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Electrophoretic separation of linear and supercoiled DNA in uncoated capillaries

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Abstract

We report electrophoretic separation of supercoiled plasmids (2–16 kilo base pairs) and linear double-stranded DNA (0.6–23 kilo base pairs) in uncoated capillaries filled with dilute hydroxyethylcellulose. Because electroosmotic flow reverses the order of elution, long plasmids spend less time in the capillary and their bandwidths are narrower than observed in coated capillaries. However, resolution is similar to that obtained in coated capillaries, because it is governed by the distribution of unresolved topoisomers. In the presence of electroosmotic flow migration of supercoiled plasmids does not follow the elastic rod model that has been observed in coated capillaries.

Keywords: DNA; Electrophoretic separation; Uncoated capillaries

1. Introduction

Capillary electrophoresis (CE) has proven to be an effective technique for DNA separation [1–3]. Barron and co-workers initially demonstrated that excellent DNA separations could be achieved in buffers containing linear polymers such as hydroxyethylcellulose (HEC), polyethylene oxide (PEO) and polyacrylamide (PAA) at concentrations well below the overlap threshold [4–6]. The low viscosity of dilute polymer solutions allows fast electrophoretic migration. Additionally, excellent resolution is obtained for the separation of larger double-stranded (ds) DNA fragments. Although the separation mechanism is entanglement of DNA with discrete polymer molecules rather than with an extended matrix [4,7–9], length-dependent separation is still observed.

There has been increasing interest in capillary electrophoresis of supercoiled (sc) plasmid DNA, driven by its emergence as a component of DNA pharmaceuticals as well as its role in many molecular biology procedures [10]. Several laboratories [11–13] have reported successful separations of sc-plasmids, plasmid/open circular DNA mixtures and sc-plasmid/linear dsDNA mixtures in dilute and semi-dilute polymer solutions. Most of these workers have employed capillaries coated to suppress electroosmotic flow (EOF).

Our own laboratory has shown that electrophoretic mobilities of sc-plasmids are less dependent on separation parameters (ionic strength, sieving polymer concentration, electric field) than are mobilities of linear dsDNA [13]. The differences arise from the different migration dynamics of the two forms of

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DNA. Plasmids migrate as elastic rods, with radius of gyration scaling approximately as N. However, dsDNAs migrate as random coils, with radius of gyration scaling approximately as $N^{0.5}$ [14]. The curiously broad sc-plasmid bands have recently been shown to result not from mobility dispersion but from the presence of unresolved topoisomers [15], each of slightly different mobility. Under some electrophoretic conditions, topoisomers can be resolved in liquid crystals [15], hydroxypropylmethyl cellulose [16] and crosslinked polyacrylamide gels [17].

Weiss and co-workers [18] have presented a general theory of bandwidths in biopolymer gel electrophoresis. They show that for polymers too large for band broadening by diffusion, broadening results from a distribution of polymer/gel entanglement times. Although their theory assumes a rigid gel-like sieving medium, Schwinefus and Morris [19] have shown that it can be used to describe band shapes and bandwidths of dsDNA in dilute polymer solutions.

Uncoated capillaries have proven to be as efficient as coated capillaries [6,20,21]. Generally, they allow fast DNA separations in dilute polymer solutions with excellent resolution and easy preparation. Because of strong EOF, net DNA migration is toward the negative electrode, and the largest DNA fragments, with the lowest electrophoretic mobilities, pass the detection window first.

UV absorbance is extensively used for detection of DNA in uncoated capillaries. Intercalator fluorescence detection has been used little, perhaps because of concern that adsorption of a positively charged mono-intercalator, which is present in the buffer, to the negatively charged silica wall would generate large and irreproducible backgrounds. This problem might not arise with the use of tightly bound bis-intercalators, because they are not added in excess. But, bis-intercalators are difficult to use and have not proven widely popular [22,23].

In this paper, we describe electrophoretic separation of linear dsDNA and sc-plasmid DNA in uncoated capillaries with fluorescence detection. Important experimental parameters are investigated and the migration dynamics are characterized through mobility scaling.

2. Experimental

2.1. Instrumentation

The capillary electrophoresis apparatus employed in these studies has been described in detail [24]. A high-voltage d.c. power supply was used to drive the electrophoresis. Separations were in fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) 20 cm (17 cm to the detector), ×75 μm I.D. and without internal coating. Injection was performed electrokinetically at the positive electrode and detection was at the negative electrode. A monointercalator dye, ethidium bromide (5 μg/ml), was added to the running buffer prior to electrophoresis. This enabled laser-induced fluorescence detection by epi-illumination with a green He–Ne laser (2 mW, 543 nm). The fluorescence signal was spatially filtered with a 100 μm diameter aperture and detected with a photomultiplier tube. The photocurrent was amplified and digitized to 12 bits at 6 samples/s and stored in an IBM-compatible computer for data analysis.

Resolution was calculated using Eq. (1):

$$R = \frac{t_1 - t_2}{w_1 + w_2} \quad (1)$$

t_1, t_2 being the migration times and w_1, w_2 the full widths at half maximum of the bands 1 and 2. To calculate bandwidths and resolution values, the individual bands were recovered by peak deconvolution using RAZOR for GRAMS/386 (Spectrum Square Associates, Ithaca, NY, USA).

2.2. Materials

The linear λ-H*ind*III restriction digest [0.6 kilo base pairs (kbp)–23 kbp], linear 1 kbp dsDNA ladder (1.6 kbp–12 kbp) and scDNA ladder (2 kbp–16 kbp) were obtained from Gibco/BRL. The DNA samples were diluted to 10 ng/μl in TBE concentrations ranging from 0.5× to 1.5× (1×TBE = 89 m*M* Tris base–89 m*M* boric acid–5 m*M* EDTA). HEC was added to the TBE buffer to generate concentrations of 0.06% to 0.25% (w/w) [HEC, number-average molecular mass ($M_n \approx 90\,000$–105 000 g/mol; Polysciences (Warrington, PA,

USA)] and the polymer solutions were shaken for at least 12 h.

2.3. Methods

Each new capillary was flushed for 3 h with 1 *M* NaOH to completely ionize the silanol groups and then flushed with water for 15 min. Before each experiment, the uncoated inner capillary wall was treated sequentially with 1 *M* NaOH, 0.1 *M* NaOH, water and finally the electrophoresis buffer HEC–TBE for 10 min each [4]. The capillary was allowed to equilibrate in the electric field for 10 min. After equilibration, the sample was electrokinetically loaded at the negative electrode at approximately 150 V/cm for 3 s. In different experiments the electric field ranged between 250 V/cm and 300 V/cm. A fan ensured air convection around the capillary to allow heat dissipation.

3. Results and discussion

Fig. 1 shows examples of electrophoretic separations of a linear λ-H*ind*III restriction digest (0.6 kbp–23 kbp) in 0.08% and 0.19% (w/w) HEC (M_n = 90 000–105 000) solution, 1× TBE, at 282 V/cm, 286 V/cm and 257 V/cm, respectively. The con-

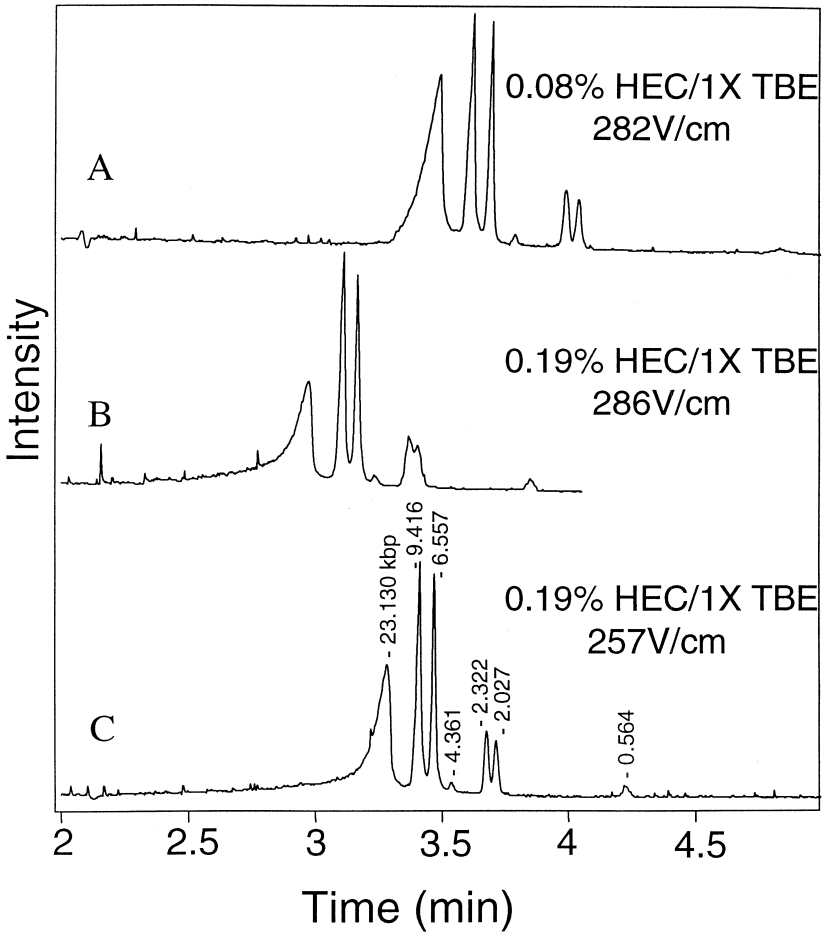

Fig. 1. Electropherograms of the λ-H*ind*III digest linear DNA (0.6 kbp–23 kbp) in HEC (M_n = 90 000–105 000), 1× TBE, injection: 150 V/cm (3 s). (A) 0.08% (w/w) HEC, 282 V/cm. (B) 0.19% (w/w) HEC, 286 V/cm. (C) 0.19% (w/w) HEC, 257 V/cm.

centration of HEC is below the overlap concentration of 0.37% (w/w) [4]. Good separations were achieved in less than 5 min for the three different experimental conditions. As observed in previous work [4] the elution order is reversed from that in coated capillaries. Fig. 1A and 1B presents separations under a higher electric field than in Fig. 1C. Fig. 1B and 1C presents separations in a polymer concentration twice as large as that in Fig. 1A.

At similar field strength (282–286 V/cm) but different polymer concentrations, the separation is much faster in 0.19% HEC (Fig. 1B) than in 0.08% HEC (Fig. 1A). However, a loss of resolution is observed for the 2.3 kbp and 2 kbp DNA fragments. The separation of DNA fragments in uncoated capillaries is faster at higher polymer concentrations. DNA fragments elute faster with the flow since more HEC molecules will hinder their electrophoretic migration. Therefore DNA migration towards the negative electrode is more rapid. If we focus on the separation achieved in Fig. 1C, the experimental conditions are the same as in Fig. 1B but with a lower electric field. With a lower electric field, the DNA fragments spend more time in the capillary, and we thus obtained a higher degree of resolution than in Fig. 1B.

We have shown that it is possible to get very good resolution and separation for a wide range of polymer concentrations.

Adsorption of non-intercalated ethidium ion to the negatively charged silica wall, if it occurs, does not generate a large background. Even if the dye ad-

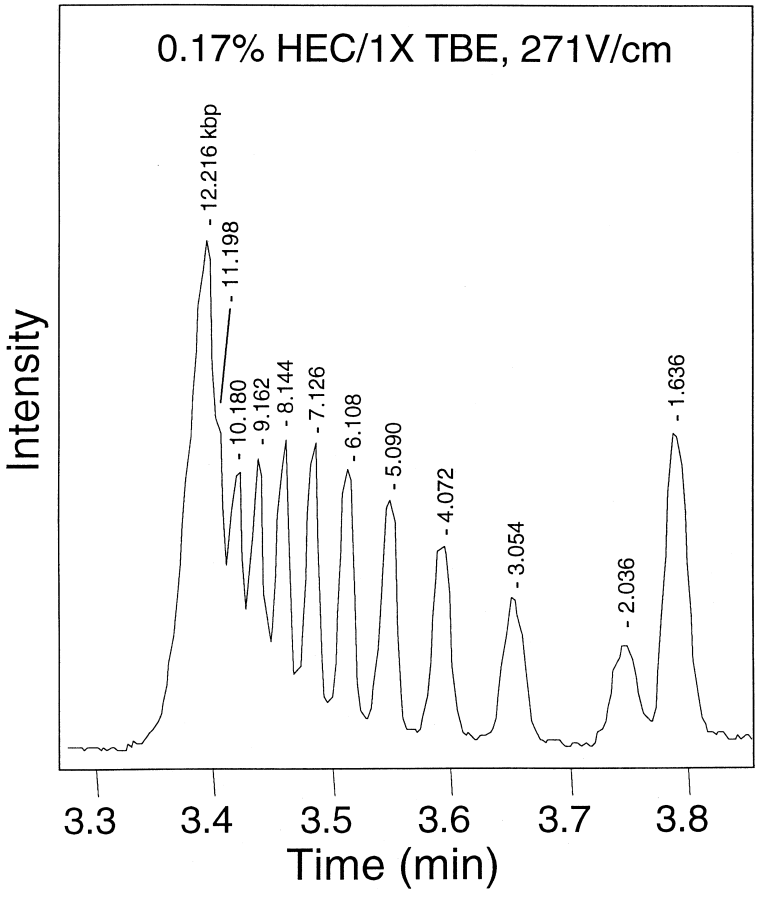

Fig. 2. Electropherogram of the linear 1 kbp dsDNA ladder (1kbp–12 kbp) in 0.17% (w/w) HEC (M_n=90 000–105 000), 1× TBE, injection: 150 V/cm (3 s), 271 V/cm.

sorbed on the wall fluoresces, the spatial filter in the optical train adequately removes out of focus background light. Because excess solution dye is in equilibrium with adsorbed dye, adsorption does not lead to irreproducible EOF.

Fig. 2 shows an electropherogram of a linear 1 kbp dsDNA ladder (1.6 kbp–12 kbp) in 0.17% (w/w) HEC (M_n=90 000–105 000) solution, 1× TBE, at 271 V/cm. The separation was completed under 4 min. Two of the fragments, 11 and 12 kbp are poorly resolved. A longer effective capillary length or a lower electric field with a lower concentration of HEC may be necessary to resolve the larger DNA fragments [25].

Fig. 3 shows examples of electropherograms of sc-plasmids (2 kbp–16 kbp) in 0.11% and 0.15% (w/w) HEC (M_n=90 000–105 000) solutions, 1× TBE, at 298 V/cm and 276 V/cm, respectively. In Fig. 3A, separation with baseline resolution was achieved under 3.1 min. Fig. 3A has a lower polymer concentration but a higher electric field than Fig. 3B. As observed, the separation represented in Fig. 3A is faster than the separation represented in Fig. 3B, even though the polymer concentration is lower.

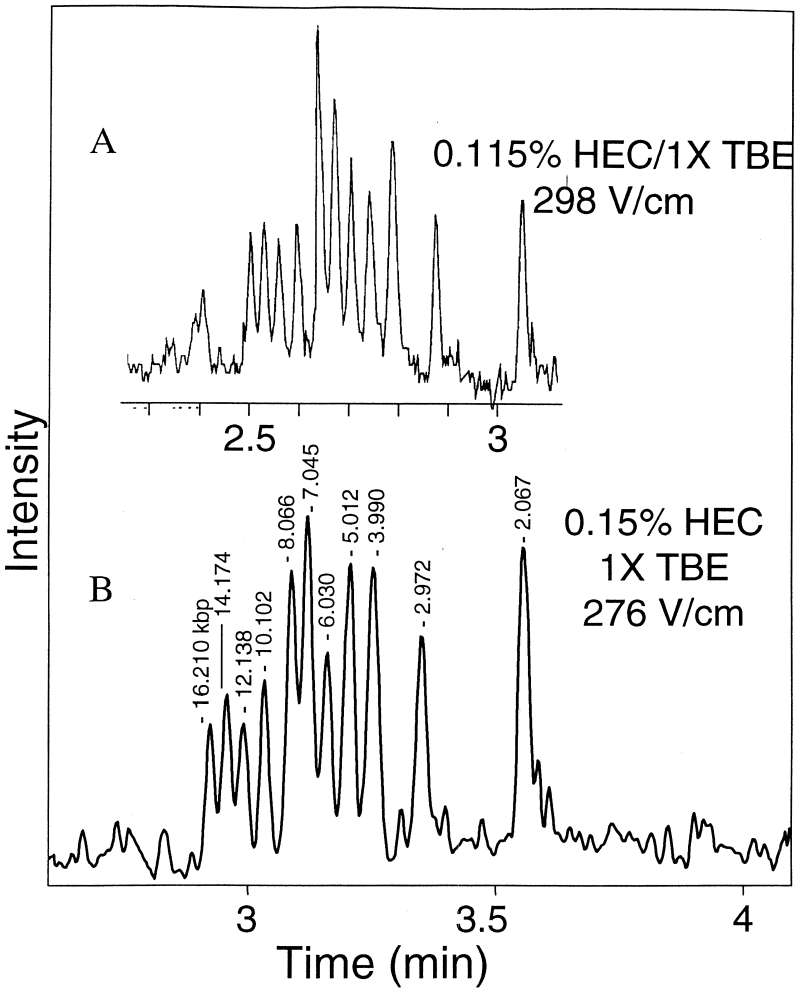

Fig. 3. Electropherograms of the supercoiled plasmid DNA ladder in HEC (M_n=90 000–105 000), 1× TBE, injection: 150 V/cm (3 s). (A) 0.11% (w/w) HEC, 298 V/cm. (B) 0.15% (w/w) HEC, 276 V/cm.

Thus, the intensity of the electric field is the dominant parameter.

As expected, the largest supercoiled DNA fragments migrate first in our uncoated system. The unmarked band that precedes the largest fragment (Fig. 3A and 3B) may be a plasmid aggregate or a decomposition product. As observed on the electropherograms in Fig. 3, the 16 kbp, 14 kbp, 12 kbp and 10 kbp fragments have much narrower bands than those reported for coated capillaries [14]. In coated capillaries, the largest fragments spend more time in the capillary and bands exhibit greater dispersion [18]. In uncoated capillaries, we then avoid this band-spreading effect. But do the narrower bands improve resolution?

In Table 1, we calculated adjacent pair resolution for the two previous plasmid separations using Eq. (1). We observe an increase in resolution as we increase the electric field and/or decrease the HEC concentration. These findings are in accord with previous results in coated capillaries [13]. Although the experimental conditions are not exactly the same, resolution for larger plasmid fragments such as 16 kbp/14 kbp is similar to those reported for coated

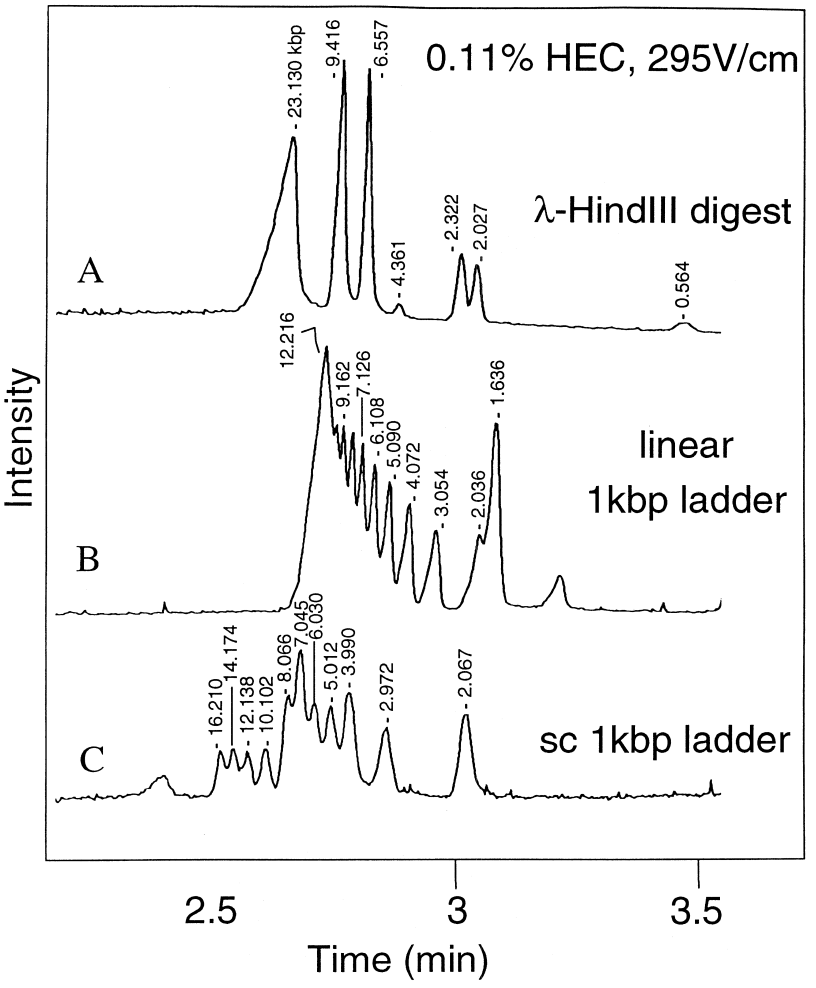

Fig. 4. Capillary electrophoresis of λ-H*ind*III digest DNA, the linear dsDNA and supercoiled plasmid DNA ladders in 0.11% (w/w) HEC (M_n = 90 000–105 000), 1× TBE, injection: 150 V/cm (3 s), 295 V/cm.

Table 1
Resolution R relative to field strength and [HEC] for supercoiled DNA calculated using Eq. (1). HEC (M_n = 90 000–105 000), 1× TBE. (A) 0.11% (w/w) HEC, 298 V/cm; (B) 0.15% (w/w) HEC, 276 V/cm

	0.11% HEC/1× TBE 298 V/cm resolution R	0.15% HEC/1× TBE 276 V/cm resolution R
14–16 kbp	0.97	0.80
12–14 kbp	0.97	0.75
10–12 kbp	1.29	0.95
8–10 kbp	1.37	1.25
7–8 kbp	1.11	0.71
6–7 kbp	1.24	0.82
5–6 kbp	1.36	0.95
4–5 kbp	1.43	0.86
3–4 kbp	2.90	1.83
2–3 kbp	5.96	4.02

Table 2
Electroosmotic flow at 300 V/cm in TBE buffer

TBE concentration	0.5×	1×	1.25×	1.5×
Mobility (10^4 cm^2/V s)	6.600	5.156	4.720	4.405

capillaries. We conclude that resolution is limited by topoisomer distribution, not band spreading generated during migration.

Fig. 4 compares representative electropherograms of linear λ-H*ind*III restriction digest, linear dsDNA and sc-plasmid DNA in 0.11% (w/w) HEC (M_n = 90 000–105 000) solution, 1× TBE, at 295 V/cm. It was previously observed in coated capillaries that sc-plasmid fragment mobilities were largely insensitive to ionic strength changes, unlike the mobilities of linear dsDNA. Thus, the mobilities of linear dsDNA could be shifted around the fairly constant sc-plasmid mobility. In the experimental conditions of Fig. 4, we observe that sc-plasmids migrate faster in EOF (and thus slower in the electrophoretic flow) which is in accord with previous results [14]. Plasmid bandwidths are greater than for linear DNA of the same size in base pair confirming previous observations in coated capillaries. However, plasmid bands in uncoated capillaries are narrower than in coated capillaries. Plasmid bands are also more symmetric, as observed in coated capillaries for low electric field [14]. Under higher electric field in

Table 3
Straight lines are obtained from a least square linear fitting of the data points

		Slope	Intercept	SE	SE slope	SE intercept	No. of data points
(A) Ln μ vs. ln N							
Linear	1.5× TBE	−0.0835	1.6803	0.0020	0.0009	0.0824	11
1 kbp ladder	1.25× TBE	−0.0778	1.7287	0.0019	0.0009	0.0081	11
	1× TBE	−0.0692	0.7634	0.0033	0.0013	0.0108	12
	0.5× TBE	−0.0873	1.9685	0.0104	0.0047	0.0383	5
	Average slope	−0.0794					
Supercoiled	1.5× TBE	−0.1352	2.1501	0.0099	0.0048	0.0422	11
1 kbp ladder	1.25× TBE	−0.1157	2.0428	0.0083	0.0040	0.0351	11
	1× TBE	−0.1119	2.1250	0.0082	0.0040	0.0355	8
	Average slope	−0.1209					
(B) Ln μ vs. ln R_g							
Linear	1.5× TBE	−0.1613	1.7847	0.0018	0.0017	0.0087	11
1 kbp ladder	1.25× TBE	−0.1502	1.8256	0.0023	0.0022	0.0110	11
	1× TBE	−0.1321	1.8428	0.0029	0.0021	0.0108	12
	0.5× TBE	−0.1605	2.0359	0.0073	0.0061	0.0296	5
	Average slope	−0.1510					
Supercoiled	1.5× TBE	−0.1352	1.7893	0.0100	0.0048	0.0295	11
1 kbp ladder	1.25× TBE	−0.1157	1.7341	0.0083	0.0040	0.0245	11
	1× TBE	−0.1119	1.8264	0.0082	0.0040	0.0248	8
	Average slope	−0.1209					

coated capillaries, plasmid bands were highly asymmetric [13].

Confirmation of our earlier description of linear dsDNA migrating as a random coil and of sc-plasmids migrating as an elastic rod is shown in Table 3 and Fig. 5. Fig. 5A and 5B show a ln–ln plot of electrophoretic mobility μ, versus N, size in bp, and radius of gyration, R_g respectively. The radii of gyration were calculated using the Porod-Kratky chain model assuming unperturbed conditions. Eq. (2) illustrates the behavior of random coils (linear dsDNAs) whereas Eq. (3) approximately represents the behavior of elastic rods (sc-plasmid DNAs).

$$R_{g,coil} = \sqrt{\frac{2aN \cdot 0.34_{nm/bp}}{6}} \quad (2)$$

$$R_{g,rod} \approx \frac{N \cdot 0.34_{nm/bp}}{2\sqrt{6}} \quad (3)$$

In Eq. (2), a is the persistence length of dsDNA, taken to be 45 nm. In Eq. (3), because the sc-plasmid DNA is circular, we choose to divide the contour length by a factor of two.

The DNA electrophoretic mobility was calculated by subtracting the contribution of EOF to the observed mobility as shown in Eq. (4).

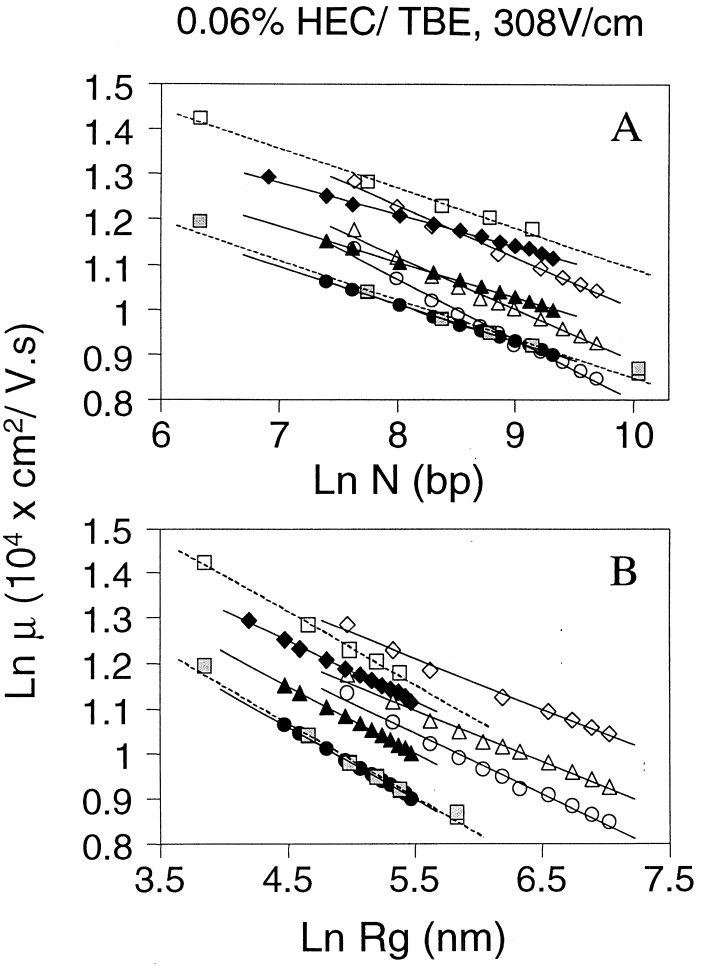

Fig. 5. Dependence of electrophoretic mobility of 1 kbp linear dsDNA ladder (♦, ▲, ●), λ-H*ind*III digest DNA (□, ■) and sc-plasmids (◇, ○, △) on chain length N and radius of gyration R_g. Conditions are 0.06% (w/w) HEC, 0.5× (□), 1× (♦, ◇), 1.25× (▲, △), 1.5× (■, ●, ○), TBE, 308 V/cm. (A) Mobility vs. chain length. (B) Mobility vs. radius of gyration.

$$\mu_{obs} = \mu_{eof} + \mu_{el} \quad (4)$$

The EOF rate was obtained by electrophoresis of Texas-Red labeled short neutral dextrans [weight-average molecular mass (M_w) = 3000] in the different TBE buffers used in our experiment (0.5×, 1×, 1.25× and 1.5×). Electroosmotic mobilities for different buffer ionic strengths are tabulated in Table 2. As the buffer ionic strength increases, the thickness of the ionic double layer increases and thus we observe a decreasing EOF.

All of the lines shown in both plots were obtained by linear regression.

The slopes of the mobility vs. size plots are different for the two kinds of DNA. For linear ds-DNA the average slope is −0.079 (R^2 = 0.993) and for sc-plasmid the average slope is −0.121 (R^2 = 0.990), giving a slope ratio of 1:1.5. The different slopes confirm the different migration behaviors of linear and scDNA. We interpret the differences as linear DNA migrating as a random coil and scDNA migrating as an elastic rod. We have previously reported slopes of −0.08 for linear dsDNA and −0.16 for sc-plasmid migrating in coated capillaries, with a slope ratio of 1:1.9 [14]. These data were obtained at slightly different HEC concentrations and at lower field strengths than those used in this work.

Buffer ionic strength affects mobility of both linear and scDNA. As the buffer ionic strength increases, polymer chains are better screened from each other's repulsive charges and thus more able to interpenetrate. In our data, the slopes of the plots of mobility vs. size decrease as the buffer ionic strength increases, thus improving the resolution of the separation. This behavior is in accord with the proposal of Sudor and Novotny that with increasing buffer ionic strength, polymer chains can be condensed to more compact forms, thus improving resolution of large deformable polymers [26].

We propose that under EOF the elastic rod approximation to sc-plasmid DNA migration dynamics begins to break down, introducing some coil-like character to the migration of the supercoiled fragments. However, the differences between the two sets of data are not sufficiently great as to require complete abandonment of the rod vs. coil picture of electrophoretic migration.

4. Conclusion

Dilute polymer solution electrophoresis of sc-plasmid DNA can be performed with fluorescence detection in uncoated as well as coated capillaries. Because larger DNA migrates past the detector before smaller ones, extraordinarily wide bands are not observed for long chain sc-plasmids. There appears to be insufficient time for the plasmid to explore the complete range of entanglement times; large sc-plasmid bands are somewhat less broad than in coated capillaries. Nonetheless, resolution of similarly sized sc-plasmids is no better than in coated capillaries. In both cases, resolution is limited by the thermal distribution of unresolved topoisomers.

In practice, the interesting case in sc-plasmid capillary electrophoresis is usually not resolution of a ladder, but resolution of a sc-plasmid from the nicked and linearized DNA of the same chain length. In uncoated as well as coated capillaries, the investigator is free to change buffer concentration, HEC concentration and field strength to optimize the separation for speed or resolution of different forms of DNA.

Acknowledgements

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