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Separation of topological forms of plasmid DNA by anion-exchange HPLC: Shifts in elution order of linear DNA

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Abstract

We sought to establish a single anion-exchange HPLC method for the separation of linear, open circular and supercoiled plasmid topoisomers using purified topoisomeric forms of three plasmids (3.0, 5.5 and 7.6 kb). However, finding one condition proved elusive as the topoisomer elution order was determined to depend on salt gradient slope. The observed change in selectivity increased with plasmid size and was most pronounced for the linear form. Indeed, the elution order of the linear 7.6 kb plasmid was reversed relative to the supercoiled form. This observation may have implications for methods used in quality control of plasmid DNA.

Keywords: Plasmid; Topoisomer separation; Anion-exchange HPLC; Slalom chromatography

1. Introduction

Plasmid DNA is increasingly being investigated in the clinic for both immunotherapeutic (vaccine) and gene therapy purposes [1]. Purity and approximate stability is most commonly analyzed by agarose gel electrophoresis [2]. However, a higher degree of sensitivity, precision and accuracy is necessary in order to determine quality of clinical-grade DNA intended for therapeutic use. Plasmids purified from *Escherichia coli* fermentation exist predominantly in the supercoiled (ccc) form, generally with varying levels of the other two topoisomers, open-circular (oc) and linear, which arise through single-stranded and double-stranded nicks, respectively. The linear plasmid form has been deemed undesirable for clinical purposes, due to a perceived increased risk of recombination events and integration into genomic DNA [3,4]; tertiary DNA structure has also been shown to influence gene expression both *in vitro* and *in vivo* [5,6]. Further, the linear form may be subject to more rapid intracellular degradation than the supercoiled and open circular forms [7]. Higher molecular weight forms, such as concatemers and catenanes, may also be present in crude plasmid preparations [8]. Consequently any method used to assess the purity of clinical-grade plasmid DNA for purposes of quality and safety must afford resolution not only by topoisomeric form (ccc, oc and linear), but also by size due to the potential presence of multimers.

Anion-exchange HPLC (AEHPLC) is a common tool for plasmid DNA analysis. Separation of plasmid molecules by AEHPLC relies on the interaction between negatively charged phosphate groups on the DNA backbone and positively charged groups on the resin stationary phase. The strength of this interaction is based not only on net charge, but also on local charge density. The overall interaction is not a distinct binding between specific charges, but rather a local attraction generated by opposing charges in close proximity [9]. With increasing

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salt concentration, DNA molecules should elute in the order of chain length (number of charged phosphates). Separation of plasmid species of equivalent charge and molecular weight is also feasible, due conformational differences. Supercoiled DNA has a higher charge density than the less constrained open circular form and therefore a stronger electrostatic attraction to the positively charged bound ligand, causing the supercoiled topoisomer to elute later than the oc form [10–12]. The chromatographic behavior of linear plasmid DNA is, however, affected by physico-chemical properties in addition to charge-density. Small double-stranded DNA fragments of up to 100 bp in length are thought to assume a straight rod conformation, the so-called persistence length [13,14]. As chain length increases the superhelical rod increasingly assumes a random coil in free solution, affording greater elasticity [15]. Therefore, when traveling through particle interstitial spaces, the linear form may be stretched by shear forces [14]. While net charge is the central factor in determining retention of linear plasmid DNA, shear forces can also affect the separation profile. Thus, elasticity sets linear plasmid DNA apart from the more static supercoiled and open circular forms. Other parameters such as dispersive forces, dipole-dipole attraction, hydrogen bonding, solvophobic repulsion, and AT content may also affect separation [14].

The objective of the work presented here was optimize AEHPLC methods for routine analysis of plasmid preparations containing the three topoisomeric forms (ccc, oc and linear), within the size range commonly encountered for therapeutic plasmids (3–12 kb. This effort was complicated by a size-dependent selectivity change of the linear plasmid form; small alterations in the slope of the salt gradient were found to cause significant changes in relative elution rates of the linear plasmid DNA.

The retention properties of AEHPLC in separating linear DNA and plasmid topoisomers has been described elsewhere [14,16,17]. However, the size- and gradient change rate-dependent reversal of elution order between the linear and supercoiled forms has to our knowledge not been previously reported.

2. Experimental section

2.1. Preparation of plasmid DNA

Plasmid pVax-1 (Invitrogen, Carlsbad, CA; 2999 bp, GC content 53%) pWRG728 [18] (5554 bp, GC content 49%) and pmpSmpCc, a derivative of pWRG728 containing also the core antigen [19], (7629 bp, GC content 49%) were transformed into competent *E. coli* HB101 (Gibco/BRL, Rockville, MD, USA) according to the manufacturer's instructions. Plasmids were produced by the GSK BioResources group. Briefly, bacterial fermentation was done in a 10 L Braun fermentor using LB broth (Gibco BRL, Rockville, MD, USA), pH 7.3, aeration at 4 slpm and a DO set point at 65%. Selection was maintained by 50 mg/L ampicillin. Bacteria were harvested in late log phase by centrifugation in a RC3B Sorvall centrifuge. Supercoiled plasmids were prepared through alkaline lysis and a chromatography procedure by using a Plasmid Giga Kit (Qiagen, Valencia, CA, USA).

2.2. Preparation of topological standards

The linear form was prepared from supercoiled stock by restriction endonucleases cleavage. *Sma I* was used to linearize pWRG728 and pmpSmpCc, whereas pVax-1 was linearized using *Eco RV* in the buffers and conditions recommended by the supplier (New England BioLabs, Ipswich, MA, USA). These enzymes cut each plasmid once in a blunt ended fashion.

Open circular topoisomers were prepared using the nickase *N.BstNB I* (New England BioLabs, Ipswich, MA, USA). Enzyme concentrations were optimized under the suggested incubation conditions. This kept the number of site-dependent phosphodiester breaks in each plasmid molecule at a minimum, increasing stability of the open-circular preparation. To each of five vials with 1 μg plasmid was added 1 μL supplied 10× buffer, *N.BstNB I* (10, 1, 0.1, 0.01 or 0.001 units) and water to 10 μL. Vials were incubated at 55 °C for 1 h and digests were quenched by addition of 1 μL 0.5 M EDTA. The reaction mixtures were denatured for 20 min at 80 °C. Finally, a 1 μL aliquot was analyzed by agarose gel electrophoresis in comparison to known quantities untreated and linearized plasmid to determine the amount of new open-circular plasmid at each dilution. The *N.BstNB I* concentration with the most open-circular plasmid, but least amount of linear plasmid, was then used to prepare reference oc material at the 1 mg scale; detectable amounts of the linear form indicated over-digestion. For scale-up the following procedure was used: 1 mg plasmid DNA, 100 μL 10× buffer, the appropriate units *N.BstNB I* (from above) and water to 1 mL were added to a 2 mL sterile tube on ice. The mixture was placed in an Eppendorf Thermomixer (Eppendorf, Westbury, NY, USA) and incubated for 1 h at 55 °C under agitation at 300 rpm. Mixtures were then quenched with 50 μL 0.5 M EDTA and heat denatured at 80 °C for 20 min. For purification of the open-circular form on the milligram scale, ultracentrifugation through a cesium chloride cushion in a Beckman Ti70 rotor (Beckman, Fullerton, CA, USA) was performed as described [20]. For the plasmids tested a typical density gradient of 4.5 mL total volume was adequate to purify about 0.5 mg of plasmid. The plasmid reaction solution was added to enough Tris–EDTA (TE) buffer, pH 8.0, containing 1.25–1.5 g/mL cesium chloride to bring the total volume to 9 mL. The plasmid-salt solution was divided into two tubes and spun at 90,000 rpm in a TLA 100.4 rotor (Beckman, Fullerton, CA, USA) for 18 h and recovered by syringe.

When necessary, purified plasmid preparations were transferred to TE buffer by dialysis in a Slide-A-Lyzer cassette (Pierce, Rockford, IL, USA) and/or ethanol precipitation prior to use.

2.3. Agarose gel electrophoresis

Samples were loaded with 6× running buffer (Promega, Madison, WI, USA) onto a 0.8% SeaKem agarose gel (BioWhittaker, Rockland, ME, USA). The gel was run at 80 V for 1 h and

then at 17 V for 22 h, in 1× Tris–borate (TBE) buffer, pH 8.0 using a Mini-Sub GT cell box (BioRad Laboratories, Hercules, CA, USA). Staining was performed with Sybr Green I (Molecular Probes, Eugene, OR, USA) and photographed using Kodak Electrophoresis Documentation and Analysis System 120 and Kodak Digital Science 1D software version 3.0.2 (Eastman Kodak, New Haven, CT, USA).

2.4. Chromatographic conditions

A TSKgel DNA-NPR (TosoHaas, Montgomeryville, PA, USA) weak anion-exchange column (4.6 mm × 75 mm) and guard column was used. The resin consisted of non-porous 2.5 μm pellicular polymethacrylate particles derivatized with diethylaminoethyl (DEAE) functional groups. The HPLC system consisted of a Waters Alliance (Waters Corporation, Milford, MA, USA) equipped with a 2690 autosampler, column heater set to 25 °C, and a 996 photodiode array detector. Chromatograms were extracted at 257 nm and Waters Millennium software v3.05.01 was used for peak integration. The equilibration buffer (A) was 20 mM Tris–HCl, pH 9.0 and the elution buffer (B) 20 mM Tris–HCl, 1 M KCl, pH 9.0. All buffers were filtered through 0.2 μm filters prior to use. In one study, HPLC grade acetonitrile (J.T. Baker, Phillipsburg NJ, USA) was used at 20% in both buffer A and B. The flow rate was 0.8 mL/min throughout. For each plasmid sample set four different gradient slopes were investigated, with an increase in buffer B of 3.5, 2.5, 1.5 or 0.5%/min, respectively.

Each sample was injected in a 10 μL volume containing 2 μg supercoiled DNA, 0.2 μg linear DNA, or 0.2 μg open circular DNA; all samples were analyzed at least twice. A linear gradient was developed from 50% to 70% buffer B, then washed with 90% buffer B for 2 min and finally re-equilibrated at 50% buffer B.

For comparative purposes relative topoisomer retention times (RRT) were estimated by the following equation:

$$\mathrm{RRT} = \frac{t_R}{t_{oc}}, \quad (1)$$

where t_R refers to the retention time of the supercoiled or linear topoisomer and t_{oc} refers to the retention times of the open circular form.

2.5. Transmission electron microscopy (TEM)

Purified plasmid was diluted to 1 μg/mL in 0.25 M ammonium acetate buffer at pH 7.7. To a 50 μL aliquot, 1.5 μL of cytochrome *C* protein at 200 μg/mL in water was added and the drop immediately placed on parafilm for 90 s. A parlodion covered copper mesh grid was touched to the surface and the sample dehydrated in 75% ethanol for 45 s and then in 90% ethanol for 5 s, after which the sample was air dried. The grids were rotary shadowcast with platinum:palladium (20:1) at an 8° angle in a high vacuum. The samples were examined in a FEI Technai 12 instrument (Phillips, Eindhoven, The Netherlands) and images recorded on sheet film.

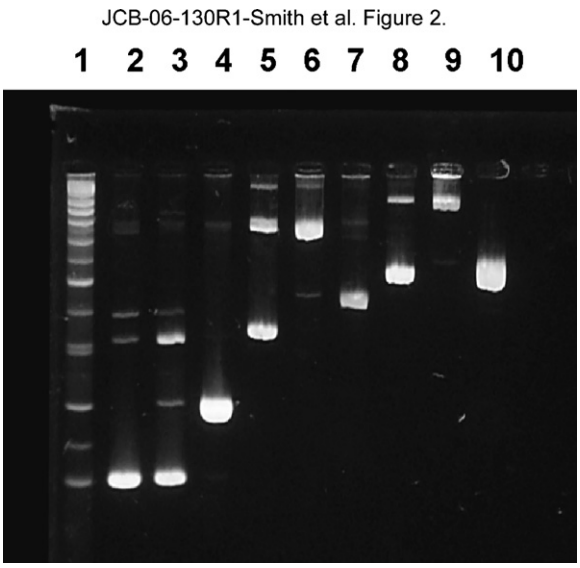

Fig. 1. Agarose gel electrophoresis (0.8%) of topological standards and supercoiled preparations. Lane 1: supercoiled molecular weight markers (Gibco BRL, Rockville, MD, USA). Lanes 2–4: 3 kb plasmid (supercoiled, open circular and linear). Lanes 5–7: 5.5 kb plasmid (supercoiled, open circular and linear). Lanes 8–10: 7.6 kb plasmid (supercoiled, open circular and linear). Topoisomeric and multimeric contaminants are visible in the supercoiled preparations (lanes 2, 5 and 8) and to a lesser extent in the open circular preparations (lanes 3, 6 and 9) due to high sample loading.

3. Results

The three topological plasmid forms (supercoiled, open circular and linear) of the plasmids used in this study (2999, 5554, and 7629 bp) were prepared enzymatically. High-resolution agarose gel electrophoresis afforded excellent resolution of bands for this plasmid size range (Fig. 1) and demonstrated that the reference preparations were pure. By AEHPLC three monomeric topoisomers were detected, as well as a trailing peak that consisted predominantly of plasmid multimers. Using a previously characterized 5.5 kb plasmid [18] we demonstrated by TEM that both concatemers and catenanes were present (Fig. 2).

Four sample sets were used to test the effects of salt-gradient on the resolution of the three plasmid topoisomers. For each gradient, supercoiled, open circular, linear, and spiked samples (oc plus ccc and linear plus ccc, respectively) were injected and monitored for changes in relative retention (Table 1). A relative gradient rate change-dependent elution of the large (7.6 kb) plasmid was unexpectedly encountered upon changing the salt gradient from a shallow 0.5%/min to 3.5%/min (Fig. 3). With increasing gradient steepness, the ccc form eluted increasingly closer to the oc peak. However, even more striking was the shift in elution order of the linear form. From eluting ahead of the ccc fraction at a gradient rate of 0.5 %/min, the elution rate of the linear form decreased relative the ccc form such that it eluted well resolved after the ccc form at a gradient change rate of 3.5 %/min. The linear form of the 5.5 kb plasmid exhibited this behavior as well (Fig. 4), although to a lesser degree; the linear form never migrated out past the back end of the supercoiled peak. In contrast to these results, the 3.0 kb linear plasmid showed no gradient rate-dependent shift (Fig. 5).

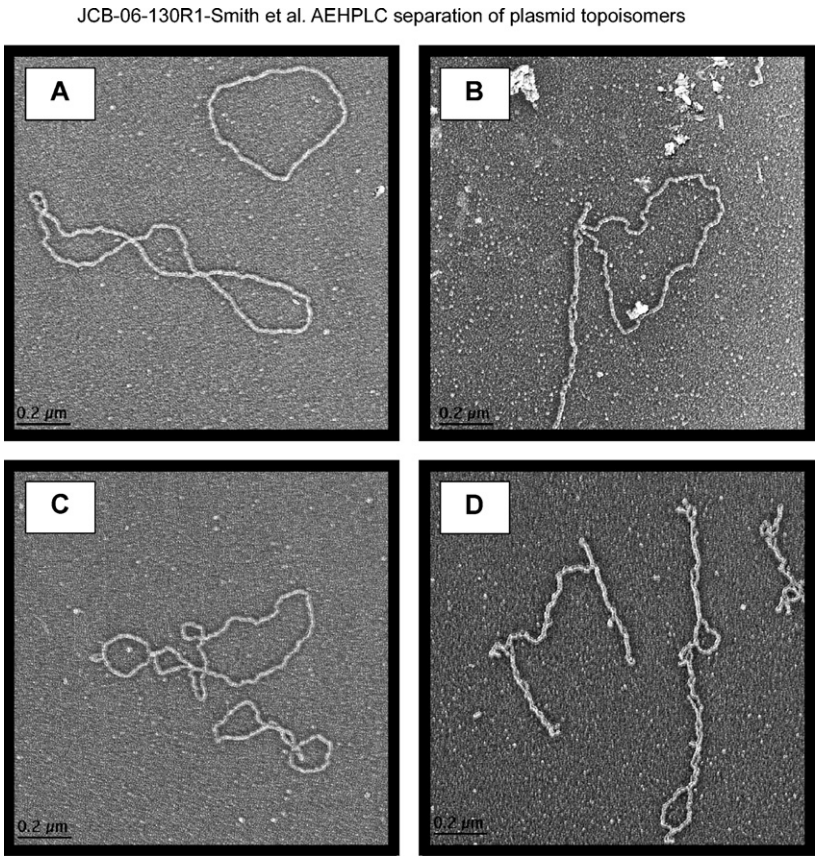

Fig. 2. TEM of plasmids from the trailing peak, eluting at 5.5 min in Fig. 4A, of AEHPLC-fractionated pWRG7128 (5.5 kb). While this peak contained some monomeric species, it consisted primarily of plasmid multimers; concatemers and catenanes were present at an approximate ration of 2.5:1. (A) Dimeric and trimeric concatemers in the open circle form. (B) Catenane with one supercoiled and one open circle. (C) Relaxed catenanes. (D) Supercoiled concatemers.

Table 1
Relative retention times (RRTs) obtained for the supercoiled and linear forms of the 3.0, 5.5 and 7.6 kb plasmid, respectively[a]

Salt gradient change rate (%/min)	RRT					
	3.0 kb		5.5 kb		7.6 kb	
	Supercoiled	Linear	Supercoiled	Linear	Supercoiled	Linear
0.5	1.11	1.11	1.12	1.06	1.12	1.06
1.5	1.09	1.10	1.08	1.08	1.10	1.11
2.5	1.05	1.04	1.08	1.07	1.09	1.14
3.5	1.08	1.07	1.08	1.08	1.07	1.16

[a] Plasmid topoisomers were eluted from a TSK gel DNA-NPR weak anion-exchange column (4.6 mm × 75 mm; particle size 2.5 mm) at a flow rate of 0.8 mL/min under the given gradient conditions. Relative retention times were derived as described under Section 2.

In order to address the cause of the elution-order shift, several factors were tested. To rule out hydrophobic factors that may influence linear DNA migration to a higher degree due to its more flexible structure [21], the same sets of runs were performed in acetonitrile-containing elution buffer. Although the relative retention times were slightly different, the results were qualitatively comparable (results not shown).

4. Discussion

The size of plasmids aimed for vaccine development purposes typically range from 3.0 to 12 kb depending on vector complexity, including such factors as the number of control elements, functional units and genes. Our goal was to optimize AEHPLC methods for routine analysis of plasmid preparations within this size range, to ultimately enable one method to be used. The weak anion-exchange TSKgel DNA-NPR column with 2.5 μm micropellicular packing used here had using conditions previously developed through DOE experiments been found to afford excellent resolution of all three topoisomers of the 5.5 kb plasmid, as well as multimeric forms, under short run times (unpublished data). The performance of non-porous stationary phases for large biopolymer separations has been shown to be superior to that of porous resins [22]. This has been determined to result primarily from favorable mass transfer properties as a consequence of DNA movement in particle interstitial spaces rather than in

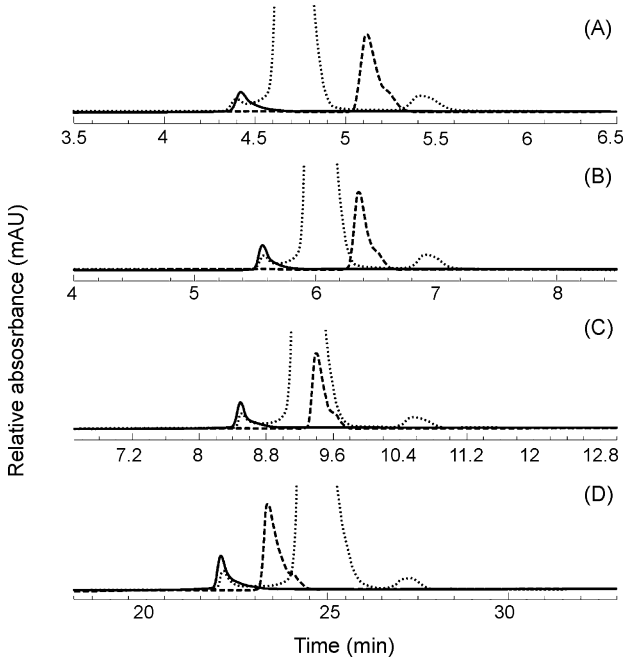

Fig. 3. Effect of gradient steepness on resolution of topological forms of the 7.6 kb plasmid, expressed as the percent change in buffer B per minute: (A) 3.5%/min; (B) 2.5%/min; (C) 1.5%/min; (D) 0.5%/min. The supercoiled form (· · ·) contained small amounts of open circular plasmid and some high molecular weight plasmid DNA in the trailing peak, as demonstrated by TEM (data not shown). The open circular form (—) eluted before the linear form (- - -). However, the elution rate of the linear topoisomer was shown to be inversely proportional to gradient steepness. Each figure shows overlayed chromatograms of the ccc, oc and linear forms, respectively.

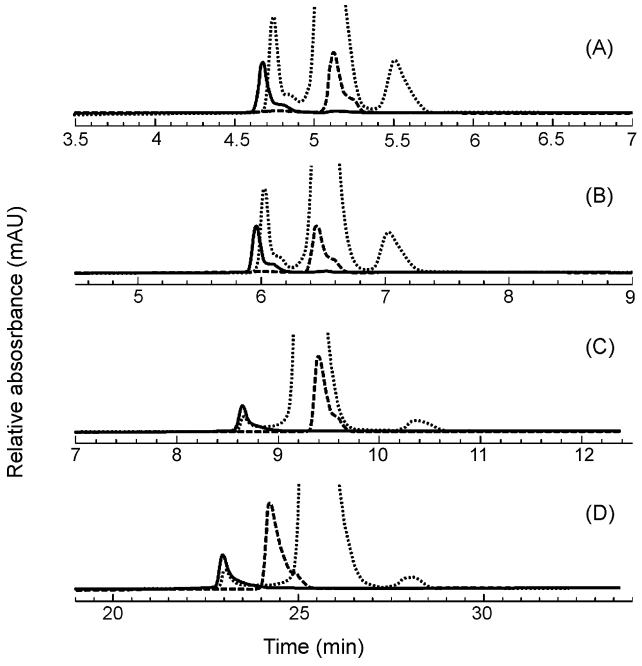

Fig. 4. Effect of gradient steepness on resolution of topological forms of the 5.6 kb plasmid, expressed as the percent change in buffer B per minute: (A) 3.5%/min; (B) 2.5%/min; (C) 1.5%/min; (D) 0.5%/min. The dotted line (· · ·) represents the supercoiled form, the solid line (—) represents the open circular form and the linear topoisomer is represented by a dashed line (- - -). Each figure shows overlayed chromatograms of the ccc, oc and linear forms, respectively.

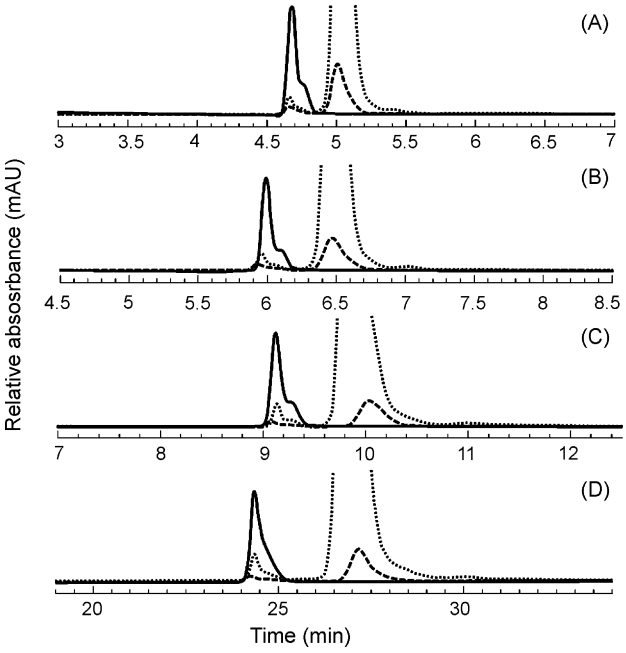

Fig. 5. Effect of gradient steepness on resolution of topological forms of the 3.0 kb plasmid: (A) 3.5%/min; (B) 2.5%/min; (C) 1.5%/min; (D) 0.5%/min. Irrespective of gradient steepness the linear form (- - -) was inseparable from the supercoiled form (· · ·), whereas the open circular form consistently eluted in front. Each figure shows overlayed chromatograms of the ccc, oc and linear forms, respectively.

large pore spaces (300–4000 Å). The latter has been shown to result in broad peaks, low sample recovery and necessitate low flow-rates and shallow gradients [14,16]. Nevertheless, we were unable to find chromatographic conditions under which all three model plasmid topoisomers could be separated.

While we were unable to find a generic method, we discovered a size-dependent change in relative elution rates. Specifically, small alterations of the salt gradient slope caused significant size-dependent changes in elution profile of the linear form. This phenomenon is illustrated in Fig. 6, where relative retention rates of the ccc and linear forms of the 7.6 kb plasmid are plotted against gradient steepness. It was further observed that relative retention time differences between oc, ccc and linear plasmids approached a minimum around a gradient change rate of 1.5%/min (Table 1). This was also close to the intersection point of migration rates for the ccc and linear form of the 7.6 kb plasmid; at a gradient rate change of 0.5 %/min the linear form elutes faster than the ccc form, whereas the elution rates are reversed at rates of 1.5 %/min and higher (Fig. 6). Finally, for all three plasmid sizes the poorest resolution was obtained at a change-rate of 1.5 %/min; under the ratios of supercoiled to open circular or linear plasmid used here the linear peak was completely obscured by the ccc peak. Consequently, if such conditions were used to assess supercoiled plasmid purity, the value would be both inaccurate and falsely elevated.

The primary parameter altered during method development was gradient slope. Hence, the observed selectivity change would appear to have resulted from either a physical modification induced by the changing mobile phase environment, or from DNA conformation. In slalom chromatography, hydrodynamic

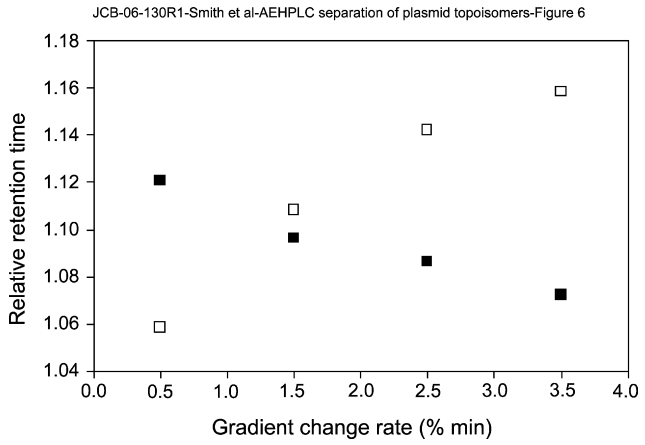

Fig. 6. Comparison of the effect of gradient rate change upon the relative retention time (RRT) of supercoiled (□) and linear (■) form, respectively, of the 7.6 kb plasmid. Retention times are expressed relative to that of the open circular topoisomer. Chromatography was performed on a TSK gel DNA-NPR weak anion-exchange column (4.6 mm × 75 mm; particle size 2.5 mm) under the conditions described in Section 2. Plasmid topoisomers were eluted by using a linear gradient slope of 3.5, 2.5, 1.5 or 0.5%/min, respectively.

forces [15] and eluant viscosity mechanisms (not equilibrium) mediate selectivity [23]. For example, Peyrin et al. reported that an increase in mobile phase viscosity improved separations of DNA fragments [23]. High molecular weight linear DNAs (10–48 kb) have been separated using 10 μm closely packed particles, where the larger molecules eluted later due to reduced mobility in interstitial space [17,24]. Separation depended upon flow rate; with higher flow, or turbulence, retention was increased [15]. Hirabayashi and Kasai concluded that separation was affected by a combination of mixed mode and hydrophobic-interaction chromatography [21]. We conclude that slalom chromatography, or possibly a mixed-mode model, likely caused the significant size- and flow-dependent elution changes observed in this study as well.

Drawing upon hypotheses formulated in previous studies [22,25], size calculations of the plasmid DNA species used were made (Table 2). All supercoiled plasmids used in this study were estimated to be less than 0.4 μm in length and the 7.6 kb open circular plasmid 0.81 μm, according to the equation of Boles et al. [22]. The supercoiled plasmids were demonstrated by TEM to approach rod form [18]. The length of the linear forms was calculated to be 1.00, 1.86 and 2.56 μm for the 3.0, 5.5 and 7.6 kb plasmids, respectively. As the stationary phase particles are 2.5 μm in diameter it might be expected that mobility

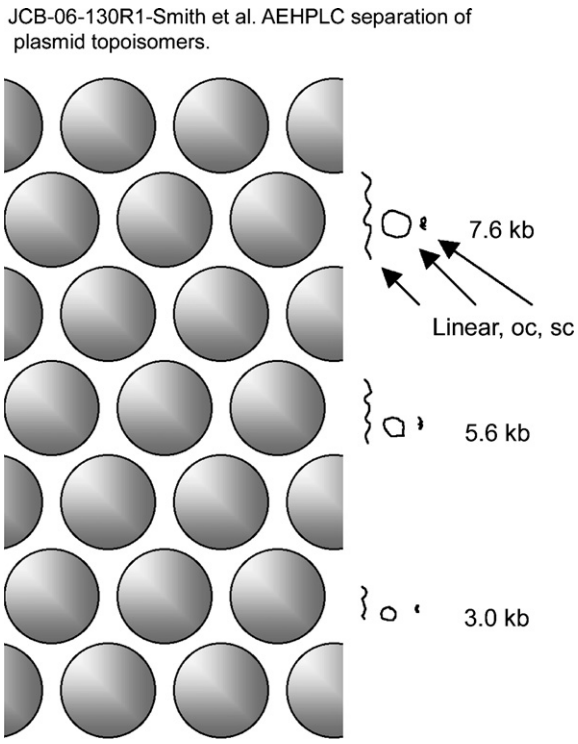

Fig. 7. Theoretical sizes of plasmid DNA topoisomers (linear, open circular and supercoiled) relative to micropellicular packing material. As in slalom chromatography, migration of larger DNA fragments may be more constrained [24]. Space between particles is enlarged.

of largest species would be more constrained than the smaller species under a given set of conditions. The pictograph of plasmid sizes and topoisomers relative the 2.5 μm particles in Fig. 7 was developed according to Hirabayashi et al. [21].

A similar inversion of mobilities amongst topoisomers is observed in agarose gel electrophoresis, where the supercoiled form typically migrates fastest due to its compact structures and high charge density, followed by the linear and open circular form, respectively [26,27]. The gel matrix here affords the tortuous path analogous to the interstitial space in the packing of an HPLC column. However, under conditions of high gel concentration and low voltage, the relative mobilities of the linear and open circular forms are reversed [26,27]. As with the present study, the effect increases with size. While properties of the agarose gel matrix clearly influences electrophoretic mobility, deformation of random coil structures or other conformational changes affecting DNA retardation are thought to be involved as well [27,28].

Table 2
Approximate size calculations for the topoisomeric forms of the plasmids used in this study

Plasmid size (kb)	Estimated superhelical length (μm)	Estimated open circle diameter (μm)	Maximum linear length (μm)
2.999	0.12	0.32	1.00
5.554	0.23	0.59	1.86
7.629	0.33	0.81	2.56

Calculation of superhelical length was based on extrapolation from 3.5 and 7.0 kb plasmids described by Boles et al. [22], who found that superhelical axis was independent of σ, or superhelical density, and approximately 41% of the total DNA length. The length of linear DNA was based on multiplying the number of base pairs by 3.35 Å/bp. To approximate the diameter of the open circular form, the linear length (circumference) was divided by π.

In summary, we have shown here that with increasing salt gradient slope in AEHPLC separation of plasmid DNA topoisomers, the linear species of plasmids 5.6 kb and above are increasingly retained on the column. The relative elution rate between the open circular and supercoiled forms remained constant under the conditions used, with open circular form eluting first. However, the linear form shifted from eluting before, to co-eluting with and eluting after the supercoiled fraction depending on gradient steepness change in the range from 0.5 to 3.5%/min. The size-dependent retention-change phenomenon appeared to have been caused by mobile phase viscosity and may thus represent a slalom chromatography, or possibly a mixed mode, mechanism. An understanding of the correlations between AEHPLC conditions, plasmid DNA size, and topoisomer resolution is clearly important in developing robust analytical methods for clinical products; robust analytical quality control methods are critical in ensuring the purity and safety of plasmid DNA intended for clinical use.

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