO-1 or YOYO-3 (Molecular Probes, Eugene, OR, USA) are diluted to 10^{-6} *M* with 0.1 × TBE (pH 8.4) and then diluted ten-fold in the same buffer (10^{-7} *M*). To this dilution, sufficient double-stranded DNA is added to obtain 9.3 to 15 bp-to-dye molecule ratio (bp:dye). The preparation is kept in the dark about half an hour prior to further dilution or injection. Several different DNA mixtures were used, including ΦX174 digested with Hae III restriction endonuclease, a 100-bp ladder and a 123-bp ladder from Gibco BRL (Toronto, Canada), λ DNA digested with Hind III endonuclease from Sigma, and double-stranded M13mp18 (Sigma) digested with Taq I endonuclease (Gibco BRL).

2.5. HPMC CE and MECC

The sample is not diluted further for CE. Sample injection is typically for 5 s at 100 V/cm, unless otherwise indicated. In general, elution at 200 V/cm with 1 × TBE buffer is used.

For MECC, samples are diluted in pH 9.4, 10 m*M* borate + 10 m*M* sodium dodecyl sulfate (SDS) buffer. There is no difference in signal sensitivity if SDS is removed from the buffer; on the other hand, its presence enhances peak sharpness. Elution using 400 V/cm is typical.

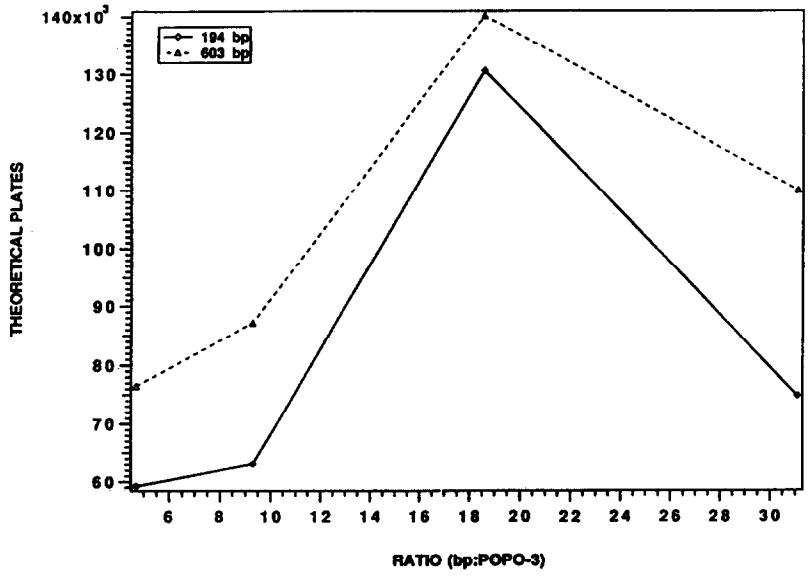

Fig. 1. Theoretical plates *versus* base pair:POPO-3 ratio for ΦX174 Hae III trimer. Data were obtained with HPMC 0.4% in 32-μm I.D. capillaries at 200 V/cm. Plate count was estimated by use of a non-linear regression analysis of each peak with a Gaussian function.

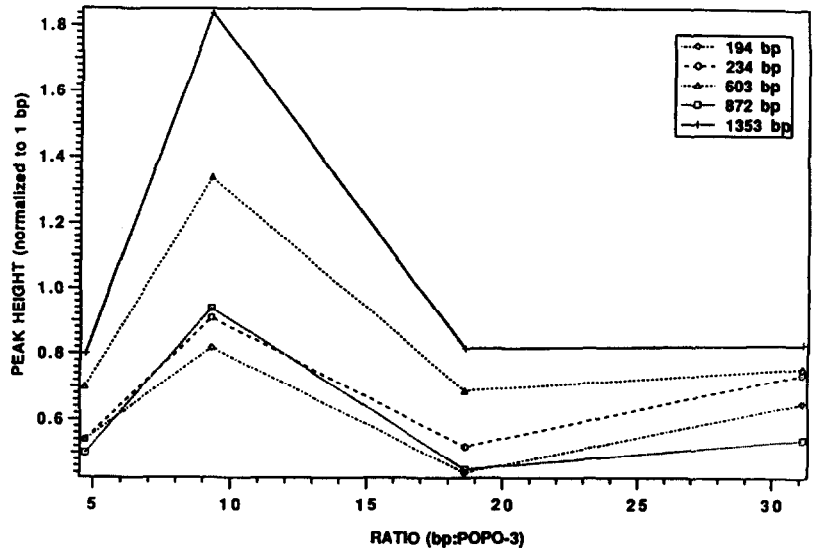

Fig. 2. Normalized peak height *versus* base pair:POPO-3 ratio. Conditions as in Fig. 1.

3. Results and discussion

The effectiveness of the double-stranded DNA separation is judged by the peak resolution, peak width, and the total time for the separation. Because there is no previous report of CE separation of double stranded DNA with intercalated POPO-3, YOYO-1 and YOYO-3, we have investigated the effect of the bp:dye ratio, ionic strength, and HPMC and polyacrylamide concentration in matrix composition.

3.1. Bp:dye ratio

The proportion of intercalating dye to DNA base pairs affects intensity and peak width of each DNA fragment. When the bp:dye ratio is changed from 3 to 33%, we observe that plate

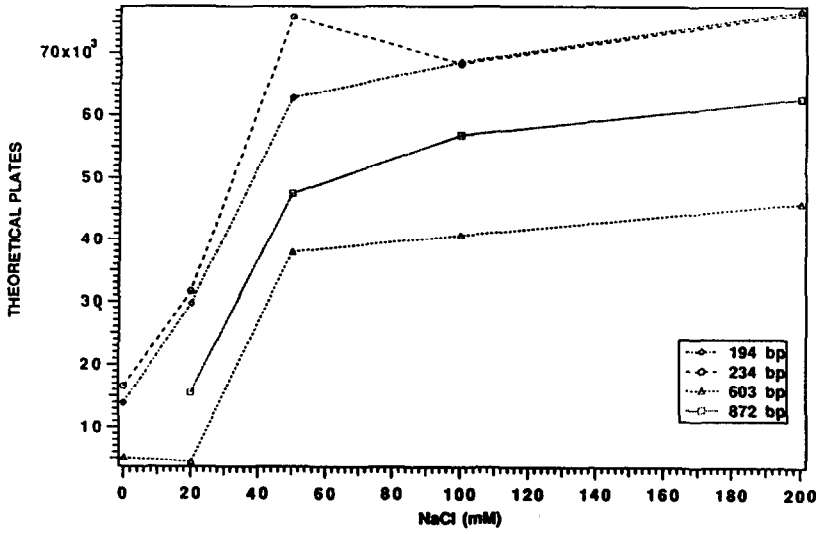

Fig. 3. Theoretical plates *versus* NaCl concentration. Conditions as in Fig. 1.

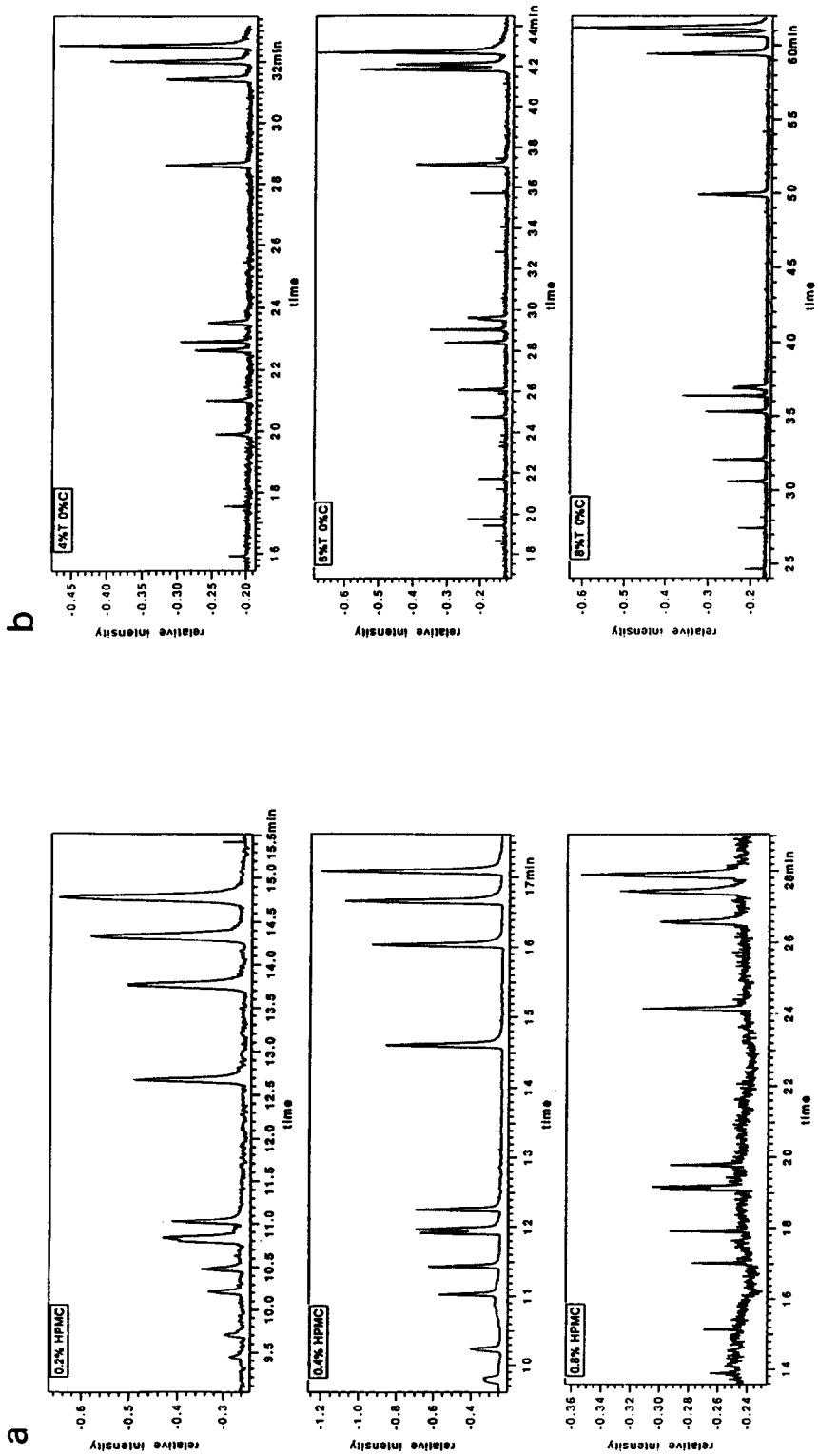

Fig. 4. Analysis of the alkylphenols at pH 10 with a lowered ionic strength. Electrolyte, 12.5 m*M* borax–50 m*M* sodium hydroxide (pH 10); [SDS] = 50 m*M*; [ACN] = 5% (v/v). Compounds as in Fig. 1.

counts are highest when one POPO-3 molecule is added for 20 base pairs of DNA for fragments 194 and 600 base pairs in length (Fig. 1).

The peak height increased linearly ($r > 0.95$) with fragment length for all fragments studied. The number of intercalated dye molecules is proportional to the fragment length. The peak height was essentially independent of the bp:dye ratio for ratios of 5, 20 and 33. However, the signal was twice as high for a bp:dye ratio of 10 (Fig. 2).

3.2. Ionic strength

The ionic strength of the running buffer (1 × TBE) affects the peak width. Ionic strength was changed by adding up to 200 m*M* NaCl in the running buffer. The theoretical plate count increased asymptotically to a limiting value (Fig. 3). The data of Fig. 3 were fit with a function of the form

$$\text{plates} = \frac{a}{\frac{b}{[\text{NaCl}]} + 1}$$

The limiting plate count ranged from 64 000 to 91 000 theoretical plates, with a mean of 81 000 ± 12 000 plates. This improvement in resolution with increasing sodium chloride concentration has been observed on slab gels. Presumably, the intercalating dye is more tightly held by the double-stranded DNA at higher ionic strength; the partition ratio of the relatively nonpolar dye would increase at higher ionic strength. As the partition ratio increases, the number of intercalating dye molecules in any particular DNA strand will increase. More importantly, the relative variance in the number of intercalated dye molecules will change due to statistical fluctuations in the amount of intercalated dye. Finally, the mobility of the dye molecules will change with concentration of intercalated dye; variation in the number of intercalated

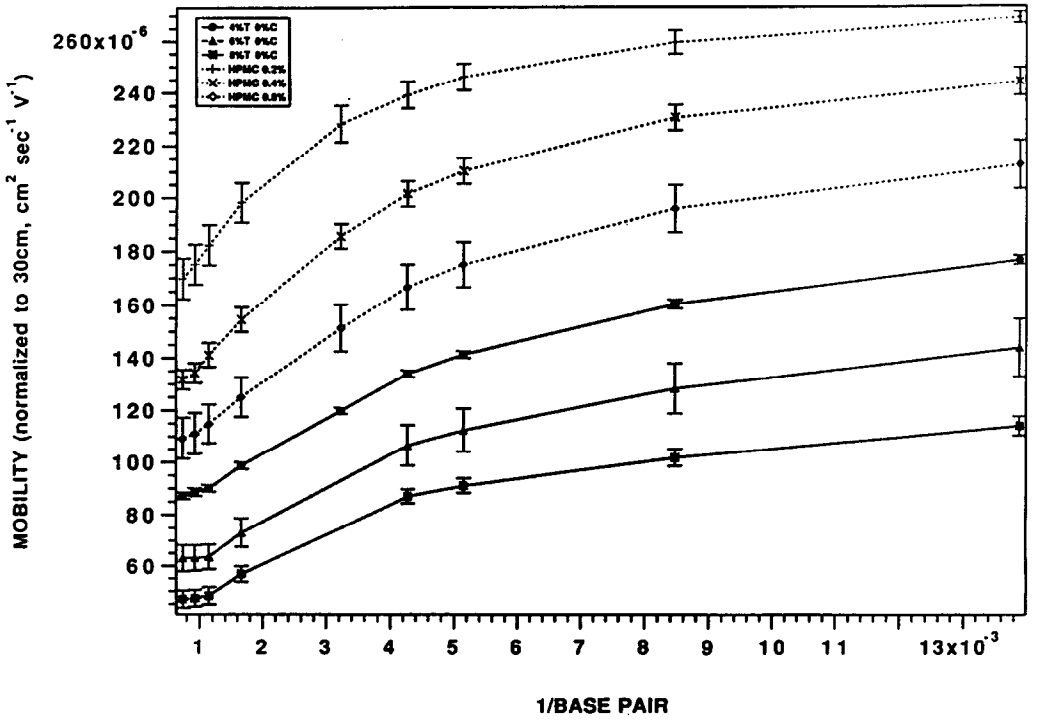

Fig. 5. Mobility *versus* reciprocal of fragment length for different polymers. The mobility was normalized to 30 cm capillary. Other conditions as in Fig. 4.

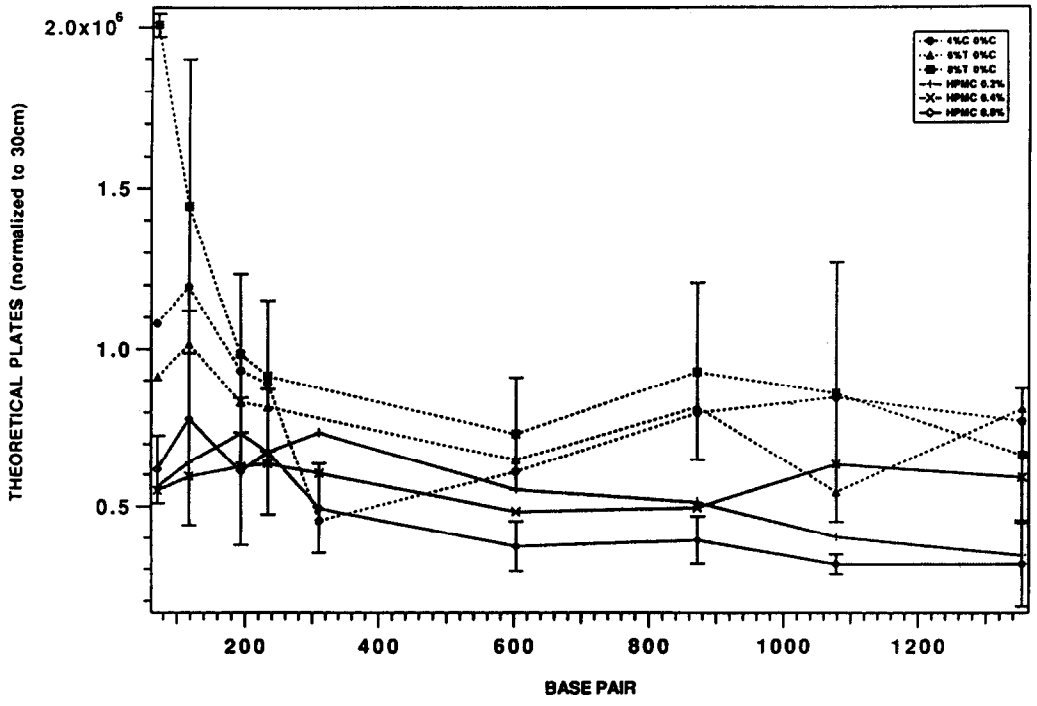

Fig. 6. Theoretical plates *versus* fragment length for different polymers. Only the standard deviation for 8%T, 0%C and HPMC 0.8% are reported for clarity. Other conditions as in Fig. 4.

dye molecules leads to variation in retention time and peak broadening. The retention time of all the fragments increases with the NaCl concentration. The increment is greater for the longer fragments. Similar results have been obtained by Nathakarnkitkool *et al.* [26], who noticed an increase in the resolution and a decrease in mobility with an increase in NaCl concentration for double-stranded DNA fragments.

3.3. Separation matrix composition

Separation of ΦX174 digested with Hae III was obtained in 0.2, 0.4 and 0.8% HPMC and in 4%T, 0%C; 6%T, 0%C; and 8%T, 0%C polyacrylamide (Fig. 4). Fig. 5 presents mobility *vs.* the inverse of the fragment length in base pairs. Highest mobility is produced in the lowest concentration HPMC; the relatively low viscosity of this medium produces relatively little retardation of the double-stranded DNA. The smallest mobility was obtained with 8%T, 0%C polyacrylamide; the high viscosity of this material produces significant retardation of the fragments.

Mobility is plotted *vs.* the inverse fragment length. Biased reptation predicts that mobility scales as

$$\mu = \alpha \cdot \left(\frac{1}{n} + \frac{1}{n^*}\right) = \frac{\alpha}{n} + \mu_\infty$$

where n is the fragment length in base pairs, α represents the extrapolated mobility of a single base pair, n^* is the fragment length for which biased reptation has produced a 50% decrease of mobility, and μ_∞ is the limiting mobility of the longest fragments. These plots reveal that the onset of biased reptation occurs for fragments roughly 500 bp in length, which is similar to the onset observed for single-stranded DNA in non-cross-linked polyacrylamide [27].

A non-linear least-squares routine was used to fit a four-parameter Gaussian function to the peaks of the electropherograms

$$I = A_0 + A_1 e^{[(t-t_0)/A_2]^2}$$

where I is the observed intensity, A_0 is the baseline signal, A_1 is the peak amplitude, t is time from injection, t_0 is retention time for the peak and A_2 is the peak width. The plate count is estimated from peak width and retention time

$$N = 2 \cdot \left(\frac{t_0}{A_2}\right)^2$$

The number of plates decreased slightly with fragment length for all of the polymers studied (Fig. 6). For non-cross-linked polyacrylamide, the number of theoretical plates reached an asymptotic value of about $0.8 \cdot 10^6$ plates for the longest fragments, whereas for HPMC the limiting plate count was about half that value. It is interesting that this trend to lower plate counts for the longer fragments mirrors the onset of biased reptation; long fragments that undergo biased reptation appear to produce similar plate counts. Presumably, the change in conformation that accompanies biased reptation produces a change in the diffusion coefficient of the fragments that matches the change in mobility.

Resolution for fragments differing in length by 10 bp was estimated as

$$R_{10} = 10 \cdot \frac{\Delta t}{2.83\overline{A_2}\Delta \text{bp}}$$

where Δt is the difference in retention time of two fragments, $\overline{A_2}$ is the average peak width for the two peaks, and Δbp is the difference in fragment length in base pairs. Note that resolution is for a 10-bp difference in fragment length; when resolution drops much below 1, fragments differing by 10 bp in length will produce overlapping peaks. Resolution decreases nearly exponentially with fragment length (Fig. 7). While resolution for fragments shorter than 300 bp is superior in non-cross-

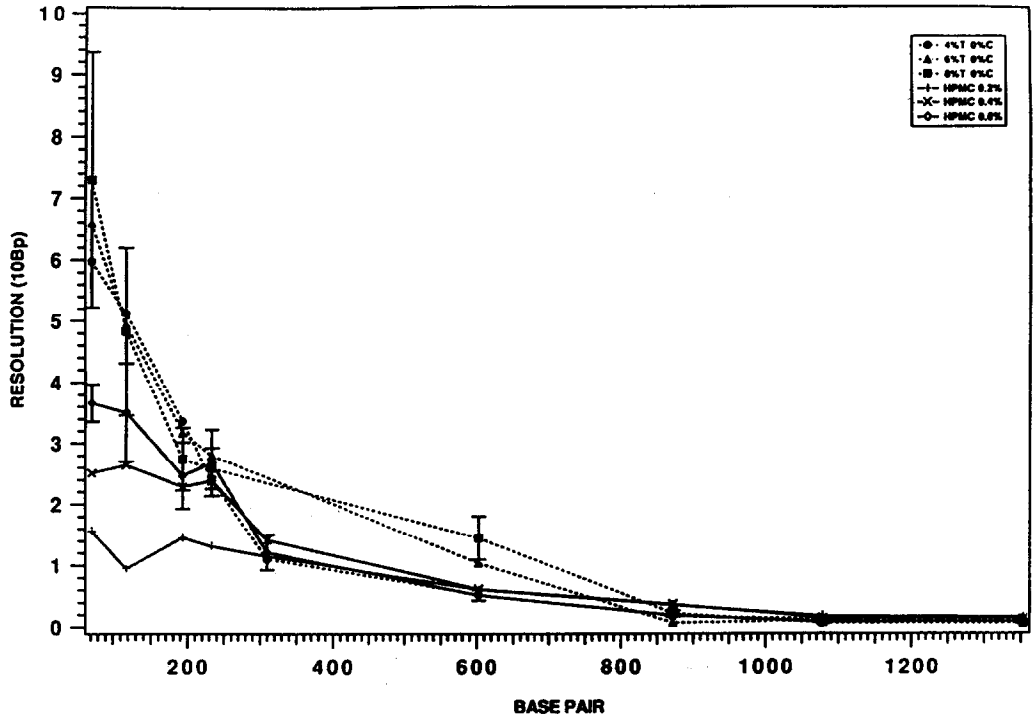

Fig. 7. Resolution *versus* fragment length for different polymers. the resolution was normalized to 10 bp. Other conditions as in Fig. 6.

linked polyacrylamide, longer fragments produce similar resolution.

In general, HPMC seems to be superior to non-cross-linked polyacrylamide for analysis of fragments longer than 300 bp. The high mobility of fragments in HPMC leads to rapid separations

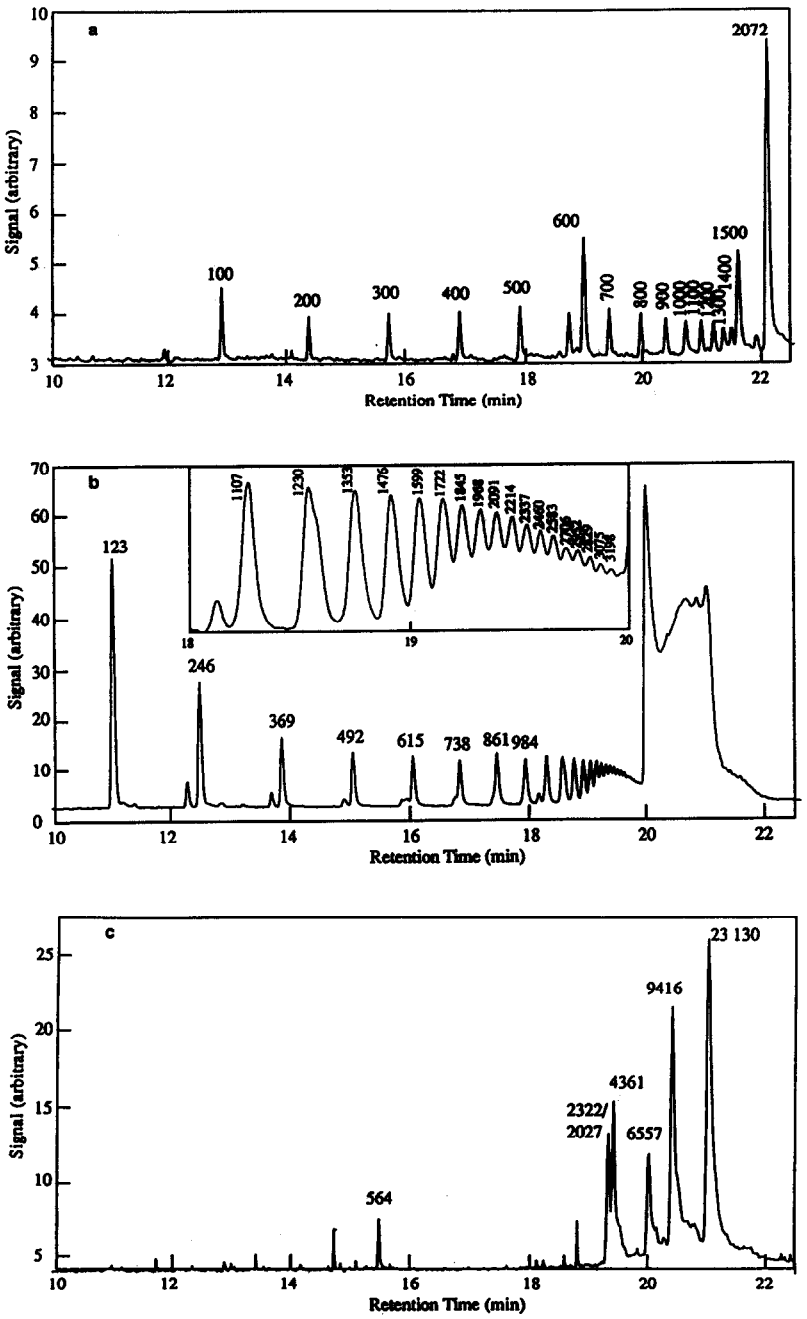

Fig. 8. Separation of (a) 100-bp ladder, (b) 123-bp ladder and (c) λ DNA Hind III intercalated with YOYO-3 on HPMC 0.4% in 10- or 32-μm I.D. capillaries at 200 V/cm with 1 × TBE, 0.1 *M* NaCl.

with no loss of resolution compared to the use of non-cross-linked polyacrylamide. In Fig. 8, we present several examples of the separation of double stranded DNA in HPMC. In Fig. 8a, a 100-bp ladder is separated in 22 min at an electric field of 200 V/cm. Note that two peaks are generated for the 600-bp fragment. This behavior is also observed in slab gels and is evident in the electropherogram supplied by the manufacturer. Fig. 8b shows a 123-bp ladder resolution extends to fragments 3798 bases in length; longer fragments generate a large peak associated with biased reptation of the plasmid used to prepare the sample. Fig. 8c presents a λ phage that has been digested with Hind III restriction endonuclease. Fig. 9 presents an electropherogram generated from double-stranded M13mp18 that has been digested with Taq I DNA restriction endonuclease.

3.4. Detection limits

These dyes undergo a significant increase in fluorescence intensity when intercalated in double-stranded DNA. Because of the large partition coefficient of these dyes (YOYO-1 $6.0 \cdot 10^8$ and YOYO-3 $1.5 \cdot 10^8$) we assume that all the dye is intercalated inside the double helix [20]. Rather than using a single double-stranded DNA fragment, we chose to use micellar capillary electrophoresis to determine detection limits. Under our experimental conditions, the Hae III digest of ΦX174 yields a single electrophoresis peak with POPO-3, YOYO-1 and YOYO-3. Table 1 gives the observed detection limit for the dyes. While micellar systems may perturb the fluorescence properties of these dyes, the micellar system minimizes adsorption of the dye on the capillary walls, producing clean separation. These detection limits should be considered as conservative.

Table 1
Detection limits of intercalating dyes

Dye	Detection limit (3σ) (mol)
POPO-3	$1.1 \pm 0.1 \cdot 10^{-21}$
YOYO-3	$1.9 \pm 0.4 \cdot 10^{-21}$
YOYO-1	$5 \pm 2 \cdot 10^{-21}$

Data were obtained in zone electrophoresis at an electric field of 400 V/cm with a 10 m*M* borate and 10 m*M* SDS buffer at pH 9.4.

These detection limits are for intercalated dye; a large number of these dye molecules can be intercalated into large fragments. For example, if the dye:DNA bp ratio is 0.05, then a single 10 000-bp DNA fragment will contain 500 dye molecules. Detection limits for large DNA molecules approach the single molecule limit in this CE instrument. These results are similar to those reported by Goodwin *et al.* [24] for analysis of neat solutions of highly dilute samples of DNA fragments labeled with intercalating dyes.

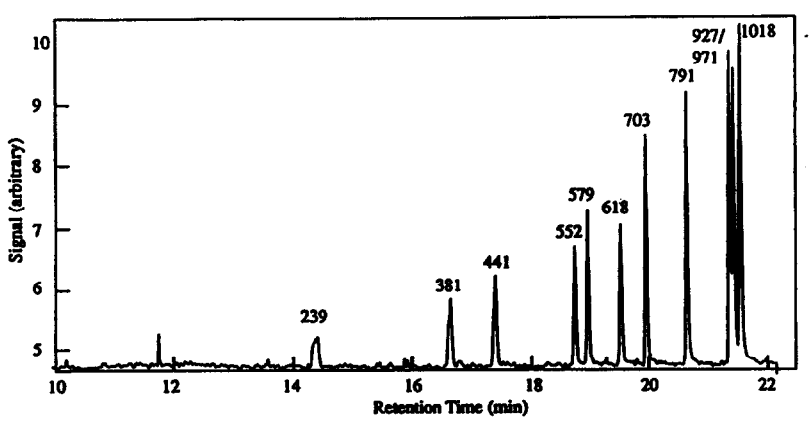

Fig. 9. Separation of Taq I digest of M13mp18. Conditions as in Fig. 8.

4. Acknowledgements

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