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Band broadening during dc and field inversion capillary electrophoresis of λ DNA in dilute hydroxyethylcellulose solutions

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Capillary electrophoresis of λ double-stranded (ds) DNA (48.5 kbp) in dilute hydroxyethylcellulose (HEC) solutions shows band spreading that cannot be explained by diffusion alone. Dispersion and asymmetry factors of λ ds-DNA bands were measured as functions of capillary length, HEC concentration and field strength. Band spreading and asymmetry can be explained by a recently developed model in which the dominant contribution is assumed to be dispersion in DNA–HEC disentanglement times. Bandwidth reduction using square-wave field inversion was also investigated. It is proposed that correlation of DNA motion is the source of band narrowing during field inversion.

Keywords: *Double-stranded DNA; capillary electrophoresis; field inversion; dispersion; band asymmetry; hydroxyethylcellulose; overlap concentration*

An attractive feature of capillary electrophoresis (CE) is high resolution. Jorgenson and Lukacs[1] early argued that for small ions, bands should be diffusion-limited Gaussians. This bandwidth is seldom achieved because finite sample introduction width, wall adsorption or finite detector window length actually limit resolution. Nonetheless, it is widely assumed that the diffusion limit is reachable in principle.

Recently, Gibson and Sepaniak[2] have shown that in semi-dilute (entangled) methylcellulose (MC) solutions, electrophoretic bandwidths of double-stranded (ds) øX-174 DNA Hae III digest fragments [72–1353 base pairs (bp)] are far too great to be diffusion-limited. The deviation from diffusion-limited widths increased with increasing chain length and increasing electric field. They attributed band broadening to dispersion in DNA–MC entanglement times by analogy with familiar chromatographic band spreading mechanisms. Deviation from a Gaussian profile has also been observed in dc gel electrophoresis.[3] In gels, the dispersion has been shown to increase with gel concentration, electric field and DNA size.[4,5]

Many investigators have demonstrated that the CE bands of ds-DNA are asymmetric, although few have commented on the phenomenon.[6–8] Tailing is easily observed even in bands reproduced as journal figures, especially if the chain length is longer than about 8–10 kbp. Close inspection often reveals less pronounced asymmetry at shorter chain lengths.

A general theory of band broadening for gel electrophoresis has been proposed.[3,9] The model is an extension of Giddings' classical treatment of chromatographic band broadening caused by kinetics of adsorption into and desorption from the stationary phase.[10] In the electrophoretic model a non-deformable polymer is assumed to entangle instantaneously with the gel matrix. Dispersion in disentanglement times leads to broad bands, which are wider on the tailing side than on the leading side. This model predicts that bands should broaden linearly with migration time and that the asymmetry factor should reach a maximum early in the migration. The model has been verified for phycoerythrin.[9]

The published chromatographic band broadening model does not strictly apply to DNA electrophoresis. A decade of video microscopy in both gels and polymer solutions shows that ds-DNA undergoes a cycle of collision with the matrix, extension, disentanglement from the matrix and collapse towards a random coil.[11–15] The extension–collapse cycle is observed in every matrix from gels[11–13] to dilute polymer solutions,[14,15] but it is not included in the Weiss model.

In chromatography, it has been commonly observed that tailing occurs whenever transfer from the mobile phase to the stationary phase is much faster than the reverse process, independent of the details of the analyte–stationary phase interaction.[10,16,17] It is reasonable to assume that if the dominant factor in electrophoretic band spreading is disentanglement kinetics, asymmetric, tailed bands should be observed, despite the existence of an extension–contraction cycle.

The situation is more complicated in DNA field inversion electrophoresis. Masubuchi *et al.*[18] have shown in gels that field inversion, under conditions which improve resolution, does not change the average extension of T4 DNA from the dc electrophoresis value. However, it is known that bandwidths decrease. Our group has shown that field inversion electrophoresis in dilute polymer solutions causes DNA bands to become narrower, but does not change mobilities greatly.[7,19] These results suggest that field inversion influences some aspect of electrophoretic dynamics other than the average geometry of DNA.

In this work, we used monodisperse λ ds-DNA (48.5 kbp) to examine electrophoretic band spreading and band asymmetry in dilute hydroxyethylcellulose (HEC) during dc and field inversion electrophoresis. We chose λ ds-DNA because it is long enough to visualize (contour length 16.5 μm) and in a size range where either dc or field inversion electrophoresis can be used.

Experimental

The CE apparatus has been described elsewhere.[7] A high voltage dc power supply (Glassman, Whitehouse Station, NJ, USA) or a high voltage amplifier (Model 609A, Trek, Medina, NY, USA) was used to provide the electrophoresis driving voltage. Power supply (dc voltage) and amplifier (dc or dc with superimposed square-wave) control voltages were generated by a personal computer fitted with a digital–analog converter. Pulsed fields are reported as percentage modulation, defined as the ratio of the ac peak voltage to the dc voltage.[19] Detection was by laser induced fluorescence of intercalated ethidium ion, excited at 543 nm. The photomultiplier tube output was amplified and digitized at 5–7 points s^{-1}.

Electrophoresis was performed in 75 μm id quartz capillaries (Polymicro Technologies, Phoenix, AZ, USA). Capillary lengths of 25.8, 16.5, and 12.5 cm with effective lengths of 22.3, 14.0 and 10.0 cm, respectively, were used. The capillary inner walls were coated with polyacrylamide to prevent electro-

osmosis.[20] HEC (M_n = 438 800) (Aqualon, Wilmington, DE, USA) was used as the separating linear polymer. HEC concentrations ranged from 0.001 to 0.09% m/m in 1X TBE (89 mM TRIS, 89 mM boric acid, 2 mM EDTA) or 0.5X TAE (45 mM TRIS, 45 mM acetic acid, 1 mM EDTA). The overlap concentration, C^*, for this HEC has been determined to be 0.09% m/m by viscosity measurements.[21] Buffers were filtered twice through 0.22 μm membrane filters before HEC was added. The solutions were stirred for 24 h to ensure complete solvation of the polymer. Ethidium bromide (2.5 ng μl^{-1}) was added to the running buffer immediately before electrophoresis.

The λ ds-DNA (48.5 kbp) was obtained from New England Biolabs (Beverly, MA, USA). Stock standard solutions of λ ds-DNA were diluted in 1X TBE to 5, 2.5 or 0.5 ng μl^{-1}. In the experiments in which TAE was used as the running buffer, stock standard solutions of DNA were diluted in 0.5X TAE to 0.5 ng μl^{-1}. The DNA solutions were electrokinetically injected at 60–100 V cm^{-1} for 3–5 s, depending on the HEC concentration.

Data analysis was performed in GRAMS (Galactic Industries, Salem, NH, USA) with the RAZOR Bayseian statistics add-on (Spectrum Square Associates, Ithaca, NY, USA). Maximum likelihood curve fitting to a log-normal function or Gaussian/Lorentzian was used to define band shapes. Field inversion electrophoresis peak shapes were usually fitted to a Gaussian/Lorentzian. An Array Basic program was used to find peak maxima and the full widths at half maximum (FWHM). Bandwidths were then converted to centimeters. Asymmetry factors[9] were calculated according to the equation

$$\Delta = \frac{w_+ - w_-}{w_+ + w_-} \quad (1)$$

where w_+ is the time from the peak maximum to the trailing edge at 50% maximum and w_- is the time from the leading edge to the peak maximum at 50% maximum. An asymmetry factor of zero indicates a symmetric band, a positive asymmetry indicates tailing and a negative asymmetry indicates fronting. Reported bandwidths and asymmetry factors are the averages of 3–10 separate measurements.

Results and discussion

Dc field electrophoresis

Fig. 1 shows representative electropherograms of λ ds-DNA at several HEC concentrations in 1X TBE at 100 V cm^{-1} (effective capillary length 14 cm). At 0.001% HEC, the band is almost symmetric about the maximum. With increasing HEC concentration, tailing becomes more pronounced and the bandwidth increases.

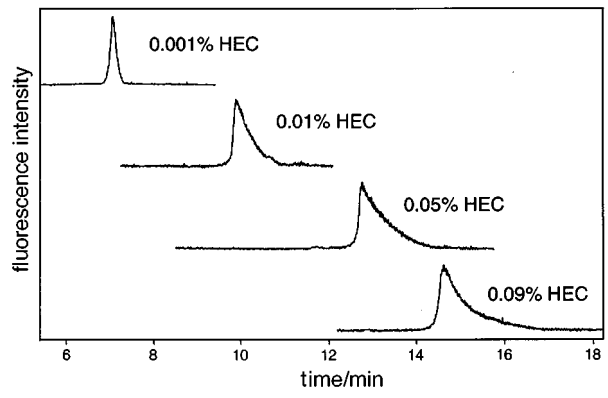

Fig. 1 Representative electropherograms of λ ds-DNA (48.5 kbp) in HEC (M_n = 438 800), 1X TBE, 100 V cm^{-1}, 16.5 cm capillary with an effective length of 14.0 cm. All peak maxima are normalized to one unit.

Bandwidths (FWHM) and asymmetry factors are shown in Fig. 2 for two field strengths, 100 and 250 V cm^{-1}, and for two capillary effective lengths. The bands were too broad and low to measure in the longer capillary at HEC concentrations above 0.05% with a field of 250 V cm^{-1}. The bandwidth increases approximately linearly with transit time, that is, the bandwidth observed in the 22.3 cm capillary is about 50% larger than that observed in the 14.0 cm capillary. At 250 V cm^{-1} asymmetric bands are observed even at 0.001% HEC and the bandwidth increases rapidly with increasing HEC concentration. Aggregation[22] was often observed in the absence of HEC or at the highest HEC concentrations at 250 V cm^{-1}. Aggregation can distort apparent bandwidths, so these data were not included in the analysis. In general, 90% confidence intervals were about ±15% of the average bandwidth value.

At both fields the same limiting asymmetry is reached. The constant maximum asymmetry is consistent with the predictions of Yarmola *et al.*[9] This plateau is reached when there have been sufficient HEC–DNA entangling collisions for a wide range of entanglement times to have been explored.

What is most striking about behavior at the two voltages is the very different dependences on HEC concentration. At 250 V cm^{-1}, the bandwidth increases approximately linearly with HEC concentration, but at 100 V cm^{-1} there is very little increase above 0.005%. Because bandwidths at 250 V cm^{-1} could not be measured at HEC concentrations above 0.05%, we do not know if a limiting bandwidth is reached at some higher concentration.

Our own measurements[14] and the theory of Hubert *et al.*[23] suggest that the number of simultaneously entangled HECs is small, in the range 1–10. It is reasonable to assume that this number increases as the DNA becomes more extended and there are more possible points of entanglement. With an increasing number of entangled HEC molecules, there will be an increasing spread of disentanglement times. At high fields a limiting bandwidth, if any, would be reached at higher HEC concentrations than we have examined.

Our observations are consistent with the two-state chromatographic model of gel electrophoresis in the Ogston regime.[3,9] However, this model cannot be directly applied to dilute or semi-dilute polymer solution electrophoresis of DNA. The gel electrophoresis model assumes a non-deformable ion migrating through an array of stationary obstacles. In the DNA case we have shown that there is a distribution of DNA–obstacle disentanglement times,[15] but in dilute or semi-dilute solution electrophoresis entangled DNA is not stationary. Instead, the obstacles move along with the DNA.[14] The DNA itself undergoes an easily seen extension–collapse cycle. However, as is frequently observed with chromatographic models,[10,16,17] the predicted band shape may be correct, even if the model is incomplete. For a qualitatively correct description, all that is necessary is that the model describe the dominant source of band broadening.

Field inversion electrophoresis

Our earlier experiments have shown that 50 Hz is close to the optimum field inversion frequency for the separation of high molecular mass ds-DNA fragments.[19] The λ ds-DNA fragment is the largest of these. Accordingly, we measured the effects of electric field strength, modulation depth, and frequency on the bandwidth and band asymmetry of λ ds-DNA. Conditions were chosen to include those known to give good separation of high molecular mass fragments.

Fig. 3 illustrates the electrophoretic behavior of λ ds-DNA in 0.001% HEC, 1X TBE at 100 V cm^{-1} dc, with square-wave

field inversion (180% modulation, effective capillary length 10.0 cm). The bandwidth decreases from the dc value, especially at the higher frequencies of 35 and 50 Hz.

The bandwidth and asymmetry data are plotted in Fig. 4(A). For comparison, the analogous data at 140% modulation are shown in Fig. 4(B). The elution profiles showed some fronting, resulting in small negative asymmetry factors. The cause of fronting is not yet understood. The measurements were repeated at 200 V cm^{-1} [Fig. 4(C) and (D)]. It proved difficult to control aggregation at 20 Hz at the higher modulation depth. The trends are the same as at 100 V cm^{-1}. Field inversion causes the bands to become narrow, especially at the higher modulation depths, and they remain nearly symmetric. At lower field inversion frequencies, DNA molecules respond to the electric field as in the dc regime and show less band sharpening effects.

The field inversion results are consistent with band spreading caused at least in part by dispersion in disentanglement times. By a mechanism still unknown, field inversion could reduce the distribution of disentanglement times of ds-DNA. This could happen if the motions of DNA are correlated by the imposition of an oscillating electric field. Video microscopy has indeed shown that the motion of ds-DNA closely follows the field cycle.[15,18] Such a correlation could result from synchronization of motions as the field direction is reversed.

Alternatively, correlation may occur through local electroosmotic flows, which are strong around the slow moving DNA poly-anion.[24] Field inversion is known to have little effect until high modulation depths are reached. High modulation depths would increase local electroosmotic flows around DNA molecules. This mechanism would become more effective as the charge on DNA increased.

To test this hypothesis, we measured the effect of field inversion on λ ds-DNA in 0.5X TAE buffer. Stellwagen *et al.*[25] have shown that in TBE with no entangling polymers, λ ds-DNA migrates as a borate–diol complex with a 20% increase in charge. Because the average extension increases rapidly with increasing DNA charge, field inversion electrophoresis in dilute polymer solutions should be more effective in TBE buffer than in TAE. As shown in Table 1 and comparison with Fig. 4, field inversion in TAE buffer results in less band narrowing than in

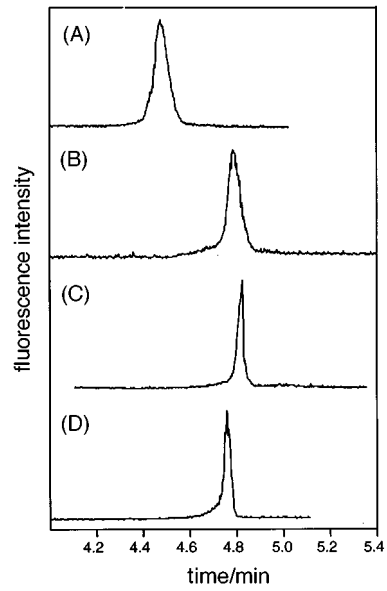

Fig. 3 Electropherograms of λ ds-DNA in 0.001% HEC solution, 1X TBE, 12.5 cm capillary with an effective length of 10.0 cm. (A) 100 V cm^{-1}; (B) 100 V cm^{-1} dc + 20 Hz square wave, 180% modulation; (C) same as (B) but with 35 Hz square wave; (D) same as (B) but with 50 Hz square wave. Peak maxima have been normalized.

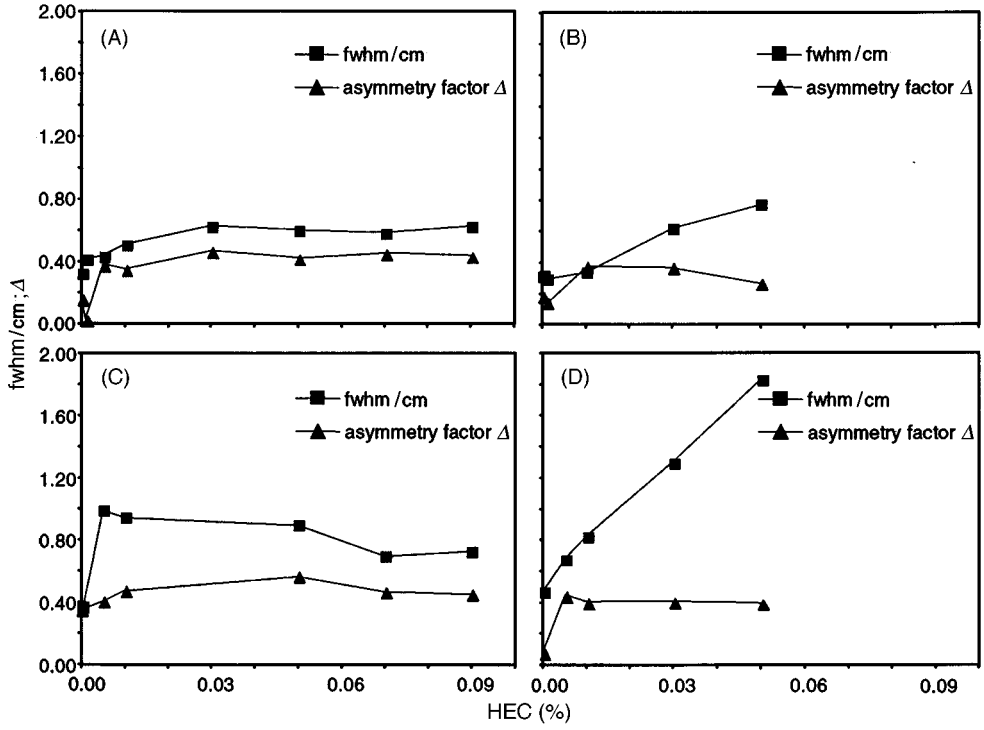

Fig. 2 FWHM and asymmetry factor as functions of HEC concentration (% m/m) for λ ds-DNA, 1X TBE. (A) 16.5 cm capillary with an effective length of 14.0 cm, 100 V cm^{-1}, 2.5 ng μl^{-1} DNA; (B) same as (A) but at 250 V cm^{-1}; (C) 25.8 cm capillary with an effective length of 22.3 cm, 100 V cm^{-1}, 5.0 ng μl DNA^{-1}; (D) same as (C) but at 250 V cm^{-1}. Each data point is the average of 3–6 separate measurements. The 90% confidence intervals were $\leqslant \pm 15\%$ of the average FWHM or average asymmetry factor.

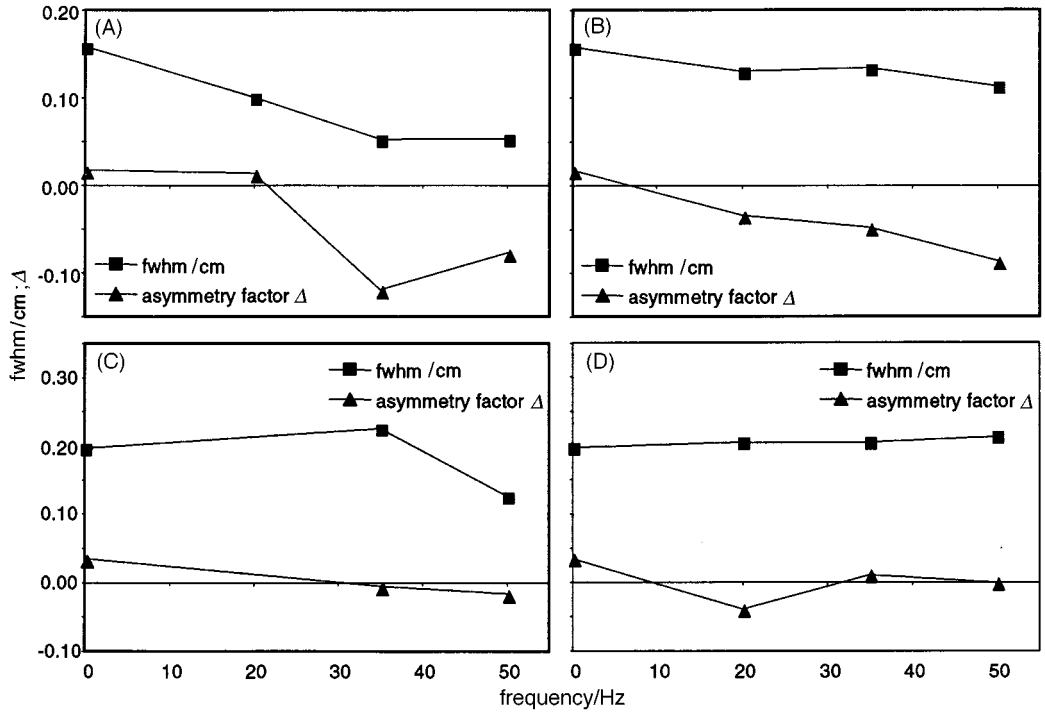

Fig. 4 Bandwidth (FWHM) and asymmetry factor as functions of field inversion frequency for λ ds-DNA (0.5 ng μl^{-1}) in 0.001% HEC, 1X TBE, 12.5 cm capillary with an effective capillary length of 10 cm. (A) 100 V cm^{-1} dc, 180% modulation; (B) 100 V cm^{-1} dc, 140% modulation; (C) 200 V cm^{-1} dc, 180% modulation; (D) 200 V cm^{-1} dc, 140% modulation. Each data point is the average of 4–6 separate measurements. The 90% confidence intervals were ⩽ ±15% of the average bandwidth.

Table 1 Dependence of λ ds-DNA bandwidth on buffer composition. 0.001% HEC, effective capillary length 10.0 cm. λ ds-DNA concentration 0.5 ng μl^{-1}

	Bandwidth*/cm	
	1X TBE	0.5X TAE
Dc constant field (100 V cm^{-1})	0.16	0.18
Field inversion (100 V cm^{-1} + 180% modulation, 50 Hz)	0.05	0.14

* Bandwidths reported as FWHM in centimeters.

TBE at 100 V cm^{-1}, 180% modulation, 50 Hz and 0.5 ng μl^{-1} λ ds-DNA. In TAE, the field inversion band width is reduced to about 75% of the dc electrophoresis value. By contrast, in TBE the field inversion band is only 33% as wide as in dc electrophoresis.

An electroosmotic explanation for correlated motion implies that field inversion should become less effective as the DNA concentration is decreased and coupling of local electroosmotic flows is reduced. Rigorous testing of this hypothesis also requires the generation of a wide range of DNA zeta potentials through variation of buffer ionic strength.

Our results confirm the qualitative applicability of the chromatographic model of band spreading to DNA capillary electrophoresis in dilute polymer solutions. The model requires extension to include DNA deformation and the motion of entangled DNA–HEC aggregates. Neither a chromatographic model nor the classical description of field inversion gel electrophoresis explains the polymer solution case. A more complete theory of dc and field inversion electrophoresis may emerge from direct measurement of entanglement–disentanglement kinetics by video microscopy. These measurements are under way and will be reported later.

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